JOURNAL OF VIROLOGY, Mar. 1974, p. 577-583
Vol. 13, No. 3
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Polymerase Activity of Pichinde Virus

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Received for publication 5 November 1973

Pichinde virus, a member of the arenavirus group, was examined for polymerase activity. Purified virus was found to contain RNA-dependent RNA polymerase but not RNA-dependent DNA polymerase activity. Since RNase but neither DNase nor actinomycin D inhibited the endogenous polymerase reaction, RNA of the virus appeared to be used as the template. The divalent cations Mg^{2+} and Mn^{2+} were required for optimal reactivity. The RNA product was partially resistant to RNase and the resistant portion had a sedimentation coefficient of 22 to 26*S* in sucrose gradients.

Polymerase enzymes have been found to be associated with the purified virions of a number of RNA viruses. RNA-dependent RNA polymerase (RNA polymerase) activity has been demonstrated with members of the orthomyxovirus (4, 10, 25), paramyxovirus (14, 15, 21), diplornavirus (6, 24), and rhabdovirus (3, 14) groups, whereas RNA-dependent DNA polymerase (reverse transcriptase) activity has been found associated with the oncornaviruses (2, 23, 27), visna virus (16), and maedi virus (17). The presence of these enzymes, which utilize the RNA genome of the virus as template, have been invaluable in understanding the sequence of events required for virus replication. We present evidence here that Pichinde virus, a member of the arenavirus group (22), possesses RNA-dependent RNA polymerase activity.

MATERIALS AND METHODS

Chemical reagents. ^{3}H-UTP was purchased from New England Nuclear Corp., and ^{3}H-TTP was obtained from Schwarz/Mann. Unlabeled ribonucleoside triphosphates (ATP, CTP, and GTP) and deoxyribonucleoside triphosphates (dATP, dCTP, and dGTP) were purchased from Sigma Chemical Co. and Miles Laboratories, respectively. Electrophoretically purified bovine pancreatic DNase and RNase were purchased from Worthington Biochemical Co., Freehold, N.J. Nonidet P-40 (NP-40) was obtained from Shell Oil Co., N.Y.

Cell culture. Baby hamster kidney (BHK-21) cells (26) were grown in Eagle minimal essential medium supplemented with 10% fetal bovine serum (FBS), 0.75 g/liter of sodium bicarbonate ($NaHCO_3$), and antibiotics (100 units of penicillin/ml and 100 μg of streptomycin/ml). Approximately 5×10^7 cells in 80 ml of growth medium were seeded into one-half gallon roller bottles, and 3 days after seeding the cells formed confluent monolayers containing approximately 5×10^8 cells per bottle.

Virus. Pichinde virus strain AN 3739 (28) was utilized in these experiments. Confluent cultures of BHK-21 cells were infected with virus at a multiplicity of infection of approximately 0.1 to 1 PFU/cell. After adsorption of the virus for 60 min at 37 C, the monolayers were washed once and 100 ml of fresh Eagle medium supplemented with 10% FBS, 1.50 g of $NaHCO_3$ per liter and antibiotics was added. Seventy-two hours after infection the extracellular fluid containing the virus was harvested. Virus was assayed by the plaque-counting method (20) and the titers were expressed as PFU per milliliter.

The murine sarcoma-leukemia virus complex (MSV-MLV) was obtained from the 78A1 cell line of transformed rat embryo fibroblasts by methods previously described (5).

Virus purification. Pichinde virus was concentrated and purified from extracellular fluids as described previously (8). The fractions from the linear 20 to 50% (wt/wt) sucrose density gradient which corresponded to densities of 1.14 to 1.18 g/cm^3 were pooled, diluted threefold in TNE buffer (0.01 M Tris, 0.1 M NaCl, 0.001 M EDTA), and pelleted by centrifugation at $47,000 \times g$ for 60 min. The pelleted virus was resuspended in 0.01 M Tris, pH 7.4, and utilized for enzyme assay.

MSV-MLV purification methods were identical to those described by Biswal et al. (5). After the final sucrose centrifugation the virus-containing fractions were pooled, dialyzed against TNE at 4 C overnight, and the dialyzed virus preparation was utilized for reverse transcriptase assay.

