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Distinctive RNA Transcriptase, Polyadenylic Acid Polymerase, and Polyuridylic Acid Polymerase Activities Associated with Pichinde Virus

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Three RNA polymerase activities were found and associated with purified Pichinde virus, a member of the Arenaviridae. A heat-labile polymerase activity which required all four ribonucleoside triphosphates for optimal activity cosedimented on sucrose gradient centrifugation with the viral ribonucleoprotein complex from detergent-disrupted virus preparations. This enzyme synthesized heteropolymers which represented about 23% of the genome RNA as determined by nucleic acid hybridization. Two relatively heat-stable polymerase activities which differed in their cation requirement and substrate specificity were recovered with the virus-associated ribosomes. These polymerase activities synthesized homopolymers of limited chain length: in the presence of 10 mM Mg^{2+}, polyuridylic acid was made, whereas in the presence of 1 mM Mn^{2+}, polyadenylic acid was made. The addition of complementary RNA synthesized with the viral transcriptase in vitro to the reaction mixture containing the polyadenylic acid polymerase activity resulted in the terminal addition of polyadenylic acid to the complementary RNA. The possible function of the ribosome-associated polymerase activities in the replication of the virus is discussed.

The genome of Pichinde virus, a member of the Arenaviridae, consists of two single-stranded RNA species (6, 27, 28). This RNA has negative polarity since it lacks the structural and functional properties of mRNA (13). In addition, considerable complementarity exists between the polysomal RNA extracted from virus-infected cells and virion RNA (12, 13). An earlier report of an RNA-dependent RNA polymerase associated with purified Pichinde virus preparations is compatible with a negative strandedness for the virion RNA (7). However, the specific activity of the RNA polymerase was relatively low when compared to that of the well characterized vesicular stomatitis virus RNA transcriptase (4, 20). In addition, the enzymatic activity associated with Pichinde virus lacked a stringent requirement for GTP as substrate. This property, which deviates from that of other known viral RNA-dependent RNA polymerases, raised doubts about the nature and origin of the enzyme.

Ribonucleoproteins of both circular and linear forms have been described in arenaviruses (22, 27). More impressive has been the demonstration of host cell ribosomes in association with the virions (6, 8, 9, 14, 28). Since RNA polymerizing activities, such as polyadenylic acid [poly(A)] polymerase and polyuridylic acid [poly(U)] polymerase, have been demonstrated to associate with ribosomes in mammalian cells (9, 17, 21, 29, 31, 32), we reasoned that similar RNA polymerizing activities might be associated with the virion-associated ribosomes. If such were the case, the existence of ribosome-associated host enzymatic activities might invalidate the conclusions concerning the origin of the previously observed viral RNA polymerase activities (7). In the present study we sought to identify and distinguish different polymerase activities associated with purified Pichinde virus.

MATERIALS AND METHODS

Virus and cells. Pichinde virus, strain AN3739, originally isolated from *Oryzomys albigularis* and passaged 12 times in baby hamster brain, was kindly supplied by Carlos Sanmartin. Virus stocks were passed twice in Vero cells and seven times in baby hamster kidney (BHK-21) cells in this laboratory (16). Experiments were conducted using virus from the last passage which titered 10^8 PFU/ml. The virus was used to infect BHK cells grown in suspension culture with a multiplicity of infection of 0.1 PFU/cell. After adsorption for 2 h at 37°C in a shaking water bath, the cells were washed twice with culture medium. The infected cells were then reseeded at 2.5×10^6 cells per ml in Joklik medium supplemented with 10% heat-inactivated fetal calf serum, 1.5 g of sodium bicarbonate per liter, 100 U of penicillin, and 100 μg of streptomycin per ml. Fresh medium was added at 24 and

48 h after infection, and the cell density was maintained at about 5×10^6 cells per ml. A titer of about 1×10^8 to 2×10^8 PFU/ml was usually reached at 72 h after infection. The medium was harvested, and then cell debris was removed by centrifugation at 8,000 × *g* for 10 min at 4°C. The virus was precipitated from the supernatant by 7% polyethylene-glycol and 0.4 M NaCl at 4°C for at least 2 h. The precipitate was collected by centrifugation at 10,000 × *g* for 30 min at 4°C. The virus precipitate was further purified by sedimentation in a discontinuous sucrose gradient and then by isopycnic centrifugation in a continuous sucrose gradient as described (16). The harvest and purification of virus were performed within 1 day, and RNA polymerase assays were performed immediately unless otherwise stated. A more than 70% loss of the transcriptase activity was observed when virus preparations were stored overnight at −20°C in the presence of 20 to 50% glycerol. Maximal activity was observed in preparations that had not been frozen and thawed (12).

Separation of Pichinde virus ribonucleoproteins by centrifugation in a sucrose gradient. A 0.3- to 1.0-mg amount of purified Pichinde virus was mixed with Nonidet P-40 and KCl to a final concentration of 0.5% and 0.5 M, respectively, for 10 min at 4°C. The virus suspension was then layered onto a 20 to 65% (wt/wt) sucrose gradient containing 0.01 M Tris-hydrochloride (pH 8.5), 0.1 M KCl, and 0.005 M $MgCl_2$. The gradient was spun in an IEC ultracentrifuge for 1 h at 100,000 × *g* at 4°C using an SB283 rotor. Twenty-drop fractions were collected. Portions of the gradient were assayed for trichloroacetic acid-precipitable radioactivity and RNA polymerase activity.

