Initiation and Direction of RNA Transcription by Vesicular Stomatitis Virus Virion Transcriptase

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The initiation of RNA transcription by the virion-bound RNA transcriptase of vesicular stomatitis virus has been examined. Multiple initiation sequences have been observed, two of which have been characterized (pppApCpGp . . . and pppGpCp . . .) suggestive of a transcription process which can start at different sites along the template RNA. By the use of sequential labeling techniques and exonucleases, it has been determined that there is a 5' to 3' direction of product RNA synthesis.

Vesicular stomatitis virus (VSV) is a rhabdovirus which possesses a virion RNA-dependent RNA polymerase (2). In vitro analyses have shown that the polymerase is responsible for repetitive transcription of the virion genome into RNA species which are complementary to the viral RNA and have a spectrum of molecular weights which principally range between 1.1×10^6 and 2×10^6 (21s to 10s) (references 3, 7, 8). It has been demonstrated that the transcription process performed by virion (VSV-1) transcriptase is not only complete but also sequential, as shown by the fact that RNA product made during short-reaction incubations will not hybridize to added VSV-defective, T-particle RNA (VSV-111 RNA), whereas some of the RNA product made in longer reaction incubations with VSV-1 will hybridize to added VSV-111 RNA (14).

Since the size of the predominant product RNA species is about one-tenth the size of the viral RNA, which is estimated to be about 4×10^6 daltons, questions arise about how and why one obtains transcription products representative of the entire genome but smaller than the template RNA. In previously reported experiments, no nuclease activity could be demonstrated using the criterion of size reduction of a small amount of added, labeled, RNA (7).

To obtain answers to these questions, studies into the initiation process of RNA transcription have been undertaken. It has been found that RNA synthesis is initiated by either ATP or GTP, but not by UTP or CTP. The process of transcription is in a 5' to 3' direction and several 5' RNA product sequences have been observed of which pppApCpGp . . . and pppGpCp . . . have been identified.

MATERIALS AND METHODS

Preparation of virus. The purification of unlabeled VSV through polyethylene glycol (PEG-carbowax 6000) precipitation and successive equilibrium and velocity sucrose gradient centrifugations has been described (1, 14). The virus preparation contained no defective T particles. After the velocity sucrose gradient centrifugation, the suspension was concentrated by centrifugation for 2 h at 35,000 rpm at 4°C in a Spinco SW41 rotor. The sucrose supernatant fluid was removed, 1 ml of 0.15 M NaCl, 0.01 M Tris-hydrochloride buffer (pH 8.0) was added to the pellet. After being left for 12 h at 4°C, the virus pellet was gently suspended by pipette, and aggregated virus was removed by centrifugation at 4°C for 20 min at 10,000 rpm in a Sorvall HB4 rotor.

Preparation of VSV RNA-ribonucleoprotein cores. Procedures for removing the virion glycoprotein and membrane protein by Triton treatment at room temperature in the presence of 1 M NaCl followed by PEG-dextran T 500 phase separation have been described (9). The dextran phase, containing the virion RNA-ribonucleoprotein core, was washed twice with 1-ml volumes of fresh PEG phases containing Triton and 1 M NaCl and finally with a 1-ml volume of a PEG phase containing 0.15 M NaCl, 0.01 M Tris-hydrochloride (pH 8.0). Usually virus preparations containing 2 to 4 mg of protein were extracted by this procedure, and this resulted in a final dextran phase containing essentially all the virion RNA, all the nucleoprotein (N), most of the virion minor proteins, as well as all of the initial transcriptase activity (9).

Synthesis of ^{32}P - α -ribonucleoside and ^{32}P - γ -ribonucleoside triphosphates. The synthesis of ^{32}P -labeled ribonucleoside triphosphates (5–20 Ci/mmol) has been described (6, 13).

Transcriptase reaction conditions and purification of transcription product RNA. Ten-fold reaction mixtures (1.25 ml) containing a ^{32}P - γ -

ribonucleoside triphosphate and a ^3H -ribonucleoside triphosphate (^3H -ATP or ^3H -GTP) were primed by 0.3 ml of fresh VSV RNA-ribonucleoprotein cores (VSV-dextran cores) and other ingredients as described previously (1, 9, 14; also see table and figure legends). After incubation at 31 C, the incubation was terminated by addition of sodium dodecyl sulfate (SDS) to 1% (wt/vol). This material was mixed with 200 μg of unlabeled cellular RNA and 1 ml of a mixture of phenol-*m*-cresol-8-hydroxyquinoline and extracted for RNA as described previously (8). RNA was separated from triphosphates and other reaction ingredients by chromatography through Sephadex G-50 and precipitated from the void volume by alcohol. To remove dextran which co-precipitated with the RNA, the pellet obtained by centrifugation was dissolved in 0.5 ml of 0.003 M EDTA, pH 7.4, and mixed with 4.5 ml of a cesium sulfate solution to give a refractive index (n) of 1.3860. This suspension was then centrifuged for 36 h at 35,000 rpm at 15 C in a Spinco 39 L rotor, and fractions were collected and assayed for refractive index and content of trichloroacetic acid-insoluble RNA. The fractions containing RNA were dialyzed against 0.003 M EDTA (pH 7.4) to remove cesium sulfate and then stored at -20 C until use.

Reaction mixtures containing ^{32}P - α -ribonucleoside triphosphates as the only labeled precursor for RNA synthesis were treated similarly.

Reaction incubations containing ^{32}P - γ -ribonucleoside triphosphates were monitored by paper electrophoresis to determine the amount of nucleoside triphosphate phosphotransferase activity, as described previously (13; P. Roy and D. H. L. Bishop, Fed. Proc., p. 1153, 1971).