Protein determinations. The concentrations of protein in purified virus preparations were determined either by the method of Lowry et al. (18) or by the method of Groves et al. (13). The latter method is a direct spectrophotometric technique for the determination of microgram quantities of protein.

RNA polymerase assay. The method used to assay vesicular stomatitis virus RNA polymerase (3)

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was slightly modified. Purified Pichinde virus was treated with 0.1% nonionic detergent (NP-40) in the presence of 0.002 M dithiothreitol (DTT) for 3 to 5 min at 4 C, and then 0.05 ml of the partially disrupted virus containing 20 to 60 μg of viral protein was added to 0.07 ml of the standard reaction mixture. The mixture contained 7 μmol of Tris-hydrochloride, pH 8.1; 8 μmol of NaCl; 1.3 μmol of $MgCl_2$; 0.2 μmol of $MnCl_2$; 1 μmol of mercaptoethanol; 0.1 μmol each of unlabeled ATP, CTP, and GTP; 0.01 to 0.1 μmol of ^{3}H-UTP (37 Ci/mmol). After incubation at 37 C (Table 1), the reaction was terminated by adding an equal volume of ice-cold 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate and 500 μg of bovine serum albumin (BSA). The samples were washed three times with ice-cold 5% trichloroacetic acid, and the washed precipitates were solubilized in 1 ml of Nuclear Chicago solubilizer (NCS). The radioactivity of each sample was measured in a Beckman LS-250 liquid scintillation counter (9).

Reverse transcriptase assay. The conditions for the reverse transcriptase assay were similar to those previously described (2, 27). Purified virus was lysed with 0.1% NP-40 in the presence of 0.05 M EDTA and 0.002 M DTT at 0 to 4 C for 15 min, and 0.05 ml of this virus lysate containing about 50 μg of viral protein was added to 0.1 ml of the reaction mixture containing 8.3 μmol of Tris, pH 8.1; 1.6 μmol of magnesium acetate; 1 μmol of β-mercaptoethanol; 0.5 μmol of EDTA; 0.05 μmol of DTT; 0.03% NP-40; 0.01 μmol each of unlabeled dATP, dCTP, and dGTP; 0.1 mμmol of ^{3}H-TTP (50 Ci/mmol). After incubation at 37 C for various time intervals, the reaction was terminated by adding 2 ml of 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate and carrier BSA solution. The acid-precipitable radioactivity of each sample was determined as previously described (5).

TABLE 1. *Properties of the Pichinde virus RNA polymerase*

Reaction mixture	^{3}H-UMP (pmol) incorporated per mg of protein
Complete[a]	80[b]
$-MgCl_2$	13
$-MnCl_2$	24
−ATP	34
−CTP	58
−GTP	76
−DTT	56
−NP40	2
−NaCl, + KCl	64
−Virus	0
+RNase (10 μg)[c]	3
+DNase (10 μg)[c]	69
+Actinomycin D (5 μg)[c]	65

[a] The standard reaction mixture contained 40 μg of viral protein and was incubated at 37 C for 30 min.

[b] The values given represent counts per minute above the counts per minute observed when the complete reaction mixtures were held at 0 to 4 C and then were acid precipitated.

[c] RNase, DNase, or actinomycin D was added to the NP-40-disrupted virus. The virus preparation containing the inhibitor was mixed gently and then added to the remainder of the standard reaction mixture.

RNA extraction. RNA was extracted from purified Pichinde virus and enzyme reaction mixtures by methods previously described (8, 9, 11). The RNA-containing material was made up to 1% with respect to sodium dodecyl sulfate (SDS) and then deproteinized with an equal volume of buffer-saturated phenol. The aqueous phase was collected and deproteinized a second time by the addition of an equal volume of chloroform-iso-amyl alcohol. The RNA in the aqueous phase was then precipitated at −20 C in 2 volumes of ethyl alcohol. The RNA was collected by centrifugation and resuspended in 0.05 M Tris, pH 7.4.

RNA-RNA annealing. RNA samples in 0.5 ml of 0.05 M Tris buffer, pH 7.4, were heat denatured by heating in a boiling water bath for 5 to 8 min. Samples were made 0.3 M with respect to NaCl and annealed under conditions previously described by Bratt and Robinson (7). At the end of annealing, $MgCl_2$ (2 μmol) and pancreatic RNase (15 μg) were added, the samples were incubated at 37 C for 60 min, and the acid-precipitable radioactivity of each sample was then determined (5, 7).

