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The 5′ Nucleotide Sequence of Vesicular Stomatitis Viral RNA

ELISABETH HEFTI AND D. H. L. BISHOP*

The Waksman Institute of Microbiology, Rutgers—The State University, New Brunswick, New Jersey 08903

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The 5′ nucleotide sequence of vesicular stomatitis viral RNA is pppApCpGp....

Vesicular stomatitis virus (VSV) is a rhabdovirus which possesses a virion RNA-dependent RNA polymerase (2). Both in vivo and in vitro studies have shown that this RNA polymerase transcribes the viral genome completely and repetitively to give messenger RNA molecules smaller than, but complementary to, the viral genome (1, 3, 4, 6, 10). RNA synthesis occurs in a 5′ to 3′ direction (12). In vitro analyses have identified four 5′ initiation sequences involving pppAp....or pppGp nucleotides (5, 11).

Although we do not know how transcription and replication are interrelated, it is of interest to identify the 5′ nucleotide sequences of not only the intracellular VSV messenger RNA but also those of the 42S viral and viral complementary RNA. We report here that the sequence of the 5′ end of VSV viral RNA is pppApCpGp.... which is one of the sequences found during in vitro transcription product analyses (5, 11).

MATERIALS AND METHODS

Reagents. ^{3}H-labeled nucleosides and [^{32}P]phosphoric acid were obtained from I.C.N., Irvine, Calif.

Preparation and purification of ^{3}H-labeled nucleoside or ^{32}P-labeled virus. The procedures used to obtain labeled virus grown in the presence of [^{3}H]uridine (25 μCi/ml, 8.0 Ci/mmol), [^{3}H]cytidine (25 μCi/ml, 36 Ci/mmol), [^{3}H]guanosine (25 μCi/ml, 16.3 Ci/mmol), [^{3}H]adenosine (25 μCi/ml, 20.8 Ci/mmol), or [^{32}P]phosphate (200 μCi/ml) have been described (1, 9). Virus preparations were purified by polyethylene glycol precipitation followed by sucrose equilibrium gradient centrifugation (10).

Extraction and purification of labeled RNA. Labeled virus preparations obtained from sucrose gradients were diluted fivefold with 0.15 M NaCl and 0.01 M Tris-hydrochloride buffer (pH 7.4), adjusted to 1% (wt/vol) sodium dodecyl sulfate, and extracted with an equivolume of a mixture of 1 part of chloroform and 4 parts of redistilled phenol, *m*-cresol, 8-hydroxyquinoline (500:70:0.5 g, previously saturated with 0.15 M NaCl, 0.01 M Tris-hydrochloride, pH 7.4). The phenol-chloroform phase was reextracted with 0.4 M NaCl, 0.01 M Tris-hydrochloride (pH 7.4) and 1% sodium dodecyl sulfate and the combined aqueous phases were extracted with a fresh chloroform-phenol mixture. After alcohol precipitation, the RNA was chromatographed through a column of 4% agarose suspended in 0.4 M NaCl, 0.01 M Tris-hydrochloride, and 0.1% sodium dodecyl sulfate. The void fractions containing the viral RNA were recovered by alcohol precipitation, dissolved in 0.001 M EDTA (pH 7.4), and stored frozen at −20 C until required.

Alkali and ribonuclease digestion conditions. Alkali digestions were performed by adjusting an RNA sample to 0.33 M KOH and incubating at 37 C for 18 h. Potassium ions were exchanged for hydrogen ions using Dowex-50 [H^+ form] ion exchange resin until pH 7 was reached (11). Ribonuclease A or T_1 digestion conditions have been described previously (9, 11, 12).

DEAE-cellulose chromatography of oligonucleotides. The resolution of nucleotides by DEAE-cellulose column chromatography has been described (5, 11, 13). Nucleotides were quantitatively recovered by ethanol precipitation of their barium salts and quantitatively converted to and recovered as their hydrogen form by use of Dowex-50 [H^+] ion exchange resin as described previously (11).

Dowex-1-formate resolution of mononucleotides. The resolution of mononucleotides by Dowex-1-formate column chromatography followed the procedure described by Hurlbert et al. (7). Nucleoside tetraphosphates were recovered from the column by stripping with 88% formic acid.

