Poliovirus-Specific Primer-Dependent RNA Polymerase Able to Copy Poly(A)

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Poliovirus-specific primer-dependent RNA polymerase able to copy poly(A)

[RNA-dependent RNA polymerase (replicase)/picornaviruses]

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ABSTRACT **A template-dependent RNA polymerase has been isolated from poliovirus-infected cells by assaying for the ability of the enzyme to copy poly(A) complexed to an oligo(U) primer. The polymerase was solubilized with detergent, and RNA was removed by precipitation with 2 M LiCl. The solubilized polymerase required both poly(A) and oligo(U) for activity and was stimulated by Mg^{2+} but was inhibited by Mn^{2+}. Poly(A) · oligo(U)-dependent poly(U) polymerase was not found in extracts of HeLa cells until about 2 hr after poliovirus infection, and then there was a linear increase in activity until about 5 hr. Analysis of the polymerase by glycerol gradient centrifugation showed that the majority of the activity sedimented at about 4 S, indicating that it was no longer complexed with high-molecular-weight RNA or cellular membranes. This poly(A) · oligo(U)-dependent polymerase activity could represent an important component of the poliovirus RNA-dependent RNA polymerase.**

An RNA-dependent RNA polymerase (replicase) is found in the cytoplasm of cells infected with poliovirus (1) as well as with other picornaviruses (2, 3) and is apparently responsible for the replication of the viral RNA genome. Previous attempts to purify the poliovirus replicase as a soluble enzyme were limited by the lack of an assay for enzymatic activity with an exogenously supplied RNA template. Studies have been restricted to the purification and characterization of the replicase complexed to the endogenous RNA template (4–7) which was found to be associated with cellular membranes (4, 8–10). A soluble replicase–template complex could be prepared by detergent treatment of the membrane fraction, followed by precipitation of the complex with 2 M LiCl (4–6). Several host and viral polypeptides were found in the precipitate, the predominant one being noncapsid viral protein 4. Previous attempts to stimulate the replicase activity by the addition of exogenous RNA were not successful (4). A template-dependent replicase has been isolated from encephalomyocarditis virus-infected cells, but it was highly unstable (11).

The detailed mechanisms involved in the replication of the single-stranded RNA genome of poliovirus are not known. The synthesis of poliovirus messenger and virion RNA (plus strands) takes place in a structure known as the replicative intermediate (12–14). The replicative intermediate consists of at least one complete strand of complementary (negative strand) RNA and approximately six nascent chains of plus strand RNA (14). The first step in the formation of the replicative intermediate should be the synthesis of a complete negative strand RNA molecule. During synthesis of the negative strand, assuming that it occurs 5′ to 3′, the initial event would be copying of the poly(A) at the 3′ end of the virion RNA to form the poly(U) found at the 5′ end of negative strand RNA (15, 16). Thus, negative strand synthesis should be initiated by a poly(A)-dependent poly(U) polymerase. This reasoning led us to investigate if poliovirus-infected cells might contain a poly(A)-dependent poly(U) polymerase. Initial attempts to use poly(A) as a template revealed no such activity, but when oligo(U) was added as a potential primer, an active poly(U) synthesis was observed. We report here the identification and partial purification of a poliovirus-specific poly(A) · oligo(U)-dependent poly(U) polymerase.

MATERIALS AND METHODS

Preparation of Infected Cell Extracts. HeLa cells were grown in suspension culture and infected with poliovirus type 1 as described (17). At 3–6 hr postinfection, 100 ml of infected cells (4×10^6/ml) were collected by centrifugation, washed once in Earle's saline, and resuspended in 10 ml of 0.01 M Tris · HCl, pH 8.0/0.01 M NaCl (TN buffer). A cytoplasmic extract was prepared by breaking the cells in a Dounce homogenizer and removing the nuclei by centrifugation (5000 × *g* for 5 min).

Isolation of the Poly(U) Polymerase. The membrane fraction of the cytoplasmic extract was deposited by centrifugation at 20,000 × *g* for 30 min. The supernatant was removed and the pellet was resuspended in 1 volume of TN buffer containing 1% (vol/vol) Nonidet P-40 (NP-40), 0.5% (wt/vol) sodium deoxycholate (DOC), and 2 M LiCl. After 15 hr at −20°, the precipitate containing single-stranded and replicative intermediate RNA was removed by centrifugation at 17,000 × *g* for 15 min. The resulting supernatant was dialyzed for 5 hr and then for 15 hr against 2-liter portions of TN buffer at 4°. One milliliter of the dialyzed supernatant was derived from about 4×10^7 infected cells.