RNA polymerase assay. The enzymatic activities of the different polymerase activities were measured as follows. The RNA transcriptase assay contained (in a total volume of 0.1 ml): 0.1 M NaCl, 0.05 M Tris-hydrochloride (pH 8.0), 0.008 M magnesium chloride, 0.001 M manganese chloride, 0.001 M each of UTP, CTP, and ATP, 10 μM of [8-^{3}H]GTP (10 Ci/mmol), and 0.002 M dithiothreitol. For poly(U) polymerase assay, similar conditions were used, except that 0.01 M manganesium chloride and 10 μM of [5-^{3}H]UTP with or without 0.001 M each of the three other substrates were employed. For poly(A) polymerase assay, magnesium chloride was omitted and 10 μM of [8-^{3}H]ATP with or without 0.001 M each of the three other ribonucleoside triphosphates was used. Whole virus or subviral fractions were used as the source of the polymerase activities. When whole virus was used, the purified Pichinde virus was mixed with one-ninth volume of 1.0% (wt/vol) Nonidet P-40 at 4°C for 5 min. The reaction was incubated at 30°C for 1 h or varying periods as described in the text. At various times, aliquots of the reaction mixture were spotted on a Whatman 3 MM paper disk. The paper disk was washed three times with 5% (wt/vol) trichloroacetic acid and 1 mM sodium pyrophosphate, and then with 50% ethanol-50% ether, and finally with ether. After the samples were dried, they were assayed for radioactivity in a toluene-based scintillation fluid using a Beckman LS-250 liquid scintillation counter.

Heat inactivation of polymerase activities. The resolution of Pichinde virus ribonucleoproteins by sucrose gradient centrifugation resulted in the recovery of two peaks of polymerase activities. Peak 1 and peak 2 were dialyzed for 4 h at 4°C against 1,000 volumes of a solution containing 0.01 M Tris-hydrochloride (pH 7.8), 0.1 M KCl, and 0.005 M $MgCl_2$. They were then placed in a water bath at 42°C. At intervals, aliquots were withdrawn and quickly chilled on ice. Aliquots of peak 1 fraction were assayed for RNA-dependent RNA polymerase activity, and aliquots of peak 2 fraction were assayed for poly(U) or poly(A) polymerase activities.

Isolation of product RNA from polymerase reaction. Ten individual 0.1-ml RNA polymerase reaction mixtures were incubated at 30°C for 1 h. The reaction mixtures were pooled and then centrifuged at 100,000 × *g* for 1 h at 4°C to pellet the ribonucleoproteins. To the supernatant, sodium dodecyl sulfate was added to 0.5% and the supernatant was then extracted with phenol-chloroform. The RNA was precipitated by the addition of one-ninth volume of 4 M NaCl and 2 volumes of ethanol. The RNA was collected by centrifugation and dried under vacuum. The RNA was then redissolved in water and stored at −50°C.

Nearest neighbor analysis. Ten individual 0.1-ml RNA polymerase reaction mixtures containing α-[^{32}P]ATP and three other respective ribonucleoside triphosphates were incubated at 30°C for 1 h. The incubations were terminated by the addition of sodium dodecyl sulfate and 200 μg of yeast tRNA. The reaction mixtures were pooled and extracted with phenol-chloroform. RNA was precipitated by the addition of one-ninth volume of 4 M NaCl and 2 volumes of ethanol. The RNA was redissolved in 0.01 M Tris-hydrochloride (pH 7.0) and dialyzed exhaustively against the buffer. The RNA was again precipitated by alcohol and collected by centrifugation. The vacuum-dried RNA was suspended in 50 μl of 0.3 N KOH and incubated at 37°C for 18 h in a tightly capped ampoule. Perchloric acid was added to neutralize the hydrolysate. Any precipitate was removed by centrifugation at 2,000 × *g* for 10 min. The ribonucleoside 2′(3′)-monophosphates were separated by electrophoresis on Whatman 3 MM paper in a pyridine-acetic acid buffer (11).

Average chain length of the product polyribonucleotides. RNA products synthesized in vitro with [^{3}H]ATP or [^{3}H]UTP were hydrolyzed by alkali as described above. The hydrolysates were chromatographed with standards of UMP, uridine, AMP, and adenosine on cellulose sheets in a buffer of isobutyric acid:NH_4OH:H_2O (24). The appropriate areas of the chromatograms were cut out and extracted with 0.01 N HCl. The eluted radioactivity was then counted in a scintillation counter.

RNA-RNA hybridization. RNA products synthesized in vitro were deproteinized by phenol-chloroform. The RNA was precipitated by ethanol and used for hybridization using the method described by Moyer and Banerjee (18). Product RNA with varying amounts of virion RNA were incubated at 37°C for 20 h in 60% formamide, 0.5% sodium dodecyl sulfate, 0.75 M NaCl, and 0.25 M Tris-hydrochloride (pH 7.4), in a final volume of 100 μl. The mixture was then diluted 10-fold to a final concentration of 0.03 M sodium

citrate and 0.3 M NaCl (pH 7.4). Pancreatic RNase A (50 μg/ml) and RNase T1 (10 U/ml) were added, and the mixtures were incubated for 30 min at 37°C. After addition of sodium pyrophosphate and trichloroacetic acid to a final concentration of 10 mM and 10%, respectively, the acid-precipitable radioactivity was determined.

Agarose gel electrophoresis. Electrophoresis of RNA was performed under denaturing conditions as described by Bailey and Davidson (1). Disc gels containing 1% agarose, borate buffer, and 5 mM methylmercury hydroxide were used. RNA samples (20 μl) were dissolved in borate buffer containing 5 mM methylmercury hydroxide, 10% glycerol, and 0.001% bromophenol blue. After electrophoresis, the gel was sectioned into 1-mm slices. Each gel slice was incubated with 0.2 ml of 10% Bio-Solv (BBS-3) solution overnight. A 10-ml volume of a toluene-based scintillation fluid containing 10% Bio-Solv was then added to each sample, and the radioactivity was measured in a scintillation counter.

