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RNA Transcription by the Virion Polymerases of Five Rhabdoviruses

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The presence of a virion-associated RNA-dependent RNA polymerase in five serologically distinct rhabdovirus isolates (vesicular stomatitis virus [VSV] Indiana, VSV New Jersey, Cocal, Chandipura, and Piry viruses) has been demonstrated. The enzyme for each virus has been shown to be a transcriptase capable of synthesizing in vitro RNA which is complementary to the viral genome. By sequence analyses it has been shown that, for each rhabdovirus, transcription is multiply initiated with specific 5′ nucleotide sequences. The results indicate that, for all five viruses, the initiation of transcription involves similar sequences (e.g., pppApCpGp . . . , pppGpCp . . . , and possibly one or two other sequences), suggesting relatedness and some genome conservation among these viruses.

Vesicular stomatitis virus (VSV Indiana strain) is a member of the group of bullet-shaped rhabdoviruses which are known to infect a wide spectrum of organisms including mammals, insects, plants, and fishes (8). It has been shown that VSV Indiana possesses a virion RNA transcriptase which is capable in vitro of transcribing the viral RNA genome completely, repetitively, and sequentially to give a spectrum of RNA molecules which are complementary to, but smaller than, the viral RNA (1-3, 5, 10). In answer to the question of whether the smaller product species arise from degradation of a complete complement RNA molecule or are made by separate initiation of individual molecules, it has been shown (11) that there are multiple, and specific, 5′ initiation sequences of which pppApCpGp . . . , and pppGpCp . . . have been identified, a third (see text) is probably pppGpGpPyp . . . (where Py is a pyrimidine), and the fourth is believed to be pppApAp-PypGp . . . or pppApAPypXpGp . . . (where X is either A, C, or U).

We have been interested in determining if any other rhabdovirus possesses a virion transcriptase and have already reported such an enzyme associated with virions of Kern Canyon virus (1). We now can report that there are viral-associated RNA transcriptases in VSV New Jersey, Cocal, Chandipura, and Piry viruses as well as in VSV Indiana. Also, we have found that transcription is multiply initiated with some identical 5′ sequences on the product RNA molecules for each of these viruses—suggesting that the viruses may be related to each other through the possession of functionally similar nucleotide sequences in their virion genomes.

MATERIALS AND METHODS

Preparation of virus. VSV Indiana and VSV New Jersey were obtained from R. W. Simpson, Rutgers University, New Brunswick, N.J. Piry, Chandipura, and Cocal viruses were provided by F. Murphy, National Communicable Disease Center, Atlanta, Ga. Each virus was cloned three times by picking a plaque from primary monkey kidney plates containing less than five plaques per plate. Virus stocks were prepared using an individual plaque isolate to infect a confluent monolayer of BHK-21 cells and harvesting the supernatant fluids 24 h later. The stocks containing infectivity titers ranging from 1×10^9 to 5×10^9 PFU per ml were mixed with fetal calf serum (20%) and stored frozen at −90 C. Virus stocks were verified by homologous and heterologous sera neutralization as described by Cartwright and Brown (7). The preparation of [^{3}H]uridine-labeled virus free from defective particles by precipitating infected BHK-21 cell supernatant fluids with polyethylene glycol, followed by equilibrium and velocity centrifugation, has been described elsewhere (10). Final virus suspensions, containing 1 to 4 mg of protein per ml of 0.15 M NaCl, 0.01 M Tris-hydrochloride buffer (pH 7.4), were stored at 4 C until required.

RNA polymerase assay conditions: synthesis of [^{32}P]α-ribonucleoside triphosphates. The synthesis of [^{32}P]α-ribonucleoside triphosphates (5 to 20 Ci/mmol) has been described (4). Polymerase assay conditions containing ^{3}H-labeled virus, three unlabeled triphosphates at optimal concentrations (5), one [^{32}P]α-ribonucleoside triphosphate, $MgCl_2$, Tris-

hydrochloride buffer, pH 8.0, dithiothreitol, and 1% Triton N101 have also been described previously (1). Reaction mixtures were incubated at 31 C, and the incorporation of ^{32}P into trichloroacetic acid-insoluble material was calculated in terms of the picomoles of [^{32}P]nucleoside monophosphate incorporated per hour per milligram of virus protein.

Synthesis of [^{32}P]γ-ribonucleoside triphosphates. The synthesis of all four [^{32}P]γ-ribonucleoside triphosphates employing the enzymes 3-phosphoglyceric phosphokinase and glyceraldehyde-3-phosphate dehydrogenase (Sigma Chemical Co., St. Louis, Mo.) was based on methodology described earlier (9), except that instead of labeling one triphosphate, all four were included in the same synthesis. Also, the reaction mixture (containing 200 mCi of [^{32}P]phosphate) was prepared in a total volume of 1 ml and the synthesis of labeled triphosphates was monitored by thin-layer chromatography using PEI cellulose F anion exchanger (11). Under these conditions approximately 50% of the label was incorporated into the γ position of the triphosphates and the specific activities of the four triphosphates were approximately equal, although during the reaction time-course purine nucleotides became labeled before the pyrimidine nucleotides. When the synthesis was complete, the reaction mixture was heated to 100 C and centrifuged at 10,000 × *g* for 10 min to remove denatured proteins, and the supernatant fluid was stored at −20 C in plexiglass containers. Transcriptase reaction mixtures employing all four [^{32}P]γ-ribonucleoside triphosphates were usually prepared to give a specific activity of 2 mCi/μmol for each triphosphate and incubated under standard conditions at 31 C for 2 h (11).

Purification of transcription product RNA, RNA annealing, nuclease and alkali digestions, and separation of oligonucleotides by DEAE cellulose column chromatography. The procedures for the purification of reaction product nucleic acids and their analyses by annealing, nuclease and alkali digestions, followed by separation of the resulting nucleotides on DEAE cellulose column chromatography, have been described (11). Reaction product nucleic acids contained ^{3}H-labeled virion template RNA as well as ^{32}P-labeled product RNA species.

