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5'-Terminal Sequences of Spring Viremia of Carp Virus RNA Synthesized In Vitro

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Sequence analyses have been undertaken on the 5' termini of the RNA species synthesized in vitro at 22°C by Spring viremia of carp virion (SVCV)-associated transcriptase by using virus grown in mammalian BHK-21 cells. SVCV product RNA was synthesized in the absence or presence of low (0.56 μM) or high (0.8 mM) concentrations of added *S*-adenosyl-L-methionine (SAM). Two major sequences obtained in the absence (or in low concentrations) of SAM have been shown to be GpppAp and GpppAmpAp(C). A minor sequence detected when a low concentration of [^{3}H]SAM was added to reaction mixtures was 7mGpppAmpAp. Larger quantities of the 7mGpppAmpAp(C) sequence, in addition to the GpppAmpAp(C) sequence, were obtained when high concentrations of SAM were used, and under these conditions no GpppAp sequences were detected. It has further been shown that with low concentrations of [^{3}H]SAM the principle in vitro methylation of adenosine in SVCV product RNA occurred at the 2'-*O*-ribose position; no methylation at the N^6-adenosine position and no internal product RNA methylation were detected. Comparison of the SVCV results to the published data on the 5'-terminal structures of the in vitro or in vivo mRNA species of vesicular stomatitis virus Indiana and vesicular stomatitis virus New Jersey suggests that the 5' sequences of transcript RNA of different rhabdoviruses may have been conserved.

Several eucaryotic and viral mRNA species have been shown to have a 5'-terminal cap structure with the sequence 7mGpppNm (21), the methylated cap structure being necessary for efficient translation (3). Sequence analyses of the in vitro- or in vivo-synthesized 5' termini of the viral complementary mRNA species of the rhabdoviruses vesicular stomatitis virus (VSV) Indiana and VSV New Jersey have identified a variety of sequences, including both uncapped, capped, and capped and methylated species (1, 2, 4, 7, 10, 11, 15, 16, 18). The similarities between the 5' termini of the in vitro-synthesized RNA of the VSV serogroup viruses raises the question of whether such sequences have been conserved during the derivation of all the various serotypes and genera of the Rhabdoviridae family.

Spring viremia of carp virus (SVCV) is a fish rhabdovirus which has a virion transcriptase with a temperature optimum of 22°C (20). The SVCV in vitro transcriptase activity is stimulated threefold by the addition of *S*-adenosyl-L-methionine (SAM) (optimum concentration, 0.8 to 1.2 mM) to reaction mixtures (20), although SAM addition has only a marginal effect on the rate of transcription by VSV Indiana. SAM has also been shown to stimulate the transcriptase activity of cytoplasmic polyhedrosis virus (9). We have chosen to study SVCV, since it is a rhabdovirus which is serologically and structurally different by comparison to members of the vesiculovirus genus of Rhabdoviridae (20). SVCV is also of interest to study in view of its ability to grow at low temperatures and productively infect both piscine and mammalian tissue culture cells (5).

In contrast to other virus systems (21) it has been suggested that 2'-*O*-methylation of the penultimate nucleotide at the 5' terminus in VSV Indiana mRNA precedes 7-methylation of the terminal guanosine (22). In addition, the recovery of the different methylated derivatives obtained in vitro varies, depending on the SAM concentrations used (22). Because of these observations, we have examined the 5'-terminal structures of the RNA species synthesized in vitro by SVCV transcriptase in the presence or absence of different concentrations of added SAM to determine whether the SVCV transcription processes resemble those of other members of the Rhabdoviridae family.

MATERIALS AND METHODS

Radioisotopes and enzymes. α-[^{32}P]ATP (4 to 23 Ci/mmol), α-[^{32}P]GTP (4 to 23 Ci/mmol), α-[^{32}P]CTP (19 Ci/mmol), α-[^{32}P]UTP (15 Ci/mmol), and *S*-ad-

enosyl-L-[*methyl*-^{3}H]methionine (64 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass. Bacterial alkaline phosphatase, nucleotide pyrophosphatase, and nuclease T2 were purchased from Sigma Chemical Co., St. Louis, Mo. Nuclease P1 was obtained from P. L. Biochemicals, Milwaukee, Wis. Reference nucleosides and nucleotides came from Sigma Chemical Co., St. Louis, Mo.

Virus and cells. SVCV was grown in BHK-21 cells and purified as described previously (19).

Synthesis and purification of RNA. RNA was synthesized in vitro, using standard transcriptase reaction mixtures (2). Unlabeled SAM was used at a concentration of 0.8 mM. When [^{32}P]GTP or [^{32}P]ATP was employed, the unlabeled concentrations of GTP or ATP, respectively, were lowered to 0.20 mM. No unlabeled SAM was included in reaction mixtures when [^{3}H]SAM (0.56 μM) was employed. All reaction mixtures were incubated at 22°C for 90 min. Under these conditions of incubation, the reaction rate was linear (20). Reactions were terminated by the addition of 1% sodium dodecyl sulfate (SDS). RNA was extracted by phenol, the aqueous phase was passed through a Sephadex G-50 column, and the RNA was precipitated from the void volume with 2 volumes of ethanol (18).

Enzymic digestion of RNA, resolution of nucleosides and nucleotides. RNase T2 digestion at 37°C involved incubating an RNA sample for 4 to 6 h in 50 mM sodium acetate buffer (pH 4.5), using 10 U of enzyme per ml of reaction mixture (10, 11). RNA in 1 mM sodium acetate buffer (pH 6.0) was digested for 2 to 4 h at 37°C with nuclease P1 (50 U/ml). Bacterial alkaline phosphatase (10 U/ml) digestions were incubated at 37°C for 2 h in 40 mM Tris-hydrochloride buffer (pH 8.0) (10). Nucleotide pyrophosphatase digestions involved incubating an RNA sample in 20 mM Tris-hydrochloride buffer (pH 7.4)–10 mM $MgCl_2$ for 1 h at 37°C. Digests were spotted on 3MM Whatman paper and analyzed either by electrophoresis or chromatography (see below). After resolution, the paper was cut into 1-cm paper strips, and the distribution of radioactivity was determined. Material was eluted from the paper by deionized water after washing the strips with toluene. RNA digests were also analyzed by DEAE-cellulose chromatography in 7 M urea (pH 8.0) or at pH 3.5 in sodium citrate buffer as described previously (4, 10, 11, 18).