RESULTS

Association of polymerase activities with virus structures. An RNA-dependent RNA polymerase activity had been demonstrated in Pichinde virus preparations (7). To determine if this enzymatic activity is associated with particulate viral structures, freshly harvested and purified virus was treated with Nonidet P-40 and KCl to a final concentration of 0.5% and 0.5 M, respectively, and centrifuged on a 20 to 65% (wt/wt) sucrose gradient containing 0.1 M KCl. The radioactivity of [^{3}H]uridine-labeled virus was recovered in a broad, fast-sedimenting peak and a slow-sedimenting peak (Fig. 1A). The fractions corresponding to the two peaks were pooled, and the RNA was extracted by phenolchloroform in the presence of 0.5% SDS and analyzed by gel electrophoresis. The slow-sedimenting peak (peak 2) was found to contain only 28S and 18S RNA (Fig. 2B), whereas the RNA recovered from the fast-sedimenting peak (peak 1) consisted mainly of the L and S RNA species of Pichinde virus. However, about 10% of radioactivity was at the 28S and 18S rRNA regions (Fig. 2A). It was concluded from these results that peak 1 contained primarily virus ribonucleoprotein structures whereas peak 2 contained mostly ribosomal structures. The RNA polymerizing activities associated with these two peaks are shown in Fig. 1B.

Characterization of the RNA polymerase activities associated with the particulate structures recovered from Pichinde virus preparations. The abilities of the RNA polymerase activities associated with the subviral particulate material to incorporate labeled ribonucleotides from reaction mixtures containing all four ribonucleoside triphosphates were examined using reaction mixtures containing both 10 mM Mg^{2+} and 1 mM Mn^{2+}. It was found that, while the peak 1 material catalyzed the incorporation of similar amounts of all four ribonucleotides, peak 2 material preferentially catalyzed the incorporation of adenosine and uridine nucleotides (Table 1).

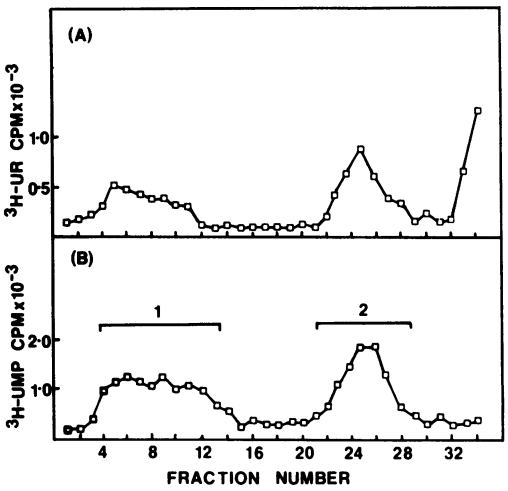

FIG. 1. *Separation of ribonucleoprotein complexes from Pichinde virion on a 20 to 65% (wt/wt) sucrose gradient. Purified Pichinde virus (0.3 mg) was treated as described in the text. Panel A shows the trichloroacetic acid precipitable [^{3}H]uridine-labeled Pichinde virus component (in counts per minute). Panel B shows the components of nonradiolabeled Pichinde virus. The individual fractions were assayed for RNA polymerase activity utilizing [^{3}H]UTP and the three other ribonucleoside triphosphates as substrate. The Mg^{2+} concentration was 10 mM and that of Mn^{2+} was 1 mM. The brackets indicate the gradient fractions hereafter routinely pooled for the source of peak 1 and peak 2 polymerase activities.*

The cation requirements for the enzyme activities associated with the two peaks were found to differ (Fig. 3). Optima of 8 mM Mg^{2+} and 1 mM Mn^{2+} were obtained for the peak 1 activity. The polymerase activity of the peak 2 material when related to cation preferences was found to differ depending on which ribonucleoside triphosphate substrate was incorporated. In the presence of all four ribonucleoside triphosphates, higher incorporation of [^{3}H]UMP was obtained between 5 and 15 mM Mg^{2+} (Fig. 3A). Addition of Mn^{2+} did not stimulate further UMP incorporation (data not shown) whereas Mn^{2+} alone had no stimulatory effect on the incorporation of [^{3}H]UMP (Fig. 3B). On the other hand, incorporation of [^{3}H]AMP was observed only in the presence of 1 mM Mn^{2+} (Fig. 3B) but not in the presence of Mg^{2+} (Fig. 3A). These observations suggest that there were two ribosome-as-

sociated polymerizing activities distinguishable on the basis of their substrate and cation requirements. Hereafter, the Mg^{2+}-dependent UMP incorporating activity is designated as the Mg^{2+}-dependent peak 2 activity, whereas the AMP incorporating Mn^{2+}-dependent activity is designated as the Mn^{2+}-dependent peak 2 activity.