Agarose gel column chromatography. A 20-fold reaction mixture primed by unlabeled VSV was incubated for 30 min at 31 C in a reaction mixture containing ^{32}P - α -UTP to label the product RNA species. The whole reaction mixture was brought to room temperature, mixed with sodium deoxycholate (1 mg/ml final concentration), NaCl (1 M final concentration), and 0.1 M Tris-hydrochloride (pH 8.0), 0.005 M 2-mercaptoethanol, 3 mg of Triton N 101 per ml, and 0.01 M MgCl_2 . The column (90 by 1 cm) was then eluted at 4 C, with the same solution and fractions collected. By this procedure the VSV RNA-ribonucleoprotein cores containing all the RNA transcriptase activity and complexes of product RNA with the virion template were separated from free product as well as radioactive triphosphates and the virion membrane protein and glycoprotein (9). The void fractions were used to prime a 20-fold standard reaction mixture containing ^3H -UTP and incubated at 31 C for 10 min. RNA was extracted and separated from triphosphates by Sephadex column chromatography as described above, precipitated with alcohol, and dissolved in 0.003 M EDTA (pH 7.4).

Nuclease and alkali digestions: separation of oligonucleotides by DEAE column chromatography. Procedures for digestion of labeled and unlabeled RNA by pancreatic ribonuclease A

or ribonuclease T_1 or by exonucleases have been described elsewhere (5, 15). Alkali digestion was performed in 0.3 M KOH for 18 h at 37 C. Potassium ions were then exchanged for hydrogen ions using Dowex-50 (H^+ form) ion-exchange resin as described previously (6). The resolution of oligonucleotides according to their net phosphate charge (at pH 8.0 on DEAE by elution with a linear gradient of lithium chloride in 7 M urea, 0.01 M Tris-hydrochloride, 0.003 M EDTA) has also been described previously (5, 18). Elution at pH 5.5 employed a sodium citrate buffer system (see figure legends).

Thin-layer chromatography. One-dimensional, thin-layer chromatography to separate purine tetraphosphates was performed using PE1 cellulose F anion exchanger (10). Precoated polyethylene plates were purchased from Brinkman Co. and washed with deionized water prior to use. After drying, samples were applied at the starting point together with marker quantities of the sodium salts of guanosine 5' tetraphosphate (ppppG) and adenosine 5' tetraphosphate (pppA) (Sigma Chemical Co.). The marker nucleotides (about 0.05 μmol) were detected under a UV lamp. After drying, Desaga paper tongues A and B (Brinkman Co.) were clamped to the top of the strip, and the chromatogram was developed by 0.75 M KH_2PO_4 adjusted to pH 3.4 with 0.75 M phosphoric acid. When buffer had reached and been absorbed by the paper tongues (about 2 h at room temperature), the resolution between the marker nucleotides was checked by UV light, and, if necessary, new tongues were applied and the chromatography was continued. When sufficient resolution was obtained, the chromatogram was dried and the positions of the marker nucleotides were determined.

The polyethylene strip was then cut up, and the distribution of ^{32}P radioactivity was determined in a scintillation counter. Ribonucleoside monophosphates were absorbed into the paper tongues during this chromatography (10).

RNA gel electrophoresis RNA annealing conditions. The conditions for annealing RNA and electrophoresis in 2% polyacrylamide gels have been described previously (3, 4, 7, 8).

RESULTS

Separation of phosphotransferase from the VSV RNA-ribonucleoprotein cores using PEG dextran phase separation. We have previously described an enzyme activity associated with budding viruses which empirically causes the randomization of the γ -phosphates of nucleoside triphosphates (13; P. Roy and D. H. L. Bishop, Fed. Proc., p. 1153, 1971). This enzyme activity, designated nucleoside triphosphate phosphotransferase, can be differentiated from virus-associated nucleoside triphosphatase—such as ATPase which degrade triphosphates to their corresponding diphosphates—since the latter is inhibited by a nonionic detergent (13). In contrast,

nucleoside triphosphate phosphotransferase is activated by a nonionic detergent (13). Since nucleoside diphosphates are potent acceptor substrates for the phosphotransferase and minor quantities of diphosphates are present in every triphosphate preparation (P. Roy and D. H. L. Bishop, *Fed. Proc.*, p. 1153, 1971), it is not known if the phosphotransferase is a nucleoside diphosphate kinase associated with, or is part of, a non-specific nucleoside triphosphatase. Nor is it known if either of these activities are related to the more recently described protein kinase also found in such virus preparations (17).

Clearly, whatever the significance of such enzyme activities, to perform transcription initiation involving ^{32}P - γ -ribonucleoside triphosphates, it was necessary to remove such activities from the virion transcriptase. A method which has proved satisfactory for the removal of most of the phosphotransferase activity from the endogenously templated virion transcriptase activity involves PEG:dextran T-500 phase separation of Triton- and NaCl-dissociated VSV virions (9). Ninety-nine percent of the initial phosphotransferase activity was removed from the transcriptase enzyme-RNA complex (VSV-dextran cores) by this procedure and recovered in the PEG phases. The VSV-dextran cores were therefore used in the transcriptase initiation studies described below. Reaction mixtures employing ^{32}P - γ -ribonucleoside triphosphates for such initiation studies were routinely monitored for phosphotransferase activity by paper electrophoresis as described previously (13). No randomization of ^{32}P to other triphosphates in such reaction mixtures was detected during 2 h of incubation at 31 C.

The incorporation of ^{32}P - γ -ribonucleoside triphosphates into product RNA by VSV transcriptase. Reaction mixtures employing ^{32}P - γ -CTP (10 Ci/mmol) and ^3H -GTP (0.4 Ci/mmol), or ^{32}P - γ -UTP (7 Ci/mmol) and ^3H -GTP (0.4 Ci/mmol), or ^{32}P - γ -GTP (5 Ci/mmol) and ^3H -ATP (1 Ci/mmol), or ^{32}P - γ -ATP (10 Ci/mmol) and ^3H -GTP (0.4 Ci/mmol) were incubated through 2 h at 31 C with VSV-dextran cores prepared as described in Materials and Methods. The ^3H -labeled triphosphates were included to monitor the net RNA synthesis. RNA was extracted from the reaction mixtures, purified from triphosphates by Sephadex column chromatography, and separated from dextran by cesium sulfate equilibrium gradient centrifugation. The recovery of labeled RNA from these reaction mixtures is given in Table 1.