Assay of Polymerase Activity. In a standard reaction, a 50-μl aliquot of enzyme was assayed in 125 μl of solution containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer (pH 8.0), 8 mM $Mg(CH_3COO)_2$, 0.08 mM [5,6-^{3}H]UTP (110–550 cpm/pmol), poly(A) (20 μg/ml), $oligo(U)_{11-19}$ (10 μg/ml), 4 mM phospho*enol*pyruvic acid, pyruvate kinase (3 international units/ml), and actinomycin D (10 μg/ml). The assay was at either 37° or 29°. The rate of UMP incorporation at the lower temperature was linear for a longer time. Enzymatic activity was measured as the amount of labeled product collected on 0.45-μm membrane filters (Millipore) after precipitation in 7% trichloroacetic acid (TCA) sodium pyrophosphate (1:3 saturated) with 200 μg of added carrier RNA. The filters were assayed in Bray's scintillation fluid (New England Nuclear) with a Beckman LS-230 scintillation counter.

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Abbreviations: replicase, RNA-dependent RNA polymerase; TN buffer, 0.01 M Tris·HCl, pH 8.0/0.01 M NaCl; NP-40, Nonidet P-40; DOC, sodium deoxycholate; DTT, dithiothreitol.

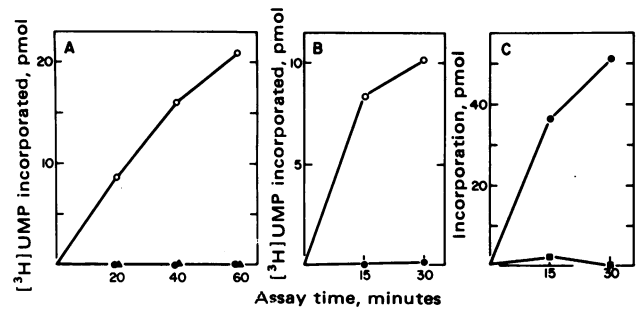

FIG. 1. The poly(A), oligo(U), and ribonucleoside triphosphate requirements of the RNA polymerase solubilized from poliovirus-infected cells. (*A*) Polymerase activity with UTP as the only added substrate. A dialyzed LiCl supernatant was prepared from cells infected for 3.5 hr and was assayed for polymerase activity at 37° as described in *Materials and Methods* except that poly(A) was used at 200 μg/ml, and 80 μM [^{3}H]UTP was the only added ribonucleoside triphosphate. The amount of labeled UMP incorporated was measured in the presence of added poly(A) (▲), oligo(U) (●), or both poly(A) and oligo(U) (○). (*B*) Polymerase activity with [^{3}H]UTP plus unlabeled GTP, CTP, and ATP. The conditions and symbols were as in *A* except that polymerase activity was measured with 80 μM [^{3}H]UTP plus ATP, GTP, and CTP, all at a final concentration of 200 μM. (*C*) Polymerase activity with [^{3}H]GTP and [^{3}H]UTP. The conditions were as in *A* except that a different preparation of enzyme was used in which 1% NP-40 and 2 M LiCl were used to solubilize the cellular membranes without added DOC. The polymerase was assayed in the presence of poly(A) and oligo(U) with either 80 μM [^{3}H]UTP (●) or 80 μM [^{3}H]GTP (■) as the only added ribonucleoside triphosphates.

Materials. All labeled nucleotides were obtained from New England Nuclear Corp. in solution in 50% ethanol; after evaporation to dryness, the solid was resuspended in sterile water at 1/10 the original volume. Unlabeled nucleotides and pyruvate kinase were obtained from Calbiochem, poly(A) was from Miles Laboratories, oligo(U)$_{11-19}$ and oligo(dT)$_{12-18}$ were from Collaborative Research, and actinomycin D was a gift of Merck Sharp and Dohme.

