JOURNAL OF VIROLOGY, Jan. 1980, p. 292-303
0022-538X/80/01-0292/12$02.00/0

Vol. 33, No. 1

# Alternate Capping Mechanisms for Transcription of Spring Viremia of Carp Virus: Evidence for Independent mRNA Initiation

KAILASH C. GUPTA AND POLLY ROY*

*Departments of Microbiology and Public Health, University of Alabama in Birmingham, Birmingham, Alabama 35294*

Two alternate mechanisms of mRNA capping for spring viremia of carp virus have been observed. Under normal reaction conditions, a ppG residue of the capping GTP is transferred to a pA moiety of the 5′ termini of mRNA transcripts. However, in reaction conditions where GppNHp is used instead of GTP, an alternate capping mechanism occurs whereby a pG residue of the capping GTP is transferred to a ppA moiety of the transcripts. The first mechanism is identical to that described previously for vesicular stomatitis virus (G. Abraham, D. P. Rhodes, and A. K. Banerjee, Nature [London] **255**:37-40, 1975; A. K. Banerjee, S. A. Moyer, and D. P. Rhodes, Virology **61**:547-558, 1974), and thus appears to be a conserved function during the evolution of rhabdoviruses. The alternate mechanism of capping indicates not only that capping can take place by two procedures, but also that the substrate termini have di- or triphosphate 5′ ends, indicating that they are probably independently initiated. An analog of ATP, AppNHp, has been found to completely inhibit the initiation of transcription by spring viremia of carp virus, suggesting that a cleavage between the $\beta$ and $\gamma$ phosphates of ATP is essential for the initiation of transcription. However, in the presence of GppNHp, uncapped (ppAp and pppAp), capped (GpppAp), and capped methylated (m7GpppAmpAp and GpppAmpAp) transcripts are detected. Size analyses of oligodeoxythymidylic acid-cellulose-bound transcripts resolved by formamide gel electrophoresis demonstrated that full-size mRNA transcripts are synthesized as well as larger RNA species. The presence of GppNHp and *S*-adenosylhomocysteine in reaction mixtures did not have any effect on the type of unmethylated transcription products. Our results favor a transcription model postulated previously (D. H. L. Bishop, *in* H. Fraenkel-Conrat and R. R. Wagner, ed., *Comprehensive Virology*, vol. 10, Plenum Press, New York, 1977; D. H. L. Bishop and A. Flamand, *in* D. C. Burke and W. C. Russell, ed., *Control Processes in Virus Multiplication*, Cambridge University Press, Cambridge, 1975; D. H. L. Bishop and M. S. Smith, *in* D. Nayak, ed., *The Molecular Biology of Animal Viruses*, Marcel Dekker, New York, 1977; P. Roy and D. H. L. Bishop, J. Virol. **11**:487-501, 1973) in which mRNA synthesis is initiated independently; they do not support a model for transcripts being synthesized by plus-strand cleavage (A. K. Banerjee, G. Abraham, and R. J. Colonno, J. Gen. Virol. **34**:1-8, 1977; A. K. Banerjee, R. J. Colonno, D. Testa, and M. T. Franze-Fernandez, *in* B. M. J. Mahy and R. D. Barry, ed., *Negative Strand Viruses and the Host Cells*, Academic Press, London, 1978).

Elucidation of the mechanism of capping is important to understanding the steps involved in the synthesis and processing of viral and cellular mRNA's. Two mechanisms of capping have been recognized (45) (see below). In one mechanism (A), the capping occurs at the onset of transcription (presumably at the level of a dinucleotide) and involves the transfer of a GMP residue from GTP to block the 5′ terminus of an elongating RNA chain. In this case an initial triphosphate terminus of the growing RNA chain is probably first hydrolyzed to a 5′ diphosphate and then capped. This mechanism is known to occur in vaccinia virus (25, 30, 31), reovirus (22, 23), cytoplasmic polyhedrosis virus (21), and HeLa cell nuclei (49).

$$\text{Gppp} + \dot{\text{p}}\dot{\text{p}}\text{PupN}\ \text{Gp-}\dot{\text{p}}\dot{\text{p}}\text{PupN} + \text{PP}_i \quad \text{(A)}$$

$$\text{G}\dot{\text{p}}\dot{\text{p}}\text{p} + \text{pNpNp} \ldots \text{G}\dot{\text{p}}\dot{\text{p}}\text{-pNpNp} \ldots + \text{P}_i \quad \text{(B)}$$

In the second mechanism, depicted in (B), a GDP residue from the capping GTP is transferred to a pN residue on the RNA chain to produce the blocked structure. This capping

process has been postulated to operate in vesicular stomatitis virus (VSV) mRNA synthesis (3) and in cellular mRNA's that contain pyrimidine caps (44). The second capping mechanism is envisaged to occur for mRNA chains generated by cleavage of a longer precursor RNA molecule. Schibler and Perry (44) have postulated that both (A) and (B) capping mechanisms may operate in mouse L cells.

Two mechanisms of VSV mRNA synthesis have been proposed based on in vitro studies. One mechanism postulates independent mRNA chain initiation (10, 13, 43) and is based on the fact that pppApApCp ... sequences (i.e., putative VSV mRNA precursors) are detected among the in vitro reaction products. The other mechanism, based on the observation that the transcription process of rhabdoviruses is sequential (2, 5, 16) and utilizes capping mechanism (B), postulates that mRNA species are generated by cleavage of precursor plus-strand RNA species (6, 7). This second mechanism is the so-called "leader" model.

Since the 3′ sequence of VSV Indiana genome is ...pCpGpU (15) and its 5′ sequence is pppApCpGp... (27), it has been suggested for the multiple mRNA initiation model of VSV transcription (see Fig. 8B) that pppApCpGp... sequences are abortive plus-strand replication attempts (10, 13). In the leader model (6) it has been suggested that pppApCpGp... sequences are the only plus-strand initiation sites, and that plus-strand RNA is subsequently cleaved into the individual mRNA species (see Fig. 8B). This latter model predicts that each mRNA initially has a 5′ sequence of pApApCpApGp..., which is then capped to give Gpp-pApApCpApGp...

In vitro studies of the SVCV transcription process have established that, as in the vesiculoviruses VSV (Indiana serotype), Chandipura, VSV (New Jersey serotype), Cocal, and Piry viruses, a variety of transcription termini are synthesized. In spring viremia of carp virus (SVCV), as in VSV, capped sequences (GpppApA) and, in the presence of *S*-adenosyl-L-methionine (SAM), capped methylated termini (GpppAmpApC and m7GpppAmpApC) have been detected (26). For the vesiculoviruses, open termini (e.g., pppApCpGp and pppApApCp and smaller quantities of pppGp termini) have been observed among in vitro reaction products.

The majority of the in vivo mRNA species of VSV (33, 39) and SVCV (P. Roy and K. C. Gupta, manuscript in preparation) have a unique nucleotide sequence [m7GpppAmpA(m)pCp(m)], where the (m) moieties are variable. In the studies of Rose (39), some VSV in vivo mRNA-size molecules were shown to have open termini (pppAp and pppGp), a result that would support the multiple initiation model of VSV mRNA initiation.

In the present communication we have addressed the question of which process of mRNA transcription is used by the rhabdovirus SVCV. We present evidence for two alternate capping mechanisms, equivalent to (A) and (B) shown above, with mechanism (B), as in VSV, being the preferred mechanism. The observation of alternate capping mechanisms and open termini which are subsequently capped and methylated supports the model of independent mRNA initiation.

