Heterogeneous 5'-Terminal Structures Occur on Vesicular Stomatitis Virus mRNAs*

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Four alternative structures occur at the 5' ends of vesicular stomatitis virus mRNAs synthesized in infected cells and are separated conveniently by a technique described here. Sixty-five to seventy per cent of the mRNA molecules have the 5' end structure m'G5'ppp5'(m)AmpAp and about 20% have a more highly modified structure m'G'ppp5'(m)AmpmAmpCp. The base of the first adenosine in each sequence is methylated in about one-half of the ends of each type and kinetic experiments suggest that the latter sequence is derived from the former by further methylations. The remaining 10 to 15% of the 5' ends are pppAp and pppGp in approximately equimolar yields. This heterogeneity with respect to 5' end structure is found within each of the vesicular stomatitis virus mRNA species examined. The mRNA molecules with 5'-triphosphate ends accumulate throughout the infection but are not found on ribosomes, suggesting that they lack a structure(s) required for ribosome recognition. In contrast to mRNA, virion RNA has a single 5' end structure, pppAp.

Since the initial discovery of an unusual, methylated oligonucleotide containing a 5'-5' pyrophosphate linkage (m5'2,7-G5'pppNmpNmpNp) at the 5' end of a small nuclear RNA (1), several reports have appeared concerning similar ends (m5'G5'ppp5'NmpNp or m5'G5'ppp5'NmpNmpNp) on viral mRNAs synthesized in vitro by virion polymerases (2, 3) or in virus-infected cells (4). Animal cell mRNAs also have similar 5' ends of the form m5'G5'ppp5'NmpNp or m5'G5'ppp5'NmpNmpNp (5). Because the viral mRNAs examined were synthesized by viruses which have double-stranded RNA or DNA genomes, it was of interest to examine the 5' ends of mRNAs transcribed from the single-stranded RNA genome (6) of vesicular stomatitis virus.

I report here the purification and structural analysis of several 5' ends obtained from individual species of VSV mRNA synthesized in VSV-infected cells. These 5' ends were isolated from the VSV mRNA species by digestion of the RNA with RNases T1, T2, and A followed by pH 3.5 ionophoresis on DEAE-paper. I have also used this procedure, which is similar to a method described previously (7), to purify the 5' end, pppAp, from the VSV virion RNA. DEAE-paper ionophoresis resolves the four 5' ends from VSV mRNA and has the further advantages of convenience and speed. Ion exchange chromatography on DEAE-cellulose in 7 M urea (8) was used to isolate 5' ends from other animal

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virus mRNAs (2, 3) but was unsuitable for VSV mRNA because all four 5' end structures which are clearly resolved by DEAE-paper ionophoresis migrate as a single peak of charge minus 5 to 6 on the DEAE-cellulose column. A report of a methylated 5' end of undetermined structure synthesized in vitro by the VSV virion polymerase has appeared recently (9).

Following infection, VSV is known to direct the synthesis of mRNA species which are complementary to the single strand of RNA within the virion (10). Purification, nucleotide sequence complexity analysis, and identification of the translational products of the VSV mRNA bands eluted from formamide-polyacrylamide gels have been reported (11, 12). These studies have shown that at least five individual mRNA species are produced in VSV-infected cells and these account for most of the coding potential of the VSV genome. Fig. 1 (inset) shows an autoradiogram of a typical formamide-polyacrylamide gel electrophoretic separation of the VSV mRNAs. VSV mRNA Bands 2 and 3 are radiochemically pure RNA species coding for the G and N proteins, respectively, while Band 4 contains two RNA species coding for the M and NS proteins. Band 1 contains mainly the 28 S mRNA which specifies the L protein (13).