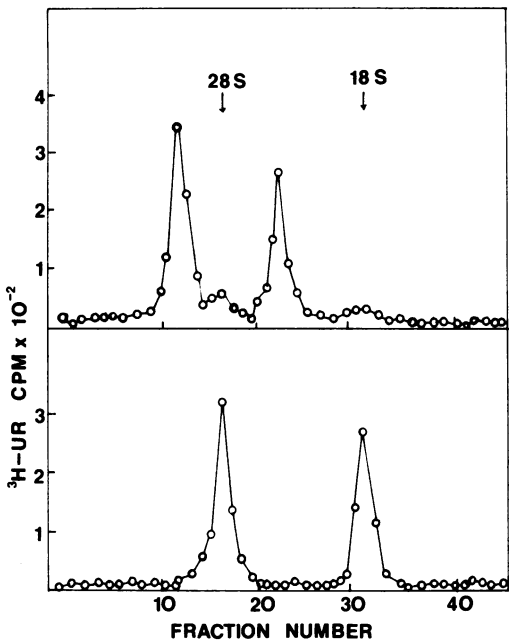

FIG. 2. *Gel electrophoresis of RNA extracted from nucleoprotein complexes from disrupted Pichinde virion. Electropherogram of RNA extracted from the fast-sedimenting (A) and the slowly sedimenting ribonucleoprotein complexes (B) recovered from the sucrose gradient shown in Fig. 1A. Gel electrophoresis was performed in 2.2% acrylamide and 0.6% agarose in aqueous buffer as described (13).*

The kinetics of the virion polymerizing activities recovered from enzyme preparations from different sources were examined. With detergent-treated whole virus preparations, the rate of incorporation of different radiolabeled substrates increased rapidly for the first 10 min of incubation. This was followed by a net decrease in total incorporation upon further incubation (Fig. 4A). By contrast, the kinetics of incorporation associated with purified peak 1 enzyme (Fig. 1) was linear for 60 min in the presence of all four ribonucleoside triphosphates (Fig. 4B); this incorporation was reduced by 90% when only any one of the four ribonucleoside triphosphates was present in the reaction mixture. The

TABLE 1. *Effect of radiolabeled substrate on the RNA polymerizing activities of Pichinde virus*[a]

Substrate	Sp act[b]	
	Peak 1	Peak 2
[^{3}H]ATP	12.1	9.8
[^{3}H]UTP	14.2	10.1
[^{3}H]CTP	11.2	1.4
[^{3}H]GTP	11.6	1.6

[a] The ribonucleoprotein components from Fig. 1B were assayed for RNA polymerizing activity as in the text except that 10 mM Mg^+ and 1 mM Mn^+ were used. Each assay condition contains the radiolabeled ribonucleoside triphosphate at 10 μM and the other three nonradiolabeled ribonucleoside triphosphates at 0.001 M.

[b] Specific activity measured in picomoles of radiolabeled ribonucleoside monophosphates incorporated per milligram of protein per hour at 30°C.

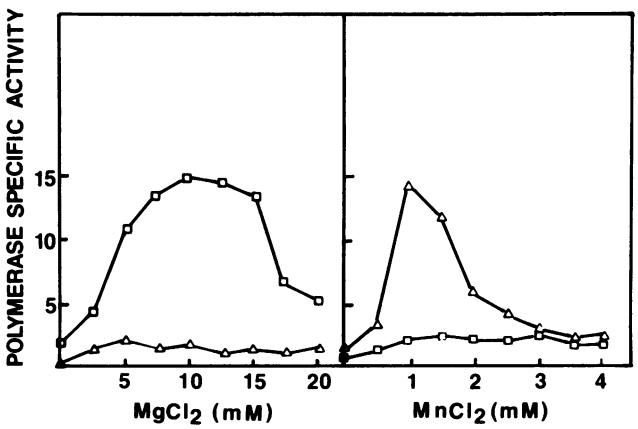

FIG. 3. *Cation requirement for peak 2 RNA polymerizing activities. The gradient fractions from peak 2 in Fig. 1B were pooled and assayed for RNA polymerase activity with various concentrations of either Mg^{2+} or Mn^{2+}. Either [^{3}H]UTP (□) or [^{3}H]ATP (△) was used as radiolabel, both in the presence of the other three ribonucleoside triphosphates. The specific activity is expressed as picomoles of radiolabeled ribonucleoside monophosphate incorporated into acid-precipitable material per milligram of protein per hour at 30°C.*

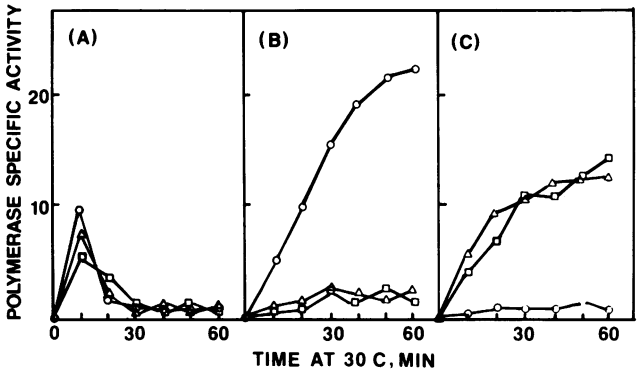

FIG. 4. *Kinetics of RNA polymerizing activities. Enzymes from different sources were employed in assays for RNA polymerase activity: (A) whole virion activated with Nonidet P-40; (B) peak 1 from Fig. 1B; (C) peak 2 from Fig. 1B. Symbols: ○, [^{3}H]GTP as radiolabel in the presence of the other three ribonucleoside triphosphates; □, [^{3}H]UTP as radiolabel with 10 mM Mg^{2+}; △, [^{3}H]ATP as radiolabel with 1 mM Mn^{2+}. No other ribonucleoside triphosphates were present in the latter two reactions. The specific activity of the enzyme is expressed as picomoles of radiolabeled ribonucleoside monophosphate incorporated per milligram of protein at 30°C.*

Mg^{2+}-dependent or Mn^{2+}-dependent enzyme activities found in peak 2 (Fig. 1) were also more stable than the whole virion enzymes. For these enzymes, the rates of the reactions were linear for at least 30 min (Fig. 4C).