## MATERIALS AND METHODS

**Chemicals.** [$\alpha$-$^{32}$P]ATP (23 Ci/mmol), [$\alpha$-$^{32}$P]CTP (23 Ci/mmol), and [$^{3}$H]UTP (30 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. [$\beta,\gamma$-$^{32}$P]ATP (120 Ci/mmol) and [$\beta,\gamma$-$^{32}$P]GTP (25 Ci/mmol) were obtained from ICN, Irvine, Calif. SAM, *S*-adenosylhomocysteine (SAH), bacterial alkaline phosphatase, RNase $T_2$, nucleotide pyrophosphatase, nucleoside triphosphates, and oligodeoxythymidylic acid-cellulose were from Sigma Chemical Co., St Louis, Mo. Nuclease P1 was from P-L Biochemicals, Milwaukee, Wis. $\beta,\gamma$-Imidoribonucleoside triphosphates (AppNHp, GppNHp, and UppNHp) were obtained from ICN, Cleveland, Ohio.

**Virus purification.** SVCV was grown on BHK-21 cell monolayers and purified as described previously (26). The final virus preparation was suspended in 10 mM Tris-hydrochloride (pH 8.0) and 30% glycerol. Nucleocapsids were prepared as described previously (11) and used directly after preparation.

**In vitro RNA transcription reactions.** Purified virus preparations, or nucleocapsids, were used to synthesize RNA in vitro using a standard reaction mixture (125 $\mu$l), containing 0.8 mM each of ATP, CTP, GTP, and UTP, as well as 70 mM of NaCl, 70 mM Tris-hydrochloride (pH 8.0), 8 mM $MgCl_2$, 0.2% (vol/vol) Triton X-100, 2 mM dithiothreitol, 0.8 mM SAM (or 1.2 mM SAH), and 20 $\mu$g of protein. Whenever labeled nucleoside triphosphates (20 to 40 $\mu$Ci of $^{32}$P or 5 $\mu$Ci of $^{3}$H) were used in the reaction, the concentration of the corresponding unlabeled compound was reduced to 0.2 mM. [$^{3}$H]SAM (5 $\mu$Ci) was used in some reaction mixtures in lieu of unlabeled SAM (26). On occasion, the imidoribonucleoside triphosphates were used instead of the corresponding normal nucleoside triphosphates. When [$\beta,\gamma$-$^{32}$P]GTP or [$\beta,\gamma$-$^{32}$P]ATP was used in reaction mixtures, [$^{3}$H]UTP was also employed to monitor the RNA synthesis. Normally reaction mixtures were incubated at 22°C for 3 h. Under these conditions the rate of RNA transcription was linear for at least 6 h (26). Reactions were terminated by adding sodium dodecyl sulfate (final concentration 1%), extracted, and purified by gel filtration and ethanol precipitation as described previously (26).

**Nuclease digestions and analyses of transcript termini.** The digestion conditions for transcripts have been described previously (26). Briefly, RNase $T_2$ digestion was carried out for 6 to 12 h at 37°C in 5

mM sodium acetate buffer (pH 4.5) with 5 to 10 U of $T_2$. Bacterial alkaline phosphatase digestion was performed at 37°C for 2 to 4 h in 10 mM Tris-hydrochloride (pH 8.0) with 0.5 U of enzyme. Nuclease P1 digestion involved incubating RNA samples for 2 h at 37°C with 20 U of enzyme in 5 mM ammonium acetate (pH 6.0). Digestion with nucleotide pyrophosphatase employed 0.08 U of enzyme in 10 mM Tris-hydrochloride (pH 7.6) and 10 mM $MgCl_2$ at 37°C for 30 min. Digests were analyzed either be DEAE-cellulose column chromatography or by paper electrophoresis at pH 3.5 (26–28, 43).

**Oligodeoxythymidylic acid-cellulose chromatography and formamide gel electrophoresis of RNA.** Oligodeoxythymidylic acid-cellulose (0.5 ml) in a 3-ml plastic syringe was used to purify SVCV polyadenylated in vitro transcripts. RNA samples were bound to the adsorbent in a buffer containing 10 mM Tris-hydrochloride (pH 7.5), 0.5 M NaCl, 1 mM EDTA, and 0.5% sodium dodecyl sulfate, washed with binding buffer and eluted with a buffer containing 10 mM Tris-hydrochloride (pH 7.5), 1 mM EDTA, and 0.05% sodium dodecyl sulfate (35), and recovered by precipitation with ethanol. The size distribution of SVCV in vitro-synthesized transcription products was determined by 3.5% polyacrylamide gel electrophoresis containing 95% formamamide, as described elsewhere (17).

**Thin-layer chromatography to resolve ribonucleoside triphosphates.** Ribonucleoside triphosphates were resolved by thin-layer chromatography by spotting 1 to 2 μl of a reaction mixture on a polyethyleneimine-cellulose thin-layer plate (10 by 20 cm; Brinkmann Instruments Inc., Westbury, N.Y.) and resolving the component ribonucleoside triphosphates with 2 M ammonium formate–2 N HCl (62:38) mixture (pH 3.5). The samples were corun with 10 to 15 nmol each of ATP, CTP, UTP, and GTP. At the end of the chromatography the positions of the unlabeled triphosphates were obtained by their absorption of UV light (280 nm), and the distribution of radioactivity was determined by autoradiography and excision and counting of the labeled triphosphates. In the solvent system used the $R_f$ values of ATP, CTP, GTP, and UTP were 0.51, 0.62, 0.25, and 0.47, respectively.

## RESULTS

**Capping in the presence of [β,γ-$^{32}$P]GTP.** It has been established that a GDP moiety from GTP is transferred during the capping process of VSV transcripts (3). To determine whether this process has been conserved during the evolution of different rhabdoviruses, the capping process of SVCV was analyzed. When [β,γ-$^{32}$P]GTP- and [$^3$H]UTP-labeled transcripts of SVCV were prepared and digested by nuclease P1 and the products were analyzed by paper electrophoresis (Fig. 1), three peaks of radioactivity were detected. The position of the pool A material corresponds to m7GpppAm, and pool B corresponds to GpppAm, as deduced in previous studies (26). The materials in pools A, B, and C were separately eluted in deionized water,

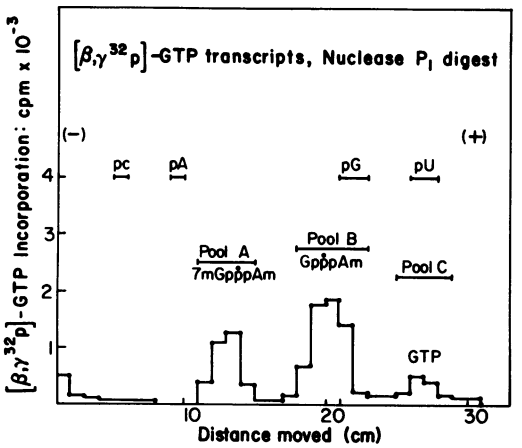


FIG. 1. *Paper electrophoresis at pH 3.5 of 5′ termini labeled with [β,γ-$^{32}$P]GTP. SVCV transcripts synthesized in the presence of [β,γ-$^{32}$P]GTP were digested with nuclease P1, and the products were electrophoresed on Whatman 3MM paper at 50 V/cm for 1 h (26). Strips (1 cm) were excised and counted in toluene-Omnifluor scintillation fluids (New England Nuclear Corp). The $^3$H counts per minute (cpm) are not presented; however, all of the $^3$H radioactivity was recovered in the position of UMP. The positions of optical quantities of marker nucleoside monophosphates are indicated by horizontal bars.*

digested with bacterial alkaline phosphatase, and again subjected to paper electrophoresis. The results obtained (data not shown) indicated that the label in either the pool A or B materials was not sensitive to alkaline phosphatase removal as expected for capped termini. By contrast, the pool C material was found to be sensitive to alkaline phosphatase, with all of its radioactivity being recovered in the position of inorganic phosphate. Since the position of the pool C material was identical to the expected position of GTP, and in view of its sensitivity to alkaline phosphatase, it was concluded that pool C was probably GTP, although whether it represented contaminant GTP, or pppG.. termini from reaction products, was not resolved.