RESULTS

Association of RNA polymerases with viral particles of VSV Indiana, VSV New Jersey, Cocal, Piry, and Chandipura viruses. Preparations of [^{3}H]uridine-labeled virions of these five rhabdoviruses were purified through polyethylene glycol precipitation followed by successive equilibrium and velocity gradient centrifugation and, after removal of sucrose, used to prime reaction mixtures containing [^{32}P]α-UTP to measure the RNA synthetic activity. The results are given in Table 1 in terms of the picomoles of [^{32}P]UMP incorporated per milligram of virus protein per hour. In each case the RNA synthesizing capability was optimal and linear at 31 C for at least 3 h as shown with VSV

TABLE 1. *RNA polymerases of five rhabdoviruses*[a]

Determination	Characteristic	Chandipura	Cocal	Piry	VSV Indiana	VSV New Jersey
RNA polymerase activity (pmol/mg of protein/h)	Complete	2,700	8,900	1,600	21,000	1,600
	−CTP	<10	<10	<10	<10	<10
	−Mg	<10	<10	<10	50	<10
	−Triton N101	<10	<10	<10	100	<10
% Of product RNase resistant: from 3-h polymerase reaction	Not annealed	18	18	22	5	20
	Self-annealed	45	32	60	14	52
	Diluted, melted, self-annealed:	5	5	8	1	3
	Diluted, melted, annealed with excess of homologous viral RNA:	96	96	100	95	91

[a] RNA polymerase reaction mixtures (1) containing [^{32}P]α-UTP (1 mCi/μmol) were primed by purified rhabdoviruses and the picomoles of [^{32}P]UMP incorporated into RNA per hour per milligram of protein was determined during incubation at 31 C (1). The incorporation of label into trichloroacetic acid-insoluble material was linear for 3 h. Control reaction mixtures lacking CTP, Mg, or Triton N101 were treated similarly. Reaction product nucleic acids extracted from such 3-h incubations were purified and the percentage of RNase-resistant ^{32}P was determined before or after self-annealing (in 0.4 M NaCl, 60 C, 24 h). A sample of the product RNA was diluted 100-fold, melted by heating at 100 C for 30 s in 0.01 M sodium phosphate, 0.005 M EDTA, pH 7.0, and then adjusted to 0.4 M NaCl and self-annealed or annealed with an excess of purified homologous viral RNA at 60 C for 4 h prior to determining the percentage of ^{32}P that was RNase resistant. RNase digestions were performed by incubating an RNA sample in 0.5 ml of 0.4 M NaCl, 0.01 M Tris-hydrochloride (or sodium phosphate buffer), pH 7.0, with 10 μg of pancreatic RNase for 30 min at 37 C and then determining the percentage of ^{32}P which remained trichloroacetic acid insoluble.

Indiana. When the reaction product nucleic acids were purified from proteins and triphosphates, melted and annealed in the presence or absence of an excess of the homologous viral RNA, it was found that the products were complementary to their respective viral genome (Table 1). These results suggested, therefore, that in each viral preparation there was a transcriptase activity which was able to make RNA complementary to the virion RNA. The RNA polymerase activity required a nonionic detergent, magnesium ions, and all four ribonucleoside triphosphates for activity as shown previously for VSV Indiana (1). The differences in specific activity of the five transcriptases may reflect different abilities of the respective enzymes, variations in the number of functional polymerase molecules per virion, or the number of enzyme-active particles in the virus preparations.

Initiation of transcription by VSV Indiana transcriptase. It has been demonstrated that preparations of VSV Indiana contain a nucleoside triphosphatase activity (e.g., ATPase) which can be inhibited by a nonionic detergent such as Triton N101 (9). In addition there is a nucleoside diphosphate kinase activity (nucleoside triphosphate phosphotransferase, P. Roy and D. H. L. Bishop, Fed. Proc., p. 1153, 1971) whose activity is revealed by a nonionic detergent (9). Empirically it has been shown that this latter enzyme randomizes the γ-phosphates between nucleoside triphosphates. Therefore, for RNA transcription initiation studies using one [^{32}P]γ-ribonucleoside triphosphate together with three unlabeled triphosphates, the phosphotransferase eventually labels all four triphosphates and so confounds a direct interpretation of the γ-phosphate incorporation data. In a previous publication we were able to obtain the removal of the phosphotransferase from the endogenously primed transcriptase activity of VSV by preparing a subviral fraction "VSV dextran cores" which contained all the initial viral RNA, ribonucleoprotein (N), minor proteins NS, A and B, much of the viral L protein, as well as all the initial viral transcriptase activity (6, 11).

With these VSV dextran cores four product RNA 5′ initiation sequences were observed (11), two starting with pppAp ... and two with pppGp.... By nearest neighbor analysis with [^{32}P]α-ribonucleoside triphosphates and determining after alkali digestion which progenitor triphosphate labeled the 5′ purine tetraphosphate, one of the 5′ adenosine initiation sequences was characterized as pppApCp.... From the [^{32}P]γ-ATP-labeled product and either pancreatic RNase digestion (hydrolyzing RNA at cytidine and uridine residues) or RNase T_1 digestion (hydrolyzing RNA at guanosine residues), the two 5′ adenosine initiation sequences were deduced to be pppApCpGp ... and pppApApPypXpGp ... (where the Xp is a purine [Pu] phosphate or a pyrimidine [Py] phosphate or nothing). Nearest neighbor analysis did not detect any pppApUp ..., pppGpUp ..., pppGpAp ..., or pppApGp ... sequences although a 5′ pppGpCp ... sequence was found. It was concluded that the two guanosine 5′ nucleotides could be pppGpCp ... and pppGpCp! ..., the cytidine cyclic phosphate (Cp!) being derived by incomplete RNase digestion (11). This question was reinvestigated, and the two 5′ guanosine nucleotides were deduced to be pppGpCp ... and pppGpGpPyp ... as described next.