The decrease in total incorporated radioactivity after incubation for more than 10 min with detergent-treated whole virus preparations suggests the presence of an RNase associated with the purified virus. This possibility was investigated by adding ^{3}H-labeled complementary RNA ([^{3}H]cRNA) synthesized with peak 1 enzyme (described below) to the preparations containing the polymerizing enzymes. As shown in Fig. 5, the RNA was rapidly degraded by nonionic detergent-treated whole virus. However, the peak 1 and peak 2 components derived by sucrose gradient centrifugation of the disrupted virus degraded the RNA much more slowly than whole virus (Fig. 5). Efforts to inhibit the RNase activity in the virus preparations by adding RNase inhibitors such as bentonite, etc., were unsuccessful.

The results of further experiments characterizing the three enzymatic activities are summarized in Table 2. The peak 1 activity required all four ribonucleoside triphosphates for maximal activity whereas the two enzymes in peak 2 functioned well when one or even three other substrates were omitted from the assay mixtures. Product synthesis by both peak 1 and peak 2 material was inhibited by RNase in the reaction mixture but uninhibited by the inclusion of DNase, actinomycin D, or α-amanitin. As a control, the Mg^{2+}- and Mn^{2+}-dependent RNA polymerase activities of uninfected BHK cell ribosomes were included.

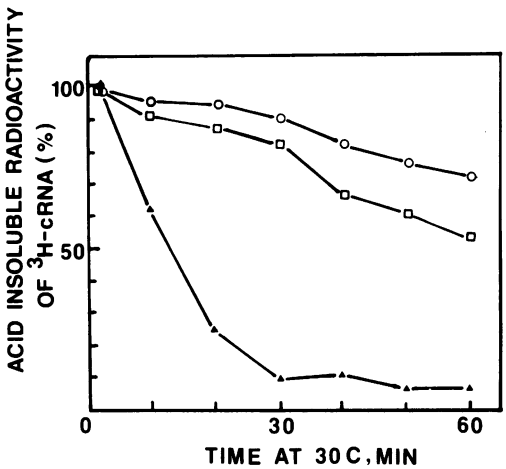

FIG. 5. *Degradation of exogenous RNA by preparations derived from purified Pichinde virus. About 5,000 cpm of [^{3}H]GTP-labeled cRNA isolated from peak 1 assay mixtures as described in the text was added to 1 A_{260} unit of disrupted whole virion (▲), or peak 1 fractions (○), or peak 2 fractions (□) in 0.01 M Tris-hydrochloride (pH 7.0), 0.1 M KCl at 30°C. At various times thereafter, aliquots were withdrawn to assay for acid-precipitable radioactivity.*

The kinetics of heat inactivation of the enzyme activities were examined to ascertain whether the three activities were resident on different molecular entities. The enzyme preparations were heated at 42°C for varying periods of time as residual enzyme activity was assayed. The heat inactivation curves of the three enzyme activities were all different (Fig. 6). The peak 1 activity was the most heat labile. The data suggest that all three enzymatic activities reside in different molecules.

TABLE 2. *Properties of RNA-polymerizing activities of Pichinde virus*

Assay conditions	Sp act[a]				
	Peak 1	Mg^{2+}-dependent peak 2	Mn^{2+}-dependent peak 2	Mg^{2+}-dependent uninfected cell ribosome	Mn^{2+}-dependent uninfected cell ribosome
Complete	15.1	10.2	9.7	17.1	11.1
− 3 NTP[b]	3.4	9.6	10.1	14.9	10.9
− CTP	2.9	9.8	9.5	16.3	12.3
+ DNase 1, 100 μg/ml	14.7	9.2	9.1	16.8	10.5
+ RNase A, 50 μg/ml	2.1	3.6	4.1	5.4	4.9
+ Actinomycin D, 20 μg/ml	13.6	11.6	10.6	15.9	11.6
+ Rifampin, 20 μg/ml	14.8	12.1	12.1	17.4	10.0
+ α-Amanitin, 1 μg/ml	14.0	10.3	10.1	16.6	9.7

[a] Specific activity measured picomoles of radiolabeled ribonucleoside monophosphates incorporated per milligram of enzyme aliquot per hour at 30°C.

[b] NTP, Ribonucleoside triphosphates.

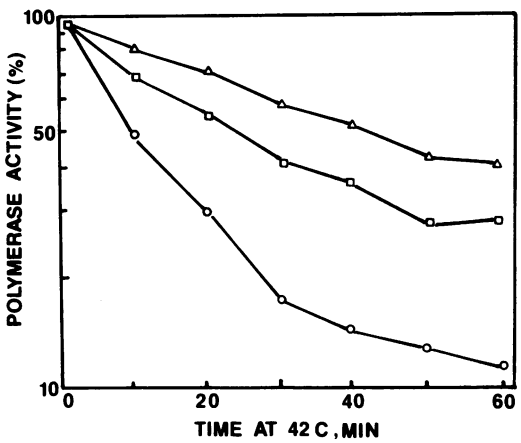

FIG. 6. *Kinetics of heat inactivation of the RNA polymerizing activities. Equal amounts of peak 1 and peak 2 fractions were incubated at 42°C. At various times, aliquots were withdrawn to assay for peak 1 RNA polymerase activity* (○), *Mg^{2+}-dependent peak 2 activity* (□), *and Mn^{2+}-dependent peak 2 activity* (△).