RESULTS

Polymerase activity of Pichinde virus. Since both Pichinde virus (28) and the oncornaviruses produce persistent infections in their natural host, initial experiments were undertaken to detect RNA-dependent DNA polymerase activity in purified Pichinde virions. Approximately 600 ml of extracellular fluid containing 7×10^7 to 10^8 PFU of Pichinde virus per ml was harvested, purified, and tested for RNA-dependent DNA polymerase and RNA-dependent RNA polymerase activity. Under conditions in which MSV-MLV significantly incorporated ^{3}H-TTP into acid-precipitable product, Pichinde virus failed to incorporate the deoxyribonucleotide into product.

On the other hand, RNA-dependent RNA polymerase activity was found to be associated with Pichinde virus. Conditions in which the enzyme activity was observed are presented in Table 1. The incorporation was appreciably decreased when the cations Mg^{2+} and Mn^{2+} were omitted from the mixture. Omission of CTP somewhat reduced the reaction, but omission of GTP did not reduce the reaction at all. It was necessary to partially lyse the virions to obtain significant incorporation of ^{3}H-UTP into product, and there was no incorporation when the virus source of both the enzyme and the template was omitted from the assay. Substitution of the monovalent cation K^+ for Na^+ at

comparable concentrations yielded less product. Only pancreatic RNase, but not DNase or actinomycin D, severely inhibited ^{3}H-UMP incorporation into acid-precipitable product. This observation provides evidence that the reaction observed was not a DNA-dependent RNA polymerase reaction since actinomycin D inhibits DNA-dependent RNA polymerase.

Several parameters were measured to determine the conditions for Pichinde virus RNA polymerase activity. Figure 1 shows that when the viral protein concentration was increased, a corresponding increase in ^{3}H-UTP incorporation into acid-precipitable product was observed. The data presented in Table 2 indicate that incorporation of ^{3}H-UTP was pH dependent with greater incorporation occurring at pH 8.5 than at pH 6.7. The effect of different concentrations of Mg^{2+} was examined, and the results are shown in Table 3. Of the concentrations tested, maximum activity was observed at 10 mM.

The RNA polymerase activity associated with viruses is more active at temperatures less than 37 C (4, 14). The activity of the RNA polymerase of Pichinde virus was compared at 32 and 37 C. The results presented in Fig. 2 show that maximum incorporation was achieved in 30 min at 37 C, whereas at 32 C maximum incorporation was not achieved until 60 min. Since the rates of reaction were similar at both temperatures, total activity was greater at 32 C than at 37 C.

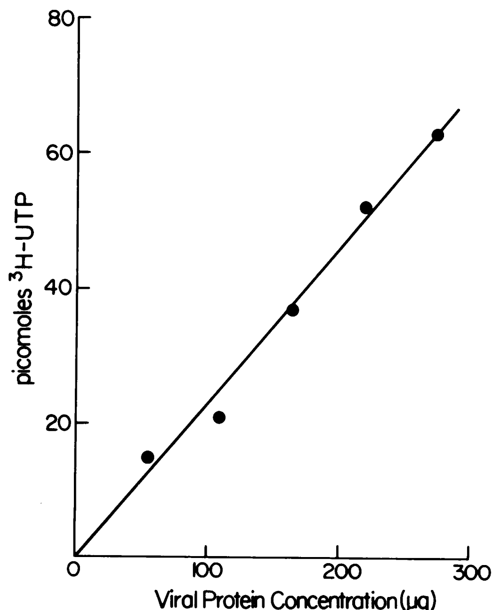

FIG. 1. *Effect of protein concentration on the RNA polymerase activity. The standard reaction mixtures containing indicated amounts of viral protein were incubated at 37 C for 30 min, and duplicate samples were analyzed for incorporation of ^{3}H-UTP into acid-precipitable material. Each point represents the average of two samples.*

TABLE 2. *Effect of pH on Pichinde virus RNA polymerase*[a]

pH	^{3}H-UMP (pmol) incorporated per mg of protein
6.4	2
6.9	6
7.5	56
8.1	80
8.5	88

[a] Standard reaction mixtures in 0.125 ml, which contained 8 µmol of Tris buffer at the indicated pH values and 60 µg of viral protein, were incubated at 37 C for 30 min. Duplicate samples were analyzed for incorporation of ^{3}H-UTP into acid-precipitable material.