Base-ratio determinations. Paper electrophoresis at pH 3.5 was employed to determine base ratios of the ^{3}H-labeled nucleoside or ^{32}P-labeled RNA samples as described elsewhere (11, 12).

RESULTS

Alkali digestion of ^{3}H-labeled nucleoside VSV RNA. Portions of VSV RNA individually labeled with each of the four ^{3}H-labeled nucleosides were subjected to alkali digestion. A sample of each digest was dissolved in scintillation fluid or evaporated to dryness and counted. It was found that the percentage of tritium exchange with water during alkali digestion varied depending upon the precursor nucleo-

side. For both the [^{3}H]guanosine- and [^{3}H]adenosine-labeled RNA, 48% of the label was recovered in water, whereas for the [^{3}H]cytidine- or [^{3}H]uridine-labeled RNA only 8% and 6%, respectively, were recovered in water. The distribution of label among the mononucleotides is shown in Table 1 in essential agreement with results reported earlier (9).

A sample of the [^{3}H]adenosine-labeled RNA digest was resolved by DEAE-cellulose column chromatography together with a pancreatic ribonuclease digest of 3 mg of chicken embryo RNA. The distribution of label relative to the mono-, di-, tri- (etc.) nucleotides is shown in Fig. 1, indicating that although most of the RNA was digested to mononucleotides, a small fraction of labeled oligonucleotides survived the alkali digestion. Evidently, to identify the terminal nucleotide, residual oligonucleotides would confuse interpretation of the results. It was found that two successive alkali digests were necessary to digest all oligonucleotides and reveal the 5′ terminal nucleotide (see below).

Identification of 5′ terminal nucleotide of VSV RNA. Mixtures of ^{32}P- and ^{3}H-labeled nucleoside VSV were digested with alkali, loaded on DEAE-cellulose, and subjected to chromatography as in Fig. 1. As soon as the labeled mononucleotides were eluted, the column was stripped by 0.7 M LiCl in 7 M urea buffer, and the resulting eluant fractions were pooled, mixed with 0.1 μmol each of 2′,3′- mixed AMP, CMP, GMP, and UMP nucleotides, and recovered as their barium salts by alcohol precipitation. After exchange of hydrogen for barium, the nucleotides were mixed with 100 μg of chicken embryo RNA, adjusted to 0.33 M KOH, and redigested for another 18 h at 37 C.

TABLE 1. *Base-ratios of labeled VSV RNA*[a]

Precursor nucleotide	[^{3}H]-adenosine (%)	[^{3}H]-guanosine (%)	[^{3}H]-cytidine (%)	[^{3}H]-uridine (%)	[^{32}P]-phosphate (%)
CMP	0.7	0.8	89.2	24.7	20.7
AMP	78.0	4.6	0.5	0.6	27.0
GMP	20.9	93.3	0.3	0.5	21.0
UMP	0.4	1.3	10.0	74.2	31.3

[a] ^{3}H-labeled nucleoside or [^{32}P]phosphate-labeled VSV RNA was obtained as described. A sample of each RNA was digested with alkali, and the products were resolved with 2′,3′- mixed mononucleotides of CMP, AMP, GMP, and UMP by paper electrophoresis at pH 3.5. The percentage of label recovered in each spot (identified by their ultraviolet light absorbance) is given.