Neither reaction involving ^{32}P - γ -UTP nor ^{32}P - γ -CTP showed any appreciable incorporation of ^{32}P into product RNA, although the reactions did exhibit a net incorporation of ^3H label into RNA. Reactions involving either ^{32}P - γ -GTP or ^{32}P -

TABLE 1. Incorporation of ^{32}P - γ - and ^3H -ribonucleoside triphosphates by VSV-dextran cores^a

Labeling conditions	^{32}P incorporation (picomole/reaction product)	^3H incorporation (picomole/reaction product)
1. ^{32}P - γ -CTP, ^3H -GTP	Less than 2	41,000
2. ^{32}P - γ -UTP, ^3H -GTP	Less than 2	38,000
3. ^{32}P - γ -GTP, ^3H -ATP	86	55,000
4. ^{32}P - γ -ATP, ^3H -GTP	352	48,000

^a Reaction mixtures containing both ^{32}P - and ^3H -labeled ribonucleoside triphosphates were incubated at 31 C for 2 h with VSV-dextran cores prepared from 2.5 mg of virus protein as described in Materials and Methods. RNA was extracted from the reaction mixtures, purified from triphosphates by Sephadex G-50 column chromatography and then from dextran by cesium sulfate equilibrium centrifugation. The amount of recovered labeled RNA is expressed in terms of the total picomoles of label incorporated. About 10% of the labeled RNA (both ^3H and ^{32}P) was lost by adherence to the centrifuge tube walls during the cesium sulfate centrifugations. It has been shown that the ratio of ATP to GTP incorporation into RNA in VSV transcription assays is about 1.4:1.0 in double-label triphosphate incorporation studies (6), in reasonable agreement with the relative ^3H incorporations observed in labeling conditions 3 and 4 given in this table.

γ -ATP resulted in the incorporation of ^{32}P into product RNA. These results suggested, therefore, that only purines are involved in the initiation of product RNA synthesis, and, from the observation that more ^{32}P - γ -ATP was incorporated than ^{32}P - γ -GTP (on the basis of the concomitant ^3H -purine monophosphate incorporation in each reaction [see legend to Table 1]), it was inferred that during these reaction incubations there were more product initiations involving ATP than GTP.

Demonstration that ^{32}P -labeled product RNA was complementary to VSV virion RNA. Samples of ^{32}P - γ -GTP- or ^{32}P - γ -ATP-labeled product RNA were mixed with 1 μg of ^3H -uridine-labeled VSV virion RNA and subjected to polyacrylamide gel electrophoresis before or after annealing at 60 C for 2 h in 0.4 M NaCl (8). The distribution of ^3H - and ^{32}P -labeled RNA for the ^{32}P - γ -ATP reaction product nucleic acids are shown in Fig. 1. Similar results were ob-

FIG. 1. Gel electrophoresis of ^{32}P - γ -ATP-labeled VSV product RNA before and after annealing to ^3H -VSV RNA. A sample (100 μl iters) of the ^{32}P - γ -ATP-labeled VSV product RNA (Table 1) was mixed with 20 μl iters of ^3H -VSV RNA ($3\text{ }\mu\text{g}$: 6×10^5 counts/min) and 10 μl iters of 4 M NaCl and then (A) subjected directly to electrophoresis in a 2% polyacrylamide gel for 2 h at 50 V and 10 mA. After slicing, the gel slices were dissolved in 0.5 ml of 30% (vol/vol) H_2O_2 at 60 C for 6 h and dissolved and counted in 15 ml of a mixture of 1 part Triton X100:2 parts toluene containing scintillation fluors. A second identical RNA sample (B) was annealed at 60 C for 2 h prior to electrophoresis. Note that the added ^3H label in VSV RNA was in great excess by comparison to ^3H product RNA label.

tained with the ^{32}P - γ -GTP reaction product nucleic acids. It was found that, prior to annealing, ^{32}P label was recovered in fast-moving RNA species as well as in species which migrated slightly slower than the free ^3H -viral RNA (i.e., VSV reaction product-template, RNA complexes). After annealing, almost all the ^{32}P and most of the ^3H label was recovered in these slow-moving species. Since it has been shown previously that annealed product-template RNA complexes of VSV migrate slower than the free viral RNA (8), these results indicate that most, if not all, of the ^{32}P - γ -ATP (and ^{32}P - γ -GTP)

labeled product RNA was complementary to the virion RNA.

Samples of ^{32}P -labeled RNA were hydrolyzed by 0.3 M KOH at 37 C for 18 h and neutralized with Dowex-50 (H^+) ion-exchange resin. To each hydrolysate were added a pancreatic ribonuclease digest of unlabeled cellular RNA and ^3H -TTP or ^3H -ATP for markers. The mixture was then loaded and resolved on DEAE by using a linear gradient of lithium chloride in 7 M urea at pH 8.0 (Fig. 2 and 3) as described in the Fig. 2 legend. It has been shown that resolution of nucleoside phosphates and oligonucleotides obtained by

FIG. 2. DEAE column chromatography at pH 8.0 of nucleotides obtained from ^{32}P - γ -ATP-labeled VSV product RNA. Columns (5 ml) of DEAE equilibrated in 7 M urea, 0.01 M Tris-hydrochloride, 0.003 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, 0.003 M EDTA (pH 8.0). Fractions (about 1.0 to 1.3 ml) were collected, and their content of unlabeled nucleotides (optical density at 260 nm) or labeled nucleotides (^3H and ^{32}P) was determined and counted in a liquid scintillation spectrometer after mixing with 15 ml of the Triton:toluene scintillation cocktail (Fig. 1). Nucleotide mixtures consisted of a pancreatic ribonuclease digest of unlabeled HeLa cellular RNA (5 mg RNA, 0.5 mg ribonuclease in 0.02 M EDTA, pH 7.0, incubated at 37 C for 30 min), together with ^3H -TTP to act as an additional marker, and the ^{32}P -labeled nucleotides. A, an alkali digest of ^{32}P - γ -ATP product was included. B, A ribonuclease T_1 digest of ^{32}P - γ -ATP RNA was chromatographed. In this latter case, the labeled RNA was mixed with 100 μg of unlabeled RNA and incubated with 10 μg of ribonuclease T_1 at 37 C for 30 min and then extracted with phenol to remove the ribonuclease. The aqueous phase, containing the nucleotides, was then mixed with ^3H -TTP and a pancreatic digest of unlabeled RNA (similarly extracted by phenol to remove the pancreatic ribonuclease). The combined mixture was then extracted five times with ether to remove phenol (which absorbs UV light at 272 nm), and final traces of ether were removed by bubbling air through the solution. The nucleotides were then adsorbed and resolved by DEAE chromatography as before. The recovery of ^{32}P between the pentanucleotides and hepta-octanucleotides is evidence to the lack of pancreatic ribonuclease digestion during chromatography of the ribonuclease T_1 digestion products of the ^{32}P -labeled RNA (compare C). C, the ^{32}P - γ -ATP-labeled RNA was included in the pancreatic ribonuclease digestion of unlabeled RNA. Note that the distribution of ^3H -labeled VSV product RNA was masked by the amount of ^3H -TTP added.