Enzyme products. The in vitro products of the enzymatic activities were analyzed by nearest neighbor analysis (Table 3). For the peak 1 activity, the transfer of α-phosphate to the nearest neighbor occurs at approximately equal frequency among the four bases, irrespective of whether α-[^{32}P]UTP or α-[^{32}P]ATP is used. Therefore, it was concluded that peak 1 enzyme synthesized heteropolymers. For the peak 2 activities, even though all four ribonucleoside triphosphates were present, the α-phosphate was transferred mainly to UMP for the Mg^{2+}-dependent activity and to AMP for the Mn^{2+}-dependent reaction. These findings suggest that homopolymers of mainly UMP or AMP were synthesized, respectively, for the Mg^{2+}-dependent and Mn^{2+}-dependent peak 2 polymerases. The Mg^{2+}-dependent peak 2 activity appears therefore to be a poly(U) polymerase whereas the Mn^{2+}-dependent peak 2 activity appears to be a poly(A) polymerase.

Heteropolymer formation by peak 1 suggests that the enzyme in this peak may be a true RNA transcriptase. If true, the product synthesized in vitro with peak 1 enzyme should be complementary to virus RNA. When ^{32}P-labeled product of the in vitro reaction was hybridized to virion RNA, up to 96% of the total RNA synthesized in vitro annealed with the RNA extracted from the virus (Table 4). The product did not reanneal significantly with rRNA or 4-5S RNA (Table 4).

The extent of the genome transcribed by the peak 1 enzyme was determined as shown in Fig. 6. Pichinde virus was grown in the presence of 100 μCi of [^{3}H]uridine per ml and 0.05 μg of actinomycin D per ml to inhibit cellular rRNA synthesis. Although preexisting nonradiolabeled rRNA would still be incorporated into the virus, little if any radiolabeled rRNA was present in the virion RNA. This virion RNA probe was used in RNA-RNA hybridizations driven by in vitro synthesized product RNA labeled with α-[^{32}P]GTP. Only about 23% of the virion RNA reannealed with the in vitro product, suggesting that the product represented only partial transcription of the genome (Fig. 7).

Homopolymer formation by the peak 2 enzymatic activities raises the question of whether they have the capability of terminal addition of ribonucleotides to exogenous RNA. The effect of adding various exogenous RNA species (at 0.1 μg) to the polymerizing reactions is shown in Table 5. There was an enhancement of about 50% in the polymerizing activity of the Mg^{2+}-dependent peak 2 enzyme when poly(U) was added to the reaction. For the Mn^{2+}-dependent peak 2 enzyme, addition of poly(A) doubled the amount of incorporation. Interestingly, a three-

TABLE 3. *Distribution of radioactivity after alkaline hydrolysis of RNA synthesized in vitro*

Enzyme source	Radiolabel used	cpm applied	Percent cpm in 2'(3')-ribomonophosphates			
			UMP	CMP	AMP	GMP
Peak 1	α-[^{32}P]UTP	8,901	29.2	21.2	28.6	21.0
Peak 1	α-[^{32}P]ATP	10,460	23.1	26.4	22.1	28.4
Mg^{2+}-dependent peak 2	α-[^{32}P]UTP	5,103	62.4	17.7	12.7	7.2
Mn^{2+}-dependent peak 2	α-[^{32}P]ATP	9,047	6.7	3.5	87.6	2.2

TABLE 4. *Hybridization of RNA synthesized in vitro by peak 1 RNA polymerizing activity with viral and nonviral RNA*

Treatment	Annealed	% Resistant to RNase digestion
None	None	12.1
Boiled, quickly chilled	None	2.2
Boiled, self annealed	Endogenous RNA	49.7
Boiled, annealed	0.5 μg Pichinde virus RNA	75.4
	1.5 μg Pichinde virus RNA	97.6
	2.5 μg Pichinde virus RNA	96.3
	5.0 μg BHK cell ribosomal RNA	3.2
	5.0 μg BHK cell 4 to 5S RNA	2.7

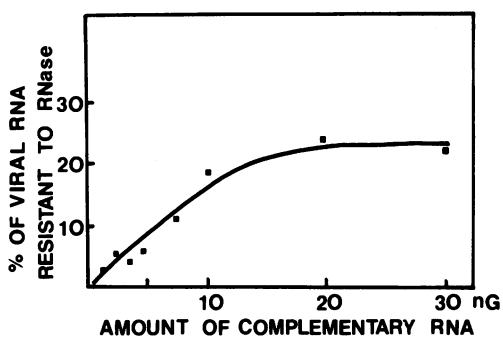

FIG. 7. *Extent of RNA genome transcribed by peak 1 RNA polymerase activity. The α-[^{32}P]GTP-labeled product RNA was isolated as described in the text. The amount of product RNA was calculated according to the specific activity of the radiolabel used. [^{3}H]uridine-labeled viral RNA with 92% of the radiolabel in the L and S RNA regions were used for hybridization. The specific activity of the viral RNA was 0.9 × 10^6 cpm/μg. About 2,000 cpm of the viral RNA was used in the RNA hybridization as described in the text.*

fold enhancement in polymerizing activity was observed when Pichinde virus cRNA synthesized in vitro by the peak 1 enzyme was added. No stimulation was observed in the peak 1 activity with any of the RNA species tested.

The relative sizes of the RNAs synthesized in the reactions incubated in the presence or absence of exogenous RNA were determined by gel filtration using Sephadex G-50 columns. De-

TABLE 5. *Effect of addition of exogenous RNA on the RNA polymerizing activities of Pichinde virus*

Exogenous RNA[a]	% of polymerizing activity		
	Peak 1	Mg^{2+}-dependent peak 2	Mn^{2+}-dependent peak 2
None	100 (20.1)[b]	100 (14.2)	100 (10.5)
28S rRNA	96	102	101
18S rRNA	98	117	98
4-5S RNA	96	96	112
Poly(U)	101	154	88
Poly(A)	102	90	197
Pichinde virion RNA	100	87	96
Pichinde virus cRNA	97	92	298

[a] A 0.1-μg amount of each RNA species was used. No significant deviation from the above data was observed when the RNA concentration was varied from 0.01 to 1.0 μg.