RESULTS

Cap structures of SVCV product RNA. RNase T2 digests of SVCV in vitro transcription product RNA were resolved by DEAE-cellulose column chromatography at pH 8.0 in 7 M urea together with an RNase A digest of optical quantities of marker chick ribosomal RNA (4, 10, 11, 18). Figure 1A presents the results of the column chromatogram for SVCV product RNA synthesized in the presence of [^{32}P]GTP and the absence of added SAM. Similar profiles of [^{32}P]-GTP-labeled nucleotides were obtained when low (0.56 μM) or high (0.8 mM) concentrations of SAM were included in the reaction mixtures (data not shown). For all samples, apart from a major peak of mononucleotides (net negative charge of 2), two other peaks of radioactivity were recovered. Peak I eluted at the leading edge of the marker tetranucleotides, whereas peak II eluted between the marker tetra- and pentanucleotides. The profile of SVCV product RNA labeled with 0.56 μM [^{3}H]SAM is given in Fig. 1B. Although this SAM concentration represented 0.1% of the optimal SAM concentration (20), the product from reactions containing 1,000-fold higher [^{3}H]SAM did not contain enough counts to give a meaningful answer, due to the low specific activity of the [^{3}H]SAM (and the prohibitory cost of using 1,000-fold more high-specific-activity [^{3}H]SAM).

For each of the ^{32}P-labeled RNase T2 digests of SVCV product RNA, the peak I and II materials were separately pooled, mixed with carrier nucleotides, and recovered as their barium salts by precipitation with ethanol at −20°C (4, 10, 11, 18). After recovery of the precipitates, the nucleotides were converted to their H^+ form for subsequent analyses. When [^{32}P]ATP was used to label SVCV product RNA (in the presence or absence of added SAM), and the RNA was digested with RNase T2, essentially similar profiles of ^{32}P-labeled oligonucleotides were obtained by DEAE-cellulose column chromatography to that shown in Fig. 1 (data not shown).

Using [^{3}H]SAM (0.56 μM) and [^{32}P]GTP (or [^{32}P]ATP) to label SVCV product RNA, it was found that the relative ratio of ^{3}H to ^{32}P label for the material in the first peak was one-tenth that found for material in the second peak, even though there were essentially similar amounts of ^{32}P-labeled nucleotides in the two peaks (Fig. 1). This result suggested that the peak I material may have contained a mixture of both nonmethylated and methylated 5′ terminus structures, the methylated varieties constituting a minor component. Alternatively, these observations could indicate that the peak II material was ten times more methylated than the peak I material. When 1,000-fold higher SAM concentrations were used (0.8 mM), as discussed above, due to the low specific activity of product RNA labeled with [^{3}H]SAM, the relative ^{3}H labeling of peaks I and II could not be accurately determined.

Resolution of peak I and II materials by pH 3.5 DEAE-cellulose column chromatography. It has been shown that the mixture of termini (pppAp, GpppAp, 7mGpppAmpAp, and pppGp) recovered in RNase T2 digests of suitably labeled VSV Indiana in vitro transcription product RNA can be partially resolved by chro-

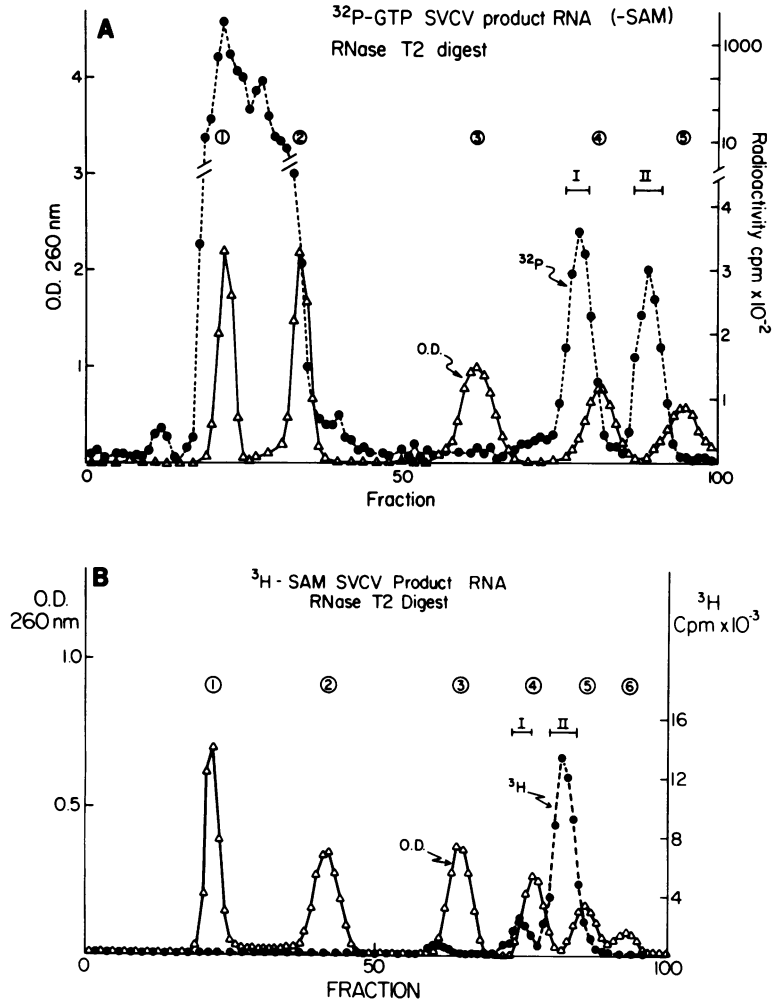

FIG. 1. *DEAE-cellulose column chromatography (pH 8.0) of a nuclease T2 digest of SVCV RNA transcripts labeled with [^{32}P]GTP in the absence of added SAM. (A) SVCV in vitro product RNA was digested with RNase T2, extracted with phenol to remove the enzyme, mixed with a pancreatic RNase digest of optical quantities of chick ribosomal RNA, and resolved by DEAE-cellulose column chromatography. Nucleotides and oligonucleotides were eluted with a linear 180-ml gradient of 0 to 200 mM LiCl in 10 mM Tris, 2 mM EDTA, and 7 M urea (pH 8.0), as described previously (18). The positions of the markers mono-, di-, tri-, tetra-, and pentanucleotides are indicated. The pooled ^{32}P-labeled nucleotides, I and II, were recovered for further analyses (see text). (B) SVCV in vitro product RNA labeled with low (0.56 μM) SAM was similarly analyzed.*

