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Purification and Properties of Poliovirus RNA Polymerase Expressed in *Escherichia coli*

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A cDNA clone encoding the RNA polymerase of poliovirus has been expressed in *Escherichia coli* under the transcriptional control of a T7 bacteriophage promoter. The poliovirus enzyme was designed to contain only a single additional amino acid, the N-terminal methionine. The recombinant enzyme has been purified to near homogeneity, and polyclonal antibodies have been prepared against it. The enzyme exhibits poly(A)-dependent oligo(U)-primed poly(U) polymerase activity as well as RNA polymerase activity. In the presence of an oligo(U) primer, the enzyme catalyzes the synthesis of a full-length copy of either poliovirus or globin RNA templates. In the absence of added primer, RNA products up to twice the length of the template are synthesized. When incubated in the presence of a single nucleoside triphosphate, [α-^{32}P]UTP, the enzyme catalyzes the incorporation of radioactive label into template RNA. These results are discussed in light of previously proposed models of poliovirus RNA synthesis in vitro.

The poliovirus genome is a 7.5-kilobase single-stranded RNA of positive polarity (23, 40). The RNA is polyadenylated (48, 61) and is covalently linked at its 5′ end to a 22-amino-acid virus-encoded protein, VPg (1, 18, 26). Following infection and translation of genomic RNA, the 52-kilodalton viral RNA polymerase (3D^{pol}) is released from the COOH terminus of the large polyprotein precursor by protease-catalyzed autocatalytic cleavage that generates the mature viral proteins (11, 20, 36, 38, 47, 54, 59).

Poliovirus RNA replication in vivo generates positive- and negative-stranded RNAs (6). Both types of RNA are linked at their 5′-terminal U residues to a tyrosine residue in VPg (2, 18, 25, 37, 39, 45). Whereas the 3′-terminal sequences of minus-strand RNA are heteropolymeric (41), the 5′-terminal sequences are composed of poly(U) which arises by transcription of the 3′-terminal poly(A) of plus-strand RNA (60). In vitro studies of viral RNA replication have followed two courses. In one approach, soluble viral RNA polymerase isolated from virus-infected cells has been incubated with viral genomic RNA (plus strand) in the presence of host cell factors (9, 12, 14, 16, 19, 56, 59). Most of these studies have detected the synthesis of minus-strand RNA only. In the second approach, crude membranous replication complexes from infected cells containing both plus- and minus-strand RNA have been used to synthesize viral RNA (10, 15, 50–53, 55). The RNA made by these complexes is mostly plus stranded.

Conflicting conclusions about the mechanism of RNA synthesis have been drawn from these studies. Highly purified preparations of viral RNA polymerase are incapable of initiating RNA synthesis in vitro with poliovirus plus-strand RNA as the template. Either oligo(U), acting as a primer (9, 16, 17, 19, 56, 58, 59), or host factor (8, 12–14), isolated from uninfected cells, is required for initiation, with the RNA polymerase acting solely as an elongating enzyme. A number of reports have demonstrated that oligo(U)-primed minus-strand RNA is identical in size to the plus-strand template (9, 12, 56, 59). Likewise, synthesis of template-sized minus-strand RNA was also demonstrated in earlier experiments in which oligo(U) was replaced with host factor (9, 12). More recent reports have demonstrated the synthesis of molecules twice the size of the template, made in the presence of either host factor (21, 22, 63, 64) or oligo(U) (28). Whereas oligo(U)-primed single- or double-length RNA is unlinked to the plus-strand template (28), double-length RNA made in the presence of host factor consists of virion template RNA covalently linked to minus-strand product RNA (21, 22, 64). Other polyadenylated RNAs could also be made double length in these reactions (21, 62).

The nature of the host factor has been a subject of controversy. Morrow et al. claimed that it is a protein kinase (32). Andrews et al. proposed that it is a terminal uridylyl transferase (TUTase) which adds UMP residues to the poly(A) tail of the poliovirus RNA (4, 5). The added U residues could snap back on the poly(A) tail to form a hairpin structure which would serve as a primer for copy-back transcription of the template by the poliovirus polymerase, resulting in the synthesis of a covalently linked dimer.