FIG. 3. DEAE column chromatography at pH 8.0 of nucleotides obtained from ^{32}P - γ -GTP-labeled VSV product RNA. DEAE column chromatography of ^{32}P - γ -GTP-labeled product RNA digested by alkali (A), ribonuclease T_1 (B), or pancreatic ribonuclease (C) was performed as described in Fig. 2.

pancreatic ribonuclease digestion on DEAE chromatography is largely related to their net phosphate charge at pH 8.0 (18). Consequently, both a pancreatic ribonuclease digest of unlabeled RNA, containing moni-, di-, tri-, tetra- (etc.) nucleotides, and a ^3H -nucleoside triphosphate were included as markers. The results presented in Fig. 2 and 3 clearly indicate that both the ^{32}P - γ -ATP and the ^{32}P - γ -GTP product RNA were labeled at the 5' terminus since the label in both cases was recovered in nucleoside tetraphosphates which chromatographed with the marker tetranucleotides.

Although theoretically and from the point of view of their net phosphate charge, pppAp and pppGp at pH 8.0 might be expected to elute with the pentanucleotides, it has been shown previously that they, in fact, elute earlier than pentanucleotides (16).

Initial sequence analyses of the 5' ends of VSV product RNA. Although both ^{32}P - γ -ATP and ^{32}P - γ -GTP labeled the 5' end of VSV transcription product RNA, neither this observation nor the recovery of label in nucleoside tetraphosphates after alkali digestion proved that the label was in the corresponding pppAp and pppGp nucleotides. It could be argued that residual phosphotransferase activity randomized the γ -phosphates on the 5' terminus of product RNA.

Digestion of the ^{32}P - γ -ATP and ^{32}P - γ -GTP reaction products by either pancreatic ribonuclease or ribonuclease T_1 confirmed that each label was associated with its progenitor nucleoside (Fig. 2 and 3). Guanosine tetraphosphate was obtained from the ^{32}P - γ -GTP-labeled product after ribonuclease T_1 digestion, whereas the corresponding digest of the ^{32}P - γ -ATP-labeled RNA did not give a nucleoside tetraphosphate (Fig. 2). Pancreatic ribonuclease digestion of either RNA sample did not give a nucleoside tetraphosphate (Fig. 2 and 3), and this result confirmed that the labeled 5' terminal nucleotide from the ^{32}P - γ -ATP reaction was in fact adenosine.

The results obtained by these experiments also indicated what initial sequences were present at the 5' ends of the product RNA species. It appeared that the principle terminus from the ^{32}P - γ -ATP reaction product had a sequence of pppAp (U or C) pGp . . . A minor peak obtained in approximately the same quantities from both nuclease digestions of the ^{32}P - γ -ATP reaction products apparently possessed a sequence pppApAp (C or U) p (A, C, or U) pGp . . . This was suggested since it occurred approximately one charge higher than the principle ^{32}P -labeled pancreatic ribonuclease digestion product, but two charges higher than the principle ^{32}P -labeled ribonuclease T_1 digestion product (Fig. 2).

From the nuclease digestions of the ^{32}P - γ -GTP

reaction product nucleic acids (Fig. 3), it was initially concluded that the principle product terminus was pppGp (G or A) p (C or U) p . . . but that there was also a minor species which had the sequence pppGp (C or U) p . . .

Prior to performing detailed sequence analyses, a method was sought for more effectively separating the 5' terminal oligonucleotides from the other nonterminal oligonucleotides. It was found that, when a pancreatic ribonuclease digest of ^{32}P - γ -ATP-labeled VSV product RNA was resolved on DEAE in 7 M urea at pH 5.5 in citrate buffer, the major ^{32}P -labeled nucleotide eluted after the pentanucleotides while the minor ^{32}P component eluted after the hexanucleotides (Fig. 4). All of the ^{32}P radioactivity added to the column was recovered in these two peaks.

To confirm that the ^{32}P nucleotide which eluted after the pentanucleotides at pH 5.5 was the same as the nucleotide which eluted prior to the pentanucleotides at pH 8.0, the following experiment was performed. Product RNA labeled by ^{32}P - γ -ATP was digested with pancreatic ribonuclease, and the oligonucleotides were resolved at pH 5.5. The post-pentanucleotide fractions, which contained the major ^{32}P -labeled nucleotide, were pooled, diluted fourfold, adjusted to pH 8.0 with KOH, and then mixed with a fresh pancreatic ribonuclease digest of unlabeled RNA and ^3H -TTP and resolved by chromatography on DEAE at pH 8.0. The ^{32}P label again eluted prior to the pentanucleotides as before (see Fig. 3), confirming that it was indeed the same terminal nucleotide.

A pancreatic ribonuclease digest of ^{32}P - γ -GTP-labeled VSV product RNA was also subjected to column chromatography at pH 5.5 with appropriate markers as shown in Fig. 4. As at pH 8.0, two ^{32}P -labeled nucleotides were eluted containing all the added label. The minor peak was retrieved just after the unlabeled hexanucleotide markers, whereas the major peak was recovered before the unlabeled heptanucleotides.