MATERIALS AND METHODS

Labeling of Viral RNAs—The procedures for labeling VSV mRNA with 32P, (10 mCi/2 × 108 infected cells) and purification of the VSV mRNA species by oligo(dT)-cellulose binding and formamide-polyacrylamide gel electrophoresis were as described previously (11) except that baby hamster kidney cells adapted to spinner culture (a generous gift from A. Banerjee) were used instead of Chinese hamster cells. Cloned virus (Indiana serotype) free of defective particles was used in all experiments. All labeling was in cells treated with 5 μg/ml of actinomycin D to suppress host RNA synthesis. Labeling of
VSV mRNA with $[^{3}P]H$adenosine or $[^{3}H]m$ganoicine (1 mCi/2 x 10^6 infected cells, 2 to 4 hours postinfection) was just as described previously for $[^{3}P]$labeling (11) except that complete medium was used. Base composition analysis of the labeled RNA by pH 3.5 paper ionophoresis showed that the labeling was greater than 97% specific for the labeled base. $[^{3}H]$Methionine labeling (1 mCi/2 x 10^6 infected cells) was in medium containing 2 x 10^{-4} M methionine. Adams and 5 x 10^{-4} M adenosine and ganoicine completely suppressed incorporation of $[^{3}H]$methyl label into mononucleotides (14). $[^{3}H]$Nucleotides and $[^{3}H]$Methionine were purchased from New England Nuclear. The protocol for labeling of virion RNA with $[^{3}P]T_1$ was as described for mRNA (11) except that the infection was allowed to proceed for 9 hours. Virion RNA (40 S) was purified by sucrose gradient sedimentation from NaDodSO_4 disrupted virions which had been purified from the cell supernatant is described previously (6).

**RNase Digestions**—Limit digestion of RNA samples (containing 5 to 10 mg of total RNA) for 5′ end isolation was for 2 hours at 37° in 5 μl of 0.05 M ammonium acetate, pH 5.2, containing 50 units/ml of RNase T2 (Calbiochem) and 90 units/ml of RNases A (Worthington) and T1 (Calbiochin). Complete venom phosphodiesterase (Boehringer-Mannheim) digestion of RNA samples was performed in 5 μl of 10 mM Tris-HCl, pH 7.8, containing 0.05 unit of enzyme. Phosphodiesterase is a 3′,exonuclease which degrades RNA to phosphates in internucleotide linkages to external phosphates (15). Fig. 2 (Lanes a and b) shows the handling of the paper and separations of oligonucleotides obtained on pH 3.5 ionophoresis. Nucleotides were located by spraying the dried paper with 30% triethylamine bicarbonate, pH 7.8, and incubation continued for an additional 30 min at 23°, followed by addition of aniline to 0.15 mM, 25% glycerol, and 0.01 unit of enzyme. Bacterial alkaline phosphatase (Boehringer-Mannheim) digestions were for 1 hour at 37° in 5 μl of 10 mM Tris-HCl, pH 7.5, containing 0.01 unit of enzyme and 5 μg of carrier RNA. Penicillium nuclease (Yamasa Biochemicals, Tokyo) digestions were for 1 hour at 37° in 5 μl of 10 mM Tris-HCl, pH 6.0, with 0.05 μg of enzyme.

5′ End Purification and Analysis—Limit RNase T1, T2, and A digests of labeled RNA were spotted on sheets (30 x 100 cm) of DEAE-paper (DE81, Whatman, Inc.) 10 cm from one end. Ionophoresis at pH 3.5 was for the times and at the voltage indicated. Barrell (15) discusses the handling of the paper and separations of oligonucleotides obtained on pH 3.5 ionophoresis. Nucleotides were located by spraying the dried paper with 30% triethylamine bicarbonate, adjusted to pH 7.5 by bubbling with CO_2 (15).

For analysis of 5′ end digestion products samples were spotted on sheets (30 x 60 cm) of Whatman No. 3MM paper and subjected to ionophoresis at 40 volts/cm for 30 to 45 min until the blue dye, xylene cyanol FF, moved from 8 to 10 cm from the origin. Marker nucleotides, about 0.5 A_m, each, were spotted on top of each applied RNA sample. The products from Spot A migrated with markers of pA<sub>G</sub>, A<sub>G</sub> and unmodified nucleotides spotted directly on top of the sample. Two-dimensional thin layer chromatography was on glass-backed thin layer cellulose plates (Brinkmann Instruments) without fluorescence indicator. Prior to cellulose thin layer chromatography, samples containing glycerol were spotted on DEAE-paper, washed with water to remove glycerol and then eluted with 30% triethylamine bicarbonate, pH 7.8.

Markers of pAp, ApA, and unmodified nucleotides were purchased from Sigma. Modified nucleotides pmG and pmA were purchased from Terra Marine Bioresearch, La Jolla, Calif. Other marker nucleotides were gifts (see "Acknowledgments").

**Periodate Oxidation and Elimination with Aniline-Periodate oxidation of 5′ end oligonucleotides A or B (about 0.05 A_m each) was for 2 hours at 23° in 10 mM periodate, pH 6.0. Rhamnose was added to a final concentration of 100 mM and incubation continued for an additional 30 min at 23°, followed by addition of aniline to 0.15 M, pH 4.5, and further incubation for 1 hour at 37°. The entire reaction was in a volume of 10 μl so that the reaction products could be spotted directly on DEAE paper and purified by pH 3.5 electrophoresis. This procedure is similar to one described previously (16).