These studies indicated therefore that the bulk of the SVCV transcripts labeled by [β,γ-$^{32}$P]GTP have sequences in which the label is protected from alkaline phosphatase removal. Since it has been shown that m7GpppAm and GpppAm sequences are labeled by [α-$^{32}$P]ATP and [α-$^{32}$P]GTP, the results with [β,γ-$^{32}$P]GTP indicate that in all probability the β-phosphate of the capping GTP is conserved in the capped structures.

**The effect of β,γ-imidoribonucleoside triphosphates on SVCV transcription reaction rates.** Due to the presence of a β,γ-imido or methylene group (which cannot be cleaved enzymatically between the β and γ phosphates),

these nucleoside triphosphate analogs have been used to study the mechanism of capping. From such studies, Furuichi (21) concluded that to obtain transcription for cytoplasmic polyhedrosis virus, the $\beta$ and $\gamma$ phosphates of ATP must be cleaved, supporting the previous findings that ppAp... is needed to form the Gp-ppAp cytoplasmic polyhedrosis virus caps. Moss and collaborators (25) in studies on vaccinia virus (where both GpppGp and GpppAp caps are formed) also showed that AppNHp completely inhibited transcription, although transcription was obtained in the presence of GppNHp. Similar results have been reported for VSV (47).

When analyses of the effects of imidoribonucleoside triphosphates were conducted with preparations of SVCV virions (Fig. 2A), it was found that AppNHp completely inhibited SVCV transcription. In the presence of GppNHp (or UppNHp) the transcription rate was 35% (or 25%, respectively) of that of a control reaction.

To test the possibility that endogenous viral GTP might be available to mitigate the effect of GppNHp, we performed similar transcription assays using purified SVCV nucleocapsids (Fig. 2B). The results obtained were comparable to those obtained with unfractionated virions, indicating that SVCV transcription in the presence of GppNHp is not due to endogenous GTP or other soluble components in purified virus preparations.

**Analyses of the reaction products made in the presence of GppNHp: studies with [$\alpha$-$^{32}$P]ATP.** To determine whether capped structures were synthesized in the presence of GppNHp, SVCV transcripts were prepared from reaction mixtures containing GppNHp and [$\alpha$-$^{32}$P]ATP as well as 0.8 mM SAM or 1.2 mM SAH. Product RNA was digested with RNase $T_2$ and analyzed by DEAE-cellulose column chromatography as described previously (26). Figure 3A (top panel) presents the elution profile of a RNase $T_2$ digest of SVCV transcripts synthesized in the presence of unlabeled SAM. In addition to the major peak of radioactivity recovered at a net charge of −2 (i.e., mononucleotides) and minor peaks of material (which were not analyzed further) eluting with or just after the −4 position, a peak of radioactivity (pool I) was observed eluting slightly ahead of the −5 charge nucleotides. Another peak of radioactivity was identified between the −5 and −6 nucleotides (pool II). The pool I and II materials were recovered (26, 27, 43) and digested with bacterial alkaline phosphatase as described previously (26–28), and the products were again resolved by DEAE-cellulose column chromatography (Fig. 3A, bottom panels). For pool I, other than some inorganic phosphate recovered at the −2 charge position and probably derived from open pppAp termini (see below), a single peak of radioactivity was observed in the position expected for m7GpppAmpA (26). For the alkaline phosphatase digest of pool II material, a single peak of radioactivity was recovered at the expected position of GpppAmpA (i.e., just before the −4 position). Based on the alkaline phosphatase sensitivity results, it was determined that approximately 20% of the ATP-labeled transcripts synthesized in the presence of SAM had open termini (i.e., with di- or triphosphates),

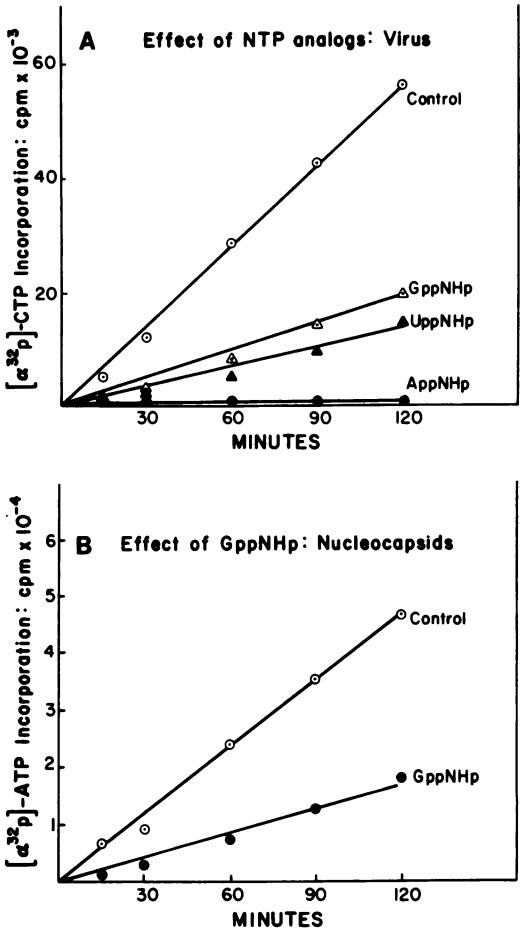


FIG. 2. *Effect of $\beta,\gamma$-imidoribonucleoside triphosphates on SVCV transcription. (A) Effect of inclusion of GppNHp, AppNHp, or UppNHp (in lieu of the corresponding nucleoside triphosphates) on the SVCV transcription process using [$\alpha$-$^{32}$P]CTP to monitor the rate of RNA synthesis. (B) Effect of GppNHp on the transcription process templated by purified SVCV nucleocapsids; the conditions were the same as those used in (A). Transcription in each case was measured by assaying the trichloroacetic acid-insoluble radioactivity.*