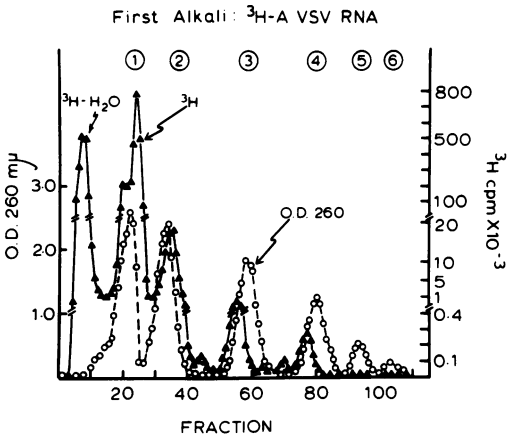

FIG. 1. *DEAE column chromatography at pH 8.0 of nucleotides obtained from an alkali hydrolysis of [^{3}H]adenosine-labeled VSV RNA. Columns (5 ml, 0.5 by 25 cm) of DEAE equilibrated in 7 M urea, 0.01 M Tris-hydrochloride, and 0.0003 M EDTA (pH 8.0) were loaded with nucleotide mixtures (see below) and eluted with a 160-ml linear gradient of LiCl (0.02 to 0.25 M) in 7 M urea, 0.01 M Tris-hydrochloride, and 0.003 M EDTA (pH 8.0). Fractions (about 0.8 ml) were collected, and their content of unlabeled nucleotides (optical density at 260 nm) or labeled nucleotides was determined by counting in a liquid scintillation spectrometer after mixing with 15 ml of the Triton:toluene scintillation cocktail (11). Nucleotide mixtures consisted of a pancreatic ribonuclease digest of unlabeled chicken embryo cellular RNA (3 mg of RNA, 0.3 mg of ribonuclease in 0.1 M NaCl, pH 7.0, incubated at 37 C for 60 min), and the ^{3}H-labeled nucleotides from a single alkali digest of the viral RNA. The alkali digest initially contained 5.84 × 10^6 counts/min of [^{3}H]adenosine-labeled RNA. Note that ^{3}H-labeled water derived by tritium exchange (see Results) eluted in the early fractions of the column. The elution positions of the mono-, di-, tri- (etc) oligonucleotides from the pancreatic ribonuclease digest are indicated.*

This second alkali digest was resolved by DEAE column chromatography at pH 8.0 with marker oligonucleotides derived from a pancreatic ribonuclease digest of 3 mg of chicken RNA. The results obtained for mixtures of ^{32}P-labeled VSV RNA plus [^{3}H]uridine-, [^{3}H]cytidine-, [^{3}H]guanosine-, or [^{3}H]adenosine-labeled VSV RNA are shown in Fig. 2. Only for the adenosine-labeled RNA were ^{3}H counts recovered in the tetranucleotide peak along with the ^{32}P-labeled material. The recovery of either label relative to the initial amount of labeled RNA was determined and the molar proportions were calculated (Table 2). The results indicated that the 5′ terminal nucleotide was pppAp.... The