**RESULTS**

To isolate potential 5′ ends from $[^{3}P]$-labeled VSV mRNA I first digested the RNA with RNases T1, T2, and A. The only nucleotides or oligonucleotides containing more than one phosphate expected to survive this digestion are 5′-phosphorylated termini or oligonucleotides resistant to RNase A because of 2′-O-methylation. The remainder of the RNA should be degraded to 3′ mononucleotides. Ionophoresis at pH 3.5 on DEAE-paper was used to separate the mononucleotides from structures containing more than one phosphate. Oligonucleotides containing increasing numbers of phosphates generally migrate closer to the origin in this system (15). Fig. 1 (Lane a) shows an autoradiogram of such a separation digested VSV mRNA with the major products (the indicated mononucleotides) farthest from the origin. In addition, a single spot is visible just above the origin. Longer times of electrophoresis reveal four spots (A, B, C, and D) above the origin (mononucleotides have migrated off the paper). These four spots are seen in digests of total VSV mRNA (Lanes b) and in digests of the individual mRNA bands eluted from the gel (Lanes c to f). A variable fraction of the applied radioactivity, 0.01 to 0.2%, always remained at the origin and was not characterized. In contrast to mRNA, only a single spot just above the origin (Fig. 1, Lane g, arrowed) is derived from RNase T1, T2, and A digests of virion RNA.

Table I shows the amounts of radioactivity found in a typical preparation of Spots A to D derived from total VSV mRNA. The relative amounts of A to D were the same for each mRNA band, but the radioactivity in A to D relative to the total radioactivity applied decreased in proportion to the increase in the size of the mRNA.

I determined the structures of Spots A to D by further digestions with alkaline phosphatase, venom phosphodiesterase, and penicillium nucleases, using $[^{3}P]$, $[^{3}H]$methyl, and $[^{3}H]$nucleotide labels. From the structures deduced it is clear that each spot must originate from the 5′ end of an RNA. For the sake of simplicity, I will refer to those structures as Spots A to D throughout the analysis.

**Phosphatase Sensitivity**—To determine the ratio of phosphates in internucleotide linkages to external phosphates in Spots A to D, I digested each with alkaline phosphatase and analyzed the products by pH 3.5 ionophoresis. Phosphatase treatment of $[^{3}P]$-labeled Spot A released 20.2% of the radioactivity as phosphate indicating a total of five phosphates, four internucleotide phosphates, and one external. Similarly, the results indicated that B must have five internucleotide phosphates and one external phosphate since 16.5% of the radioactivity migrated like phosphate after phosphatase treatment. In contrast to A and B, all $[^{3}P]$ radioactivity in Spots C and D migrated like phosphate after phosphatase treatment, showing that they did not contain phosphate in internucleotide linkage.

**Venom Phosphodiesterase Digestion Products**—Venom phosphodiesterase is a 3′-exonuclease which degrades RNA to 5′-mononucleotides and will release a 3′-phosphorylated terminus as a nucleoside 3′,5′-diphosphate (15). Fig. 2 (Lanes a to d) shows the products of complete venom phosphodiesterase digestion of Spots A and B with and without prior phosphatase treatment. The products from Spot A migrated with markers of pmG, pAm, pAp, and P<sub>c</sub> (Lane a) while those derived from Spot B were coincident with markers of pmG, pAm, pCp, and P<sub>c</sub> (Lane c). After phosphatase treatment of Spots A and B, the venom phosphodiesterase digestion products did not contain radioactivity migrating with pAp or pCp markers (Lanes b and d) indicating that pAp and pCp were the respective 3′ termini of Spots A and B.

To identify further the products of venom phosphodiesterase digestion of Spots A and B, each nucleotide was eluted from the paper ionophoretic separation and run on a two-dimensional thin layer cellulose chromatography system which separates methylated and unmethylated nucleotides. Separation of the complete venom phosphodiesterase digestion products of Spot A is shown in Fig. 3a with analysis of individual
FIG. 1 (left). Autoradiograms of ionophoretic separations of 5' ends from limit RNase T1, T2, and A digest of VSV RNAs. Digested samples were spotted on DEAE-paper and subjected to pH 3.5 ionophoresis for (a) 4 hours at 30 volts/cm or (b) 12 hours at 30 volts/cm. Lanes c to f show separations (12 hours, 30 volts/cm) of limit digests of mRNAs from gel bands 1-4, respectively. Lane g shows the separation of a limit digest of VSV virion RNA (6 hours, 30 volts/cm). Origins are on a line between the two spots at the bottom of each lane. To ensure purity prior to characterization, each spot was eluted from the paper, redigested with RNases 'T1, T2, and A' and again subjected to pH 3.5 ionophoresis on DEAE-paper. The inset shows an autoradiogram of a formamide-polyacrylamide gel electrophoretic separation of VSV mRNAs. The electrophoresis procedure was described previously (11).