These observations gave rise to a relatively simple purification procedure to separate the 5' nucleotides from other nucleotides, namely, chromatography at pH 8.0 followed by chromatography at pH 5.5.

Sequence analyses of the 5' terminal oligonucleotides of VSV transcription product RNA labeled by ^{32}P - α -ribonucleoside triphosphates. Reactions run in the presence of ^{32}P - α -ribonucleoside triphosphates (UTP, CTP, GTP, or ATP) were incubated with VSV-dextran cores for 2 h at 31 C, and the reaction product nucleic acids were purified as described in Materials and Methods.

^{32}P - α -CTP-labeled RNA. RNA obtained from a reaction containing ^{32}P - α -CTP (1.3×10^7

FIG. 4. DEAE column chromatography at pH 5.5 of nucleotides obtained from ^{32}P - γ -ATP- or ^{32}P - γ -GTP-labeled RNA. A pancreatic ribonuclease digest of unlabeled RNA and ^{32}P - γ -ATP-labeled RNA (A) was mixed with ^3H -TTP and adjusted to pH 5.5 with HCl. The mixture was applied to a 5-ml column of DEAE equilibrated in 7 M urea, 0.02 M sodium citrate, 0.003 M EDTA, pH 5.5, and eluted with a 160-ml linear gradient of LiCl (0.02 to 0.20 M). Fractions were collected and monitored for optical density and content of radioactivity. A digest of ^{32}P - γ -GTP-labeled RNA was analyzed similarly (B).

counts/min) was digested by pancreatic ribonuclease, and the resulting oligonucleotides were separated by DEAE-7M urea column chromatography at pH 8.0 together with a pancreatic digest of unlabeled cellular RNA and ^3H -TTP for markers. The result is shown in Fig. 5. Fractions expected to contain the 5' terminal nucleotide sequences were pooled (pools A and B) and mixed with 0.1 μmol of each of the four 2',3' ribonucleoside monophosphates to act as a coprecipitant. To the mixtures were added 0.1 ml of a saturated solution of barium chloride and 5 volumes of ethanol. After storage at 4 C for 2 h, the flocculant precipitate formed by the insoluble

barium nucleotides was collected by centrifugation at 10,000 rpm at 4 C in a Sorvall HB4 rotor. Less than 1% of the initial label of either pool remained in the supernatant fluids, which were therefore discarded.

The precipitates were redissolved in 5 ml of deionized water and reprecipitated with ethanol to remove all traces of urea. After a second centrifugation, the barium was exchanged for hydrogen utilizing Dowex-50 (H^+) ion-exchange resin, and the nucleotides were neutralized to pH 7.0 by addition of KOH.

Pool A: tetra-pentanucleotides. A sample of pool A (tetra-pentanucleotides) was hydrolyzed

FIG. 5. DEAE column chromatography at pH 8.0 of nucleotides obtained from pancreatic ribonuclease digestion of ³²P- α -CTP-labeled VSV product RNA. A reaction mixture containing ³²P- α -CTP and primed by VSV-dextran cores was incubated at 31 C for 2 h. The ³²P-labeled RNA was purified by Sephadex G50 column chromatography and cesium sulfate equilibrium centrifugation as before. A sample of the RNA (1.3×10^7 counts/min) was mixed with unlabeled RNA and digested by pancreatic ribonuclease, loaded, and then resolved on DEAE at pH 8.0, together with ³H-TTP. Two pools of nucleotides were obtained as indicated: pool A tetra-pentanucleotides, pool B penta-hexanucleotides.

by alkali and then loaded and eluted on DEAE at pH 8.0 together with a pancreatic digest of unlabeled RNA and ³H-TTP for markers. The distribution of ³²P-labeled nucleotides is shown in Fig. 6. Approximately 22% of the ³²P was recovered with the tetranucleotides. This result indicated that cytidine had labeled the nucleoside tetraphosphate(s).

Another sample of the ³²P- α -CTP pool A nucleotides was chromatographed at pH 5.5 on DEAE together with a pancreatic ribonuclease digest of unlabeled RNA and ³H-TTP for markers (Fig. 7). Approximately 25% of the ³²P label recovered from the column was present in a distinct peak which eluted after the pentanucleotides (compare Fig. 4). This result indicated that the cytidine phosphate was the nearest neighbor to the 5' terminal adenosine, i.e., a 5' terminal sequence of pppApCp . . . Confirmation of this suggestion was obtained by thin-layer chromatography of the alkali-digested, ³²P- α -CTP-labeled tetra-pentanucleotides (pool A, Fig. 5).

A small amount of ³²P was recovered from the pH 5.5 column in the hexanucleotide isopleth. The amount recovered was too low to analyze further but indicated that CTP had probably labeled the minor guanosine 5' nucleotide.

Separation of purine tetraphosphates by thin layer chromatography. A procedure has been described for the separation of purine tetraphosphates by two-dimension thin-layer chromatography (TLC) using PEI plates (10). Using

one-dimensional TLC and guanosine and adenosine 5' tetraphosphates, we have obtained a similar separation of purine tetraphosphates.

A sample of the ³²P- α -CTP-labeled nucleotides (pool A, the tetra-pentanucleotides obtained from the pH 8.0 column, Fig. 5) was hydrolyzed by alkali, neutralized with Dowex-50 (H⁺) ion-exchange resin, and chromatographed together with ppppG and ppppA for markers. It was found that most of the label was recovered in adenosine tetraphosphate (pppAp). A small amount was found in a slower-moving component which could have been guanosine tetraphosphate (pppGp).

Pool B: penta-hexanucleotides. When the ³²P- α -CTP-labeled penta-hexanucleotides (pool B, Fig. 5) were chromatographed on a DEAE column at pH 5.5, a small peak of labeled nucleotides was recovered close to the unlabeled heptanucleotides (Fig. 7B). When the same pool B material was digested by alkali and then subjected to thin-layer chromatography, labeled guanosine tetraphosphate was obtained. These results suggested therefore that there was a sequence pppGpCp . . . present among these nucleotides. The same labeled pppGp was obtained by alkali digestion of the ³²P-labeled nucleotide associated with the unlabeled heptanucleotides of the pH 5.5 column (Fig. 7B).