Several caveats apply to this model. It has never been shown that the minus-strand sequences in these dimers are covalently linked directly to the 3′ end of the poly(A) tail. In addition, dimers of subgenomic fragments of poliovirus RNA could be synthesized by the viral polymerase even in the absence of host factor (27). It was proposed that base-paired structures at the 3′ end of these RNAs would act as template primers for copy-back synthesis by the polymerase. Crude host factor preparations were shown to contain a nuclease activity that could randomly cleave poliovirus RNA to generate products, some of which were nearly full-length but lacked the poly(A) tail (22). Copy-back transcription of these subgenomic molecules by the polymerase gave rise to near-dimer-length molecules. Nuclease cleavage and elongation could not be relevant to RNA replication in vivo because the poly(A) tail would be lost by this mechanism. In this regard, double-length RNA has also been detected in poliovirus-infected cells (42). Most of these molecules, however, were devoid of poly(A) sequences, consistent with the notion that they were aberrant products, arising by cleavage and extension of RNA templates upstream of the poly(A) tail.

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The role of VPg in in vitro RNA synthesis with purified or partially purified preparations of RNA polymerase and host factors has not been unequivocally demonstrated; evidence both for (7, 31, 33, 34) and against (3, 62) its involvement has been reported. However, in a second approach in which crude replication complexes isolated from infected cells were used to direct poliovirus RNA synthesis (10, 15, 50–53, 55), evidence strongly suggesting that VPg participates in this reaction was obtained (50–53, 55). On the basis of these studies, a model was proposed whereby most RNA synthesis, both plus and minus strand, is primed by VPg or VPg precursors, with double-length molecules serving only as the initial template for the first round of RNA synthesis (50).

Although much new information will undoubtedly emerge from these studies, the inherent complexity of replication complexes from infected cells suggests an additional approach to determining the mechanism of viral RNA replication, i.e., to express all viral proteins potentially involved in RNA replication in nonmammalian hosts and use them to reconstruct authentic viral replication complexes in vitro. Several recent reports have described the expression of poliovirus RNA polymerase from cDNA in *Escherichia coli* (35, 43, 46). Fusion proteins of the polymerase linked to the viral 3C protease were expressed which then underwent cleavage, releasing active polymerase. Limited characterization of partially purified preparations indicated that the enzyme possessed catalytic properties similar to that of enzyme obtained from virus-infected eucaryotic cells. In this communication, we report the cloning into a bacterial expression vector of a cDNA encoding the poliovirus RNA polymerase not linked to any additional amino acids except the N-terminal methionine. Large quantities of enzymatically active protein were produced and purified to near homogeneity. Analysis of the RNA products made in the presence of the purified enzyme suggested that the enzyme possessed catalytic properties not previously detected in other poliovirus polymerase preparations obtained either from infected cells or *E. coli*.

MATERIALS AND METHODS

Construction of plasmids. We initially tried to express the poliovirus RNA polymerase by cloning the gene into pRC23, a plasmid containing the p_L promoter of bacteriophage lambda in front of a consensus Shine-Dalgarno sequence (11). We could not detect either polymerase protein or enzymatic activity after temperature shift induction of cultures harboring the recombinant plasmid. The polymerase gene was therefore inserted into an expression plasmid regulated by T7 RNA polymerase. Details of these constructions are described below.

Plasmids pT7PV1-5 was a gift from E. Wimmer. This plasmid contains a full-length cDNA copy of the poliovirus type 1 (Mahoney strain) genome placed 2 nucleotides downstream of a T7 RNA polymerase promoter (57). At the 3′ end of the cDNA is an 84-nucleotide dA-dT tract followed by a unique *EcoRI* site. An *Asu*II-*Eco*RI fragment (nucleotide 6012 to 3′ end) containing most of the polymerase gene (the gene starts at nucleotide 5987) was cut from the plasmid and ligated to a pair of complementary oligonucleotides that rebuilt the 5′ end (nucleotides 5987 to 6011) and added adjacent *Nde*I and *Eco*RI sites immediately upstream. The reconstructed gene was inserted into *Eco*RI-digested pRC23 to generate pRC-POL. Digestion of pRC-POL with *Nde*I and *Bam*HI released an *Nde*I-*Nde*I fragment (5′ end to nucleotide 6428) and an *Nde*I-*Bam*HI fragment which contained the remainder of the polymerase gene linked to the *Eco*RI-*Bam*HI sequence from pRC23. The *Nde*I-*Nde*I and *Nde*I-*Bam*HI fragments were ligated and inserted into pT7K digested with *Nde*I and *Bam*HI. pT7K, a derivative of the pET translation vectors of Rosenberg et al. (44) in which a kanamycin resistance gene has replaced the ampicillin resistance gene, is a plasmid containing a strong T7 RNA polymerase promoter upstream of the authentic Shine-Dalgarno sequence and starting ATG of T7 bacteriophage gene 10, as well as a transcription termination site downstream of the *Bam*HI site. The ATG forms half of the *Nde*I site. Because the *Nde*I-*Nde*I fragment can be inserted in either orientation, two plasmids were generated: pT7-POL, the correct orientation for translation of the poliovirus polymerase gene, and pT7-POL-Nde/rev, the translation of which is aborted by several stop codons.