FIG. 2 (right). Autoradiograms of pH 3.5 paper ionophoretic separations of degradation products of Spots A-D. Total venom phosphodiesterase digestion products of Spot A before (Lane a) and after (Lane b) phosphate treatment. Total venom phosphodiesterase digestion products of Spot B before (Lane c) and after (Lane d) phosphatase treatment. Phosphatase-treated samples were purified by pH 3.5 ionophoresis on DEAE-paper prior to digestion with venom phosphodiesterase. Lanes e and f show the mobilities of the 32P-labeled products obtained from Spots A and B after periodate oxidation and aniline treatment. The circles indicate their mobilities prior to the reaction. Lanes g and h show the products of phosphodiesterase digestion of the periodate-oxidized and aniline-treated Spots A and B. The circles indicate their mobilities prior to the reaction. Lanes i and j show the products of venom phosphodiesterase digestion of Spots C and D with radioactive markers of PP, and P, shown in Lane k. Positions of nonradioactive markers are indicated where appropriate. Origins are on a line between the two spots at the bottom of each lane and the dotted circles indicate the positions of the blue dye.

nucleotides eluted from Lane a of the paper electrophoretic separation (Fig. 2) shown in Fig. 3, b, c, and d. The nucleotides coincident with pm'G and pAp on paper electrophoresis migrated exactly with these markers in both dimensions of the chromatography system, confirming their identities. However, the radioactivity previously coincident with the pAm marker separated into two spots, one of which migrated exactly with pAm and another which moved faster in both dimensions (Fig. 3e). Since the latter nucleotide has the mobility of pA on pH 3.5 paper ionophoresis (pA and pAm have the same mobilities in this system) and moved faster than pAm in both dimensions of the chromatography system, it was tentatively identified as a base methylated 2'- O-methyl adenosine (pmAm) since an additional methylation would cause faster migration in both dimensions of the chromatography system. The same venom phosphodiesterase digestion products in the same ratios were derived from Spot A purified from each VSV mRNA band. The two-dimensional analysis of total venom phosphodiesterase digestion products of Spot B derived from total VSV mRNA gave a pattern identical with that from Spot A except that pCp replaced pAp. Thus the same nucleotides are apparently present in A and B except for the different 3'-phosphorylated termini. The complete quantitations of the products of venom phosphodiesterase digestion of Spots A and B are given in Table II and will be discussed later. Further confirmation of the identities of the modified nucleotides was obtained as described below.

[3H]Methyl and 3H-Nucleoside Labeling—Table I shows that Spots A and B were labeled with [3H]methyl from [methyl-3H]methionine, [3H]adenosine, and [3H]guanosine. Clear separations of Spots C and D were not obtained in the 3H-labeling experiments, and thus the 3H radioactivity is given for both together. Adenosine and guanosine labels were found in these spots, but the methyl label is apparently due to background since a direct analysis (see below) showed that they did not contain methylated nucleotides.

To determine the identities of the modified nucleotides in Spots A and B, each 3H-labeled spot was combined with 32P-labeled A or B and digested with venom phosphodiesterase. The products were separated on the two-dimensional chromatography system as in Fig. 3. Each nucleotide was located by autoradiography, eluted, and the 3H and 32P radioactivity determined. The results (Table III) show clearly that the nucleotides tentatively identified as pm'G, pAm, and pmAm show specific 3H-nucleoside as well as [3H]methyl labeling. The ratio of [3H]methyl to 32P-labeling in the spot tentatively identified as pmAm is about 2-fold higher than that in pm'G and pAm, confirming that it is a dimethyl adenosine.
elimination with aniline (16), a procedure which specifically obtained by periodate oxidation of A and B followed by phosphatase-resistant product ran with an ApA marker. Since external phosphates and one internucleotide phosphate. The to determine the ratio of internucleotide to external phosphates. After digestion with phosphatase, 80% of the radioactivity migrated as phosphate (Fig. 2, Lane R), indicating four phosphorylated ends already established.