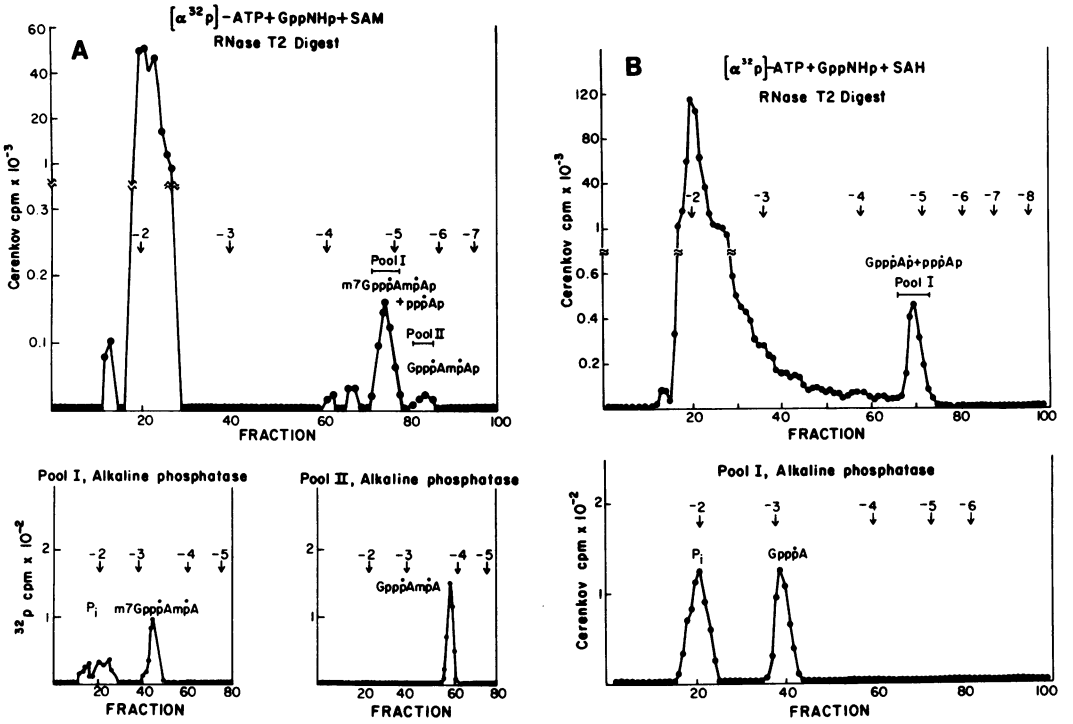


FIG. 3. *DEAE-cellulose column chromatography of SVCV reaction products labeled by [α-$^{32}$P]ATP in the presence of GppNHp and (A) SAM or (B) SAH. SVCV transcription reaction products were prepared as described previously (26), using reaction mixtures containing 0.8 mM GppNHp in lieu of GTP, [α-$^{32}$P]ATP, and unlabeled 0.8 mM SAM or 1.2 mM SAH. The reaction products were purified, digested with RNase $T_2$, and (top panels) analyzed by DEAE-cellulose column chromatography (26). For both columns the indicated fractions were pooled, recovered, and digested with alkaline phosphatase, and the products were reanalyzed by DEAE-cellulose column chromatography (bottom panels). The positions and origin of label in the designated nucleotides are based on our previous analyses of SVCV transcript termini and the precursor nucleoside triphosphate used in these experiments.*

whereas 80% of the termini were methylated, capped, or both.

When SVCV transcripts were synthesized in the presence of [α-$^{32}$P]ATP and SAH and the products were digested with RNase $T_2$ and analyzed by DEAE-cellulose chromatography, the elution profile shown in Fig. 3B (top panel) was obtained. Apart from a peak of labeled mononucleotides, a single major peak of termini was obtained eluting at the −5 position (pool I). Alkaline phosphatase digestion of the recovered pool I materials (Fig. 3B, bottom panel) gave both inorganic phosphate and a peak of material eluting in the position of GpppA (25). In part the label recovered as inorganic phosphate reflects the fact that the nearest neighbor of the GpppA residue is pA [i.e., the pool I material contained GpppAp(A) sequences]. However, as will be shown below, some open pppAp termini were probably also present in these pool I termini.

The results presented above for SVCV transcripts synthesized in the presence of GppNHp and SAM (or SAH) and labeled with [α-$^{32}$P]-ATP indicate that capped termini are synthesized which behave like those identified previously for SVCV (i.e., m7GpppAmpAp, GpppAmpAp, and pppAp [SAM reactions], or [SAH reactions] GpppAp and pppAp).

**Analyses of the reaction products made in the presence of GTP or GppNHp: studies with [β,γ-$^{32}$P]ATP.** Since the β,γ-imidoribonucleoside triphosphates cannot be enzymatically cleaved between the β and γ phosphates, the possibility that the β phosphate from ATP-labeled termini was included in the capped structure was examined for reactions containing GTP or GppNHp.

SVCV transcription products were first prepared from reaction mixtures containing unlabeled GTP, [β,γ-$^{32}$P]ATP, and SAM or SAH. The reaction products were purified, digested with RNase $T_2$, and resolved by DEAE-cellulose column chromatography (Fig. 4). Apart from a

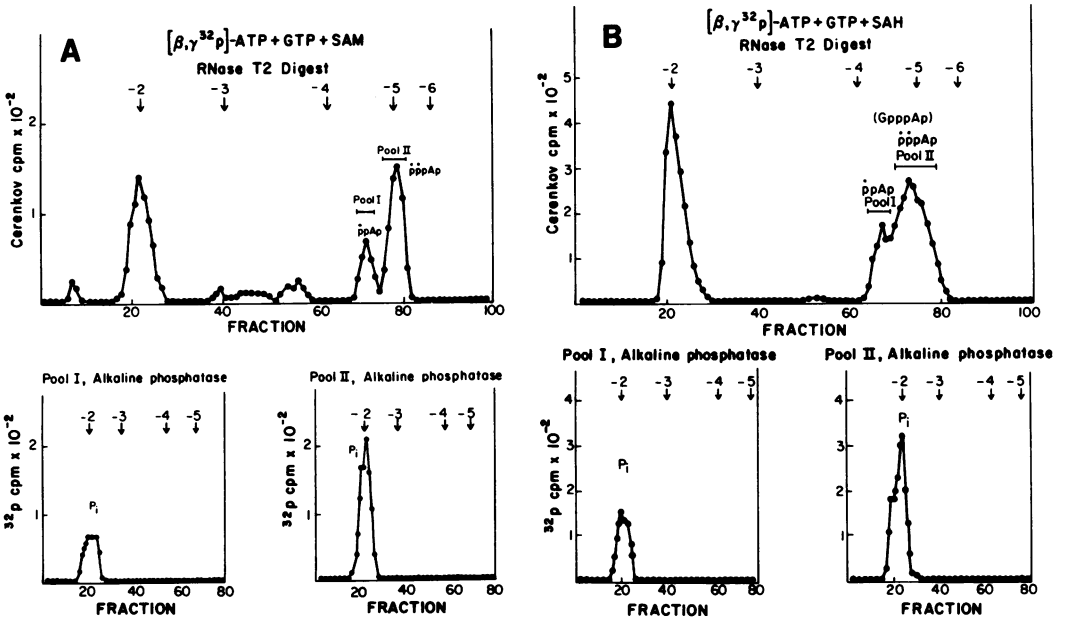


FIG. 4. *DEAE-cellulose column chromatography of SVCV transcripts synthesized in the presence of [β,γ-$^{32}$P]ATP, unlabeled GTP, and (A) SAM or (B) SAH. SVCV transcription reaction products were prepared as described previously (26) using reaction mixtures containing GTP, [β,γ-$^{32}$P]ATP, and either 0.8 mM SAM or 1.2 mM SAH. The reaction products were purified, digested with RNase $T_2$, and (top panels) analyzed by DEAE-cellulose column chromatography. For both columns the indicated fractions were pooled, recovered, and digested with alkaline phosphatase, and the products were reanalyzed by DEAE-cellulose column chromatography (bottom panels). The positions and origin of label in the designated nucleotides are based on our previous analyses of SVCV transcript termini and the precursor nucleoside triphosphate used in these experiments (26).*

peak of radioactivity eluting at the −2 position, two additional significant peaks of radioactivity were obtained from the SAM reaction, pool I eluting between the −4- and −5-charged nucleotides, and pool II eluting with the −5 nucleotides (Fig. 4A, top panel). Both pools I and II were separately recovered and digested with alkaline phosphatase, and the products were again analyzed by DEAE-cellulose column chromatography (Fig. 4A, bottom panels). Both materials were found to be totally sensitive to alkaline phosphatase, indicating that they contained open termini which, from their elution characteristics, are probably equivalent to ppAp (pool I) and pppAp (pool II; 27, 44).

Similar analyses of the transcripts synthesized in the presence of GTP, [β,γ-$^{32}$P]ATP, and SAH were undertaken (Fig. 4B). As in the analyses of the reaction products synthesized in the presence of the methyl donor, the analyses of the SAH reaction products indicated that only open labeled termini were present (ppAp and pppAp).

SVCV reaction products were also prepared in the presence of GppNHp, [β,γ-$^{32}$P]ATP, and SAM or SAH and similarly analyzed (Fig. 5). For SVCV products synthesized in the presence of SAM, a peak of material was recovered in the RNase $T_2$ digest eluting at a −2 position, while three peaks of radioactivity were found after the −4-charged nucleotides (Fig. 5A, top panel). Two of these peaks (pools I and II) were recovered; the third contained too low an amount of radioactivity for analysis. Both pools of nucleotides were analyzed by alkaline phosphatase digestion. DEAE-cellulose column chromatography of the products revealed that, in addition to inorganic phosphate, both peaks yielded a resistant species which eluted from the column in the position of m7GpppAmpA (Fig. 5A, bottom panels). The fact that both pools I and II yielded the same terminus probably reflects the fact that the pools of nucleotides were made with respect to the major labeled peaks and that the labeled m7GpppAmpAp residue overlapped both of them. From the results obtained it was deduced that the percentage of open termini labeled with [β,γ-$^{32}$P]ATP was approximately 70% (taking into account the point that the capped termini should only be labeled by one of the labeled phosphates from the precursor triphosphate, whereas the open termini would be labeled by one or two phosphates).