Dextran cores of VSV were used to prime reaction mixtures containing either [^{32}P]α-GTP or [^{32}P]γ-GTP (11). The reaction mixtures were incubated at 31 C for 2 h, and the product RNA was purified and digested with pancreatic RNase. The ^{32}P-labeled digest was mixed with an RNase A and T_1 digest of [^{3}H]uridine-labeled RNA and resolved by DEAE cellulose column chromatography (Fig. 1). From the [^{32}P]γ-GTP-labeled product (Fig. 1A), two peaks (A and B) corresponding to the two 5′ guanosine initiation sequences were observed. The amounts of ^{32}P present in these two peaks were 31% for A and 69% for B (i.e., a ratio of 1:2.2). The equivalent eluants from the [^{32}P]α-GTP-labeled product (Fig. 1B) were separately pooled to recover the A or B 5′ sequences, and the nucleotides were recovered by precipitation from alcohol as their barium salts (11) and then digested with alkali. The recovery of guanosine tetraphosphate from each digest was determined by separating the ^{32}P-nucleoside monophosphates from the guanosine tetraphosphate by DEAE cellulose chromatography (11). The ratio of pppGp recovered from pool A and pool B was 1:4.3, respectively. Since the relative amount in pool B was almost exactly double that obtained in the [^{32}P]γ-GTP product, it was concluded that the 5′ initiation sequence of the pool B was probably pppGpGpPyp ... whereas that in the pool A was pppGpCp.... The absence of 3′ cyclic phosphates in either of the [^{32}P]γ-guanosine-labeled 5′ nucleotides was indicated in an experiment in which the pool A and pool B nucleotides (Fig. 1A) were recovered as described previously (11), redigested with pancreatic RNase, and rechromatographed on DEAE. Both samples gave a single peak of ^{32}P radioactivity eluting from the chromatograms

as before, suggesting that neither contained 3' cyclic phosphates.

Since all rhabdoviral preparations we have examined contain a phosphotransferase activity (unpublished observations), there were two alternative methods available to us for studying transcription initiation with these viruses; either to prepare subviral particles free from the phosphotransferase or use reactions in which all four ribonucleoside triphosphates were labeled in their γ-phosphate position. The latter procedure was chosen and, although it required greater levels of radioactivity, fewer reaction analyses were involved. The procedure was tested by using VSV Indiana virions as described below.

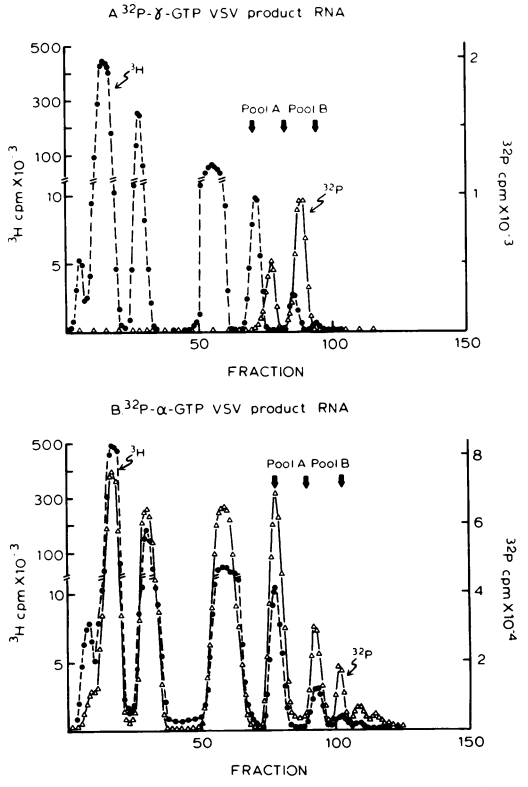

FIG. 1. *DEAE column chromatography of nucleotides from [^{32}P]γ-GTP- or [^{32}P]α-GTP-labeled VSV transcription product RNA. Transcription reaction mixtures (1) containing [^{32}P]γ-GTP (10 Ci/mmol) or [^{32}P]α-GTP (5 Ci/mmol) were primed by [^{3}H]uridine-labeled VSV dextran cores (6) and incubated at 31 C for 2 h. RNA was extracted and purified from the reaction products (11). A pancreatic RNase and RNase T_1 digest of [^{3}H]uridine-labeled VSV virion RNA was prepared by incubating 500 μg of E. coli RNA containing 2 $\times$ 10^7 counts/min of VSV RNA with 50 μg of pancreatic RNase and 50 μg of RNase T_1 at 37 C for 30 min. After addition of SDS, the digest was extracted three times with phenol to remove the nucleases, the phenol was removed by extraction with ether, and the ether was finally removed by passing nitrogen through. The ^{32}P-labeled VSV reaction product RNA was melted by heating at 100 C for 90 s and fast cooling, then digested at 37 C for 30 min with pancreatic RNase in the presence of carrier RNA at an RNA-to-RNase ratio of 1:10. The ^{32}P digest was mixed with a portion of the ^{3}H digest and chromatographed on DEAE cellulose as described previously (11). (A) [^{32}P]γ-GTP digest; (B) [^{32}P]α-GTP digest. The indicated fractions were pooled as described in the text.*

A reaction mixture containing all four ribonucleoside triphosphates was primed by VSV Indiana and incubated at 31 C for 2 h, and the nucleic acids were extracted as described previously (11). Portions of the RNA were digested by alkali, pancreatic RNase or RNase T_1, and the nucleotides were resolved with suitable markers by DEAE cellulose column chromatography (Fig. 2). Alkali digestion gave one peak of ^{32}P label eluting with the tetraphosphates (pppAp, pppGp). Upon digestion by pancreatic RNase, two major peaks were eluted: the first (62% of the label) was recovered between the tetra- and pentanucleotides and corresponded to pppApCp and pppGpCp (11), whereas the second (38% of the label) eluting between the penta- and hexanucleotides corresponded to pppApApPyp and pppGpGpPyp (11).