These results indicated that the major ³²P- γ -GTP-labeled nucleotide possessed a sequence pppGpCp . . . (see Discussion).

³²P- α -UTP-labeled RNA. A reaction mixture

FIG. 6. Alkali digestion of pancreatic ribonuclease derived tetra-pentanucleotides obtained from ³²P- α -CTP-labeled VSV product RNA. The pool A tetra-pentanucleotides of Fig. 5 were mixed with unlabeled ribonucleoside monophosphates and purified as described in the text. A sample was digested by KOH (see Materials and Methods), neutralized, and resolved with pancreatic ribonuclease-derived, unlabeled oligonucleotides and ³H-TTP for markers (Fig. 2). Approximately 22% of the ³²P was recovered with the tetra-nucleotides. Note that the ³²P-labeled mononucleotide peak showed the presence of both adenosine and guanosine monophosphates eluting slower than the pyrimidine monophosphates.

containing ³²P- α -UTP and templated by VSV-dextran cores was incubated at 31 C for 2 h, and the RNA was extracted and purified as described in Materials and Methods. A sample of the RNA (10⁷ counts/min) was digested with pancreatic ribonuclease and resolved by DEAE column chromatography in 7 M urea at pH 8.0 as described in Materials and Methods. Fractions containing the tetra-pentanucleotides or penta-hexanucleotides were pooled, and the nucleotides were purified by barium precipitation as described above. When half of each sample was subjected to alkali hydrolysis, neutralized, and chromatographed on DEAE at pH 8.0 with a pancreatic digest of unlabeled RNA and ³H-TTP as markers, no label was recovered in the tetranucleotide peak for either sample.

These results indicated, therefore, that there were no pppApUp... or pppGpUp... sequences among the product RNA species.

When the remaining sample of each pool was chromatographed on DEAE at pH 5.5, no peaks corresponding to the 5' termini were observed (see Fig. 7).

³²P- α -GTP-labeled RNA. VSV transcription product RNA labeled by ³²P- α -GTP during 2 h of incubation at 31 C was obtained and purified as described in Materials and Methods. A sample (1.2 $\times$ 10⁷ counts/min) was digested with pancreatic ribonuclease and resolved by DEAE

column chromatography together with a pancreatic ribonuclease digest of unlabeled RNA and ³H-TTP for markers. The tetra-pentanucleotides (pool A) and penta-hexanucleotides (pool B) were collected and purified from urea by barium precipitation as described above. After alkali digestion, neutralization, and resolution at pH 8.0 on DEAE with appropriate markers (see Fig. 6), both pools gave a labeled peak in the tetranucleotide isopleth, and the only nucleoside tetraphosphate found in each case was pppGp, as identified by one-dimensional PEI thin-layer chromatography. The guanosine tetraphosphate obtained from the pool B nucleotide was about eightfold more abundant than that derived from the pool A nucleotides. It was concluded, therefore, that there were no pppApGp... sequences amongst the product nucleotides.

When the pool A, GTP-labeled nucleotides were resolved by chromatography at pH 5.5 on DEAE, two small peaks of ³²P were observed in addition to the tetra- and pentanucleotides (Fig. 8A). A small peak of ³²P nucleotides cochromatographed with the hexanucleotides; however, since the amount of label therein was very small, it was not analyzed further. It was presumed that this nucleotide contained the minor ³²P- γ -GTP-labeled nucleotide (see Fig. 3, 4).

A second ³²P-labeled peak was also observed after the main pentanucleotide peak. This ma-

FIG. 7. DEAE column chromatography at pH 5.5 of tetra-penta-, or penta-hexanucleotides obtained from pancreatic ribonuclease digestion of ^{32}P - α -CTP labeled VSV product RNA. The pooled nucleotides (Fig. 5 pools A and B) were adjusted to pH 5.5 and separately resolved on DEAE together with pancreatic ribonuclease digests of unlabeled RNA and ^3H -TTP for markers (see Fig. 4). A; ^{32}P -labeled tetra-pentanucleotides; B; ^{32}P -labeled penta-hexanucleotides.

terial (see Fig. 8A) was collected and purified by barium precipitation, digested with alkali, and neutralized, and the nucleoside 2',3' monophosphates were resolved by paper electrophoresis (5). The ratio of ^{32}P label recovered in CMP, AMP, GMP, and UMP was 200:20:120:30, respectively.

This result was compatible with the previous observation that ribonuclease T_1 digestion of ^{32}P - γ -ATP-labeled RNA gave an oligonucleotide which behaved like a trinucleotide hexaphosphate, i.e., pppAp (U or C) pGp. Since it has

been shown that cytidine and not uridine is the nearest neighbor to the terminal adenosine, these results suggest that the nucleotide sequence is pppApCpGp. . .

When the pool B penta-hexanucleotides were resolved by DEAE column chromatography at pH 5.5 (Fig. 8B), a small peak of ^{32}P label was recovered just before the heptanucleotides. In view of its origin and recovery at pH 5.5 with the heptanucleotides, it was presumed that this nucleotide was the same as the principle ^{32}P - γ -

FIG. 8. DEAE column chromatography at pH 5.5 of tetra-pentanucleotides or penta-hexanucleotides obtained by pancreatic ribonuclease digestion of ³²P-α-GTP-labeled VSV product RNA. A reaction mixture containing ³²P-α-GTP and primed by VSV-dextran cores was incubated at 31°C for 2 h. The ³²P-labeled RNA was purified by Sephadex G-50 column chromatography and cesium sulfate equilibrium centrifugation. A sample of the RNA (1.2×10^7 counts/min) was digested by pancreatic ribonuclease together with unlabeled RNA and resolved with ³H-TTP as a marker by DEAE column chromatography at pH 8.0. The tetra-pentanucleotides (pool A) and penta-hexanucleotides (pool B) were collected (see Fig. 5) and purified from urea and salt. Samples of pool A (A) or pool B (B) nucleotides were resolved by pH 5.5 DEAE column chromatography as described in Fig. 4. From the nucleotides resolved in A, a small peak of label was collected eluting from the chromatograms after the pentanucleotides as shown.