matography at pH 3.5 on DEAE-cellulose columns (10, 11). Therefore, to determine whether there was more than one terminus in either the peak I or peak II materials recovered from SVCV product RNA, portions of both (labeled by [^{32}P]ATP or [^{32}P]GTP in the presence of 0.56 μM [^{3}H]SAM, or 0.8 mM unlabeled SAM) were resolved by chromatography on DEAE-cellulose at pH 3.5 (Fig. 2). For all the peak II samples, a single peak of radioactivity (c) was obtained (see Fig. 2B). For the peak I materials, labeled by 0.56 μM [^{3}H]SAM and [^{32}P]ATP (or [^{32}P]GTP), pH 3.5 chromatography yielded one major ^{32}P-labeled peak (b) and, based on the ^{32}P distribution, a minor peak (a) labeled by [^{3}H]SAM, [^{32}P]ATP, and [^{32}P]GTP (Fig. 2A). For peak I materials labeled in the presence of 0.8 mM SAM by [^{32}P]ATP, two major labeled termini (a, b) were obtained from the pH 3.5 chromatogram (Fig. 2D). However, for peak I materials labeled in the presence of 0.8 mM SAM by [^{32}P]GTP, only one major peak (a) was obtained (Fig. 2C).

The results obtained by pH 3.5 DEAE-cellu-

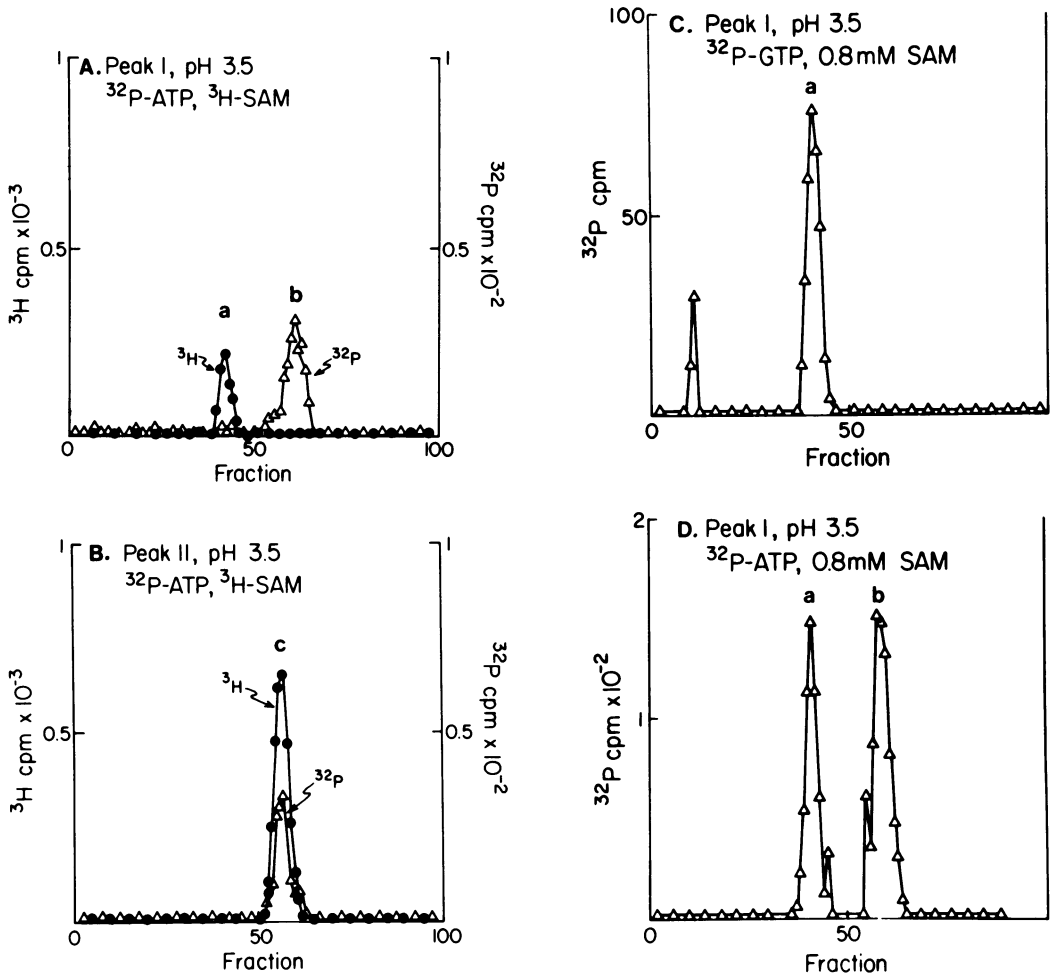

FIG. 2. *DEAE-cellulose column chromatography (pH 3.5) of peak I and peak II nucleotides. A linear 160-ml gradient of LiCl (50 to 250 mM) in 50 mM sodium citrate buffer (pH 3.5) was used to resolve the nucleotides. Peak I nucleotides labeled by [^{32}P]ATP and [^{3}H]SAM (A), or [^{32}P]GTP in the presence of unlabeled 0.8 mM SAM (C), or [^{32}P]ATP in the presence of unlabeled 0.8 mM SAM (D), or peak II nucleotides labeled by [^{32}P]ATP and [^{3}H]SAM (B) were obtained from pH 8.0 DEAE-cellulose column chromatograms (Fig. 1) as described previously (4, 10, 11, 18).*

lose column chromatography are compatible with the peak II material containing primarily one terminus (c) labeled by GTP, ATP, and [^{3}H]SAM, whereas the peak I material produced in the presence of 0.56 μM SAM contained a small amount of a methylated terminus (a) but considerably more nonmethylated structures (b). In the presence of 0.8 mM SAM, peak I materials contained at least two termini (a, b), one of which (b) was labeled by [^{32}P]ATP but not [^{32}P]GTP, whereas the other (a) was labeled with [^{32}P]ATP and [^{32}P]GTP.