The RNase T_1 digest gave three peaks on elution by the salt gradient. The first represented pppGp (29% ^{32}P), the second represented pppApCpGp (51% ^{32}P), and the third represented pppApApPypGp (20% ^{32}P) (or pppApApPypXpGp ...). These identifications are based upon the analyses reported previously (11; see above).

In confirmation of earlier studies, there was no transcription initiation by pyrimidine nucleotides as evidenced by the fact that no nucleoside tetraphosphate was recovered from the pancreatic RNase digest.

The relative proportions of 5' initiation sequences were calculated from these pancreatic and RNase T_1 digests on the assumption that there were only four initiation sequences present (11, see Discussion). From these calculations it was concluded that there was 51% of the ^{32}P in the 5' initiation sequence pppApCpGp ..., 20% in pppApApPypGp ... (pppApApPypXpGp...), 38% - 20% = 18% in pppGpGpPyp..., and 62% - 51% = 11% in pppGpCp ... sequences.

Initiation of transcription by Piry transcriptase. A reaction mixture containing all four [^{32}P]γ-ribonucleoside triphosphates was primed by Piry virus, and the reaction product nucleic acids were purified and digested by

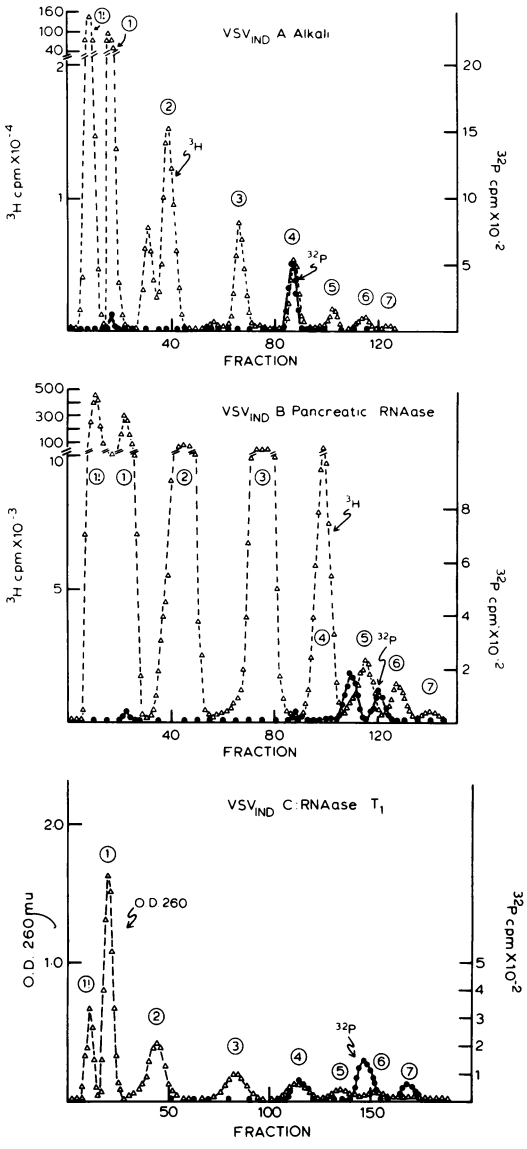

FIG. 2. *DEAE cellulose column chromatography of nucleotides obtained from [^{32}P]γ-ribonucleoside triphosphate-labeled VSV Indiana transcription product RNA. Transcription reaction mixtures (1) containing all four [^{32}P]γ-ribonucleoside triphosphates (2 Ci/mmol) were incubated with [^{3}H]uridine-labeled VSV Indiana (VSV$_{Ind}$) at 31 C for 2 h. RNA was extracted and purified from the reaction products (11). (A) Sample of the labeled RNA was digested by alkali (0.3 M KOH, 18 h at 37 C), neutralized, mixed with ^{3}H marker nucleotides (see below), and subjected to DEAE cellulose column chromatography (11). (B) Sample of labeled RNA was heat denatured by heating at 100 C for 30 s and digested with pancreatic RNase in the presence of carrier amounts of cell RNA (400 μg of RNA, 40 μg of pancreatic RNase, 37 C, 30 min) prior to mixing with ^{3}H marker nucleotides and resolving by DEAE cellulose column chromatography. (C) Sample was treated as in (B) except that RNase T$_1$ was employed in lieu of the pancreatic RNase. Also after digestion, the mixture was extracted by phenol to remove the RNase, the aqueous phase was mixed with a pancreatic digest of 4 mg of E. coli RNA (similarly extracted by phenol), the combined mixture was extracted five times with ether to remove phenol, and then ether was removed by bubbling air through the solution. The nucleotides were then absorbed and resolved by DEAE chromatography as before (11). In (A) and (B) the marker nucleotides were obtained by pancreatic and RNase T$_1$ digestion of [^{3}H]uridine-labeled VSV viral RNA (100 μg of RNA, 10 μg of RNase T$_1$, 5 μg of pancreatic RNase, 37 C, 30 min). The products were purified from nucleases by phenol extraction as described above and used in portions of about 2 × 10^6 counts per min per column analysis. A peak of incompletely digested cyclic mononucleotides (1!) was observed eluting at the front of the chromatogram. The other oligonucleotides, mono-(1), di-(2), tri-(3), etc., are indicated. No significant amounts of label were recovered from the columns by stripping with 1 M LiCl.*

alkali, pancreatic RNase, or RNase T$_1$ (Fig. 3). The alkali digest gave a peak of labeled nucleoside tetraphosphates which were recovered with the tetranucleotides. The pancreatic digest gave a peak of label (equivalent to 93% of the ^{32}P), recovered between the tetra- and pentanucleotides and which therefore corresponded to pppPupPyp.... There was about 7% of the ^{32}P recovered between the penta- and hexanucleotides, corresponding to a pppPupPupPyp ... sequence.