TABLE 3. *Effect of different concentrations of $MgCl_2$ on Pichinde virus RNA polymerase activity*[a]

$MgCl_2$ concn (mM)	^{3}H-UMP (mol) incorporated per mg of protein
0	12
2	27
4	48
6	58
8	59
10	71
20	43

[a] The standard reaction mixture (0.125 ml) containing 20 µg of viral protein and different concentrations of $MgCl_2$ was utilized. Duplicate samples of the various reaction mixtures were incubated at 37 C for 30 min, and the acid-precipitable activity of each sample was determined.

Association of RNA polymerase activity with infectious Pichinde virus. To determine if the RNA polymerase was a part of the Pichinde virion, partially purified virus was centrifuged to equilibrium in a sucrose density gradient and fractions were simultaneously tested for infectivity and polymerase activity (Fig. 3). Over 99% of the infectious virus and all of the polymerase activity were found in fractions corresponding to densities of 1.10 to 1.20 g/cm^3. Maximum virus titers were observed at densities of 1.15 to 1.16 g/cm^3, and the maximum polymerase activity was found at the

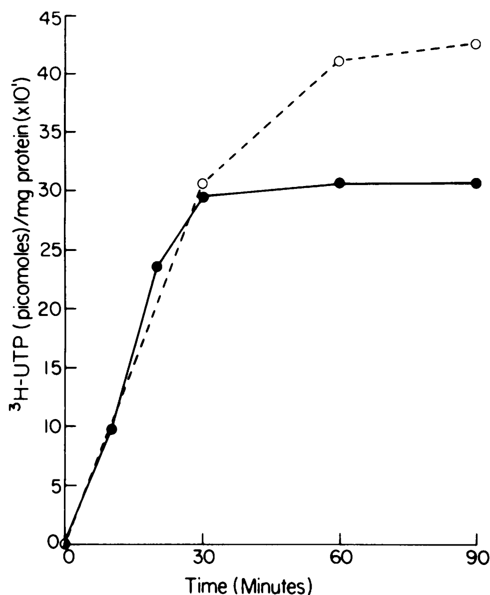

FIG. 2. *Effect of temperature on polymerase activity. Standard reaction mixtures containing 60 µg of viral protein were incubated at either 32 or 37 C. At indicated times, duplicate samples were taken and acid-precipitable radioactivity of each was determined. Each point represents the average of two samples. Symbols: ●, 37 C; O, 32 C.*

density of 1.15 g/cm^3. Extracellular fluids from uninfected BHK-21 cells, which were concentrated and purified as described for virus-containing fluids, were centrifuged and tested for RNA polymerase activity. No enzyme activity was observed in any of the fractions tested (Fig. 3).

Properties of the RNA polymerase product. In preliminary experiments to characterize the RNA polymerase product, a portion of the product was found to be resistant to RNase. Heat denaturation followed by rapid cooling resulted in a substantial increase in RNase sensitivity, whereas reannealing decreased the RNase sensitivity. The product was characterized by centrifugation in a sucrose density gradient (Fig. 4). The polymerase reaction product was separated into two major peaks of radioactivity, a broad peak corresponding to 22 to 26*S* and a second peak at 4 to 6*S*. The faster sedimenting 22 to 26*S* RNA was found to be partially resistant to RNase digestion, whereas the 4 to 6*S* material was completely digested by RNase.

DISCUSSION

Single-stranded RNA viruses may be placed into one of three groups with respect to endogenous polymerase activity. There are those in which the virion contains reverse transcriptase, those in which the virion contains RNA polymerase, and those viruses which do not possess any polymerase activity. The RNA genome of the viruses which do not possess enzyme activity appears to serve a messenger function in the initial events of replication. Viruses of the picornavirus and arbovirus groups have these characteristics. Rhabdoviruses, orthomyxoviruses, and paramyxoviruses possess RNA polymerase, and the RNA genome of these viruses appears to be complementary to the viral mRNA (1). Transcription of at least a portion of the virus genome into complementary RNA which serves an mRNA function is re-