It has been shown previously for vesicular stomatitis virus (VSV) Indiana termini that the order of elution by pH 3.5 DEAE-cellulose column chromatography is 7mGpppAmpAp, pppAp, and pppGp, and that GpppAp is not resolved from pppAp by this procedure; also it has been demonstrated that these nucleotides cannot be resolved by pH 8.0 DEAE-cellulose column chromatography and are all eluted in peak I (10, 11). Compared with the results obtained previously for VSV Indiana termini (10, 11), it appears from the pH 3.5 DEAE-cellulose elution characteristics obtained for SVCV product labeled in the presence of 0.56 μM SAM that the peak I material (original tetranucleotide isoplith sample) contained a small amount of a methylated structure (a) which eluted in the position where 7mGpppAmpAp is recovered (11,

12). In addition, peak I material contained nonmethylated material(s) (b) which eluted from the column where both GpppAp and pppAp are known to elute (11). No pppGp structures were detected for SVCV product termini by this procedure. For SVCV product labeled in the presence of 0.8 mM SAM, it appeared that [^{32}P]ATP labeled the same two peaks (a and b), although [^{32}P]GTP only labeled peak a. The lack of peak b labeling by [^{32}P]GTP (high SAM) indicates that there are no GpppAp sequences in the peak I (high SAM) product, suggesting that peak b (high SAM) may contain pppAp or other ATP but not GTP-labeled termini.

The peak II material recovered from the SVCV product has no counterpart in the in vitro termini analyzed in these laboratories for VSV Indiana (4, 10, 11, 18). To analyze it further, peak II materials were subjected to alkaline phosphatase digestion.

Effect of alkaline phosphatase treatment on peak I and peak II termini. The results of the pH 3.5 DEAE-cellulose column chromatography suggested that the peak II material probably contained a single terminus labeled by [^{32}P]ATP, [^{32}P]GTP, and [^{3}H]SAM. To characterize it further, portions of the variously labeled peak II samples, synthesized in the presence of labeled SAM (0.56 μM), were digested with alkaline phosphatase, phenol extracted to remove the enzyme, and rechromatographed at pH 8.0 on DEAE-cellulose with a RNase A digest of optical quantities of chicken embryo ribosomal RNA. For samples labeled with [^{32}P]ATP and [^{3}H]SAM (Fig. 3A), or [^{32}P]GTP and [^{3}H]SAM (Fig. 3B), a single major peak of dual-labeled material was recovered eluting just before the marker trinucleotides. Little of the initial radioactivity was recovered in the position of free phosphate (i.e., eluting with the mononucleotides). These results indicate that probably all of the original donor radioactive phosphate residues from ATP and GTP were protected from removal by alkaline phosphatase. The results are compatible with the conclusion (see below) that the peak II material was GpppAmpAp(C).

When the peak I material, labeled with 0.56 μM [^{3}H]SAM, was similarly treated with alkaline phosphatase, extracted with phenol, and rechromatographed on DEAE-cellulose at pH 8.0 with marker oligonucleotides, a single peak of ^{3}H radioactivity was recovered eluting just after the dinucleotides (Fig. 4C). Studies on VSV Indiana and vaccinia virus (11, 23) in vitro-synthesized RNA labeled with [^{3}H]SAM have shown that the terminus 7mGpppAmpA elutes in this position. Phosphatase digests of peak I material labeled with [^{32}P]GTP in the presence

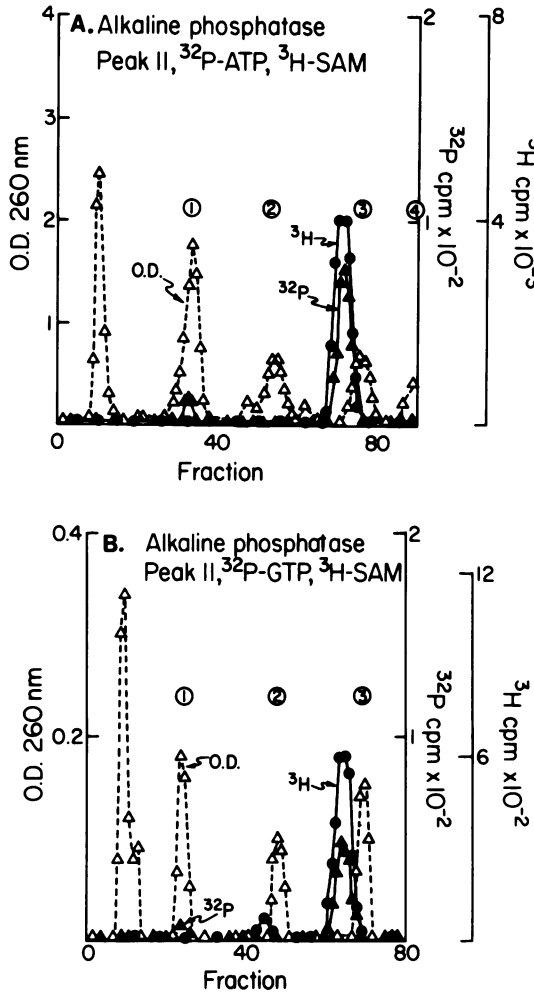

FIG. 3. *DEAE-cellulose column chromatography of peak II nucleotides after digestion with bacterial alkaline phosphatase. The conditions for elution of nucleotides were the same as those used in Fig. 1. (A) Peak II nucleotides (Fig. 1) were obtained from SVCV product RNA labeled with [^{3}H]SAM and [^{32}P]ATP; (B) peak II nucleotides were obtained from SVCV product RNA labeled with [^{3}H]SAM and [^{32}P]GTP. Nucleotides were digested with alkaline phosphatase as described in the text.*

or absence of 0.56 μM SAM (Fig. 4A) indicated that 80% of the radioactivity was recovered eluting with the dinucleotides (like GpppA) (10, 11); the rest eluted as free phosphate. For alkaline phosphatase-treated peak I materials labeled with [^{32}P]ATP (in the presence or absence of 0.56 μM SAM), 40% of the radioactivity eluted with the dinucleotides (like GpppA), and the rest eluted as free phosphate (data not shown). Very little of the ATP or GTP (0.56 μM SAM) phosphatase-treated peak I termini eluted in the