Digestion of each product confirmed the elimination of m'G. Thus Spots A and B have both a 3'-hydroxyl end as well as the 3'-monophosphate. Since this nucleotide comes from internal positions in Spots A and B (see below) and alkali or RNase T2 did not cleave at these positions, one of the methylations is presumably a 2'-ribose methylation and the other is probably a base methylation.

Location of pm'G in Spots A and B—Spots A and B were digested partially with venom phosphodiesterase and the products separated by two-dimensional thin layer chromatography. Fig. 3 shows this analysis for Spot A. Both A and B gave two products, pm'G and another which streaked somewhat in the second dimension but ran as a single spot just slower than the parent A or B spot on pH 3.5 DEAE-paper ionophoresis. Since venom phosphodiesterase digests most readily from a nonphosphorylated 3' end, and releases 5'-mononucleotides, the specific release of pm'G from A and B indicates that pm'G is linked by its 5'-phosphate and has a free 3'-hydroxyl end on both A and B.

Further evidence for free 2',3'-hydroxyls on m'G was obtained by peridode oxidation of A and B followed by elimination with aniline (16), a procedure which specifically removes one nucleoside from the 3' end of an oligonucleotide with free 2',3'-hydroxyls. The 3P-labeled products of this reaction migrated faster than the untreated A or B spots on pH 3.5 paper ionophoresis (Fig. 2, Lane e). A subsequent digestion of each product confirmed the elimination of m'G. Thus Spots A and B have both a 3'-hydroxyl end as well as the 3'-monophosphorylated ends already established.

Structure of Spot A—The 3P-labeled product obtained after elimination of m'G from Spot A was treated with phosphatase to determine the ratio of internucleotide to external phosphates. After digestion with phosphatase, 80% of the radioactivity migrated as phosphate (Fig. 2, Lane g), indicating four external phosphates and one internucleotide phosphate. The phosphatase-resistant product ran with an ApA marker. Since

5'...
The venom phosphodiesterase digestion products of Spots A and B labeled with [3H]methylmethionine, [3H]adenosine, or [3H]guanosine were separated by two-dimensional thin layer chromatography as shown in Fig. 5, scraped off the thin layer, and radioactivity determined in a scintillation spectrometer. Radioactivity from 3'- and 3'-P-labeled Spots A and B was combined prior to enzyme digestion and thin layer chromatography, so that the digestion products could be located by autoradiography. The numbers in parentheses are the ratios of [3H]methyl to 3P label in the nucleotides.

| Label          | Radioactivity in pm'G | pmAm | pmAm | pAp |
|----------------|-----------------------|------|------|-----|
| CHCl3 [3H]Methionine | 485                  | 221  | 865  | 13  |
| [3H]Adenosine   | (0.98)                | (1.1) | (2.2) |     |
| [3H]Guanosine   | 491                   | 201  | 393  | 948 |
| CHCl3 [3H]Methionine | 666                  | 5    | 144  | 305 |

pAp is the 3'-phosphorylated terminus of Spot A and the sum of the molar yields of pmAm and pmAm in the total digestion products of Spot A is about one relative to pAp (Table II), the only structure consistent with the results is ppp(p(m)Am)pAp with only partial base methylation of the first adenosine. Since m'G is linked in Spot A by a 5'-phosphate, the complete structure must be m'G5'ppp5'(m)Am(p)Ap. This structure is consistent with the yields of total venom phosphodiesterase digestion products except for the high yield of phosphate which is probably due to phosphatase activity. The phosphatase nature of the 5'-5' linkage was also confirmed directly as described below for Spot B.

**Structure of Spot B**—Elimination of m'G from Spot B rendered the 3P-labeled product 65% phosphatase-sensitive indicating two internucleotide phosphates and four external phosphatases (Fig. 2, Lane h). Since pCP is the 3'-phosphorylated terminus of Spot B and the only internal nucleotides are pAm and pAm (Table II), the only structure consistent with the yields of the nucleotides is m'G5'ppp5'(m)Am(p)mAm(p)mAm(p)Cp with the location of the adenosine base methylations uncertain.