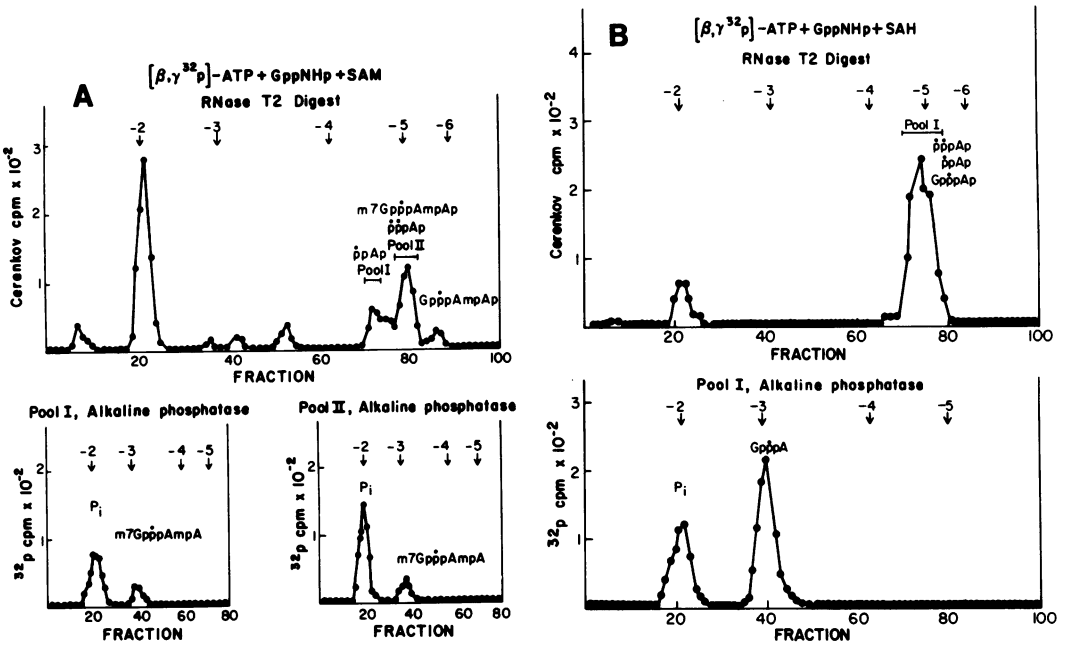


FIG. 5. *DEAE-cellulose column chromatography of SVCV transcripts synthesized in the presence of [β,γ-$^{32}$P]ATP, GppNHp, and (A) SAM or (B) SAH. SVCV transcription reaction products were prepared as described previously (26) using reaction mixtures containing [β,γ-$^{32}$P]ATP, 0.8 mM GppNHp in lieu of GTP, and either 0.8 mM SAM or 1.2 mM SAH. The reaction products were purified, digested with RNase $T_2$, and (top panels) analyzed by DEAE-cellulose column chromatography. For both columns the indicated fractions were pooled, recovered, and digested with alkaline phosphatase, and the products were reanalyzed by DEAE-cellulose column chromatography (bottom panels). The positions and origin of label in the designated nucleotides are based on our previous analyses of SVCV transcript termini and the precursor nucleoside triphosphate used in these experiments (26).*

The results obtained with GppNHp, in contrast to those obtained with GTP, suggested that [β,γ-$^{32}$P]ATP labeled the capped termini in the GppNHp (SAM) reaction products. Note that the third peak of termini recovered just before the −6-charged nucleotides (Fig. 5A, top panel) corresponds in elution properties to GpppAmpAp (26), although whether in fact this material contained that sequence was not determined.

When SVCV reaction products were synthesized in the presence of GppNHp, [β,γ-$^{32}$P]ATP, and SAH, recovered, and digested with RNase $T_2$, the profile shown in Fig. 5B (top panel) was obtained. Other than material recovered at a −2 charge, a broad peak of radioactivity was recovered in the vicinity of the −5-charged nucleotides (pool I). The pool I material was recovered and digested with alkaline phosphatase, and the products were again resolved by DEAE-cellulose column chromatography (Fig. 5B, bottom panel). Although part of the label was recovered as inorganic phosphate (−2 charge), the majority was recovered as an alkaline phosphatase-resistant terminus eluting with the −3-charged nucleotides, i.e., in the position of GpppA. Again these results indicate that in the presence of GppNHp and SAH radioactivity was incorporated into capped structures from the [β,γ-$^{32}$P]ATP precursors. From the results obtained it was determined that there were approximately 30% open termini labeled by [β,γ-$^{32}$P]ATP in the presence of SAH (assuming that only one labeled phosphate was incorporated into the capped termini and one or two into the open [di- or triphosphate] termini). The significance of the increased capping efficiency in the presence of SAH, in comparison to SAM, is not known.

**Identification of the methylated capped structures synthesized in the presence of GppNHp.** SVCV transcripts were also prepared from reaction mixtures containing GppNHp, unlabeled ATP, and [$^3$H]SAM. The transcripts were digested with RNase $T_2$, and the digests were resolved by DEAE-cellulose column chromatography (Fig. 6). Two major peaks of radioactivity were recovered (Fig. 6A) at the expected positions of m7GpppAmpAp (pool I) and GpppAmpAp (pool II). The pool I material was

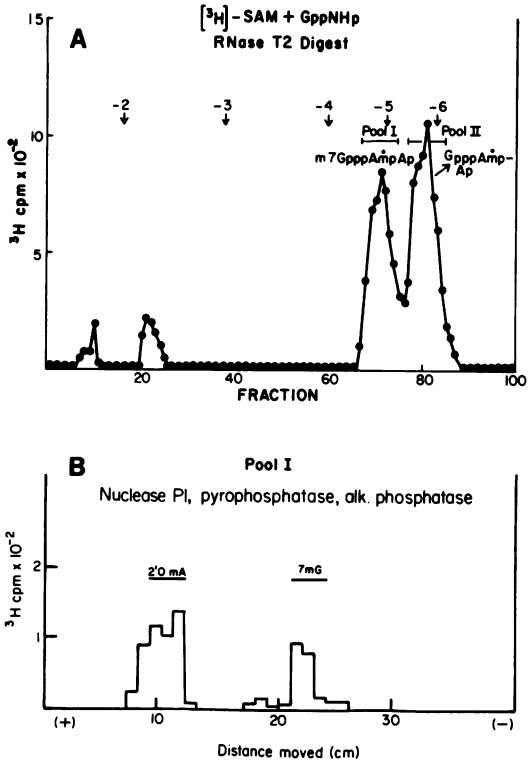


FIG. 6. *DEAE-cellulose column chromatography of SVCV transcripts synthesized in presence of [$^3$H]SAM and 0.8 mM GppNHp. SVCV transcription reaction products were prepared as described previously (26) using reaction mixtures containing [$^3$H]-SAM and 0.8 mM GppNHp in lieu of GTP. The reaction products were purified, digested with RNase $T_2$, and (A) chromatographed on DEAE-cellulose as described previously (26). The indicated fractions were pooled, and the pool I materials were recovered, digested sequentially with nuclease P1, pyrophosphatase, and alkaline phosphatase; the products were resolved by paper electrophoresis at pH 3.5 (B; 26). The positions and origin of label in the designated nucleotides are based on our previous analyses of SVCV transcript termini and the radioactive precursor used in these experiments. The positions of optical marker nucleotides in the electropherogram are indicated by horizontal bars.*

recovered and digested with nuclease P1, nucleotide pyrophosphatase, and then alkaline phosphatase, and the methylated nucleosides were resolved by paper electrophoresis at pH 3.5 (26). The radioactivity was almost equally distributed between m7G and 2'-*O*-methylribose adenosine (Fig. 6B).