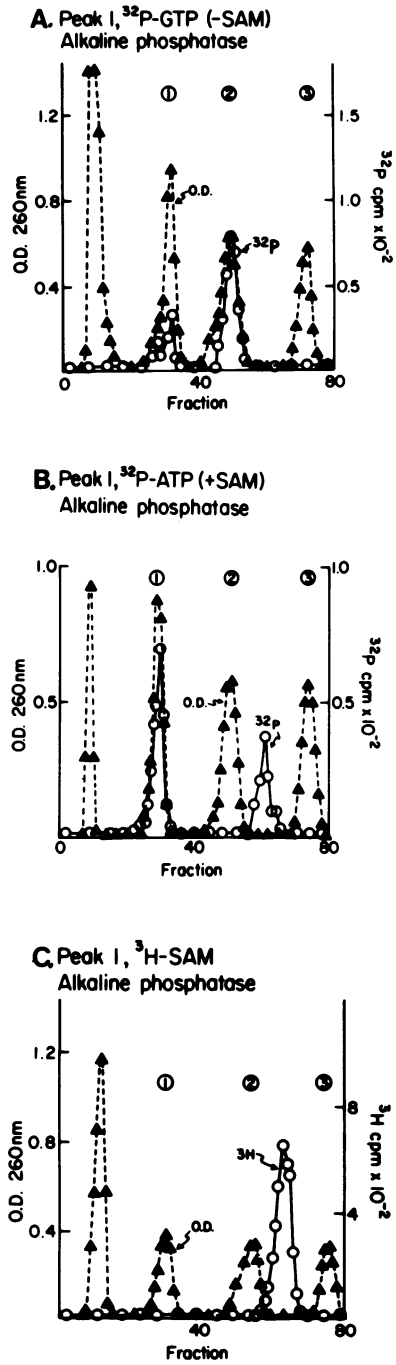

FIG. 4. *DEAE-cellulose column chromatography of peak I nucleotides after digestion with bacterial alkaline phosphatase. The conditions for the elution of nucleotides are described in Fig. 1. Peak I nucleotides came from SVCV product labeled by: (A) [^{32}P]GTP in the absence of SAM; (B) [^{32}P]ATP in the presence of 0.8 mM unlabeled SAM; or (C) [^{3}H] SAM.*

position where m7GpppAmpA was recovered, indicating that this [^{3}H]SAM-labeled structure, presumably also labeled by GTP (and ATP, see below) was a minor component of the reaction products. For phosphatase-treated peak I materials labeled by [^{32}P]ATP in the presence of 0.8 mM SAM, 40% of the radioactivity eluted between the di- and trinucleotides (i.e., like 7mGpppAmpA), whereas the rest was recovered as free phosphate (Fig. 4B) and almost no label was recovered eluting with the dinucleotides (i.e., like GpppA). Comparable analyses with peak I materials labeled with [^{32}P]GTP (0.8 mM SAM) resulted in 84% of the radioactivity eluting in a single peak between the di- and trinucleotides. The rest was recovered as free phosphate (see Fig. 4B). Again, almost no label was recovered eluting with the dinucleotides.

Previous similar studies on alkaline phosphatase-treated termini recovered from VSV Indiana product RNA species indicated that GpppA elutes with the marker dinucleotides, and 7mGpppAmpA elutes just after the dinucleotides (10, 11). Thus, the present results obtained with phosphatase-treated SVCV peak I materials synthesized in the presence of low concentrations of SAM indicate that there were structures present which were equivalent in labeling and elution characteristics to both GpppAp (major component) and 7mGpppAmpAp (minor component). For the SVCV peak I materials synthesized in high SAM concentrations, termini with the characteristics of 7mGpppAmpAp were found (but not GpppAp), as well as termini labeled by ATP but not labeled by GTP.

Since the relative proportion of ^{32}P recovered as free phosphate in the mononucleotides of the phosphatase digests of [^{32}P]ATP-labeled peak I termini (high, or low or no SAM) was greater than that recovered with or after the dinucleotide peak, it is possible that the peak I material also contained other termini (e.g., pppAp) in addition to GpppAp (no, or low SAM) and m7GpppAmpAp (low or high SAM). Such conclusions are supported by the data shown in Fig. 2.

SVCV transcription product RNA labeled by [^{32}P]CTP and [^{32}P]UTP. When SVCV transcription product was synthesized in the presence of CTP, the product digested with RNase T2 and resolved by DEAE-cellulose column chromatography with marker nucleotides, both peak I and peak II type materials were obtained (data not shown). The ratio of radioactivity in the two peaks was 0.60 (peak I) to 0.40 (peak II). Alkaline phosphatase treatment of both materials resulted in all of the radioactivity eluting from subsequent DEAE-cellulose

columns as free phosphate (data not shown). Similar results were obtained for the SVCV product synthesized in the presence of 0.8 mM SAM, except that the ratio of ^{32}P in the two peaks was 0.55 (peak I) to 0.45 (peak II). For both samples, alkaline phosphatase treatment resulted in all the ^{32}P being recovered as free phosphate. When the peak I (high SAM) material labeled by CTP was rechromatographed on DEAE-cellulose at pH 3.5, one-fourth of the radioactivity was recovered in peak a, the remainder being recovered in peak b (see Fig. 2).

For SVCV transcription product labeled by [^{32}P]UTP, no labeled peak I or II materials were obtained in the presence or absence of added SAM. Thus the results obtained with the CTP-labeled product suggest that the peak II terminus (no or high SAM) has a CTP-labeled phosphate residue which is accessible to alkaline phosphatase removal [as in GpppAmpAp(C)]; a similar conclusion was reached for the high SAM peak a material [as in m7GpppAmpAp(C)] and peak b material [as in pppAp(C)].

Identification of the methylated nucleotides. To further characterize the SVCV termini, product RNA labeled in vitro with [^{3}H]-SAM was purified and digested with nuclease P1 and alkaline phosphatase, and the digest was resolved by paper electrophoresis together with optical quantities of pC, pA, pG and pU (8). Two nuclease- and phosphatase-resistant termini were identified (III and IV, Fig. 5A), one (III) in the vicinity of the marker pG and the other (IV), which was a minor component, between the markers pA and pG.