pT7-POL(TRP$^-$), a plasmid lacking a TGG sequence coding for a tryptophan residue at amino acid 5 of the normal protein, was accidently generated during the cloning of pT7-POL. The loss of the triplet was confirmed by DNA sequencing. pT7-POL(AvrII) was generated by cutting pT7-POL with *Avr*II (poliovirus nucleotide 7205), filling in the overhangs with Klenow DNA polymerase, and religating the blunt ends. This procedure generates a stop codon, which results in the translation of a truncated protein lacking the 53 COOH-terminal amino acids.

Each of the plasmids was used to transform *E. coli* BL21(DE3)(LysS) cells which contain a chromosomal copy of the gene coding for T7 RNA polymerase under the control of the *lacUV5* promoter and a plasmid (chloramphenicol resistant) expressing a low level of T7 lysozyme, which inhibits basal levels of the T7 polymerase (30, 49). Induction by isopropyl-β-D-thiogalactopyranoside (IPTG) overproduces the T7 polymerase, which in turn transcribes the sequences inserted into the pT7 plasmids.

Purification of poliovirus RNA polymerase. Cells transformed with pT7-POL (approximately 2 liters) were grown in LB broth in the presence of kanamycin (25 μg/ml) and chloramphenicol (10 μg/ml) to an optical density at 600 nm of 1.0, at which time IPTG was added to a final concentration of 1.0 mM. After 3 h at 37°C, the cells were harvested by centrifugation and the pellet was either stored at −20°C or suspended immediately in 20 ml of 50 mM Tris (pH 8.0)–0.5 mM EDTA–1.0 mM dithiothreitol (DTT)–0.1% Nonidet P-40 (buffer I) per liter of original culture. After 10 min at 0 to 4°C, the suspension was sonicated at 0°C in a Soniprep 150 (MSE Corp.) for 5 to 10 cycles of 1 min each until it was no longer viscous and then centrifuged at 25,000 × *g* for 20 min. The pellet was reextracted in 10 ml of buffer I containing 1.0% Nonidet P-40 and 1.0 M NaCl. After 10 min at 0°C, the suspension was centrifuged as before. The two supernatants were pooled and dialyzed for several hours against 2 liters of 50 mM Tris (pH 8.0)–1.0 mM DTT–0.1% Nonidet P-40–10% glycerol (buffer A). Any precipitate was removed by centrifugation, and the clear supernatant was loaded onto a column (1.6 by 12 cm) of phosphocellulose (Whatman P-11) equilibrated in buffer A. After the column had been washed with 75 to 100 ml of buffer A, a linear gradient of 0 to 1.0 M KCl in buffer A (total volume, 100 ml), was applied. Fractions were monitored for poly(U) polymerase activity (see below). Active fractions (eluting between ca. 0.25 and 0.35 M KCl) were pooled, dialyzed against buffer A–50 mM KCl for several hours, and applied to a Mono Q HR 5/5 FPLC column (Pharmacia, Inc.) equilibrated in this buffer. After being washed, the column was developed with a 20-ml linear gradient of 50 to 500 mM KCl in buffer A. Fractions were

analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and assayed for enzymatic activity after portions had been diluted 1:20 in buffer A (undiluted enzyme is saturating for the assay); active fractions were pooled, dialyzed against buffer A, and loaded onto a poly(U)-Sepharose (Pharmacia) column (1.6 by 8 cm) equilibrated with this buffer. After a 10-ml wash, the column was developed with a linear gradient (100 ml) of 0 to 400 ml KCl in buffer A. Active fractions (assayed by diluting portions as above) containing only a single visible Coomassie blue-stained band of protein with an apparent molecular mass of about 55 kilodaltons were pooled, glycerol was added to a final concentration of 20%, and the enzyme was stored at −70°C. Partially purified enzyme can be safely stored at all stages of purification at −70°C in the presence of 10% or more glycerol.