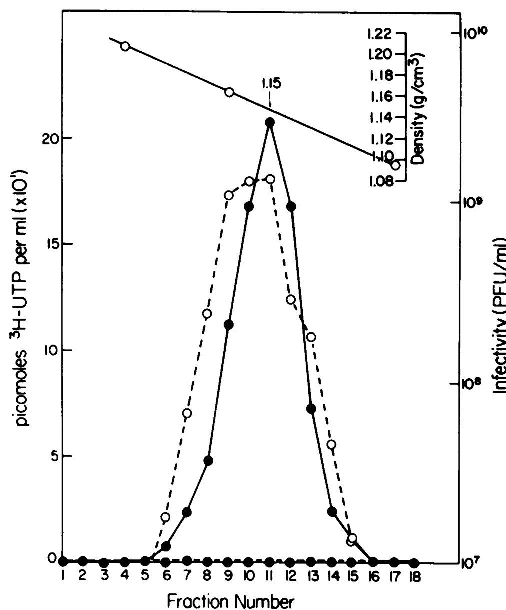

FIG. 3. *Association of RNA polymerase with infectivity. Pichinde virus was centrifuged on 20 to 50% (wt/wt) sucrose gradients at 35,000 rpm for 2 h at 4 C in a Spinco SW 50.1 rotor. Fractions (0.25 ml) were collected from the bottom, 0.05-ml samples were taken from each fraction for measurement of infectivity, and the density of every fourth fraction was determined as previously described (20). Each fraction was diluted to less than 10% sucrose by adding TNE buffer, and the virus was pelleted by centrifugation (147,000 × g, 60 min). The pelleted virus was resuspended in 0.1 ml of 0.01 M Tris buffer, pH 7.4, and this virus preparation was utilized in the standard reaction mixture. The temperature of incubation for the reaction was 32 C for 60 min. In some experiments, extracellular fluids from uninfected cells were centrifuged and tested for polymerase activity by identical methods. Symbols: ●——●, polymerase activity; ●---●, extracellular fluid from uninfected cells; O——O, density; O----O, infectivity.*

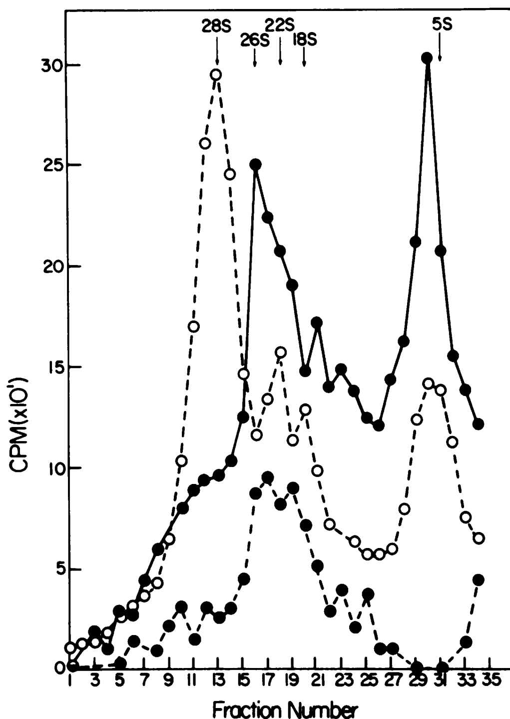

FIG. 4. *Sedimentation of the RNA polymerase reaction product of Pichinde virus. A 0.2-ml sample from the complete reaction product (Table 1) was layered on a 5 to 20% (wt/wt) linear sucrose gradient in TNE buffer, and centrifuged at 43,000 rpm for 3.5 h at 4 C in a Spinco SW 50 rotor. Fractions were collected by bottom puncture of the tube, and one-half of each fraction was tested for RNase sensitivity prior to acid precipitation. HeLa cell ribosomal RNA and purified Pichinde virion RNA were centrifuged in separate tubes and used as markers (19). Symbols: (●——●), acid-precipitable radioactivity without RNase treatment; (●---●), acid-precipitable radioactivity after RNase treatment; (O---O), RNA extracted from Pichinde virus.*

quired for replication of this group of viruses. The presence of RNA polymerase in Pichinde virus suggests that the arenaviruses may belong to the latter class. The inability to isolate infectious RNA from Pichinde virus (M. Carter, unpublished observation) is compatible with this observation since the correlation between the presence of RNA polymerase and the inability to demonstrate infectious RNA has been explained on the basis of a functional role of the polymerase enzyme which is lost in RNA purification (1).