Both materials III and IV were eluted from the paper, and portions digested with pyrophosphatase and the products were again analyzed by paper electrophoresis, using optical quantities of the markers pU, pG, pA, pC, mA, and 7mG. Most (94%) of the label in the material III digest was recovered (Fig. 5C) in the position of the pA marker. A small amount (3%) was recovered at the origin (where 7mG-5′-phosphate should be located), and another 3% of the label was recovered with the marker mA. None of the label was found in the location of the 7mG marker. Another portion of the material III was digested with pyrophosphatase and then alkaline phosphatase and resolved by paper chromatography (13) with optical quantities of 6mA, 2′-*O*-mA, 2′-*O*-mG, 6($\gamma\gamma$-dimethylallylamino)-purine ribonucleoside, and 7mG (Fig. 5B). Although the system did not resolve 6mA from 2′-*O*-mA, almost all of the radioactivity was recovered in the location of the monomethylated adenosine residues; none was recovered with the dimethylated derivative which was located near the solvent front. When similar analyses were performed with [^{32}P]GTP-labeled product, pyrophosphatase digestion of material III yielded a labeled pG residue with less than 15% of the recovered label associated with pA or 7mG-5′-phosphate (Fig. 6A).

After pyrophosphatase digestion of [^{3}H]SAM-labeled material IV (Fig. 5D), radioactivity was recovered at the origin (25%), and with the pA (55%), mA (13%) and 7mG (7%) markers. Due to the small amounts of ^{3}H radioactivity available, no further analyses were performed on this sample of material IV. Similar analyses of [^{32}P]GTP-labeled material IV yielded, after pyrophosphatase treatment, radioactive 7mG-5′-phosphate, with less than 15% of the label associated with the pG or pA markers (Fig. 6B). Although for the [^{3}H]SAM-labeled material IV after pyrophosphatase digestion the ^{3}H label was not equally distributed between the adenosine and the guanosine derivatives (as expected for a m7GpppAm structure), it is likely that the material IV sample was contaminated with some material III components (Fig. 5A).

Another portion of the [^{3}H]SAM-labeled total SVCV product RNA was digested with nuclease P1, then pyrophosphatase, and then alkaline phosphatase, to obtain the labeled nucleosides. Paper electrophoresis of the products, run with markers 7mG, 2′-*O*-mA and 6mA, indicated that almost all of the labeled nucleosides were adenosine derivatives (96%). A small proportion of the label (4%) was recovered in the vicinity of the 7mG marker (Fig. 7A). When the labeled adenosine derivatives were recovered and digested with 90% formic acid at 100°C for 2 h to hydrolyze the ribose moiety, and the products were resolved by paper chromatography (14, 17) with optical quantities of 6-methyl-adenosine and 6($\gamma\gamma$-dimethylallylamino)-purine ribonucleoside (Fig. 7B), all of the radioactivity was recovered near the solvent front, indicating that the methylation of the adenosine residues was located in the ribose moiety (presumably 2′-*O*-mA).

The analyses of the [^{3}H]SAM-labeled product termini described above did not preclude the possibility that there were both internal RNA methylation sites (as in B77 avian sarcoma virus, 6) and/or adenine-base methylated derivatives in the termini. To investigate these questions, a sample of [^{3}H]SAM-labeled total product RNA was digested with RNase T2, and the distribution of radioactive label was determined after chromatography on DEAE-cellulose together with an RNase A digest of optical quantities of chick ribosomal RNA. None of the ^{3}H label was recovered with the mononucleotides, whereas