RESULTS

Identification of the Polymerase. The poly(U) polymerase was first detected in a solubilized membrane fraction of poliovirus-infected HeLa cells. Membranes were prepared from a cytoplasmic extract of cells infected for 3.5 hr and resuspended in a solution of 1% NP-40, 0.5% DOC, and 2 M LiCl. All single-stranded and replicative intermediate RNA was allowed to precipitate at −20° and then removed by centrifugation. After the LiCl was removed from the resulting supernatant by dialysis, a polymerase was found that synthesized poly(U) with poly(A) as the template, oligo(U) as the primer, and labeled UTP as the only substrate (Fig. 1*A*). Both poly(A) and oligo(U) were required for activity (Fig. 1*A* and Table 1), suggesting that the polymerase was only active on a duplex structure of template and primer. Replacing the oligo(U)$_{11-19}$ primer with an oligo(dT)$_{12-18}$ primer resulted in a complete loss of activity (Table 1). When all four ribonucleoside triphosphates were present, addition of both poly(A) and oligo(U) was still required for activity (Fig. 1*B*), suggesting that all of the endogenous RNA template was removed by LiCl precipitation. There was no significant incorporation of the noncomplementary nucleotides GMP (Fig. 1*C*) or CMP (data not shown) in the presence of added poly(A) and oligo(U). Thus, the dialyzed LiCl supernatant does not support a nonspecific terminal addition of ribonucleotides to either homopolymer. When Mg^{2+} was replaced by Mn^{2+}, less than 10% of the original polymerase activity remained and Mn^{2+} inhibited the activity stimulated by Mg^{2+} (Table 1). Mn^{2+} is also known to inhibit the endogenous replicase activities of poliovirus and Mengovirus (1, 2). The poly(U) polymerase did not show an absolute requirement for dithiothreitol (DTT) but its activity was stimulated by about 50% in the presence of added DTT (data not shown).

Table 1. Template, primer, and divalent cation requirements for polymerase activity

Additions	[^{3}H]UMP incorporated, pmol	% of control
Experiment 1		
Poly(A), oligo(U)$_{11-19}$	10.4	100
None	<0.5	<5
Oligo(U)$_{11-19}$	<0.5	<5
Poly(A)	0.5	5
Oligo(dT)$_{12-18}$	<0.5	<5
Oligo(dT)$_{12-18}$, poly(A)	<0.5	<5
Experiment 2		
8 mM $MgCl_2$	23.5	100
None	<0.5	<2
8 mM $MnCl_2$	1.9	8
8 mM $MgCl_2$, 5 mM $MnCl_2$	2.5	11

The poly(U) polymerase in the dialyzed LiCl supernatant was prepared and assayed as described in *Materials and Methods* with the following modifications. In experiment 1, the assay mixture contained poly(A) (20 μg/ml), oligo(U)$_{11-19}$ (10 μg/ml), oligo(dT)$_{12-18}$ (10 μg/ml), or combinations as indicated. In experiment 2, a different enzyme preparation was used and the divalent cation concentrations were as indicated. For each assay in experiment 2, poly(A) (20 μg/ml), oligo(U)$_{11-19}$ (10 μg/ml), and 10 mM DTT were present. The assays were for 30 min at 29°.

Poly(A) · oligo(U) could stimulate poly(U) synthesis in crude cytoplasmic extracts and most of the activity sedimented with the cellular membranes. Various procedures were used to solubilize the activity from the membrane pellet. Using 1% NP-40 and 2 M LiCl without added DOC resulted in a 30% increase in activity, but a precipitate was formed after the final dialysis step that contained most of the polymerase activity (data not shown). When 2 or 4 M LiCl was used without added detergent, only about half of the activity was recovered compared to that found with NP-40/DOC/2 M LiCl. Using 2 mM EDTA in combination with 2 M LiCl resulted in even lower yields.

The Poly(U) Polymerase Is Poliovirus-Specific. The poly(A) · oligo(U)-dependent poly(U) polymerase was not detectable in the dialyzed LiCl supernatant prepared from uninfected HeLa cells and first became evident about 2 hr after poliovirus infection (Fig. 2). The increase in enzyme activity coincided with the start of detectable virus-specific RNA synthesis and continued linearly until 5 hr after infection. Similar results were reported for endogenous poliovirus replicase activity, but this activity reached a maximum value much earlier and then decreased with kinetics similar to those found for the rate of RNA synthesis within the infected cell (1, 4, 7). Assuming that the poly(A) · oligo(U)-dependent activity reflects the activity of the poliovirus replicase, these results suggest that the decrease in the endogenous activity at late infection times may not result from an inherent instability in the replicase itself. Rather, the decreased activity may result from a degradation of the endogenous RNA template or from the loss of some other factor required to initiate RNA synthesis.