To determine the complete structure of Spot B, I removed m'G by periodate oxidation and elimination with alkaline and digested the 3P-labeled product with penicillinase, P1 digested RNA to 5'-mononucleotides regardless of base or ribose modifications and also has a 3'-phosphatase activity (18). The products of P1 digestion and relative molar yields were P (1), pC (1), pmAm (1), pppAm (0.6), and pppAm (0.4). The structures of the two triphosphates, which just separated on pH 3.5 paper ionophoresis were determined by digestion with venom phosphodiesterase which gave the products PP, pAm or pAp, and pmAm and pAp. PP was identified by its rapid mobility on pH 3.5 paper ionophoresis as described below for the PP, derived from Spots C and D. The fraction of radioactivity migrating with markers of pAp and PP, and digested D to pGp and PP (Fig. 2, Lanes i and j) indicating structures of pppAp and pppGp for C and D, respectively. I eluted the radioactivity which migrated with the pAp and pGp markers on pH 3.5 paper electrophoresis and ran it in the two-dimensional thin layer chromatography system described earlier. The radioactivity was again exactly coincident with pAp and pGp markers indicating that neither nucleotide was modified.

The potential 5' end spot derived from VSV virion RNA (Fig. 1, Lane g) was totally phosphatase-sensitive and gave venom phosphodiesterase digestion products of pAp and PP, exactly as shown for Spot C derived from mRNA. Thus its structure must be pppAp. In these analyses of Spots C, D, and the virion RNA 5' end, venom phosphodiesterase digestion always produced a variable amount of P, presumably due to phosphatase activity in the enzyme preparation. The method used here to determine the structures of the tetraphosphates was first described by Roblin (19). VSV virion RNA is about 10,000 nucleotides long (20), and the pppAp recovered from the RNA was about 0.035% of the total radioactivity indicating that at least 85% of the RNA molecules have the 5' end pppAp.

**Origin of Spots A to D**—RNases T1, T2, and A leave 3'-phosphates when they cleave RNA. Therefore the free 2',3'-hydroxyl ends on A and B indicate a terminal origin for these oligonucleotides. Since the m'G is linked 5'-5' to the other nucleotides within A and B, these oligonucleotides must originate from the 5' end of the RNA. The other spots, C and D, both contain 5'-triphosphates and, therefore, must also be 5'-terminal.

The fraction of radioactivity in the sum of Spots A to D is consistent with A to D being alternative 5' ends on each mRNA species. For example, the fraction of 3P radioactivity in Spots A to D derived from a single VSV mRNA species (Band 3) was 0.0031. Since Band 3 RNA is a single mRNA species 1550 nucleotides long (11), this fraction of the radioactivity is equivalent to that expected from a single oligonucleotide containing five phosphates derived from Band 3 mRNA (0.0031 x 1550 nucleotides). Thus, the sum of the yields of 5' ends A to D (each of which contains four to six phosphates) is approximately equivalent to 1 mol/mol of RNA.

The possibility that Spots A to D had an origin other than from VSV mRNA was ruled out by the following experiments. First they were found only in mRNAs obtained from the regions of the formamide polyacrylamide gel containing the discrete VSV mRNA bands, and not in RNA eluted from the interband regions. Second, they cannot originate from host RNAs whose synthesis escaped inhibition by actinomycin D since the amount of 3P, incorporated by mock-infected, actinomycin D-treated cells into oligo(dt)-cellulose binding RNA was less than 1.0% that seen in infected cells. Furthermore, this RNA from uninfected cells did not contain detectable levels of these 5' ends.

To determine whether synthesis of these alternative 5' ends was a peculiarity of virus growth in a particular cell line, I isolated VSV mRNA from VSV-infected Chinese hamster ovary cells. Analysis of the 5' ends showed identical 5' ends A to D in the same ratio found in baby hamster kidney cells. In addition, the same extent of partial methylation was found in Spots A and B.

**Functional Significance of 5' Ends**—To examine the possible functional significance of the different 5' ends, I analyzed 5' ends from mRNAs obtained from the polyribosomal and
nonribosomal fractions of VSV-infected cells. Fig. 4 shows the polyribosome profiles from uninfected (A) and VSV-infected cells at 4 hours postinfection (B). VSV mRNA was purified from the indicated regions of the gradient by phenol extraction, ethanol precipitation, and oligo(dT)-cellulose binding. The RNA was then digested with RNases T1, T2, and A, and the products separated by pH 3.5 DEAE-paper ionophoresis. Fig. 4, Lane a, shows the 5' ends derived from total mRNA, while Lanes b and c show the 5' ends derived from nonribosomal and polyribosomal regions, respectively. The striking result was that those mRNAs on ribosomes contained only the 5' ends A and B while those mRNAs containing 5' ends C and D were exclusively in the non-ribosomal fraction. If 5% of those mRNAs with 5'-triphosphate ends C and D had been on polyribosomes, I would have detected them.