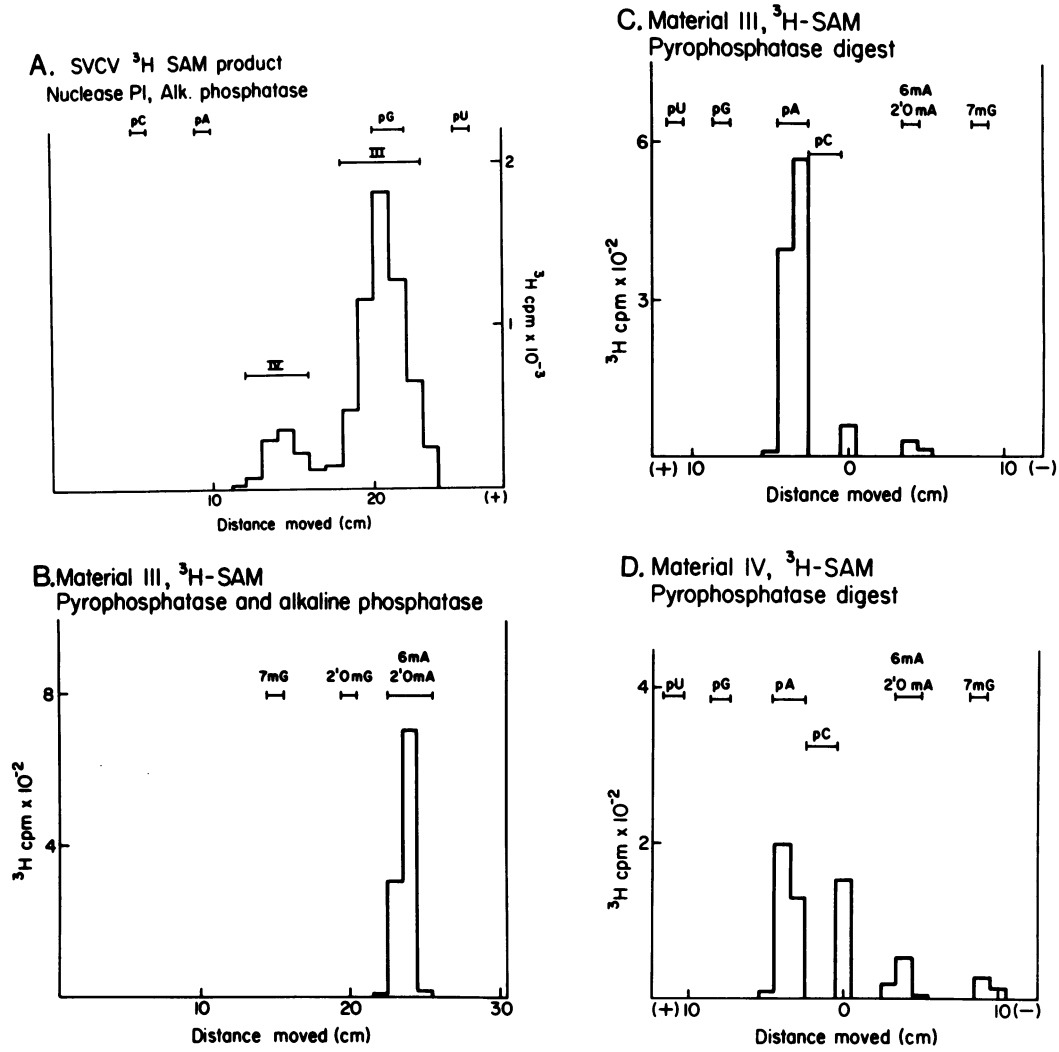

FIG. 5. *Identification of methylated nucleotides in SVCV product RNA. (A) [³H]SAM-labeled SVCV in vitro product RNA was digested with nuclease P1 and then bacterial alkaline phosphatase. The digest was resolved by paper electrophoresis (50 V/cm) at pH 3.5, together with optical quantities of the 5'-monophosphate nucleotides, pC, pA, pG, and pU. The final positions of the marker nucleotides were obtained by using short-wave UV light. The distribution of radioactivity was then determined, and the peak IV and III nucleotides were recovered as described in the text. (B) The material III nucleotides (see A) were digested with pyrophosphatase and then alkaline phosphatase, and the products were resolved by ascending paper chromatography (13), using optical quantities of marker 7-methyl guanosine (7mG), 2'-O-methyl guanosine (2'OmG), 6-methyl adenosine (6mA), 2'-O-methyl adenosine (2'OmA) and 6 (γγ-dimethylallylamino)-purine ribonucleoside (6mm aminopurine, which was recovered at the solvent front). The solvent system was isopropanol-water-NH₃ (7:2:1).*

10% was recovered with the tetranucleotides (equivalent to peak I in Fig. 1) and 90% was recovered between the tetra- and pentanucleotides (peak II, Fig. 1). These results indicated that, at least for product labeled in the presence of 0.56 μM SAM, no internal nucleotides were methylated.

Taken together with the results described above, it was concluded that the material III, representing the majority of the ³H label incorporated from radioactive SAM (0.56 μM), was a G-end blocked (capped) terminus which contained 2'-*O*-mA. The relative lack of 7mG recovered from the material III sample indicates that the cap structure was not 7mG. The analyses on the minor material IV component sug-

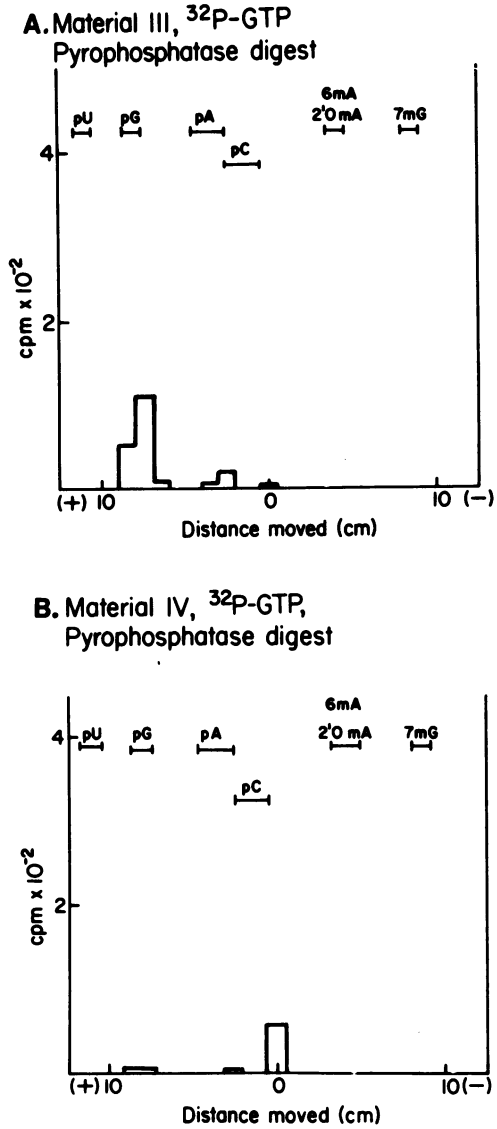

FIG. 6. *Identification of the guanosine-methylated derivatives in SVCV termini. SVCV product RNA labeled by [^{32}P]GTP in the presence of 0.56 μM SAM was digested with nuclease P1 and alkaline phosphatase and resolved by paper electrophoresis at pH 3.5 as described for Fig. 5A. The ^{32}P-labeled materials III and IV were recovered, digested with pyrophosphatase, and resolved by paper electrophoresis with marker nucleotides as described for Fig. 5C. The result obtained for [^{32}P]GTP-labeled material III is given in A, and that obtained for [^{32}P]GTP-labeled material IV is given in B.*

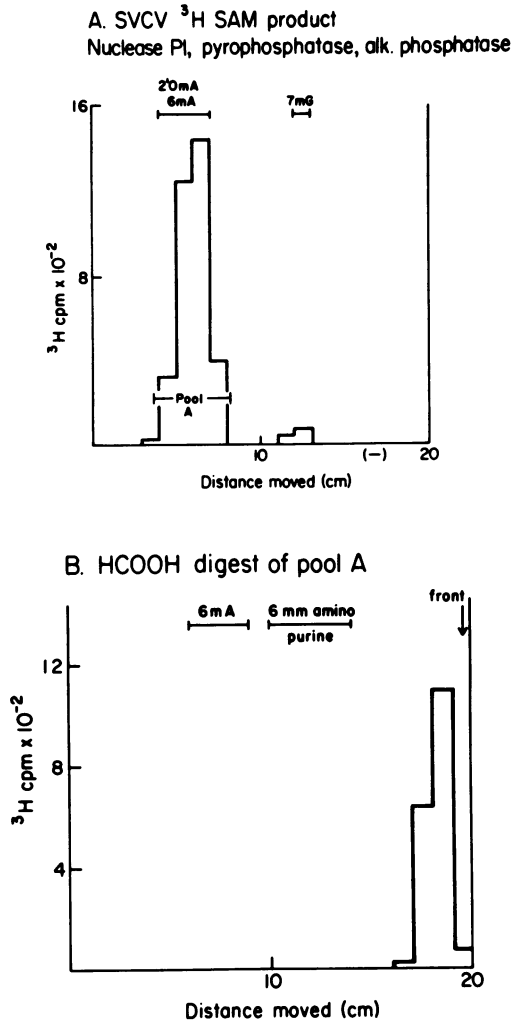

FIG. 7. *Identification of the methylated components of SVCV product RNA. (A) SVCV product RNA labeled by [^{3}H]SAM was sequentially digested with nuclease P1, pyrophosphatase, and alkaline phosphatase and resolved by paper electrophoresis at pH 3.5 with marker 7-methyl guanosine (7mG), 2'-O-methyl adenosine (2'OmA), and 6-methyl adenosine (6mA) nucleosides. The pool A nucleosides were recovered, digested with formic acid, and resolved with 6mA and 6mm aminopurine ribonucleoside markers by ascending paper chromatography in methanol-hydrochloric acid-water (7:2:1), following the procedure of Randerath and Randerath (14, 17). For additional markers, adenine and 6-methyl adenine were run in a parallel lane to that containing the digest.*

gest that it is a capped terminus containing both 7mG and methylated adenosine residues, presumably 2'-*O*-mA (i.e., 7mGpppAm).

