# THE 5' SEQUENCES OF VSV IN VITRO TRANSCRIPTION PRODUCT RNA (+ SAM).

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SUMMARY: A variety of different 5' initiation sequences have been characterized for the in vitro transcription product RNA synthesized by vesicular stomatitis viral transcriptase. In the absence of S-adenosyl-L-methionine (SAM), the predominant 5' sequences are pppApCp.. and $G^{5'}$pppApAp... In the presence of SAM, the predominant 5' sequences are $m^7$GpppAmpApCp... and pppApCp..

## INTRODUCTION

Vesicular stomatitis virus, Indiana serotype, possesses a virion RNA dependent RNA polymerase capable of transcribing in vitro and in vivo the entire genome (1-3). Certain viral complementary RNA species function as mRNA in in vitro translation system (4-6). In previous communications we reported that at least four distinct 5' initiation sequences (pppApCpGp..., pppApApPyp-XpGp..., pppGpCp... and pppGpGpPyp, where Py=pyrimidine and X is A,C, or U) can be detected among the in vitro transcription product RNA (7,8). In addition, some product species have capped 5' ends which quantitatively become methylated when S-adenosyl-L-methionine (SAM) is included in the reaction mixture (9). Studies by Banerjee and associates have recently characterized the 5' sequences of certain VSV mRNA species formed in vivo or in vitro and shown that the 5' sequence $m^7$GpppAmpAp... is common to the smaller (13-18S) mRNA species (10,11). Rose has found that the 5' sequences $m^7$Gppp(m)AmpAp..., $m^7$Gppp(m)Amp(m)Amp.. as well as pppGp.. and pppAp.. occur on in vivo 13-18S mRNA size molecules (12). We present here the analyses of the 5' sequences of the product RNA species synthesized in vitro by our strain of VSV Indiana, in the presence or absence of SAM.

## MATERIALS AND METHODS

[$\alpha$-$^{32}$P]Ribonucleoside triphosphates were obtained from N.E.N., Boston,

Mass. S-adenosyl-L-methionine, venom phosphodiesterase, and alkaline phosphatase were obtained from Boehringer Mannheim, New York. Ribonuclease $T_2$ and various nucleotides were obtained from Sigma, St. Louis, MO., $m^7G$ 5' phosphate was obtained from Terra-Marine Bioreasearch, La Jolla, Calif. VSV transcription assays were incubated, and their reaction products purified and analysed as reported previously (3,9). Venom phosphodiesterase digestion involved incubating a 100 µl sample in 0.02 M tris-HCl buffer, 5mM sodium phosphate buffer, pH 7.4, with 1 unit of enzyme for 4 hr at 37° C. Ribonuclease $T_2$ treatment in 1 ml of 0.05 M sodium acetate buffer, pH 4.5, containing 3 mM EDTA and 10 units of enzyme, involved incubating samples at 37° C for 5 hours.

## RESULTS

Analyses of VSV transcription product 5' sequences, synthesized in the absence of SAM. VSV transcription product RNA, synthesized in the absence of SAM, but presence of either [$\alpha$-$^{32}$P]CTP, [$\alpha$-$^{32}$P]ATP or [$\alpha$-$^{32}$P]GTP, was purified, digested with ribonuclease $T_2$ and the proceeds resolved by DEAE cellulose column chromatography at pH 8.0, together with optical quantities of a pancreatic ribonuclease digest of chick embryo RNA (7). Labeled nucleotides were obtained with the mononucleotide and tetranucleotide isopliths (9). The latter were recovered and resolved by DEAE cellulose column chromatography using a 0.05 M to 0.30 M LiCl gradient in 0.05 M sodium citrate buffer, pH 3.5 (Fig. 1-3, left side). In each case two major peaks of labeled nucleotides were recovered and subjected to alkaline phosphatase treatment to remove exposed 3' or 5' phosphates (Fig. 1-3, left side inserts). The second major peak of the pH 3.5 chromatograms (fractions 65-80) was shown by thin layer chromatography on PEl cellulose to be pppGp (7). The first peak (fractions 40-55) was determined to be a mixture of pppAp (labeled by CTP and ATP but not GTP) and GpppAp (labeled by ATP and GTP but not CTP). Despite various attempts, it was not possible to separate these two nucleotides by DEAE column chromatography. However it was possible to separate pppAp from GpppAp by paper electrophoresis at pH 3.5 (12) and thereby demonstrate that ATP and CTP (but not GTP) labeled the pppAp nucleotide, while half the ATP labeled phosphates of the GpppAp sequence were phosphatase sensitive. Venom phosphodiesterase treatment of the GTP labeled structure yielded only pG so that it could be deduced that the sequence was $G^{5'}$pppAp(A).