Total VSV mRNA was approximately equally distributed between the ribosomal and non-ribosomal regions of the gradient and the ratio of 5' sequence A to B was the same in both fractions indicating no ribosomal preference for mRNAs having either of these ends. The ribosomal distinction between mRNAs with and without the 5'-5' linkage appeared to be absolute in that the triphosphate ends constituted 13% of the 5' ends in total mRNA, less than 1% of the 5' ends of ribosome-associated mRNAs, and 32% of the 5' ends of non-ribosome-associated mRNAs.

Kinetics of 5' End Synthesis—To determine whether the 5'-triphosphate ends were only on newly synthesized mRNA molecules, VSV-infected cells were pulse-labeled with ³²P for 10 min to 1 hour at the indicated times (Table IV) between 2 to 4 hours postinfection. These experiments showed that 10 to 15% of the newly synthesized mRNAs had 5' ends pppAp and pppGp just as in mRNA labeled throughout infection. Also, the 5' sequence B was not seen in pulses shorter than 1 hour at several times from 1 to 4 hours postinfection indicating that the additional modifications in B occur slowly. Since in short and long labeling periods the ratio of mRNAs having 5'-triphosphate ends to those having the 5'-5' linkage is approximately constant, mRNAs with either type of 5' end are probably equally stable. Thus, the mRNAs having the 5'-triphosphate ends apparently accumulate throughout the infection and do not attach to ribosomes.

DISCUSSION

I have shown that individual species of VSV mRNA contain four alternative 5' end structures. These four structures are probably common to each VSV mRNA species since they are derived from each of the VSV mRNA bands purified by formamide-polyacrylamide gel electrophoresis, and two of these bands contain pure mRNA species (11, 12).

The major 5' end sequences, m⁷G₅'ppp₅'(m)AmpAp and m⁷G₅'ppp₅'(m)AmpmAmpCp are probably related in that the latter is derived from the former by additional methylation of the second adenosine, indicating a basic sequence ApApCp common to each VSV mRNA 5' end. The relationship of those 5' ends containing 5'-5' linkages to those with 5'-triphosphates is uncertain. A possible explanation for the multiple 5' ends is that the specificity for the initiating nucleotide is not absolute and only partial modification occurs after initiation. For example, each mRNA species may be initiated 95% of the time with pppAp and 5% of the time with pppGp. The pppAp ends may then be further modified to form m⁷G₅'ppp₅'(m)AmpAp and m⁷G₅'ppp₅'(m)AmpmAmpCp, while the pppGp ends escape this modification.

A recent report of a methylated 5' end structure resistant to alkaline phosphatase on VSV mRNA synthesized in vitro by the virion polymerase (9) suggests that at least some of the modifications seen on VSV mRNAs synthesized in vivo may be made by an enzyme (or enzymes) within the virion. In contrast to this recent report, Roy and Bishop have determined sequences of pppApCpGp and pppGpCpGp at the 5' ends of VSV mRNAs synthesized by the virion polymerase in vitro (21).

Table IV

| Labeling period | Counts per min in 5' ends |
|-----------------|--------------------------|
| A   | B   | C   | D   |
| 10 min | 1084 | 11  | 91  | 79  |
| (2 hrs 50 min to 3 hrs PI) | (86) | (1) | (7) | (6) |
| 30 min | 1500 | 29  | 111 | 125 |
| (2.5-3 hrs PI) | (85) | (2) | (6) | (7) |
| 1 hr | 1397 | 88  | 126 | 78  |
| (2-3 hrs PI) | (82) | (5) | (7) | (5) |
| 3 hrs | 2754 | 592 | 204 | 211 |
| (1-4 hrs PI) | (73) | (16) | (5) | (6) |
These sequences appear unrelated to the major 5' end sequences on VSV mRNA in vivo. However, since the in vitro products described by Roy and Bishop did not have the discrete sizes of the VSV mRNAs, it is possible that artificial initiation was occurring. Alternatively, the discrepancy may indicate mRNA processing in vivo. Clearly, further analysis of the in vitro 5' end sequences is required to determine the relationship of these sequences and modifications to those produced in vivo. In contrast to the multiple 5' ends on VSV mRNA, I find only pppAp at the 5' end of the virion RNA, confirming the results of Hefti and Bishop (22).