Analysis of the RNA Product. The product synthesized *in vitro* in the presence of added poly(A), oligo(U), and labeled UTP was completely converted to acid solubility by digestion

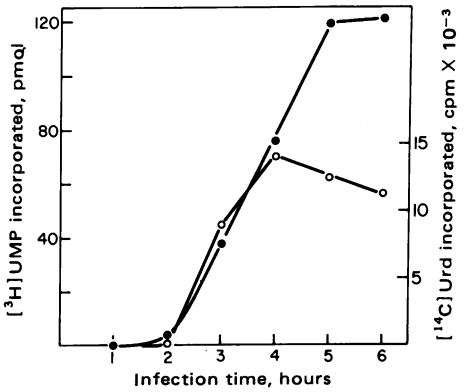

FIG. 2. The appearance of poly(A)-oligo(U)-dependent poly(U) polymerase activity in HeLa cells after poliovirus infection. Aliquots of a poliovirus-infected culture containing 5 μg/ml of actinomycin D were taken at the indicated times and a dialyzed LiCl supernatant was prepared at each time point using 1% NP-40 and 2 M LiCl without added DOC. Assays for polymerase activity (●) were at 37° for 30 min as described in *Materials and Methods* with poly(A) (200 μg/ml) and oligo(U) (10 μg/ml) and 80 μM [^{3}H]UTP as the only ribonucleoside triphosphate. RNA synthesis (○) in the infected culture was measured by adding [^{14}C]Urd (0.5 μCi/ml) to a portion of the culture and measuring the amount of the radioactivity incorporated into acid-insoluble material in 0.1-ml aliquots of the culture taken at the indicated times.

with either ribonuclease or 0.5 M NaOH but was not affected by deoxyribonuclease digestion (Table 2). When [α-^{32}P]UTP was used as the substrate, a nearest neighbor analysis indicated that the polymerase product was poly(U) because the α-^{32}P in labeled UMP residues was only transferred to other UMP residues (Table 3). If UMP residues were ever incorporated next to AMP or any other nucleotide, incorporation must have occurred at a frequency of less than about $\frac{1}{1000}$. In addition, the above results rule out the possibility that UMP residues are being incorporated into poliovirus double-stranded RNA by a repair mechanism similar to that recently proposed by Yin (7).

Glycerol Gradient Centrifugation of the Polymerase. The poly(U) polymerase in the dialyzed LiCl supernatant was analyzed by centrifugation on a linear 5–20% glycerol gradient (Fig. 3). The majority of the polymerase activity sedimented

Table 2. Digestion of the labeled product with RNase, DNase, and NaOH

Digestion conditions	% of control
Untreated	100
DNase I	98
Takadiastase + RNase A	6
0.5 M NaOH, 20 min at 80°	3

The reaction conditions were as described in *Materials and Methods* except that [α-^{32}P]UTP was used as the labeled substrate. For nuclease digestion, the product was first purified by chromatography of the reaction mixture on a Sephadex G-50 column in 0.4 M triethylamine carbonate. The void volume of the column, which contained the labeled product [presumably hydrogen-bonded to poly(A)], was lyophilized and digested with pancreatic DNase I (Worthington) in 5 mM $MgSO_4$/100 mM sodium acetate (pH 5.0) at 25° or with takadiastase plus RNase A (20). Prior to digestion with takadiastase plus RNase A, the purified product was incubated at 100° for 2 min and then rapidly cooled. To test for alkali sensitivity, a control sample was precipitated with TCA, and a test sample was placed in NaOH and heated as indicated. After cooling, sufficient TCA was added to neutralize the NaOH and give a final concentration of 5% TCA. The treated and untreated samples were then filtered and assayed for radioactivity.

Table 3. Nearest neighbor analysis of the product synthesized with added poly(A), oligo(U), and [α-^{32}P]UTP

3′-Nucleotide	cpm	% of total
UMP	9446	99.7
GMP	<10	<0.1
AMP	<10	<0.1
CMP	<10	<0.1

The reaction conditions were the same as in *Materials and Methods* except that the substrate was [α-^{32}P]UTP. The product was precipitated in 5% TCA, digested with 0.4 M KOH at 37° for 20 hr, neutralized by passing it through a Chelex 100 column (H^+ form) (Bio-Rad), and analyzed by high-voltage electrophoresis at pH 3.5 on Whatman 3MM paper with ^{32}P-labeled ribonucleoside 3′-monophosphate markers in parallel lanes.

at about 4 S relative to the 4.6S sedimentation marker (bovine serum albumin, molecular weight 68,500). This indicated that the NP-40/DOC/2 M LiCl treatment solubilized the majority (about 80%) of the poly(U) polymerase activity and that the polymerase was no longer complexed with either high molecular weight RNA or cellular membranes. The size of the poly(U) polymerase is apparently within the molecular weight range of the two stable noncapsid poliovirus-specific proteins, 3 (70,000) and 4 (57,000).