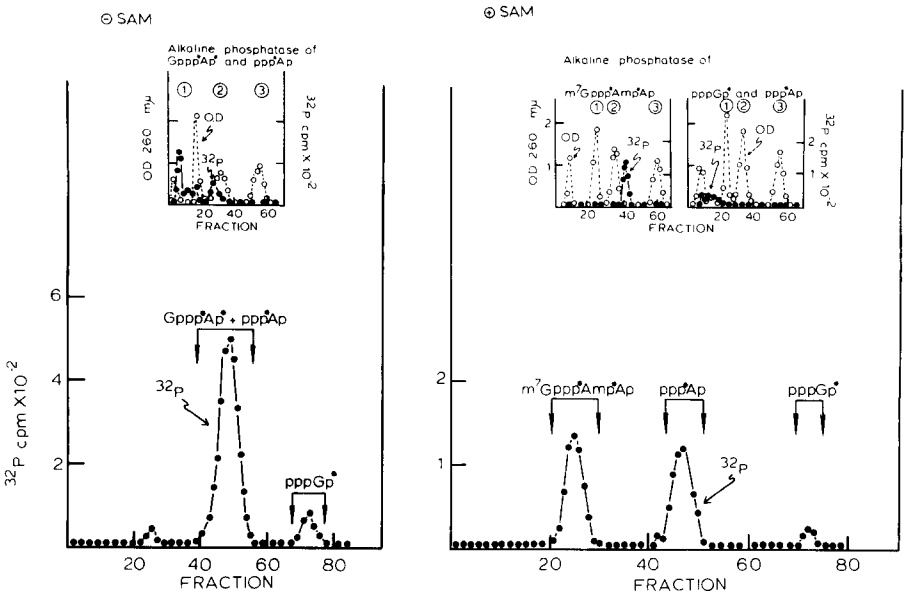


Fig. 1. Analyses of [α-$^{32}$P]ATP labeled VSV transcription product 5' termini. The 5' termini obtained by ribonuclease $T_2$ digestion of VSV transcription product RNA labeled by [α-$^{32}$P]ATP in the absence (left side) or presence (right side) of SAM, were purified as described in Text and resolved by DEAE column chromatography at pH 3.5. The indicated fractions were recovered, treated with alkaline phosphatase and the proceeds resolved by DEAE column chromatography at pH 8.0 with marker nucleotides derived from pancreatic ribonuclease digest of chick embryo RNA (respective inserts).

These results are compatible with our previous observations using [γ-$^{32}$P] and [α-$^{32}$P]ribonucleoside triphosphates that 5' termini possessing the sequences pppApCpG.., pppApApPypXpGp..., pppGpCp... and pppGpGpPyp.. were present among VSV product species. In these present analyses, the additional sequences $G^{5'}$pppAp(A) and trace amounts of pppGp(A) have been demonstrated.

Analyses of VSV transcription product synthesized in the presence of SAM. Similar analyses to those described above were undertaken on VSV transcription product synthesized in the presence of SAM. As before, the nucleotides recovered in the tetranucleotide isoplith of the pH 8.0 chromatograms were subsequently resolved by DEAE cellulose chromatography at pH 3.5 (Fig. 1-3, right side). The three peaks of labeled material recovered from each column were treated with alkaline phosphatase (Fig. 1-3, right side, inserts). It