RNase T$_1$ digestion gave three discernible peaks, one (44% of the label) eluting with the tetranucleotides (therefore equivalent to pppGp), one (48% of the label) recovered between the penta- and hexanucleotides (equivalent to pppApPypGp ...), and a third found in the vicinity of the septanucleotides. This third peak corresponded to about 8% of the ^{32}P label and was presumed to be equivalent to the larger size pancreatic RNase digest product and therefore with a possible sequence of pppApApPypGp ... (or pppApApPypXpGp ...).

It was concluded that the guanosine tetraphosphate had a pyrimidine neighbor (pppGpPyp), and that no product initiations concurred with pyrimidine nucleotides.

To determine by nearest neighbor analyses the penultimate pyrimidine in the 5′ nucleotide obtained by pancreatic RNase digestion (i.e., pppPupPyp ...), reaction mixtures were prepared using [^{32}P]α-UTP or [^{32}P]α-CTP to label the product RNA. After purification of the product, digestion by pancreatic RNase, and recovery of the tetra-pentanucleotides by bar-

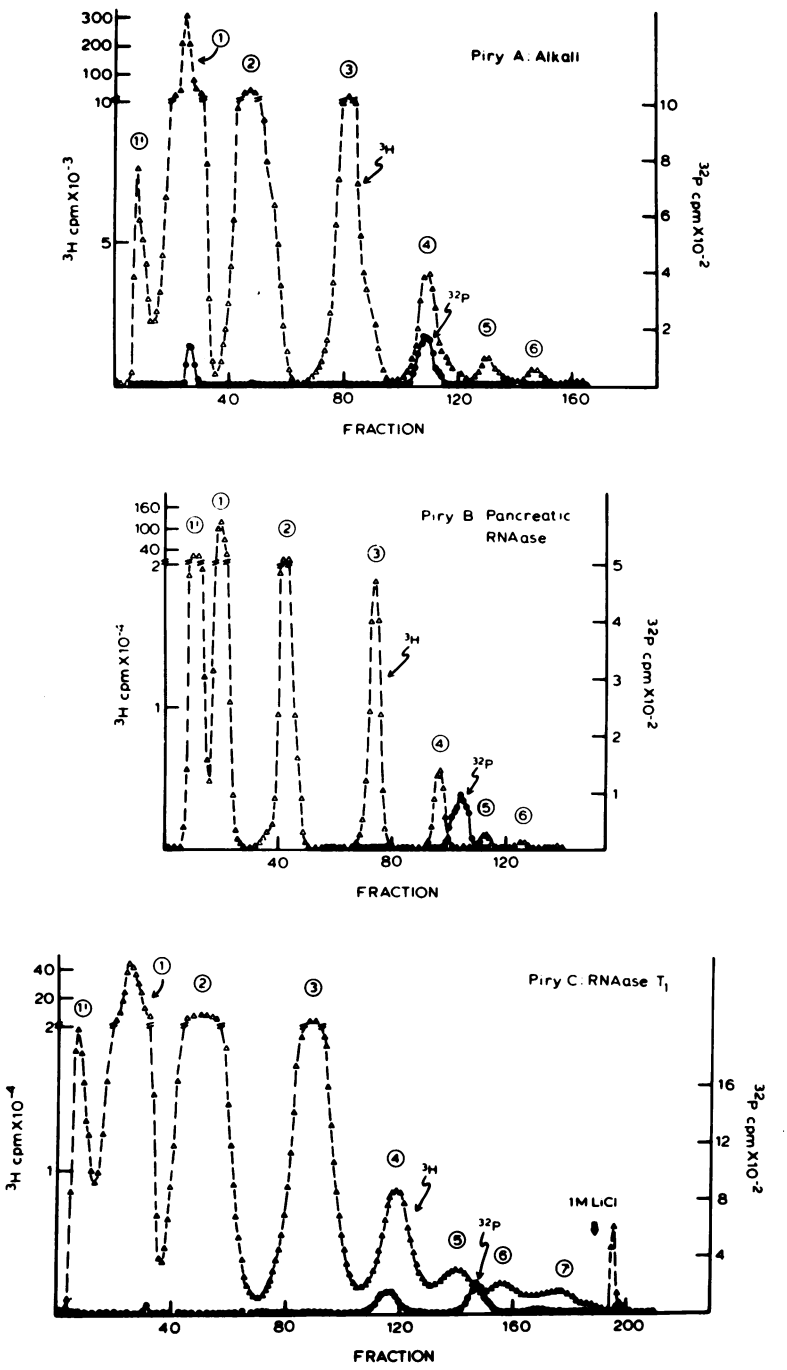

FIG. 3. *DEAE cellulose column chromatography of nucleotides obtained from [^{32}P]γ-ribonucleoside triphosphate-labeled Piry virus transcription product RNA. Transcription product RNA labeled by [^{32}P]γ-ribonucleoside triphosphates from Piry virus-primed reaction mixtures was prepared, purified, and analyzed by (A) alkali digestion, (B) pancreatic RNase digestion, and (C) RNase T_1 digestion as described in Fig. 2. In the latter column a small amount of ^{3}H and ^{32}P was eluted by a 1 M LiCl wash.*

ium precipitation (Fig. 4), these nucleotides were digested by alkali and a labeled nucleoside tetraphosphate was sought. Only in the case of the [^{32}P]α-CTP reaction were labeled purine nucleoside tetraphosphates recovered, suggesting that the sequences pppGpCp ... and pppApCp ... were present among the product species.