Enzymatic assays. Poly(A)-dependent oligo(U)-primed poly(U) polymerase activity was measured at 30°C for 30 min in 25-μl reaction mixtures containing 1.0 to 2.5 μl of undiluted or diluted (see above) enzyme, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 8.0), 10 μM UTP, 5 mM DTT, 3.5 mM magnesium acetate, 20 μg of rifampin per ml, 1.0 μg of oligo(U_{15-30}), 2.5 μg of poly(A), and 0.5 to 2.5 μCi of [α-^{32}P]UTP. Reactions were stopped with 10 mM EDTA, and the labeled product was collected by precipitation on GN-6 membrane filters (pore size, 0.45 μm; Gelman Sciences, Inc.) with ice-cold 10% trichloroacetic acid in the presence of 100 μg of carrier RNA. The filter radioactivity was counted by measuring Cerenkov radiation. RNA polymerase activity was measured in 50-μl reaction mixtures containing 1 or 2 μl of poly(U)-Sepharose-purified enzyme, 50 mM HEPES (pH 8.0), 0.4 mM each ATP, CTP, and GTP, 15 μM UTP, 5 mM DTT, 3.5 mM magnesium acetate, 0.1 mM $ZnSO_4$, 0.25 to 0.5 μg of poliovirus plus-strand RNA synthesized by T7 RNA polymerase (see below), 50 U of RNasin (Promega Biotec), and 2.5 to 10 μCi of [α-^{32}P]UTP. When included, 12.5 ng of oligo(U_{15-30}) was added. When globin mRNA replaced poliovirus RNA, 0.05 to 0.25 μg was added. Some reactions were done in the absence of added ATP, CTP, and GTP; in these cases the unlabeled UTP concentration was reduced to 1.0 μM. After incubation at 30°C for 60 min, reactions were terminated by addition of EDTA to 10 mM; the mixture was phenol-chloroform extracted, and the RNA was subjected to three successive ethanol precipitations in the presence of 2.5 M ammonium acetate and 20 μg of carrier RNA.

Gel electrophoresis and protein concentration. RNA was analyzed by electrophoresis on 1.0% agarose gels after glyoxal denaturation (29). SDS-PAGE (10% acrylamide; acrylamide/bisacrylamide ratio, 30:0.8) was performed as described by Laemmli (24). The protein concentration was measured by using the Bio-Rad protein assay (Bio-Rad Laboratories).

Preparation of poliovirus RNA, globin RNA, and oligo(U). Plus-strand poliovirus RNA was made by transcription of *Eco*RI-digested pT7PV1-5. T7 RNA polymerase (200 to 400 U; Bethesda Research Laboratories, Inc.) was incubated with 10 μg of DNA for 1 h at 37°C in the buffer obtained from the manufacturer to which was added DTT to 10 mM, RNasin to 1,000 U/ml, and 0.4 mM each ATP, CTP, GTP, and UTP in a total volume of 200 μl. To monitor RNA synthesis, [α-^{32}P]UTP (2.5 to 5.0 μCi) was occasionally included. This amount of radioactive label did not interfere with the subsequent analysis of RNA synthesized in the presence of the poliovirus polymerase; these reactions were done with the labeled nucleotide present at 100- to 200-fold-higher specific activity. The reaction was terminated by adding EDTA to 20 mM, SDS to 1%, and proteinase K to 200 μg/ml. After 15 min at 37°C, the reaction mixture was phenol-chloroform extracted, and the RNA was ethanol precipitated. Following oligo(dT)-cellulose chromatography, the poly(A)$^+$ RNA was collected by ethanol precipitation and dissolved in 200 μl H_2O. About 50 μg of total RNA was usually synthesized by the T7 polymerase, of which about half was poly(A)$^+$. Globin mRNA was prepared by lysis of rabbit reticulocytes (PelFreeze Biologicals) lysis in ice-cold H_2O followed by proteinase K digestion in the presence of SDS (200 μg of proteinase K per ml, 1% SDS, 10 mM EDTA, 20 mM Tris [pH 8.0]) for 1 h at 37°C, phenol-chloroform extraction, and purification of the poly(A)$^+$ RNA by binding to and elution from two successive oligo(dT)-cellulose columns. Oligo(U_{15-30}) was prepared by incubating poly(U) (50 ml at 200 μg/ml) in 0.1 N NaOH for 35 min at 37°C. After neutralization with 10 ml of 1.0 M Tris (pH 8) and 0.4 ml of 12 N HCl, $MgCl_2$ was added to 1.0 mM followed by 250 U of calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals), and the reaction was incubated for 1 h at 37°C. Proteinase K to 50 μg/ml and EDTA to 5 mM were added; after a further 30 min the reaction mixture was phenol-chloroform extracted; and the oligo(U) was ethanol precipitated after addition of $MgCl_2$ to 15 mM and $LiCl_2$ to 0.2 M. After being washed in 80% ethanol, the oligo(U) was dissolved in 0.5 mM EDTA (pH 8.0) at a concentration of 0.8 mg/ml. Polyacrylamide gel analysis of ^{32}P-end-labeled oligo(U) indicated an average length of 15 to 30 nucleotides (data not shown).