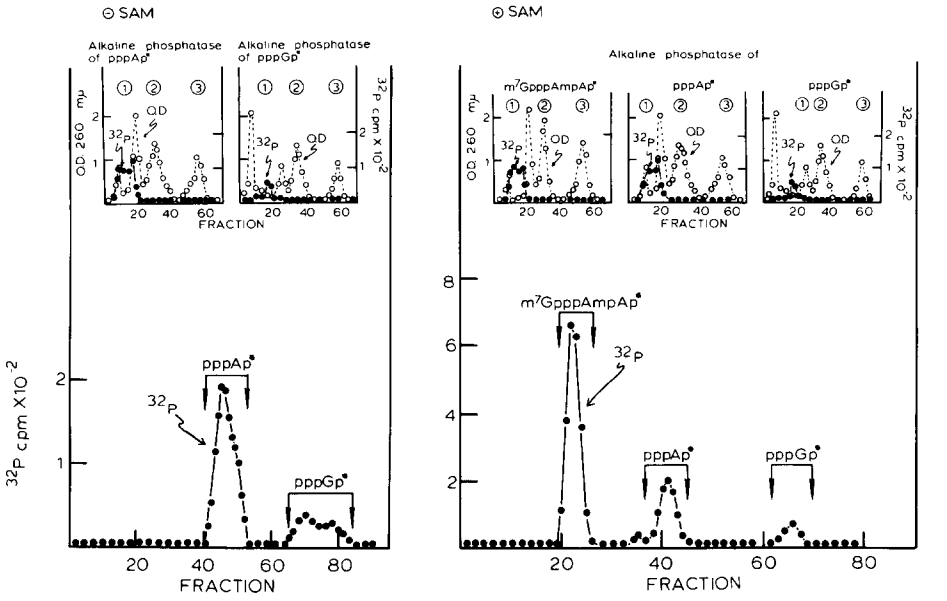


Fig. 2. Analyses of [α-$^{32}$P]CTP labeled VSV transcription product 5' termini. The preparation, purification, and analyses of the 5' termini obtained from [α-$^{32}$P]CTP labeled RNA synthesized in the absence (left side) or presence (right side) of SAM were similar to those described in Fig. 1.

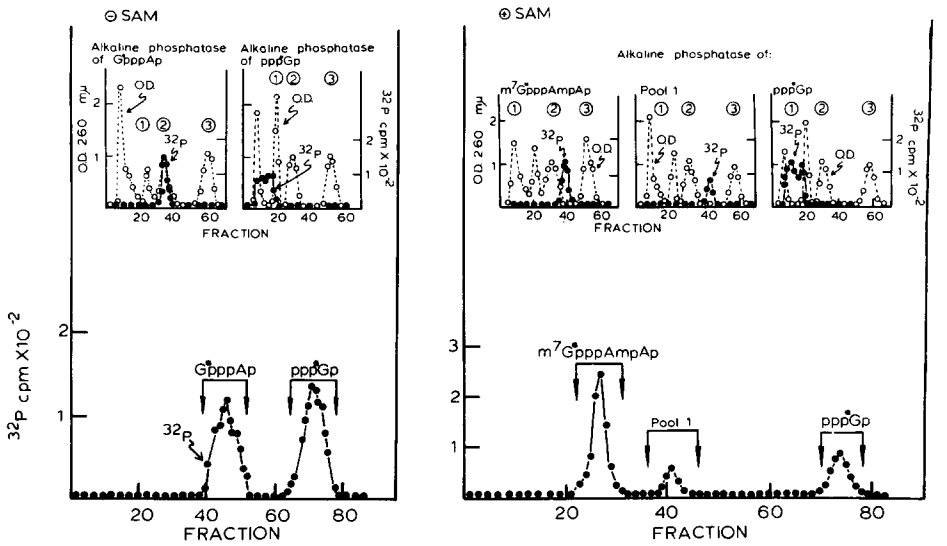


Fig. 3. Analyses of [α-$^{32}$P]GTP labeled VSV transcription product 5' The preparation, purification and analyses of the 5' termini obtained from [α-$^{32}$P]GTP labeled RNA synthesized in the absence (left side) or presence (right side) of SAM were similar to those described in Fig. 1.

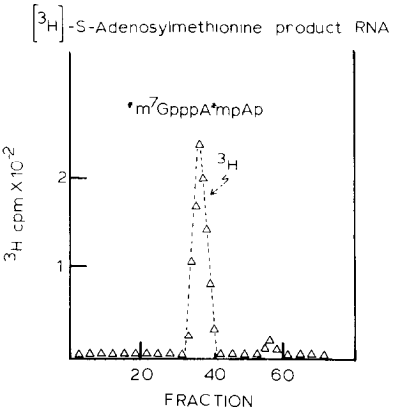


Fig. 4. Analyses of [$^3$H]S-Adenosyl-L-methionine labeled VSV transcription product 5' termini. Product RNA synthesized in the presence of $^3$H-SAM was digested with ribonuclease $T_2$ and resolved on DEAE cellulose at pH 8.0. The 5' termini were recovered and then resolved by chromatography on DEAE cellulose at pH 3.5.

was again found that the material in fractions 60 and 80 of the pH 3.5 column was pppGp (labeled by GTP, ATP and CTP).