In conclusion, therefore, three sequences have been identified in Piry transcription product RNA: pppApCpGp ... (48%), pppGpCp ... (44%), and pppApApPypGp (8%) (or pppApApPypXpGp ...).

Initiation of transcription by Chandipura transcriptase. Chandipura transcription reaction product nucleic acids labeled by [^{32}P]γ-ribonucleoside triphosphates were prepared as described for VSV Indiana and Piry viruses. Upon digestion by alkali, nucleoside tetraphosphates were identified by DEAE cellulose column chromatography (Fig. 5). Pancreatic RNase digestion of the product gave 91% of the label in pppPupPyp ... sequences and 9% in pppPupPupPyp ... sequences, whereas RNase T_1 digestion gave evidence of initiation sequences starting with pppGp ... (27%), pppApPypGp ... (68%), and possibly pppApApPypGp (5%) (or pppApApPypXpGp). The pyrimidine moiety of the pancreatic digest product was identified as cytidine by nearest neighbor analysis (Fig. 6) as described for Piry virus.

It was concluded that there were two to four identifiable 5′ initiation sequences among Chandipura transcription products, namely, pppApCpGp ... (68%), pppGpCp ... (91 − 68 = 23%), and possibly both pppApApPypGp ... (5%) (or pppApApPypXpGp) and some pppGpGpPyp (9% − 5% = 4% or 27% − 23% = 4%).

Initiation of transcription by Cocal transcriptase. When Cocal transcription reaction product nucleic acids were labeled by [^{32}P]γ-ribonucleoside triphosphates and analyzed as described for VSV Indiana, Piry, and Chandipura viruses, three initiation sequences were identified pppGpCp ... (13%), pppApCpGp ... (78%), and pppGpGpPyp ... (9%). The data obtained were essentially identical to those found for the other three viruses and so are not shown.

Initiation of transcription by VSV New Jersey transcriptase. Reaction product RNA was prepared for VSV New Jersey using all four [^{32}P]γ-ribonucleoside triphosphates as described for VSV Indiana. Upon alkali digestion, nucleoside tetraphosphates were recovered from the nucleic acids. Pancreatic RNase revealed the presence of a pppPupPyp ... 5′ initiation

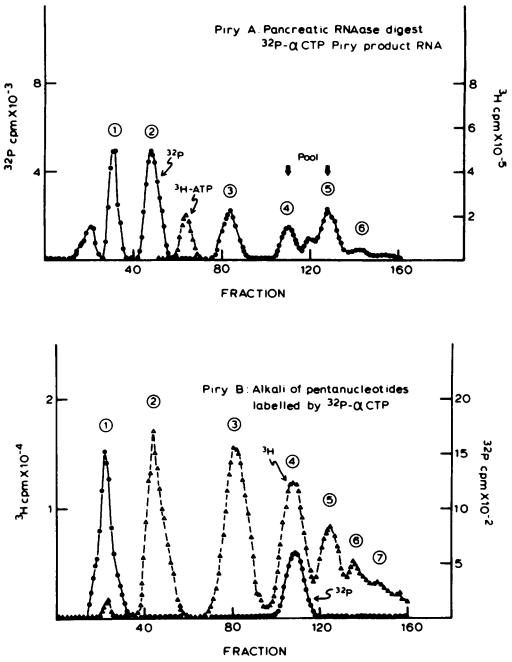

FIG. 4. *DEAE cellulose column chromatography of nucleotides obtained by pancreatic RNase digestion of [^{32}P]α-CTP-labeled Piry transcription product RNA. A reaction mixture containing [^{32}P]α-CTP and primed by Piry virus was incubated at 31 C for 2 h. The labeled RNA was purified, mixed with unlabeled RNA, and digested by pancreatic RNase (400 μg of RNA, 40 μg of pancreatic RNase, 37 C, 30 min), then (A) loaded and resolved on DEAE together with [^{3}H]ATP as a marker. The tetra-pentanucleotides were pooled, mixed with unlabeled ribonucleoside monophosphates, and recovered by barium precipitation as described previously (11). After exchange of barium for hydrogen-utilizing Dowex-50 (H^+) ion-exchange resin, the nucleotides were hydrolyzed by alkali and then resolved (B) with a pancreatic digest of [^{3}H]guanosine-labeled VSV viral RNA by DEAE cellulose column chromatography. Note that almost no marker [^{3}H]mononucleotides were obtained but that [^{3}H]guanosine-labeled dinucleotides (UG, CG) and larger [^{3}H]oligonucleotides were recovered.*

sequence (70% of the ^{32}P) as well as other larger sequences which were poorly resolved from each other. Some 15% of the label was recovered by the 1 M LiCl wash, although whether it represented underdigested or large 5′ purine oligonucleotide sequences was not determined. RNase T_1 digestion of the reaction product RNA gave a small amount (5%) of label recovered with the tetranucleotides (presumably pppGp), a major peak (70% of the ^{32}P) recovered between the penta- and hexanucleotides (pppApPypGp ...), as well as 20% of the label recovered in the 1 M LiCl wash. Identification of the penultimate pyrimidine of the 5′ oligonucleotide by