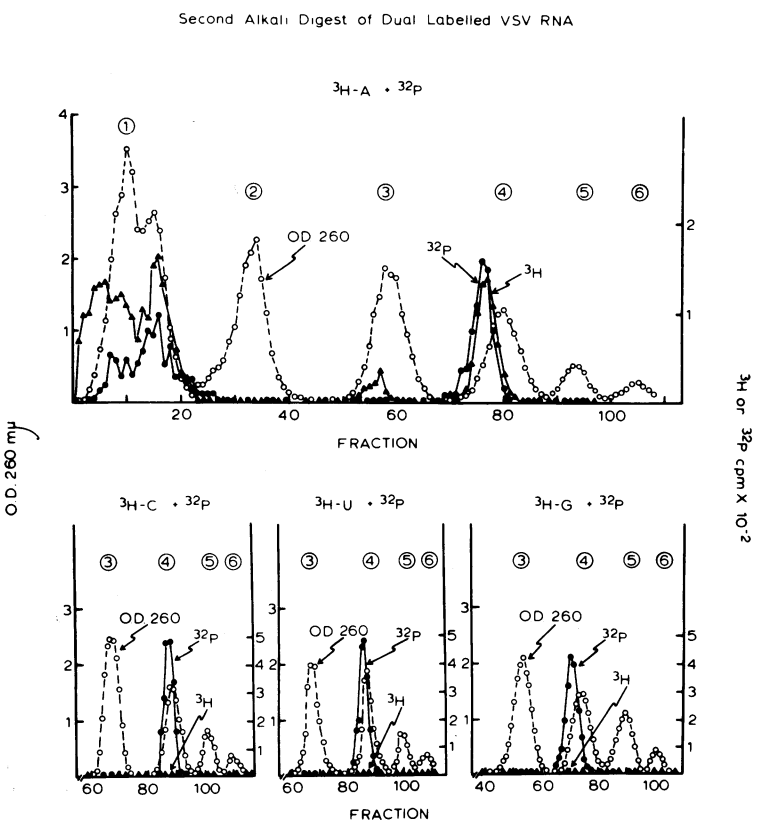

FIG. 2. *DEAE column chromatography at pH 8.0 of nucleotides obtained after two alkali digests of ^{32}P-labeled VSV RNA plus ^{3}H-labeled nucleoside VSV RNA. Mixtures of 8.7 × 10^{6} counts/min of [^{3}H]adenosine-labeled RNA plus 3.0 × 10^{6} counts/min of ^{32}P-labeled VSV RNA, 6.8 × 10^{6} counts/min of [^{3}H]cytidine-labeled RNA plus 7.8 × 10^{6} counts/min of ^{32}P-labeled VSV RNA, 8.0 × 10^{6} counts/min of [^{3}H]guanosine-labeled RNA plus 8.0 × 10^{6} counts/min of ^{32}P-labeled VSV RNA, or 6.7 × 10^{6} counts/min of [^{3}H]uridine-labeled RNA plus 7.7 × 10^{6} counts/min of ^{32}P-labeled VSV RNA were treated with two cycles of alkali digestion as described in Results and the second digests were resolved on DEAE columns with suitable marker nucleotides as described in Fig. 1.*

fact that adenosine tetraphosphate is recovered in the tetranucleotide isoplith (i.e., somewhat earlier than its net phosphate charge would predict) has been documented in previous communications (5, 11).

Identification of the penultimate nucleotide at the 5′ end of VSV RNA. A sample of [^{3}H]adenosine-labeled VSV RNA was digested with pancreatic ribonuclease to degrade the RNA at cytidine and uridine residues and the resulting oligonucleotides were resolved by DEAE-cellulose chromatography at pH 8.0 (Fig. 3). The region of the chromatogram (i.e., the tetra-, pentanucleotides) expected to contain pppApPyp (where Py is a pyrimidine residue) was pooled (5, 11) and a portion was subjected to alkali digestion and chromatographed on DEAE-cellulose. The number of ^{3}H counts per minute obtained in the tetranucleotide peak was equivalent to that expected for a single terminus of pppApPyp in the original digest.

A similar pancreatic digest of ^{32}P-labeled VSV resolved at pH 8.0 was used to give nucleotide pools corresponding to the tetranucleotides, tetra-pentanucleotides, penta-hexanucleotides, or hexa- and larger nucleotides. Each pool was subjected to alkali digestion but only the tetra-pentanucleotides gave a ^{32}P-labeled pppAp nucleotide upon subsequent alkali digestion (data not shown).

A second portion of the [^{3}H]adenosine-labeled tetra-pentanucleotide pool (Fig. 3) was chromatographed at pH 10.0 together with a pancreatic ribonuclease digest of 3 mg of chicken embryo RNA. Considerable resolution

TABLE 2. *Recovery of 5' nucleotides from alkali digests of labeled VSV RNA*[1]

Labeled RNA	Counts/min recovered in tetranucleotides	Mole equivalent per mole of RNA
(1) [^{3}H]adenosine	650	0.86
[^{32}P]phosphate	760	3.2
(2) [^{3}H]cytidine	19	0
[^{32}P]phosphate	2,000	3.8
(3) [^{3}H]guanosine	22	0
[^{32}P]phosphate	2,279	3.5
(4) [^{3}H]uridine	0	0
[^{32}P]phosphate	2,066	4.2

[a] Mixtures of (1) 8.7×10^6 counts/min of [^{3}H]adenosine-labeled RNA plus 3.0×10^6 counts/min of ^{32}P-labeled VSV RNA, (2) 6.8×10^6 counts/min of [^{3}H]cytidine-labeled RNA plus 7.8×10^6 counts/min of ^{32}P-labeled VSV RNA, (3) 8.0×10^6 counts/min of [^{3}H]guanosine-labeled RNA plus 8.0×10^6 counts/min of ^{32}P-labeled VSV RNA, or (4) 6.7×10^6 counts/min of [^{3}H]uridine-labeled RNA plus 7.7×10^6 counts/min of ^{32}P-labeled VSV RNA were treated with two cycles of alkali digestion (Fig. 2), and the ^{3}H or ^{32}P counts per minute recovered in the tetranucleotides were computed. The total nucleotides present in VSV RNA have been determined (11,200, reference 10) so that the count equivalent for 1 mol of phosphate obtainable from 1 mol of RNA could be calculated for each digest (taking into consideration the ^{32}P decay during the analysis time course). Likewise on the basis that VSV RNA contains 3,100 adenosine, 3,500 uridine, 2,100 cytidine, and 2,500 nucleotides (10), the count equivalent from 1 mol of adenosine, guanosine, cytidine, and uridine could be calculated after correcting for the tritium exchange (e.g., 48% for each alkali digest for adenosine, see Results). The recovery of each label is given in terms of the mole equivalent obtained from 1 mol of RNA. The molar ratio of [^{3}H]-adenosine to [^{32}P]phosphate in mixture 1 is 1:3.7.