Antibody production and immunoprecipitation. Antibody generated against poliovirus RNA polymerase obtained from infected eucaryotic cells was a gift from E. Wimmer. To prepare antibody against the recombinant enzyme, we inoculated two rabbits at 2-week intervals with 100 μg each of poly(U)-Sepharose-purified RNA polymerase that had been dialyzed against Dulbecco phosphate-buffered saline–1 mM DTT. One week after the third injection, serum was collected and stored at −20°C. To determine whether the sera could specifically immunoprecipitate the RNA polymerase in crude extracts, 10 ml of BL21(DE3)(LysS) cells transformed with either pT7-POL or pT7-POL-Nde/rev was grown in LB broth to an optical density at 600 nm of 1.0, pelleted, and suspended in minimal medium (M9 plus 0.5% glucose) containing IPTG at 1.0 mM. After 20 min at 37°C, rifampin was added to 20 μg/ml followed by [^{35}S]methionine to 5 μCi/ml 20 min later. After an additional 1 h at 37°C, the cells were pelleted, resuspended, and boiled in 0.5 ml of 0.6% SDS–20 mM Tris (pH 8.0)–1 mM EDTA–2 mM DTT. Aliquots (20 μl) were diluted with 500 μl of NETS buffer (20 mM Tris [pH 8.0], 1 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40)–0.5% bovine serum albumin to which was added 5 μl of either immune serum, nonimmune serum, or antibody against the eucaryotic enzyme. After 3 h at 4°C, 200 μl of protein A-Sepharose suspension (Pharmacia) (diluted 1:10 in NETS buffer) was added, and the mixture was incubated for an additional 1 h. After pelleting, the gel beads were washed three times with NETS buffer and boiled in Laemmli buffer, and the solubilized proteins were resolved by SDS-PAGE. The labeled proteins were detected by overnight autoradiography of the dried gel.

Materials. [α-^{32}P]UTP was obtained from Amersham Corp. Restriction and other enzymes were obtained from Bethesda Research Laboratories, Inc.; Boehringer Mannheim, Biochemicals; New England BioLabs, Inc.; and Pro-

mega Biotec. The sources of chromatographic media are identified in the text. All other chemicals were obtained from Boehringer Mannheim Biochemicals and Sigma Chemical Co.

RESULTS

Cloning and enzyme purification. To express the viral polymerase in *E. coli* without the need for cleaving a precursor polypeptide, a restriction fragment containing most of the polymerase gene was ligated to a synthetic pair of oligonucleotides that rebuilt the 5′ end of the gene and added an ATG codon immediately upstream of the triplet coding for glycine. Insertion of this construct into a bacterial expression vector under the tight transcriptional control of T7 RNA polymerase generated the plasmid pT7-POL (Fig. 1; see Materials and Methods for details of this construction). Translation of the RNA generated from this plasmid would produce poliovirus RNA polymerase containing only a single additional amino acid, the N-terminal methionine.