These results indicate that, in the presence of GppNHp and SAM, the capped methylated structures normally found in SVCV reaction products were also present when the GTP analog was employed in lieu of GTP. Note that the pool II nucleotides from the [$^3$H]SAM products were not analyzed, since previous studies have indicated that capped structures found in SVCV reaction products eluting in that position lack a methylated G residue (i.e., they are GpppAmpAp sequences).

**Size of SVCV RNA transcripts synthesized in vitro.** To ensure that SVCV transcription in the presence of GppNHp and SAH gave full-size transcripts, we have analyzed the reaction product RNA by polyacrylamide gel electrophoresis using denaturing conditions. An autoradiogram is shown in Fig. 7 of SVCV in vitro mRNA transcripts synthesized under normal reaction conditions (Fig. 7, left lane), in vivo mRNA prepared as described elsewhere (Fig. 7, center lane; Roy and Gupta, manuscript in preparation), and in vitro transcripts synthesized in the presence of ($\alpha$-$^{32}$P)CTP, GppNHp, and SAH

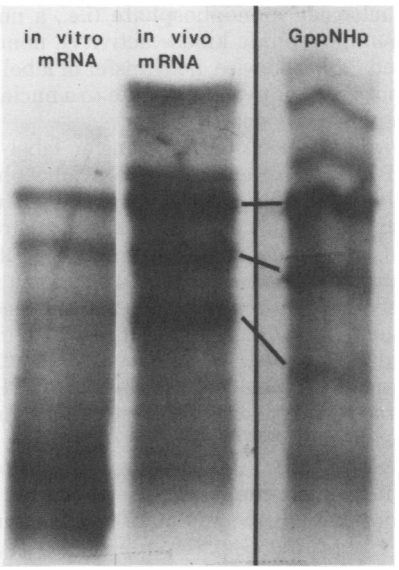


FIG. 7. *Formamide–3.5% polyacrylamide gel electrophoresis of SVCV in vitro transcripts and in vivo mRNA. SVCV mRNA (center lane) was prepared from virus-infected cells (40; Roy and Gupta, manuscript in preparation); the polyadenylic acid-containing species were selected by oligodeoxythymidylic acid-cellulose column chromatography and resolved by formamide–3.5% polyacrylamide gel electrophoresis together with the polyadenylic acid-containing in vitro transcripts of SVCV reaction products from control [$\alpha$-$^{32}$P]CTP-labeled reactions (left lane). Total [$\alpha$-$^{32}$P]CTP-labeled SVCV transcripts from GppNHp reaction products (right lane) were likewise resolved by electrophoresis in a formamide–3.5% polyacrylamide gel. The latter gel was electrophoresed at 20 mA for 22 h; the former gel was electrophoresed at 20 mA for 16 h. The corresponding positions of the SVCV mRNA species are indicated.*

(Fig. 7, right lane). Although the GppNHp gel was run longer than the other gel, it is clear that not only are SVCV transcripts made in the presence of GppNHp (comparable in length to those found in control reactions as well as those obtained from infected cells), but also that some larger-size product RNA species were present.

**The lack of transfer of label from [$\beta,\gamma$-$^{32}$P]ATP to GppNHp.** It has been shown previously that virions of many enveloped viruses, including the rhabdovirus VSV, have phosphotransferase activities capable of exchanging the $\gamma$-phosphates (but not the $\beta$- or $\alpha$-phosphates) of nucleoside triphosphates (29, 42). With a $\gamma$-phosphate-labeled nucleoside triphosphate and unlabeled nucleoside diphosphate (29), the $\gamma$-phosphate can be exchanged between the nucleotides (so that the enzyme functions as a nucleoside diphosphate kinase). In experiments designed to determine whether label was transferred from a $\gamma$-phosphate-labeled triphosphate to a nucleoside monophosphate (i.e., a nucleoside monophosphate kinase activity), none was detected (29). Likewise, no transfer of label from free phosphate or pyrophosphate to a nucleoside triphosphate was detected (42).

The ability of SVCV to transfer label from [$\beta,\gamma$-$^{32}$P]ATP to other nucleoside triphosphates, including GppNHp, has been examined using polyethyleneimine-cellulose thin-layer chromatography to resolve and quantitate the labeled triphosphates. After 4 h of incubation in a reaction mixture containing all the normal reaction ingredients including SAM and the four unsubstituted nucleoside triphosphates, 4% and 22% of the initial ATP label was recovered in GTP and CTP, respectively, with the rest being found in the positions of ATP and UTP (UTP, however, was too close to ATP to resolve in the chromatographic system employed). In a reaction mixture containing SAM and all of the usual ingredients except that GppNHp replaced GTP, 13% of the initial label was recovered as CTP; the rest was recovered in the position of ATP and UTP. No radioactive spot was detected in the position where GTP chromatographed. These results indicate therefore that there was no detectable transfer of label from ATP to form GTP in reaction mixtures containing GppNHp, and that it is unlikely that the transfer of label from ATP to capped structures in the presence of GppNHp is through the intermediary of GTP formed by a nucleoside mono- or diphosphate kinase reaction.

## DISCUSSION

The results presented above indicate that capping of SVCV transcripts involving either the addition of GDP (preferred capping mechanism, Fig. 8A), or, in the presence of GppNHp, the addition of GMP (alternate capping mechanism, Fig. 8A). Based on the fact that there can be incorporation of label from [$\beta,\gamma$-$^{32}$P]ATP into protected capped and capped methylated structures, as well as the demonstration that open ATP-labeled termini [pppAp(A)] are present among the reaction products of SVCV (26) as well as VSV (14, 43), it is most probable that the substrates for capping are initially triphosphate termini on growing RNA chains. It is possible that a subsequently derived diphosphate (or even monophosphate) terminus is an intermediate, although until such a terminus is shown to be a precursor to an eventual capped RNA chain, that question will remain open.

In studies examining the fate of [$\beta,\gamma$-$^{32}$P]ATP in reaction mixtures containing GppNHp, we have not obtained evidence for the transfer of label to GTP even though label was transferred to CTP (29, 43; and probably UTP). These results indicate that the label obtained in capped structures when GppNHp and [$\beta,\gamma$-$^{32}$P]ATP is used is not derived from an initial pyrophosphate exchange from [$\beta,\gamma$-$^{32}$P]ATP to form GTP and subsequent inclusion by the preferred capping scheme shown in Fig. 8A. Note also that in the GTP and [$\beta,\gamma$-$^{32}$P]ATP reactions (Fig. 4) no label was recovered in the termini. Although we cannot totally exclude the possibility that the transcription enzyme might exchange a pyrophosphate from ATP to GppNHp uniquely at the capping site, we have no evidence for transfer of label from ATP to GppNHp in the total reaction mixture, and we believe that the results obtained are compatible with the interpretation given above.