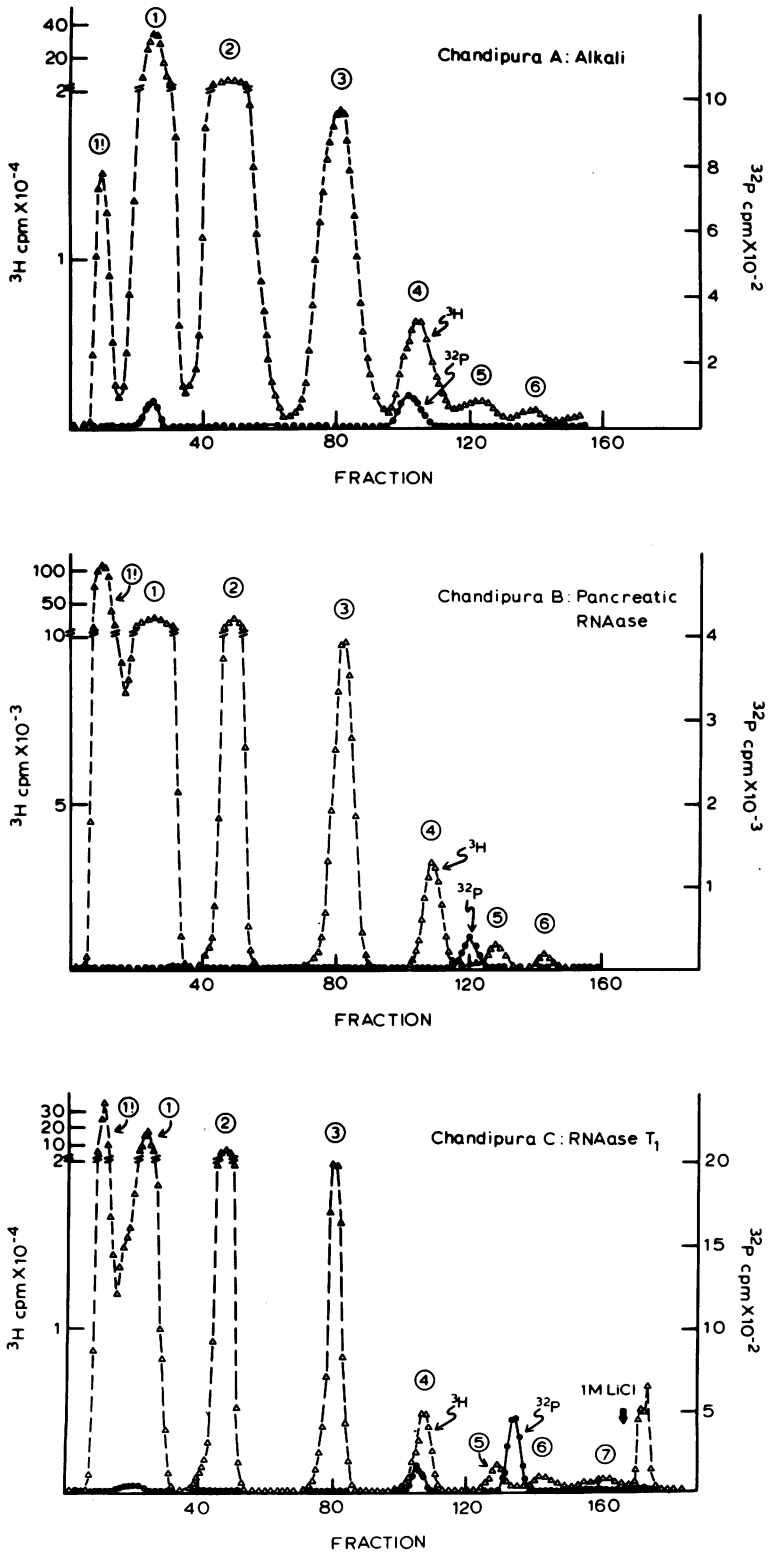

FIG. 5. *DEAE cellulose column chromatography of nucleotides obtained from [^{32}P]γ-ribonucleoside triphosphate-labeled Chandipura virus transcription product RNA. Transcription product RNA labeled by [^{32}P]γ-ribonucleoside triphosphates from Chandipura virus-primed reaction mixtures was prepared, purified, and analyzed by (A) alkali digestion, (B) pancreatic RNase digestion, and (C) RNase T_1 digestion as described in Fig. 2.*

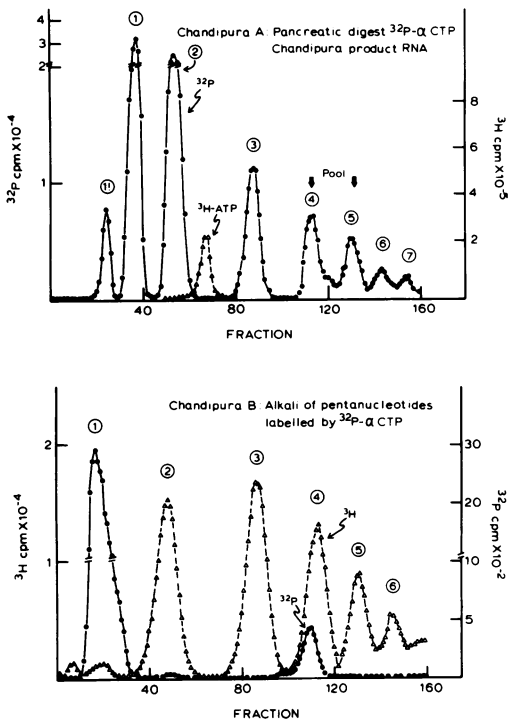

FIG. 6. *DEAE cellulose column chromatography of nucleotides obtained by pancreatic RNase digestion of [^{32}P]α-CTP-labeled Chandipura transcription product RNA. A reaction mixture containing [^{32}P]α-CTP and primed by Chandipura virus was incubated at 31 C for 2 h, the purified RNA was digested by pancreatic RNase, and the nucleotides were resolved by DEAE (A) from which the tetra-pentanucleotides were recovered and hydrolyzed by alkali before being analyzed by DEAE (B) as described in Fig. 4.*

nearest neighbor analysis proved that it was a cytidine residue, and although the 5′ sequence pppApCpGp ... was therefore deduced, the small amount of pppGp recovered from the T_1 digest of the [^{32}P]γ-ribonucleoside triphosphate-labeled product precluded an identification of its nearest neighbor.