DISCUSSION

The analyses described in this paper indicate that a variety of termini are present on SVCV product RNA species synthesized in vitro at

22°C. When [^{3}H]SAM (0.56 μM) was included in the reaction mixtures, some of the product termini were found to be methylated. Most of the methylation sites were shown to involve 2'-*O*-mA residues; a few were 7mG residues. In the presence of a more than 1,000-fold higher concentration of SAM (0.8 mM), it has been deduced that a larger proportion of the total termini have 7mG residues (compare Fig. 4C and 4B).

The relative amounts of the materials III and IV (Fig. 5A), when compared to the relative amounts of the ^{3}H-labeled peak I and II samples (see Fig. 1) obtained by RNase T2 digests of [^{3}H]SAM-labeled product RNA, suggest that the methylated component in peak I is equivalent to the methylated derivative in material IV, whereas the peak II methylated derivative is equivalent to the material III sample. In support of these conclusions, the peak I derivative, synthesized in the presence of labeled SAM, ATP, and GTP, yielded, after alkaline phosphatase treatment, a structure labeled by all three precursors and which eluted from DEAE-cellulose in the position of 7mGpppAmpA (Fig. 2A, 4C). By comparison, material IV has been shown to contain both 2'-*O*-mA (Fig. 5D) and 7mG-5'-phosphate (Fig. 5D, 6B), agreeing with the conclusion that peak I was equivalent to material IV. Although material IV was found to have twice as much mA-5'-phosphate as 7mG-5'-phosphate (Fig. 5D), the problem of it being contaminated with material III (Fig. 5A), which does not have 7mG residues but does have 2'-*O*-methyl adenosine residues, leads us to rely on the DEAE-cellulose studies of peak I materials which indicate that the SAM, ATP, and GTP-labeled peak I material yielded, after phosphatase treatment, a compound which eluted in the position of 7mGpppAmpA (11).

The fact that the peak II terminus was labeled by [^{3}H]SAM, [^{32}P]ATP, [^{32}P]CTP, and [^{32}P]-GTP and yielded, after alkaline phosphatase treatment, a structure which eluted from DEAE-cellulose columns later than 7mGpppAmpA, and was labeled by SAM, ATP and GTP (Fig. 3), is compatible with it having the sequence GpppAmpA, the lack of a positively charged 7mG residue accounting for the later elution characteristic. The results obtained with [^{32}P]-CTP as a precursor indicate that not only is peak II material labeled by CTP, but its CTP-labeled phosphate is accessible to alkaline phosphatase, as in the sequence GpppAmpAp(C). By comparison, the material III obtained after nuclease P1 and alkaline phosphatase treatment has been shown to contain 2'-*O*-mA (Fig. 5B, C, and 7B) as well as G-5'-phosphate residues (Fig. 6A), but lacks 7mG residues (Fig. 6A). Thus, material III must have the sequence GpppAm. Since both material III and peak II are the major methylated products of their respective analyses and they contain similar constituents, the most reasonable conclusion is that they are equivalent to each other and that material III is GpppAm, and peak II is GpppAmpAp(C). β Elimination will, however, be needed to prove that the G residue is a capping nucleoside.

Although we have no direct evidence to prove that peak II synthesized in the absence of SAM is GpppAmpAp, if the indirect evidence has been correctly interpreted, then it suggests that a methyl donor such as SAM is present within SVCV virions. This suggestion is supported by the observation that with the methylation antagonist *S*-adenosyl-L-homocysteine in reaction mixtures, no peak II materials are obtained by using either labeled ATP or GTP as precursors (K. Gupta and P. Roy, unpublished data).

The results obtained with SVCV product synthesized in vitro in the presence of 0.56 μM SAM at 22°C have been interpreted to indicate that there are a small number of 7mGpppAmpAp termini present as well as a 20-fold larger number of GpppAmpAp termini (based on the ^{3}H label ratio of peaks I and II). In addition, there are a large number of unmethylated peak Ib termini, some with the structure GpppAp (no or low SAM), and others labeled by ATP but not GTP, e.g., pppAp (high SAM). The fact that the peak Ib (high SAM) was labeled by CTP suggests that some of the peak Ib (high SAM termini) could be pppAp(C) sequences, as found in VSV Indiana transcripts (18). Whether pppAp(A) sequences are also present has not been determined (18). Product analyses with β,γ-[^{32}P]ATP precursors will be needed to provide direct evidence for the existence of pppAp termini.

The fact that in high SAM concentrations no GpppAp (peak Ib) termini were found (Fig. 2C), whereas m7GpppAmpAp termini (peak Ia) were present, suggests that in high SAM concentrations GpppAp termini are quantitatively methylated, a conclusion obtained previously from studies with VSV Indiana (1, 11).

One question raised by these studies is why the GpppAmpAp termini are not further methylated (e.g., to give m7GpppAmpAp termini) when high concentrations of SAM are employed. Although for VSV Indiana it has been suggested that adenosine methylation precedes cap methylation (22), the results obtained for SVCV could be interpreted to indicate that prior adenosine methylation precludes subsequent cap methylation. Alternatively, it is possible that with high

SAM, GpppAp sequences are converted to GpppAmpAp sequences and that some, but not all, GpppAmpAp sequences are methylated to give m7GpppAmpAp sequences. These possibilities, as well as the effect of low temperatures and host cell influence (in vitro and in vivo) upon transcript composition, will require further investigation.

It is noteworthy that similar transcript termini to those identified for SVCV have been detected on VSV Indiana and VSV New Jersey in vitro and in vivo viral complementary RNA species (1, 2, 4, 7, 10, 11, 15, 16, 18). This suggests that the viral genome sequences specifying the 5′ ends of rhabdovirus viral complementary RNA species may have been conserved among the Rhabdoviridae family.

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