Our results do not support the leader model of mRNA synthesis for rhabdoviruses (depicted in Fig. 8B), since in that model the putative mRNA substrate only has a monophosphate terminus. Although our results do not exclude the possibility of RNA cleavage and subsequent capping of cleaved RNA chains, we know of no direct proof that rhabdovirus-cleaved plus-sense RNA is subsequently capped. In fact, from studies involving SVCV reaction mixtures lacking UTP we have obtained direct evidence for the synthesis of capped and capped methylated oligonucleotides, and, secondly, for reaction mixtures lacking UTP and GTP, we have obtained methylated oligonucleotides (Gupta and Roy, manuscript in preparation). Since the cleavage model requires leader RNA to be entirely synthesized before mRNA sequences are produced (Fig. 8B), these additional results also do not support the leader RNA model for rhabdovirus mRNA transcription. As discussed elsewhere (10, 13), we believe that the in vitro synthesis of

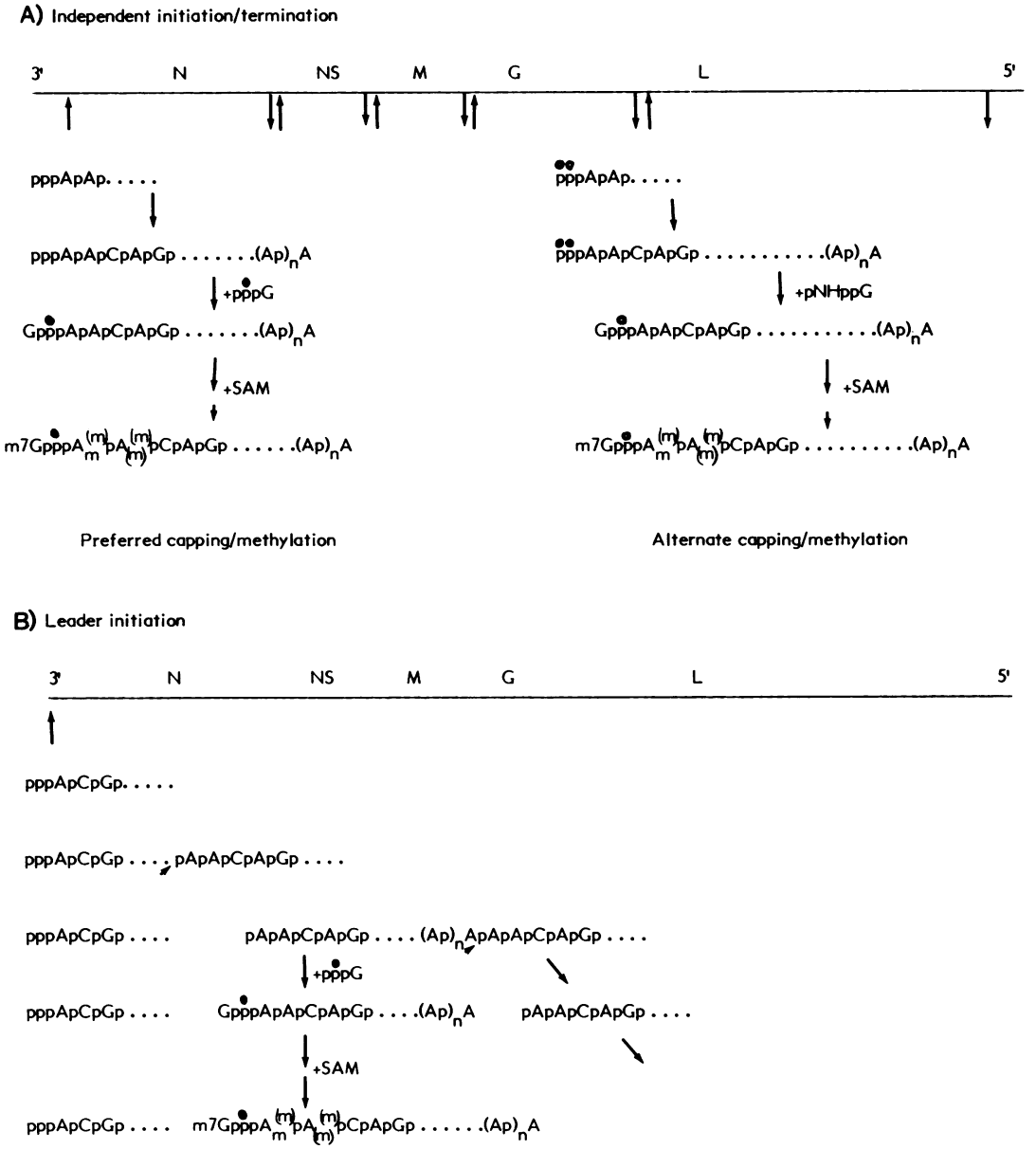


FIG. 8. *Mechanisms of rhabdovirus mRNA transcription. (A) Model of mRNA transcription involving independent initiation and termination (10, 13) by either the preferred or alternate capping procedures. (B) The "leader model" of rhabdovirus mRNA transcription with a unique site of mRNA initiation (6). The 5' and 3' sequences shown, the origins of the phosphates in the cap structures, and the gene sequence reflect the data obtained from in vitro or in vivo studies for VSV, SVCV, or both (see text; 1-10, 12-16, 18-20, 24, 26-28, 32-34, 36-43, 47, 48; Roy and Gupta, manuscript in preparation).*

VSV product RNA species having pppApCpGp termini or, for SVCV, pppAp(C) termini, is best explained by abortive in vitro replication attempts. In this regard it is of particular interest that SVCV product synthesized in the presence of GppNHp includes some RNA species which are apparently larger (in formamide denaturing gels) than the SVCV mRNA species. Work is in progress to characterize these larger species and to determine their complexities and 5'- and 3'-terminal sequences.

ACKNOWLEDGMENTS

We thank D. H. L. Bishop for his counsel and constructive

criticism during the course of this work.

This work was supported by Public Health Service grant AI-13686 from the National Institute of Allergy and Infectious Diseases.