Since the mRNAs which have 5'-triphasphate ends are not ribosome-bound in vivo, the 5'-5' linkage, or methylation, or both, may play a role in ribosome recognition of mRNA. This interpretation of the results requires the assumption that the nucleotide sequences adjacent to the 5'-triphasphate ends are identical with those adjacent to the 5' ends containing m'G. It is possible, however, that mRNAs having the triphosphate ends were initiated incorrectly and lack other sequences required for ribosome recognition. Thus, further work on determination of the nucleotide sequences adjacent to the various 5' ends is required before a role in ribosome recognition can be ascribed directly to the 5'-5' linkage, or methylation, or both. However, it is interesting to note that recent work has shown that methylation is required for efficient translation of reovirus and VSV mRNAs in a cell-free protein-synthesizing system derived from wheat embryo (23). However, it was also concluded (23) from drug inhibition experiments that mRNA methylation occurs on ribosomes suggesting that unmethylated RNA will bind to ribosomes. This result appears inconsistent with my finding that mRNAs with 5'-triphasphate ends are not attached to ribosomes.

Since many mRNAs in eukaryotic cells have the 5'-terminal structures m'GpppNmpNp or m'GpppNmpNmpNp (5), it is interesting that a fraction of each VSV mRNA species is also 2'-O-methylated at the second as well as the first adenosine. Thus, similar heterogeneous modification may occur on individual cellular mRNAs.

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Note Added in Proof—Moyer et al. (24) recently reported the structure of a single methylated 5' terminus derived from total VSV mRNA synthesized in vivo. The structure reported is similar to the major 5'-terminal structure reported here but lacks partial methylation. Insensitivity of the techniques these authors used to purify and analyze the structure might explain the discrepancies between their results and mine.

REFERENCES

1. Ro-Choi, T. S., Reddy, R., Choi, Y. C., Rai, N. B., and Hennings, D. (1974) Fed. Proc. 33, 1548
2. Wei, C. M., and Moss, B. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 318-322
3. Furuichi, Y., Morgan, M., Muthukrishnan, S., and Shatkin, A. J. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 362-366
4. Furuichi, Y., Muthukrishnan, S., and Shatkin, A. J. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 742-745
5. Adams, J. M., and Cory, S. (1975) Nature 255, 28-33
6. Huang, A. S., and Wagner, K. H. (1966) J. Mol. Biol. 22, 381-384
7. Konrad, M., Toivonen, J., and Nierlich, D. P. (1972) Nature New Biol. 238, 231-233
8. Tener, G. M. (1967) Methods Enzymol. 12, 398-404
9. Rhodes, D. P., Moyer, S. A., and Banerjee, A. K. (1974) Cell 3, 327-333
10. Huang, A., Baltimore, D., and Stamper, M. (1970) Virology 42, 946-957
11. Rose, J. K., and Knipe, D. (1976) J. Virol. 15, 1004-1011
12. Moyer, S. A., Abraham, G., Adler, R., and Banerjee, A. K. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 362-366
13. Fujimoto, M., Kuninaka, A., and Yoshino, H. (1974) Agric. Biol. Chem. 38, 1555-1561
14. Barrell, B. (1971) in Procedures in Nucleic Acid Research (Cantor, G. C., and Davies, D. R., eds) Vol II, pp. 775-779, Harper and Row, N. Y.
15. Furuichi, Y., Muthukrishnan, S., and Shatkin, A. J. (1975) Methods Enzymol. 12, Sect. B, 243-246
16. Walker, R. T., and Raj Bhandary, U. L. (1975) Nucl. Acid Res., 61-69
17. Fujimoto, M., Kuninaka, A., and Yoshino, H. (1974) Agric. Biol. Chem. 38, 1555-1561
18. Knipe, D., Rose, J. K., and Lodish, H. F. (1975) J. Virol. 13, 62-72
19. Barrell, B. (1971) in Procedures in Nucleic Acid Research (Cantor, G. C., and Davies, D. R., eds) Vol II, pp. 775-779, Harper and Row, N. Y.
20. Furuichi, Y., Muthukrishnan, S., and Shatkin, A. J. (1975) Methods Enzymol. 12, Sect. B, 243-246
21. Menninger, G., and Steinschneider, A. (1967) Methods Enzymol. 12, Sect. B, 243-246
22. Adams, J. M., and Cory, S. (1975) Nature 255, 28-33
23. Both, G. W., Banerjee, A. K., and Shatkin, A. J. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 1189-1193
24. Moyer, S. A., Abraham, G., Adler, R., and Banerjee, A. K. (1975) Cell 5, 59-67
Heterogeneous 5'-terminal structures occur on vesicular stomatitis virus mRNAs.
J K Rose

J. Biol. Chem. 1975, 250:8098-8104.

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