The first peak of material obtained from each column (fractions 20 to 30) was determined to be $m^7G^{5'}$pppAmpAp(C) on the basis of the following observations. (a) For only the CTP labeled material was the label phosphatase sensitive. (b) Venom phosphodiesterase treatment of the GTP labeled material only yielded $m^7G$ 5' monophosphate as determined by pH 3.5 paper electrophoresis with optical quantities of $m^7G$ 5' phosphate (10,12). (c) Venom phosphodiesterase followed by alkaline phosphatase digestion of the ATP labeled material yielded equal amounts of free phosphate and material which coelectrophoresed at pH 3.5 with optical marker nucleotides of ApA (AmpA) but not ApG, ApC or ApU.

The second peak of material from the pH 3.5 chromatograms of the ATP or CTP labeled product was deduced from the phosphatase results to be pppAp, this was confirmed by PEl thin layer chromatography. However for the GTP labeled product, a small peak of material was obtained which upon phosphatase treatment yielded a structure which was recovered between the di- and

Table 1. The percent occurrence of various 5' terminal nucleotides among VSV *in vitro* transcription product RNA.

| Sequence | No SAM | With SAM |
|---|---|---|
| pppApCpGp... | 25-35% | 20-30% |
| pppApApPyp... | 5-10% | Not known |
| GpppApAp.... | 30-40% | Not detected |
| $m^7$GpppAmpApCp.. | Not detected | 40-50% |
| pppGpCp... | 5-15% | 5-10% |
| pppGpAp.. | 5-15% | 5-10% |
| pppGpGp | 10-15% | 5-10% |
| Unidentified* | Not detected | 5-15% |

The range of percent occurrence of various 5' terminal nucleotides found among VSV *in vitro* transcription product RNA was calulated from Fig. 1-3 and previous observations (7,8). The structure of the capped and methylated nucleotide found in Fig. 3, 4 is not known (*).

trinucleotides. The small amounts of material available precluded further analyses.

Product termini were also isolated from VSV *in vitro* transcription analyses incubated in the presence of [$^3$H]S-adenosyl-L-methionine. One major peak (in the tetranucleotides) and a second minor peak between the tetra- and pentanucleotides (8%) was observed following chromatography at pH 8.0. Two peaks were obtained upon chromatography at pH 3.5 (Fig. 4). Phosphatase treatment of the major peak from the pH 3.5 chromatogram yielded a nucleotide which chromatographed just after the dinucleotides. Venom phosphodiesterase followed by alkaline phosphatase treatment yielded material which upon electrophoresis was recovered with the ApA nucleotide (presumably AmpA) and an equal number of counts were obtained with $m^7$G (10,12). These results were compatible with the structure $m^7G^{5'}$pppAmpAp(C). The minor peak was not analyzed further due to insufficient amounts of radioactivity.

## DISCUSSION

The results obtained indicate that there are several sites on the genome of VSV RNA where *in vitro* transcription can initiate. The proportions of the various 5' terminal sequences have been computed from these and previous analyses and are given in Table 1. Without SAM, the major sequences are GpppAp(A).. and pppApCpGp... With SAM the major sequences are $m^7$GpppAmpAp(C) and pppApCpGp.. These and our previous observations could be interpreted to indicate that the capping and methylation process is selective and involves the conversion of pppApApCp.. sequences to GpppApApCp by capping and $m^7$Gppp-AmpApCp by the subsequent process of methylation (assuming Py=C). Whether capping can occur after transcription has proceeded beyond several nucleotides is under investigation. The fact that a second major *in vitro* 5' terminus (pppApCpGp..) is neither capped nor methylated may well relate to *in vitro* replication attempts by the virion enzyme templated by the 3' terminus of the viral RNA. If so, then this would predict that the 5' of the 42S viral complementary plus strand RNA is pppApCpGp.. (like that of the 42S viral strand), and that the viral 3' sequence is ...CpGpU$_{OH}$ (9). These predictions are under investigation. The significance of the minor amounts of other *in vitro* product 5' termini is not known and although the presence of another capped and methylated component may reflect the $m^7$GpppAmpAmpPyp.. sequence identified by Rose (12), it may represent a different capped and methylated structure relevent to the 28S VSV mRNA species.

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