DISCUSSION

The results presented indicate that there are virion-associated RNA transcriptases in preparations of VSV Indiana, VSV New Jersey, Piry, Chandipura, and Cocal viruses. Also it has been shown that for each virus the transcription process is initiated by purine nucleotides, that the three phosphates of the progenitor triphosphates are conserved on at least some of the product RNA, that there was no detectable transcription initiation by pyrimidine nucleotides, and that all five viruses have at least one product 5′ RNA sequence in common (pppApCpGp ...), whereas four of the viruses have a second 5′sequence in common (pppGpCp ...). The results obtained indicate that Piry and Chandipura viruses could have another 5′ sequence like one of VSV Indiana (i.e., pppApApPypGp ... or pppApAp-PypXpGp ...), and Chandipura and Cocal viruses may have a pppGpGpPyp ... initiation sequence in common with VSV Indiana.

The five viruses examined possess different transcriptase-specific activities possibly due to genetic reasons or differences in the amounts of active transcriptase per virion. It has been shown previously that VSV Indiana is able to transcribe in vitro the viral genome completely (5). For VSV Indiana, VSV New Jersey, and Cocal viruses, the minimal extent of transcription based on the [^{3}H]RNase resistance of the virion RNA in the self-annealed reaction product nucleic acids varied from 75% for VSV Indiana to 5% for New Jersey and Cocal viruses (Piry and Chandipura products were not analyzed). Consequently, since VSV Indiana transcriptase is more active and the transcription process more complete than with the other viruses, it is possible that additional 5′ initiation sequences exist for the other rhabdoviruses which were not identified in the analyses presented here. We also do not know if all the VSV Indiana 5′ initiation sequences have been identified, nor if the same 5′ sequence is present on different product molecules, nor if some of the product RNA species synthesized by complete virions lost their γ-phosphates through nucleoside triphosphatase activity.

The conclusion that RNA transcription is multiply initiated for five rhabdoviruses suggests that, if the function of a rhabdovirus transcriptase is to supply an infected cell with viral mRNA, then the production of this RNA is through the synthesis of separate mRNA species rather than by the synthesis of a polycistronic message and subsequent degradation to unit sizes or processing of a polycistronic protein complement into its functional moieties.

Genome homology of rhabdoviruses. Serologically it has been shown that, among these five rhabdoviruses, there is some antigenic relationship between whole virions of VSV Indiana and Cocal viruses presumably due to similarities between their surface glycoproteins (7). In hybridization studies between the intracellular mRNA specified by one virus and the viral RNA of a heterologous virus, we have found no more than 10% RNA homology between VSV Indiana and Cocal viruses and even less for all the other viruses which have been studied here. These hybridization experiments will be reported in a

subsequent communication. Also, we have shown that the virion proteins of VSV Indiana, VSV New Jersey, Chandipura, and Piry viruses can each be distinguished by protein gel electrophoresis (J. F. Obijeski, Marchenko, D. H. L. Bishop, Cann, and Murphy, J. Gen. Virol., in press), indicating that differences can be detected at the macromolecular level between these viruses. In contrast, the present studies indicate that common RNA 5′ initiation sequences are found for these viruses and, although such a result does not unequivocally prove relatedness, it can be interpreted as indicating that these viruses may have had a common ancestor. Although through evolution most of their genomes have changed, the sequences which specify initiation of RNA transcription may have been conserved.

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LITERATURE CITED

1. Aaslestad, H. G., H. F. Clark, D. H. L. Bishop, and H. Koprowski. 1971. Comparison of the ribonucleic acid polymerases of two rhabdoviruses, Kern Canyon virus and vesicular stomatitis virus. J. Virol. **7**:726-735.
2. Baltimore, D., A. S. Huang, and M. Stampfer. 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. II. An RNA polymerase in the virion. Proc. Nat. Acad. Sci. U.S.A. **66**:572-576.
3. Bishop, D. H. L. 1971. Complete transcription by the transcriptase of vesicular stomatitis virus. J. Virol. **7**:486-490.
4. Bishop, D. H. L., J. F. Obijeski, and R. W. Simpson. 1971. Transcription of the influenza ribonucleic acid genome by a virion polymerase. I. Optimal conditions for in vitro activity of the ribonucleic acid-dependent ribonucleic acid polymerase. J. Virol. **8**:66-73.
5. Bishop, D. H. L., and P. Roy. 1971. Properties of the product synthesized by vesicular stomatitis virus particles. J. Mol. Biol. **58**:799-814.
6. Bishop, D. H. L., and P. Roy. 1972. Dissociation of vesicular stomatitis virus and relation of the virion proteins to the viral transcriptase. J. Virol. **10**:234-243.
7. Cartwright, B., and F. Brown. 1972. Serological relationships between different strains of vesicular stomatitis virus. J. Gen. Virol. **16**:391-398.
8. Knudson, D. L. 1973. Rhabdoviruses. J. Gen. Virol. **20**(Suppl):105-130.
9. Roy, P., and D. H. L. Bishop. 1971. Nucleoside triphosphate phosphotransferase. A new enzyme activity of oncogenic and non-oncogenic "budding" viruses. Biochim. Biophys. Acta **235**:191-206.
10. Roy, P., and D. H. L. Bishop. 1972. The genome homology of vesicular stomatitis virus and defective T particles and evidence for the sequential transcription of the virion ribonucleic acid. J. Virol. **9**:946-955.
11. Roy, P., and D. H. L. Bishop. 1973. Initiation and direction of RNA transcription by vesicular stomatitis virus virion transcriptase. J. Virol. **11**:487-501.