Pichinde virus matures by budding from surface membranes of the infected cell (22). Since host cell ribosomes are incorporated into the virion, it can be surmised that other host cell-derived macromolecules may also be incorporated into the virion. Although it appears likely that the RNA polymerase detected in Pichinde virus is unique for the virus, the enzyme could theoretically be a host cell contaminant which does not play a role in virus replication. Data beyond those presented in this report will be required to resolve this point.

The RNA polymerase activity associated with Pichinde virus is quite similar to that observed for other viruses. The enzyme can utilize the RNA of disrupted virus particles as template, requires Mg^{2+}, functions optimally at alkaline pH, and incorporates the nucleotides into acid-insoluble product more effectively at 32 C than at 37 C. The polymerase associated with Pichinde virus was similar to the polymerase associated with influenza virus in that Mn^{2+} was required for maximum activity (4, 10, 25). The RNA polymerase activity of Pichinde virus was different from that reported in other viruses, since the nucleotide GTP was not required for maximum incorporation of labeled nucleotide into reaction product (4, 10, 15, 21, 25). However, a similar observation has been made for dGTP in endogenous reverse transcriptase of Rous sarcoma virus (27) which may be due to the presence of endogenous dGTP in the reaction mixture.

The association of RNA polymerase with Pichinde virus was demonstrable by isopycnic centrifugation in sucrose density gradients. The enzyme activity and virus infectivity were recovered in the same region of the gradient, and no enzyme activity was detected in fractions of gradients containing purified fluids from uninfected cultures. The peak of maximum polymerase activity and peak of infectivity were not coincident (see Fig. 3). Previously, we had observed a similar discordance of virus particles, or radiolabeled virus and virus infectivity (8). The peak of maximum RNA polymerase activity thus appears to correspond to the peak of maximum virus particles.

The RNA within Pichinde virus served as template for the endogenous polymerase. Velocity sedimentation of the product in sucrose gradient revealed two major components. One component corresponded to 22 to 26*S* in size and was partially RNase resistant, whereas the other component corresponded to 4 to 6*S* in size and was completely destroyed by RNase. The RNA of Pichinde virus can be separated into at least five components. Two components, 31 and 22*S*, appear to be of viral origin, and three components, 28, 18, and 4*S*, appear to represent ribosomal RNA of host cell origin (8, 9). Data

acquired in this study are not sufficient to conclude whether or not there is preferential utilization of different components of the viral RNA by the enzyme. The 4 to 6*S* product of the polymerase reaction could represent either small transcripts released from a larger template or the product of transcription of the 4 to 6*S* RNA found in the virion. The larger components of the virion RNA probably serve as the template for the 22 to 26*S* product. In other virus systems (14, 21) the RNA polymerase product which was associated with the RNA template in an RNase-resistant form was found to have a sedimentation coefficient less than that of the endogenous viral RNA template. This would suggest that the 22 to 26*S* product of Pichinde virus polymerase would have as its template the 31 or 28*S* components of the viral RNA. If the 28*S* RNA is within ribosomes, the 31*S* component of viral RNA would act as the template. Resolution of these speculations will require further experimentation.

The lack of reverse transcriptase in Pichinde virus supports the observation that lymphocytic choriomeningitis virus, the prototype member of the arenavirus group, also lacks reverse transcriptase activity (23). The arenaviruses share certain properties with the tumor viruses in that they produce chronic, persistent infections in their natural hosts and can readily produce persistent infection in cell culture. The lack of reverse transcriptase activity in virions from productively infected cells does not necessarily preclude a need for the enzyme in establishing or maintaining a persistent infection. Recently, Furman and Hallum (12) reported that Newcastle disease virus harvested from persistently infected L cells acquired reverse transcriptase activity which was not found in the wild-type virus. Examination for reverse transcriptase of Pichinde virus and lymphocytic choriomeningitis virus harvested from persistently infected cultures would be of interest in evaluating the possible role of reverse transcriptase in chronic infections.

ACKNOWLEDGMENT

This research was supported in part by Public Health Service Research grant AI-10125 and training grant 5T1AI74 from the National Institute of Allergy and Infectious Diseases, and by grant Q-376 from the Robert A. Welch Foundation.

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