DISCUSSION

In the search for a poliovirus replicase, previous studies have depended on the activity of the enzyme with endogenous viral RNA as template. Attempts to stimulate activity with added viral RNA have failed. We have approached the problem by assaying for a model activity of the replicase. Reasoning that minus strand synthesis involves poly(U) synthesis as its first step, we assayed for poly(A)-dependent poly(U) synthesis. Because we had previously had success assaying reverse transcriptase with polymer templates and oligomer primers (18), when poly(A) by itself did not stimulate poly(U) synthesis, we added oligo(U) as a potential primer.

Our ability to assay poly(A) · oligo(U)-dependent poly(U) synthesis in poliovirus-infected cells, but not in uninfected cells, suggests that we have demonstrated a new virus-related enzymatic activity. Whether this is the replicase itself, or even a portion of the replicase, remains to be shown. A suggestion that

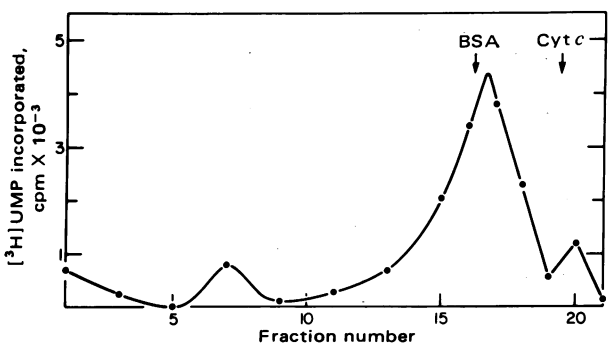

FIG. 3. Glycerol gradient centrifugation of the poly(A)-oligo(U)-dependent poly(U) polymerase. A dialyzed LiCl supernatant was prepared from poliovirus-infected cells (5.5 hr) and a 0.7-ml portion was layered on a linear 5–20% (vol/vol) glycerol gradient (10.5 ml) prepared in TN buffer. The gradient was centrifuged in a Beckman SW-41 rotor at 35,000 rpm at 4° for 19 hr and was collected from the bottom in 0.5-ml fractions which were assayed for 60 min at 29° as described in *Materials and Methods* with 10 mM DTT added. The sedimentation markers [^{125}I-labeled bovine serum albumin (BSA) and horse heart cytochrome *c* (Cyt *c*)] were run under identical conditions in a parallel gradient.

the LiCl supernatant fraction can copy heteropolymers as well as poly(A) has been obtained: poliovirion RNA will support incorporation of labeled UMP or GMP in this fraction in the presence of all four ribonucleoside triphosphates and oligo(U) (J. B. Flanegan and D. Baltimore, unpublished data).

We previously found that poliovirion RNA is not infectious if its 3′-poly(A) has been removed (19). If the replicase does initiate minus strand synthesis by making poly(U), it may be unable to copy poly(A)-deficient RNA, thus explaining the requirement of poly(A) for infectivity.

The dependence of poly(U) synthesis on an oligo(U) primer suggests that the replication of poliovirus RNA could also be dependent on a preformed primer. A protein has been found covalently linked to the 5′ termini of poliovirion and replicative intermediate RNAs (20, 21). Such a 5′-terminal protein, possibly with one or more nucleotides attached, could be a natural primer for synthesis of plus strand RNA. Whether such a model is relevant to minus strand synthesis will depend on whether there is a protein linked to the 5′ ends of minus strands.

The requirement for a preformed primer has recently been reported for another viral RNA polymerase. Plotch and Krug (22) found that the endogenous activity of the influenza virion RNA transcriptase was stimulated 100-fold in the presence of certain specific dinucleoside monophosphates. These were shown to act as primers for the synthesis of RNA by the viral transcriptase. Thus, certain RNA polymerases, like all DNA polymerases, may be primer-dependent enzymes.

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