## LITERATURE CITED

1. **Abraham, G., and A. K. Banerjee.** 1976. The nature of the RNA products synthesized *in vitro* by subviral components of vesicular stomatitis virus. Virology **71:** 230-241.
2. **Abraham, G., and A. K. Banerjee.** 1976. Sequential transcription of the genes of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. **73:**1504-1508.
3. **Abraham, G., D. P. Rhodes, and A. K. Banerjee.** 1975. Novel initiation of RNA synthesis *in vitro* by vesicular stomatitis virus. Nature (London) **255:**37-40.
4. **Abraham, G., D. P. Rhodes, and A. K. Banerjee.** 1975. The 5'-terminal structure of the methylated mRNA synthesized *in vitro* by vesicular stomatitis viruses. Cell **5:**51-58.
5. **Ball, L. A., and C. N. White.** 1976. Order of transcription of genes of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. **73:**442-446.
6. **Banerjee, A. K., G. Abraham, and R. J. Colonno.** 1977. Vesicular stomatitis virus: mode of transcription. J. Gen. Virol. **34:**1-8.
7. **Banerjee, A. K., R. J. Colonno, D. Testa, and M. T. Franze-Fernandez.** 1978. Mechanism of RNA synthesis *in vitro* by vesicular stomatitis virus, p. 249-259. *In* B. M. J. Mahy and R. D. Barry (ed.), Negative strand viruses and the host cells. Academic Press, London.
8. **Banerjee, A. K., S. A. Moyer, and D. P. Rhodes.** 1974. Studies on the *in vitro* adenylation of RNA by vesicular stomatitis virus. Virology **61:**547-558.
9. **Banerjee, A. K., and D. P. Rhodes.** 1973. *In vitro* synthesis of RNA that contains polyadenylate by virion-associated RNA polymerase of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. **70:**3566-3570.
10. **Bishop, D. H. L.** 1977. Virion polymerases, p. 117-278. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 10. Plenum Press, New York.
11. **Bishop, D. H. L., S. U. Emerson, and A. Flamand.** 1974. Reconstitution of infectivity and transcriptase activity of homologous and heterologous viruses: vesicular stomatitis (Indiana serotype), Chandipura, vesicular stomatitis (New Jersey serotype), and Cocal viruses. J. Virol. **14:**139-144.
12. **Bishop, D. H. L., and A. Flamand.** 1975. The transcription processes of animal RNA viruses, p. 95-152. *In* D. C. Burke and W. C. Russell (ed.), Control processes in virus multiplication. Cambridge University Press, Cambridge.
13. **Bishop, D. H. L., and M. S. Smith.** 1977. Rhabdoviruses, p. 167-180. *In* D. Nayak (ed.), The molecular biology of animal viruses. Marcel Dekker, New York.
14. **Chang, S. H., E. Hefti, J. F. Obijeski, and D. H. L. Bishop.** 1974. RNA transcription by the virion polymerases of five rhabdoviruses. J. Virol. **13:**652-661.
15. **Colonno, R. J., and A. K. Banerjee.** 1976. A unique RNA species involved in initiation of vesicular stomatitis virus RNA transcription *in vitro*. Cell **8:**197-204.
16. **Colonno, R. J., and A. K. Banerjee.** 1977. Mapping and initiation studies on the leader RNA of vesicular stomatitis virus. Virology **77:**260-268.
17. **Duesberg, P. H., and P. K. Vogt.** 1973. Gel electrophoresis of avian leukosis and sarcoma viral RNA in formamide: comparison with other viral and cellular RNA species. J. Virol. **12:**594-599.
18. **Ehrenfeld, E.** 1974. Polyadenylation of vesicular stomatitis virus messenger RNA. J. Virol. **13:**1055-1060.
19. **Ehrenfeld, E., and D. F. Summers.** 1972. Adenylate-rich sequences in vesicular stomatitis virus messenger ribonucleic acid. J. Virol. **10:**683-688.
20. **Franze-Fernandez, M. T., and A. K. Banerjee.** 1978. In vitro RNA transcription by the New Jersey serotype of vesicular stomatitis virus. I. Characterization of the mRNA species. J. Virol. **26:**179-187.
21. **Furuichi, Y.** 1978. Pretranscriptional capping in the biosynthesis of cytoplasmic polyhedrosis virus. Proc. Natl. Acad. Sci. U.S.A. **75:**1086-1090.
22. **Furuichi, Y., M. Morgan, S. Muthukrishnan, and A. J. Shatkin.** 1975. Reovirus messenger RNA contains a methylated, blocked, 5'-terminal structure: m7G(5')ppp(5')GmpCp. Proc. Natl. Acad. Sci. U.S.A. **72:**362-366.
23. **Furuichi, Y., and A. J. Shatkin.** 1977. 5' termini of reovirus mRNA: ability of virus cores to form caps post-transcriptionally. Virology **77:**566-578.
24. **Galet, H., and L. Prevec.** 1973. Polyadenylate synthesis by extracts from L cells infected with vesicular stomatitis virus. Nature (London) New Biol. **243:**200-203.
25. **Gershowitz, A., R. F. Boone, and B. Moss.** 1978. Multiple roles for ATP in the synthesis and processing of mRNA by vaccinia virus: specific inhibitory effects of adenosine ($\beta,\gamma$-imido)triphosphate. J. Virol. **27:**399-408.
26. **Gupta, K. C., D. H. L. Bishop, and P. Roy.** 1979. 5'-Terminal sequences of spring viremia of carp virus RNA synthesized in vitro. J. Virol. **30:**735-745.
27. **Hefti, E., and D. H. L. Bishop.** 1975. The 5'-sequence of VSV viral RNA and its *in vitro* transcription product RNA. Biochem. Biophys. Res. Commun. **66:**785-792.
28. **Hefti, E., and D. H. L. Bishop.** 1975. The 5'-sequences of VSV *in vitro* transcription product RNA (± SAM). Biochem. Biophys. Res. Commun. **68:**393-399.
29. **Hefti, E., P. Roy, and D. H. L. Bishop.** 1975. The initiation of transcription by influenza virion transcriptase, p. 307-326. *In* B. W. J. Mahy and R. D. Barry (ed.), Negative strand viruses. Academic Press, London.
30. **Moss, B., A. Gershowitz, C. M. Wei, and R. Boone.** 1976. Formation of the guanylated and methylated 5' termini of vaccinia virus mRNA. Virology **72:**341-351.
31. **Moss, B., S. A. McAir, M. J. Ensinger, R. F. Boone, and C.-M. Wei.** 1976. Modification of the 5'-terminals of mRNAs by viral and cellular enzymes. Progr. Nucleic Res. Mol. Biol. **19:**63-81.
32. **Moyer, S. A., and A. K. Banerjee.** 1975. Messenger RNA species synthesized *in vitro* by the virion associated RNA polymerase of vesicular stomatitis virus. Cell **4:**37-43.
33. **Moyer, S. A., and A. K. Banerjee.** 1976. *In vivo* methylation of vesicular stomatitis virus and its host-cell messenger RNA species. Virology **70:**339-357.
34. **Moyer, S. A., M. J. Grubman, E. Ehrenfeld, and A. K. Banerjee.** 1975. Studies on the *in vivo* and *in vitro* messenger RNA species of vesicular stomatitis virus. Virology **67:**463-473.
35. **Poulson, R.** 1978. Isolation, purification and fractionation of RNA, p. 333-367. *In* P. R. Stewart and D. S. Letham (ed.), The ribonucleic acids. Springer-Verlag, New York.
36. **Rhodes, D. P., G. Abraham, R. J. Colonno, W. Jelinete, and A. K. Banerjee.** 1977. Characterization of vesicular stomatitis virus mRNA species synthesized in vitro. J. Virol. **21:**1105-1112.
37. **Rhodes, D. P., and A. K. Banerjee.** 1976. 5'-Terminal sequences of vesicular stomatitis virus mRNAs, synthesized in vitro. J. Virol. **17:**33-42.
38. **Rhodes, D. P., S. A. Moyer, and A. K. Banerjee.** 1974. *In vitro* synthesis of methylated messenger RNA by the virion-associated RNA polymerase of vesicular stomatitis virus. Cell **3:**327-333.
39. **Rose, J. K.** 1975. Heterogeneous 5'-terminal structures occur on vesicular stomatitis virus mRNAs. J. Biol. Chem. **250:**8098-8104.
40. **Rose, J. K., and D. Knipe.** 1975. Nucleotide sequence

complexities, molecular weights, and poly(A) content of the vesicular stomatitis virus mRNA species. J. Virol. **15**:994-1003.

41. **Rose, J. K., H. F. Lodish, and M. L. Brock.** 1977. Giant heterogeneous polyadenylic acid on vesicular stomatitis virus mRNA synthesized in vitro in the presence of *S*-adenosylhomocysteine. J. Virol. **21**:683-693.
42. **Roy, P., and D. H. L. Bishop.** 1971. Nucleoside triphosphate phosphotransferase. A new enzyme activity of oncogenic and non-oncogenic "budding" viruses. Biochim. Biophys. Acta **235**:191-206.
43. **Roy, P., and D. H. L. Bishop.** 1973. Initiation and direction of RNA transcription by vesicular stomatitis virus virion transcriptase. J. Virol. **11**:487-501.
44. **Schibler, U., and R. P. Perry.** 1976. Characterization of the 5'-termini of hnRNA in mouse L cells: implications for processing and cap formation. Cell **9**:121-130.
45. **Shatkin, A. J.** 1976. Capping of eukaryotic mRNAs. Cell **9**:645-653.
46. **Shimothno, K., and K. Miura.** 1976. The process of formation of the 5'-terminal modified structure in messenger RNA of cytoplasmic polyhedrosis virus. FEBS Lett. **64**:204-208.
47. **Testa, D., and A. K. Banerjee.** 1979. Initiation of RNA synthesis *in vitro* by vesicular stomatitis virus: role of ATP. J. Biol. Chem. **254**:2053-2058.
48. **Toneguzzo, F., and H. P. Ghosh.** 1976. Characterization and translation of methylated and unmethylated vesicular stomatitis virus mRNA synthesized in vitro by ribonucleoprotein particles from vesicular stomatitis virus-infected L cells. J. Virol. **17**:477-491.
49. **Wei, C.-M., and B. Moss.** 1977. 5'-Terminal capping RNA by guanylyltransferase from HeLa cell nuclei. Proc. Natl. Acad. Sci. U.S.A. **74**:3758-3761.