[b] Numbers within parentheses indicate specific activities measured in picomoles of radiolabeled ribonucleoside monophosphates incorporated per milligram of enzyme aliquot per hour at 30°C.

proteinized product of the endogenously templated peak 1 reaction was found in the excluded volume (Fig. 8A). However, only 35% of the synthesized product of the endogenous reaction using the Mg^{2+}-dependent peak 2 activity (Fig. 8B) and 38% of the product of the Mn^{2+}-dependent peak 2 activity were recovered in the excluded volume (Fig. 8C). The remainder of the product of these two latter reactions was recovered in the included volume, indicating that mostly small RNA molecules were synthesized by the peak 2 enzymatic activities. When Pichinde virus cRNA was added to the Mn^{2+}-dependent peak 2 reaction, 80% of the RNA product was removed in the excluded volume (Fig. 8D), suggesting that the radiolabeled ribonucleotide was added to the virus cRNA.

The sizes of the RNAs in the excluded volume were further characterized by electrophoresis in 1% agarose gels containing 5 mM methylmercury hydroxide. The peak 1 enzyme synthesized RNA of a broad size range, from 4.5 × 10^5 to 2.1 × 10^5 daltons (Fig. 9A). Although the size range of the RNA species synthesized was constant throughout the study, the number of species varied. For the endogenously templated Mg^{2+}-

dependent peak 2 reaction products (Fig. 9B) and Mn^{2+}-dependent peak 2 reaction products (Fig. 9C), the materials in the excluded volumes recovered from Sephadex G-50 chromatography were found to be associated with 28S and 18S rRNA only, suggesting terminal addition of ribonucleotides to the RNA component of the ribosomes. Large polynucleotides recovered from the excluded volume of the exogenously templated peak 2 Mn^{2+} reaction products were found to be associated with the viral cRNA and rRNA species (Fig. 9D). No apparent increase in the length of cRNA species was observed by gel electrophoresis. These results suggest that AMP was terminally added to the Pichinde virus cRNA.

The lengths of the added ribonucleotides were estimated by deriving the ratio of product nucleotides to nucleosides. The polynucleotides from the product synthesized by the Mg^{2+}-dependent peak 2 enzyme which were included or excluded by Sephadex filtration were both found to be seven or eight nucleotides in length. The polynucleotides synthesized by the Mn^{2+}-dependent peak 2 enzyme were determined to be

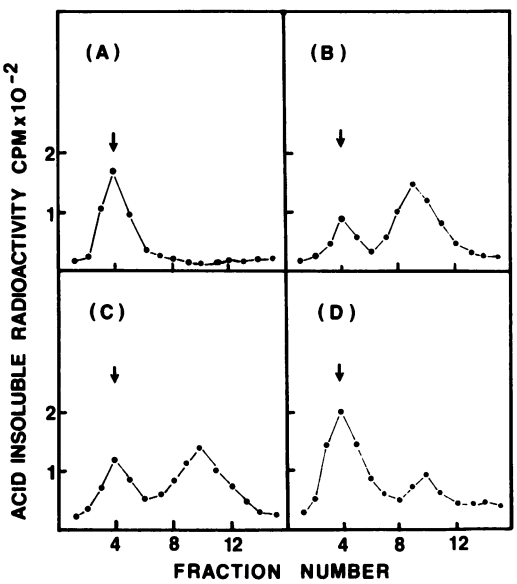

FIG. 8. *Gel filtration of product RNA on Sephadex G-50 column. The product RNA was isolated from assay mixtures as described in the text. The RNA was applied to a Sephadex G-50 column previously equilibrated with 0.01 M Tris-hydrochloride (pH 7.4)–0.1 M NaCl. It was then eluted with the same buffer, and acid-precipitable radioactivity was assayed. The gel filtration profiles of product RNA from (A) peak 1, (B) Mg^{2+}-dependent peak 2, (C) Mn^{2+}-dependent peak 2, and (D) Mn^{2+}-dependent peak 2 activity in the presence of exogenously added Pichinde cRNA are shown. The arrow indicates the void volume.*

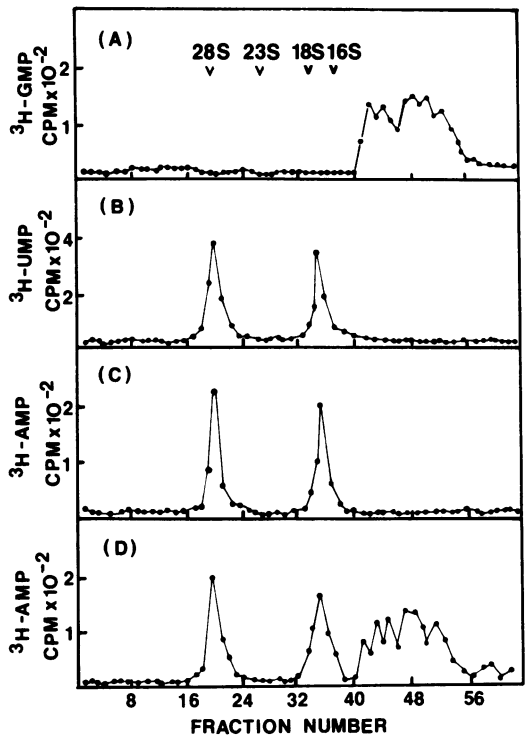

FIG. 9. *Agarose gel electrophoresis in 5 mM methylmercury hydroxide. The excluded volumes of Fig. 7 were analyzed by gel electrophoresis in 1% agarose with 5 mM methylmercury hydroxide as noted in the text. The electrophoretic profile of (A) peak 1, (B) Mg-dependent peak 2, (C) Mn^{2+}-dependent peak 2, and (D) Mn^{2+}-dependent peak 2 activity in the presence of exogenously added Pichinde cRNA are shown.*

10 to 12 nucleotides long, and this was true for apparently free forms (included volume) as well as those attached to rRNA or Pichinde virus cRNA species.