of the tetra-pentanucleotides was obtained. The reason for choosing pH 10 was due to an observation we have made that chromatography of the pppAp nucleotide is affected by pH changes to a greater extent than tetranucleotides obtained by pancreatic ribonuclease digestion. For instance, at pH 7.0 pppAp is eluted after the tetranucleotides, whereas at pH 9.0 it elutes before the tetranucleotides, as can be seen in one of the chromatograms (Fig. 4) in which a batch of urea buffer possessing a pH of 7.8 was used.

A mixture of ^{32}P- and [^{3}H]cytidine-labeled VSV RNA was subjected to pancreatic ribonuclease digestion and the tetra-pentanucleotides were recovered and chromatographed at pH 10.0 (Fig. 4). Of the various peaks resolved by the pH 10.0 chromatography, only the pool 2 nucleotides gave a ^{32}P-labeled pppAp upon subsequent alkali digestion (Fig. 4). Although the alkali digest contained labeled pppAp and (presumably) ^{3}H-labeled CMP in equimolar proportions, there were evidently more ^{32}P-labeled mononucleotides than expected (the ^{32}P-labeled pppAp to ^{32}P-labeled mononucleotide ratio was 4:3). Did these mononucleotides contain ^{32}P-labeled UMP in molar equivalent proportions to the pppAp? To answer this question, the alkali digest of the pool 2 nucleotides was chromatographed on Dowex-1-formate ion exchange resin, the CMP, AMP, GMP, UMP, and pppAp were resolved, and the label therein was computed (Table 3). Very little ^{32}P-labeled UMP was detected, although a molar equivalent of ^{32}P-labeled CMP was present. Consequently, the 5' terminal nucleotide sequence was deduced to be pppApCp. . . .

The third nucleotide at the 5' terminus of VSV RNA. A ribonuclease T_1 digest of ^{32}P-labeled VSV RNA was chromatographed at pH 8.0 on DEAE-cellulose (Fig. 5) and pools of nucleotides were made corresponding to the

TABLE 3. *Dowex-1-formate chromatography of alkali digest of nucleotide pool containing 5' terminal pancreatic ribonuclease fragment*[a]

Nucleotides	Counts recovered
^{3}H-labeled CMP	600
^{32}P-labeled CMP	144
^{3}H-labeled AMP	0
^{32}P-labeled AMP	122
^{3}H-labeled GMP	0
^{32}P-labeled GMP	71
^{3}H-labeled UMP	0
^{32}P-labeled UMP	35
^{32}P-labeled pppAp	534

[a] The pooled nucleotides obtained from pH 10.0 DEAE-cellulose chromatography of the tetra-pentanucleotides of a pancreatic digest of [^{3}H]cytidine- and [^{32}P]phosphate-labeled VSV RNA (Fig. 3) were digested with alkali, neutralized, mixed with 2 μmol each of 2',3'- mixed mononucleotides (CMP, AMP, GMP, and UMP), and resolved by Dowex-1-formate column chromatography. The elution and identification of the mononucleotides were monitored by their absorbance at 280 and 260 nm and the counts per minute content was computed. The column was stripped by 88% formic acid to recover the pppAp.