GTP-labeled 5' terminal nucleotide (see Fig. 3, 4).

The elution profile at pH 5.5 of the pool B, ³²P-labeled nucleotides also gave evidence of a split peak for the hexanucleotide isopleth. Al-

though its position was identical to where the minor ³²P-γ-ATP 5' terminal nucleotide eluted (Fig. 2, 4), the resolution from other ³²P-labeled pentanucleotides was insufficient to allow further analysis.

³²P- α -ATP-labeled RNA. A reaction mixture containing ³²P- α -ATP and templated by VSV-dextran cores was incubated at 31 C for 2 h, and the RNA was extracted and purified as described in Materials and Methods. A sample of the RNA (10⁷ counts/min) was digested with pancreatic ribonuclease and resolved by DEAE column chromatography in 7 M urea with appropriate markers (see Fig. 5). The tetra-penta-nucleotides (pool A) and penta-hexanucleotides (pool B) were purified from urea as described previously. Alkali digestion of each pool and subsequent resolution by PEI thin-layer chromatography gave only pppAp. The recovery of adenosine tetraphosphate was about fivefold greater from the pool A nucleotides than from the pool B nucleotides. These results suggested that there were no pppGpAp... sequences among the product RNA.

Resolution of pool A nucleotides on DEAE at pH 5.5 gave a small peak of ³²P label eluting after the pentanucleotides (as in Fig. 8). This result was compatible with the elution pattern of the principle ³²P- α -ATP-labeled 5' nucleotide (Fig. 2, 4). No peak was observed in the hexanucleotides of the pH 5.5 chromatogram of the pool A nucleotides.

When the pH 8.0-derived, pool B penta-hexanucleotides were resolved by chromatography on DEAE at pH 5.5, no label was recovered with the heptanucleotides, and the hexanucleotides only showed a shoulder on the slower eluting side. This material was not analyzed further.

The direction of product RNA synthesis by VSV transcriptase. To determine the direction of product RNA synthesis, an experiment was devised in which use could be made of the fact that particular exonucleases digest RNA from either the 3' or 5' end of the molecule. If the initially synthesized end of a product RNA could be labeled with one isotope (³²P) and the other was subsequently labeled with an alternate isotope (³H), then digestion of the double-labeled product species with either spleen or venom exonucleases should indicate which isotope was present at the 3' or 5' end of the RNA molecule and hence the temporal direction of product RNA synthesis.

To obtain RNA labeled in such a manner, a double-pulse-labeling experiment was devised. A reaction mixture containing ³²P- α -UTP was incubated at 31 C for 30 min with unlabeled VSV and other reaction ingredients. The mixture was brought to room temperature, mixed with sodium deoxycholate, NaCl and glycerol, and then loaded and resolved on a column of 4% agarose as described in Materials and Methods. The void fractions, which contained tem-

plate-product complexes and all the initial transcriptase activity but no free product RNA species or ³²P- α -UTP (9), were used to initiate a second reaction mixture containing ³H-UTP to label subsequent product RNA synthesis. RNA was extracted and purified from this latter reaction mixture, and the free product RNA was separated from residual product-template complexes by 4% agarose column chromatography as before.

The ³²P-, ³H-labeled free product RNA obtained from the double-pulse-labeling experiment was digested with either spleen phosphodiesterase and phosphatase (to remove terminal phosphates from the 5' end of the product RNA), or venom phosphodiesterase (Table 2). Since venom phosphodiesterase digests RNA from the 3' end of the molecule and the ³H label was solubilized first through its use, it was concluded that the direction of product RNA synthesis was in a 5' to 3' mode. This was confirmed by the spleen phosphodiesterase selective digestion of the ³²P portion of the labeled product RNA (Table 2).

DISCUSSION

In the studies reported here, it has been shown that product RNA synthesis by VSV virion transcriptase can be initiated by either ATP or GTP, the former being the preferred starting material. Since the sequences identified are unique (i.e., pppApCp... and pppGpCp... but not pppApGp... or pppApUp... or pppGpUp... or pppGpAp...) and no terminal UTP or CTP was detected, the results suggest that the direction of product RNA synthesis is in a 5' to 3' mode. Such a conclusion was corroborated by the double-pulse-labeling experiment in which it was shown that the initial RNA synthesized was at the 5' ends of the product species.

Initiation sequences starting with ppAp... Pancreatic ribonuclease digestion of ³²P- γ -ATP-labeled product RNA identified two ³²P-labeled terminal nucleotides, one of which has been shown to be pppApCp..., the other was suspected to be pppApAp (C or U)p...; however, the amount of this second 5' nucleotide was much smaller than the pppApCp... terminus, and, since it was poorly resolved from the hexanucleotides on pH 5.5 column chromatography, its sequence has not been confirmed or studied further.

Sequence analyses involving ³²P- α -ribonucleoside triphosphates have identified that the nearest neighbor to the pppApCp... sequence is guanosine. This result agreed well with the ribonuclease T₁ digest of the ³²P- γ -ATP-labeled RNA and we conclude that the sequence is therefore pppApCpGp... By comparison, the

TABLE 2. *Direction of product RNA synthesis by VSV transcriptase^a*

Time (min)	Spleen phosphodiesterase (% initial label)		Venom phosphodiesterase (% initial label)	
	³ H	³² P	³ H	³² P
0	100	100	100	100
10	92	82	71	90
20	83	66	43	74
30	69	49	32	51
60	45	32	21	28