DISCUSSION

This study demonstrates the existence of three RNA polymerase activities associated with the Pichinde virus preparation: a viral nucleocapsid-associated transcriptase activity and ribosome-associated poly(U) polymerase and poly(A) polymerase activities. The transcriptase activity has been shown to transcribe a heterogeneous size range of cRNA species. Its product RNA has been demonstrated to be heteropolymers as indicated by nearest neighbor analyses. Complete complementarity appears to exist between the virion RNA and the transcriptase product RNA.

The viral ribosome-associated RNA polymerase activities have been differentiated into two enzyme activities with different ribonucleotide and cation requirements: a Mg^{2+}-dependent

poly(U) polymerase and a Mn^{2+}-dependent poly(A) polymerase. The enzymes function either by terminally adding ribonucleotides to preexisting polynucleotides or by synthesizing short, free chains. Neither enzyme copied the exogenous virion RNA for the synthesis of cRNA (Table 5). The presence of poly(U) polymerase and poly(A) polymerase has been demonstrated in nuclear, cytoplasmic, and ribosomal fractions of mammalian cells (9, 17, 21, 29, 31, 32). The properties of the virion-associated enzymes studied in this communication were similar to those of the ribosome-associated activities of uninfected BHK cells (Table 2) (17). The role of these enzymes in the replication of the Pichinde virus is not known. Stretches of uridine or adenosine sequences have not been demonstrated in the genome RNA of Pichinde virus. However, the poly(A) polymerase did utilize in vitro exogenously added Pichinde virus cRNA as a substrate. It should be noted that the experiments were done with cRNA synthesized by the viral ribonucleoprotein core separated on a sucrose gradient; this cRNA was then added to the gradient fractions containing virion-associated ribosomes. A more ideal situation might have been to employ detergent-disrupted whole virus where the viral ribonucleoprotein core and the ribosomes would be in close proximity and would enhance any possible cooperative effect of the transcriptase and the poly(A) polymerase. However, negligible amounts of polymerizing activity were observed under these conditions, possibly due to the presence of ribonuclease in the virus preparation.

An unusual association exists between arenaviruses and ribosomes in that ribosome-like structures are incorporated into the virion, there is a close proximity between ribosomes and the site of virus replication, and there is an unusual aggregation of ribosome-like structures in the cytoplasm of virus-infected cells (19, 23). Pichinde virus mRNA isolated from polysomal fractions of cells infected with the virus contain polyadenylated sequences (13). These circumstances raise the possibility that the poly(A) polymerase associated with the ribosomes might function in vivo to direct the synthesis of the 3'-terminal polyadenylated sequences of the cRNA transcribed from the viral RNA. Utilizing a mutant cell with a temperature-sensitive defect in the 60S ribosomal particles, it was shown that the protein synthesizing function of ribosomes was not required for the initiation of replication of Pichinde virus (14). However, in this cell mutant the poly(A) polymerase and poly(U) polymerase associated with the ribosomes were active and not temperature sensitive (unpublished observation). Thus, the experiments in temperature-sensitive cells do not exclude a transcriptional role of the ribosome in the replication of the virus.

The presence of ribosomes in Pichinde virus raised several considerations regarding the detection of a virus-specific transcriptase. The presence of the three enzymes can affect the outcome of the assay using unfractionated virus as the source of polymerase activity. The relative activity of the poly(A) polymerase and poly(U) polymerase will be dictated, in part, by the relative amount of ribosomes in the virion. In our experience, the amount of virion-associated ribosomes varies with passage of virus and methods of cultivating the virus, i.e., monolayer cultures versus suspension. In the majority of the virus preparations, we found that about 30 to 40% of the total [^{3}H]uridine label isolated from purified virus was in the ribosomes. In some virus preparations, especially those prepared in suspension cultures, up to 50% of the [^{3}H]uridine label was present in the ribosome fraction. In contrast, less than 5% of the label was in the ribosome fraction in a few instances where the preparations contained unusually high titers of virus. The amount of poly(U) polymerase and poly(A) polymerase generally paralleled the amount of virion-associated ribosomes. Consistent demonstration of the RNA transcriptase activity required the fractionation of subviral ribonucleoprotein core on sucrose gradients, a condition which also exhibits minimal ribonuclease activity. Moreover, the transcriptase activity was found to be labile during storage whereas the poly(U) and poly(A) polymerases were relatively stable (12). Consequently, using stored virus as the enzyme source, which is a common laboratory practice, could result in the detection of only the ribosome-associated activities (30). It is stressed that freshly purified virion should be used.

We found the specific activity of Pichinde virus RNA transcriptase to be relatively low when compared to the transcriptase of vesicular stomatitis virus. However, the activity of the transcriptase of Pichinde virus was in the same range as the transcriptase described for mumps, rabies, Sendai, and measles viruses (3, 10, 15). The reason for only 23% of the genome being transcribed by the Pichinde virus transcriptase is not known. It may reflect performing the in vitro reaction at less than optimal conditions or the lack of a transcription factor which is present in abundance in infected cells but absent in the virion (5). In this context, it should be noted that enucleation did inhibit the replication of Pichinde virus. This latter possibility could be

verified by experiments of primary transcription in infected cells.

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