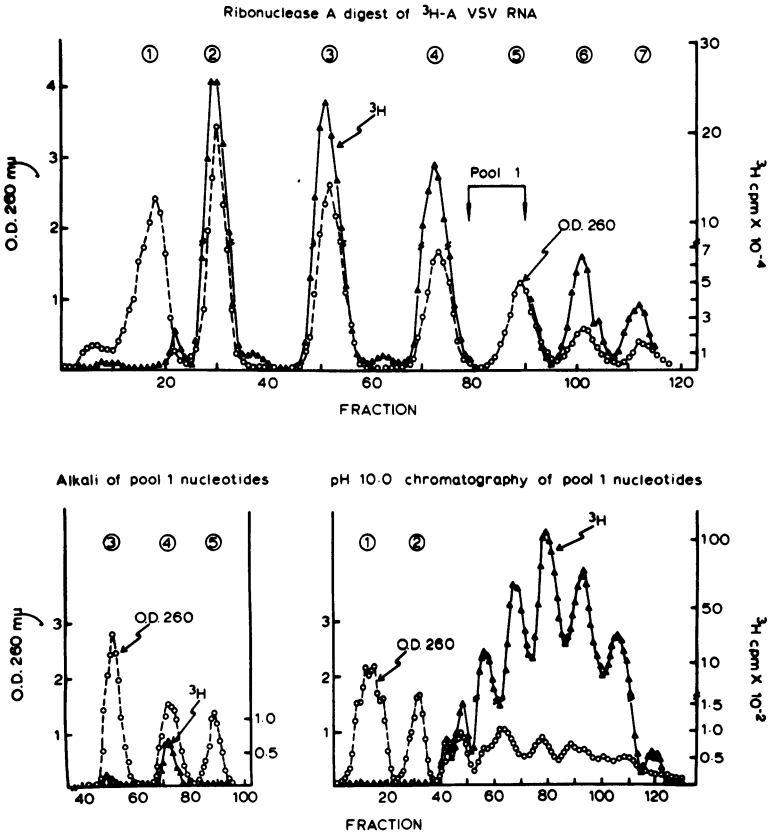

FIG. 3. *DEAE column chromatography of a pancreatic ribonuclease digest of [³H]adenosine-labeled VSV RNA. [³H]adenosine-labeled VSV RNA (5 × 10⁶ counts/min) plus 3 mg of chicken embryo fibroblast RNA were digested with pancreatic ribonuclease and resolved by column chromatography (upper panel) as described in Fig. 1. The indicated fractions were pooled and a portion was either digested with alkali and resolved at pH 8.0 by DEAE chromatography (lower left panel) or resolved without digestion by DEAE chromatography with a pancreatic ribonuclease digest of chicken embryo fibroblast RNA at pH 10.0 (lower right panel). The apparent resolution of the chicken embryo fibroblast oligonucleotides at pH 10.0 does not reflect resolution of oligonucleotides according to their net charge, but rather appears to relate to some extent to their base composition.*

expected position of nucleotides of sequence pppGp, pppXpGp, pppXpYpGp, pppXpYpZpGp, etc. (5, 11). Each pool was subjected to alkali digestion and the digests were resolved by DEAE chromatography. Only for the pool expected to contain pppXpYpGp was a labeled nucleoside tetraphosphate obtained. Consequently, the 5′ terminal nucleotide sequence of VSV RNA was deduced to be pppApCpGp...

DISCUSSION

In light of the recent discovery that the messenger polarity strands of the double-stranded RNA genomes of cytoplasmic polyhedrosis virus (9) and human reovirus (K. Miura, K. Watanabe, M. Sugiura, and A. T. Shatkin, Proc. Nat. Acad. Sci. U.S.A., in press) possess modified 5′ nucleotides, presumed to consist of a 2′-*O*-methyl group on the ribose moiety of the nucleotide, we were interested to know if VSV also contains a modified 5′ nucleotide. Evidence has been presented that the modified ribose prevents alkali, ribonuclease T_1, or pancreatic ribonuclease digestion at the phosphodiester bond between the modified ribose and the next nucleotide, presumably by preventing 2′,3′-cycle phosphate intermediates (8; K. Miura et al., in press). The presence of four phosphates in a nucleotide residue obtained after alkali digestion is, therefore, not sufficient to identify the 5′ terminal nucleotide since the sequence could be