^a A standard reaction mixture (2.50 ml) containing ³²P- α -UTP (0.2 mCi/ μ mol) was incubated with unlabeled VSV at 31 C for 30 min (1). The reaction products were then resolved by chromatography through 4% agarose (9) as described in Materials and Methods. From the void volume were obtained the virion RNA-product complexes, also containing the viral ribonucleoprotein and transcriptase enzyme activity but lacking free product RNA and triphosphates (9). A portion of these complexes were used to prime a second reaction mixture containing ³H-UTP (1 mCi/ μ mol) to label subsequent product RNA synthesis. After incubation at 31 C for 10 min, unlabeled RNA was added, and mixture was extracted by phenol. RNA was separated from triphosphates by Sephadex G-50 column chromatography and recovered by alcohol precipitation. The RNA was then chromatographed on a 25-ml column of 4% agarose, and the void fractions containing template-product complexes were discarded. From the included fractions, free, labeled RNA was collected by alcohol precipitation, dried and dissolved in 1 ml of 0.001 M EDTA, pH 7.4 (9,050 ³H counts/min, 7,120 ³²P counts/min). Samples of the RNA were digested at 37 C with venom phosphodiesterase (0.2 ml RNA, 0.4 ml of 0.5 mg enzyme per ml of 0.005 M MgCl₂, 0.1 M NH₄HCO₃). Samples were removed at intervals to determine the residual acid-insoluble radioactivity (expressed as the percentage of the initial present). Another sample of the original double-labeled RNA was similarly digested by a mixture of alkaline phosphatase and spleen phosphodiesterase (0.2 ml RNA, 0.1 ml of 0.05 mg alkaline phosphatase per ml of 0.01 M Tris-hydrochloride, pH 7.4, 0.4 ml of 0.5 mg of spleen phosphodiesterase per ml of 0.1 M ammonium acetate, pH 6.0).

second ³²P- γ -ATP-labeled terminus could have the sequence pppApAp (C or U)p (A, C or U)pGp..., although this can only be regarded as speculative until better isolation and a detailed sequence analysis have been achieved.

Initiation sequences starting with pppGp... Apparently, among the reaction product nucleic acids synthesized *in vitro*, there are fewer 5' sequences initiated with GTP than

with ATP. From the results obtained, two GTP-derived 5' nucleotides were observed, the major one apparently having the sequence pppGpCp... The sequence of the minor 5' nucleotide may also be pppGpCp... due to the presence of a cyclic phosphate on the cytidine of the minor component (pppGpCp!) caused by incomplete or inhibited digestion by ribonuclease and resulting in the nucleotide having one less phosphate charge by comparison to the other nucleotide. However, until the minor component can be obtained and purified in greater amounts, this conclusion is only speculative.

The behavior of 5' terminal nucleotides on DEAE column chromatography. It was noted that the ³²P- γ -ATP and ³²P- γ -GTP-labeled 5' terminal nucleotides derived by pancreatic ribonuclease digestion of the product RNA behaved in an unexpected fashion on chromatography at pH 5.5 in comparison to their behavior at pH 8.0. The elution of the ³²P- γ -ATP-labeled 5' nucleotides after the penta- or hexanucleotides at pH 5.5 was not expected. At pH 5.5, oligonucleotides are expected to lose a single phosphate charge by comparison to their behavior at pH 8.0, and this is confirmed by the observation that the various oligonucleotides eluted at a lower ionic strength than at pH 8.0. However, it was expected that the 5' nucleotide would lose two charges and, for the principal ³²P-labeled nucleotide, would elute earlier than the unlabeled tetraphosphates. This expectation was not realized since it eluted after the pentanucleotides. Furthermore, ³H-TTP (which, like the unlabeled oligonucleotides, would be expected to lose one phosphate charge at pH 5.5 and consequently would elute near the unlabeled dinucleotides, as at pH 8.0) eluted nearer the trinucleotides at pH 5.5. Although pH 5.5 was selected because it is above the pKs of the nucleoside amine groups and below that of the secondary phosphate, it was apparent that the behavior of the ³²P- γ -ATP- and ³²P- γ -GTP-labeled nucleotides were anomalous even with regard to each other as well as by relation to the unlabeled oligonucleotide markers. In unpublished observations we have found that the dinucleotide ApGp on chromatography at pH 8.0, and even more at pH 5.5, elutes substantially after the dinucleotides ApCp or ApUp, so that the different chromatographic behavior of pppApCp and pppGpCp might be a reflection of the guanine content.

It was observed that ³H-TTP eluted at pH 5.5 closer to the unlabeled trinucleotide marker by comparison to its behavior on pH 8.0. Also it was noted that, at pH 8.0, both nucleoside triphosphates and nucleoside tetraphosphates, on the basis of their net phosphate charge, elute earlier than expected. Careful examination of

the elution properties of pppAp by comparison to pppGp, indicate that, by comparison to the unlabeled tetranucleotide marker, the guanine nucleotide eluted slightly later than the adenine nucleotide.

The significance of multiple initiation sequences of VSV product RNA synthesis. The observation that more than one product initiation sequence is present suggests that the VSV virion transcriptase is able to start RNA synthesis at more than one site along the template strand. This result is compatible with the observation, both in vivo (11, 12) and in vitro (7, 8,) that the mRNA (i.e. the RNA synthesized from, and complementary to the virion RNA) is smaller than the viral RNA. The synthesis of smaller mRNA molecules suggests that either it is transcribed as a complete unit and cut posttranscription by an endonuclease (which could be the transcriptase itself), or that it is transcribed in segments. In the latter case one would predict multiple initiations of product RNA synthesis. Although such initiation sequences might be initially identical for a certain number of nucleotides, our observations are compatible with their being not only multiple initiation but also different product initiation sequences.

These observations raise an interesting question concerning the transcription process, namely, whether there are any stop signals for the transcriptase to recognize and cease RNA elongation. Although it is too premature to speculate further, it should be borne in mind that both start and stop signals could be relevant to the functions of the various minor proteins associated with the virion RNA-ribonucleoprotein complex (9).

To confirm by an alternate procedure that there are several product initiation sequences and not mistakes in transcription at one site, it will be necessary to selectively isolate the product RNA formed from different regions of the virion genome. A possibility to achieve this has been opened through the recent demonstration that one of the defective particles formed in VSV infections is homologous to only one-third to one-quarter of the infectious VSV genome (15). Such experiments are being currently undertaken.

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