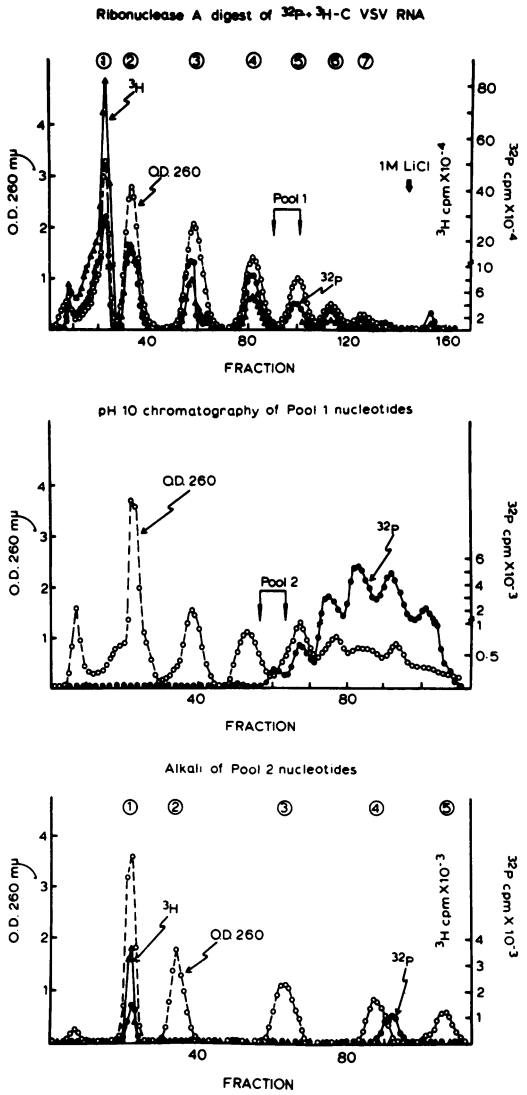

FIG. 4. *DEAE column chromatography of a pancreatic ribonuclease digest of [^3H]cytidine and ^{32}P-labeled VSV RNA. A mixture of [^3H]cytidine (5.2 × 10^6 counts/min) and ^{32}P-labeled (5.2 × 10^6 counts/min) VSV RNA was subjected to pancreatic ribonuclease digestion and the products were resolved by DEAE column chromatography at pH 8.0 (upper panel). The indicated fractions were pooled and chromatographed at pH 10.0 (lower right panel). A portion of the indicated fractions from the pH 10.0 chromatogram was subjected to alkali digestion and the digest was subjected to DEAE chromatography at pH 7.8 (lower left panel). The recovery of ^3H-labeled CMP in the alkali digest was equivalent to 1 mol of ^3H-labeled mononucleotide per original mole of ^3H-labeled RNA. The recovery of ^{32}P in the tetranucleotides was equivalent to 4 mol (as expected for pppAp), although the ratio of ^{32}P-labeled monophosphates to ^{32}P-labeled pppAp was 3 to 4.*

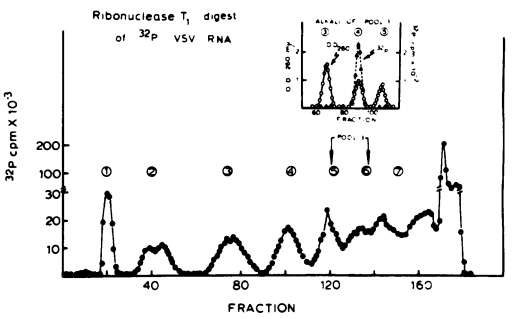

FIG. 5. *DEAE column chromatography of a ribonuclease T_1 digest of ^{32}P-labeled VSV RNA. A sample of ^{32}P-labeled VSV RNA (2.5 × 10^6 counts/min) was digested with ribonuclease T_1 and the products were resolved by DEAE column chromatography at pH 8.0. The indicated fractions were pooled, and the nucleotides were recovered and digested with alkali. This digest was then chromatographed at pH 8.0 (inset) with marker nucleotides derived from a pancreatic ribonuclease digest of 1.5 mg of chicken embryo fibroblast RNA. After fraction 160, 1 M LiCl was used to strip the column.*

pppXp or ppX*pYp, where X* indicates a nucleotide with a modified ribose.

We have sought another approach to settle this question which involved using both base and phosphate-labeled RNA and comparing the molar ratios of labels in the 5′ nucleotide residue. Our results indicate that there is a pppAp sequence and not a ppA*pAp or other dinucleotide sequence.

It is surely more than coincidental that the 5′ nucleotide sequence of the VSV viral RNA (pppApCpGp. . .) is the same as one of the 5′ initiation sequences we have found in in vitro transcription analyses for VSV (Indiana or New Jersey serotypes) as well as Chandipura, Cocal, and Piry viruses (5). To determine whether this pppApCpGp sequence is related to replication attempts in the in vitro system, we will have to identify the 5′ terminal nucleotide sequence of the 42S viral complementary RNA (which is no mean task) as well as those of the intracellular VSV messenger RNA species.

ACKNOWLEDGMENTS

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