When IPTG-induced BL21(DE3)(LysS) cells harboring pT7-POL were lysed and analyzed by SDS-PAGE, a Coomassie blue-stained protein band of about 55 kilodaltons was observed (Fig. 2, lane 4). This band was not observed in uninduced cells (lane 3), nor was it observed in cells harboring a plasmid (pT7-POL-Nde/rev) which contained the polymerase gene with a 5′-terminal fragment inserted in reverse orientation—a construction that generates numerous stop codons (lanes 1 and 2). Extracts prepared from sonically treated IPTG-induced cells harboring pT7-POL demonstrated significant poly(A)-dependent oligo(U)-primed poly (U) polymerase activity (Table 1); as expected, no such activity was detected in extracts prepared from cells containing pT7-POL-Nde/rev (data not shown). Under the conditions of the assay used, little or no activity was detected in pT7-POL extracts in the absence of either oligo(U) or poly(A) (data not shown). We purified the viral polymerase by following a protocol similar to one used previously to purify the enzyme from virus-infected eucaryotic cells (5). Bacteria pelleted from approximately 2 liters of induced culture were disrupted by sonication in a low-salt buffer, and the pellet was reextracted with a high-salt buffer in the presence of detergent. About one-third to one-half of the total polymerase is solubilized by this procedure. Although the pellet can be solubilized by urea treatment and enzymatic activity can be recovered after dialysis (data not shown), we have not used this renatured enzyme in subsequent experiments. The pooled supernatants were dialyzed and applied to a phosphocellulose column. To avoid precipitation of the enzyme, dialysis was limited to a few hours. After elution of the enzyme from the phosphocellulose column, active fractions were pooled, dialyzed, and applied to an FPLC Mono Q column (anion exchanger). Active fractions eluted from this column were dialyzed and applied to a poly(U)-Sepharose column. Enzyme eluted from this column in a peak, the first two-thirds of which contained only a single band of protein free of other detectable impurities, as determined by using Coomassie blue-stained gels (Fig. 3B). The remaining one-third eluted with small amounts of a few lower-molecular-weight polypeptides. Only the purest fractions were pooled. A typical purification profile is shown in Fig. 3A and Table 1. The initial phosphocellulose chromatography step results in extensive purification of the enzyme; most of the bacterial proteins in the crude extract do not bind to the column in the loading buffer and are collected in the flowthrough fractions, whereas

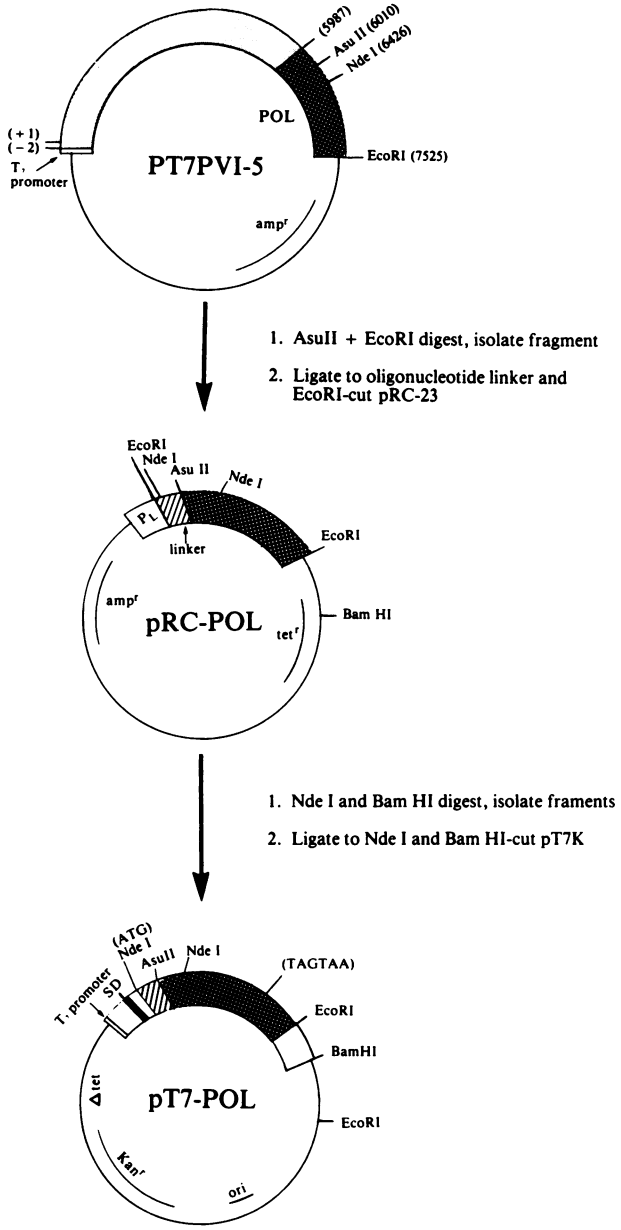

FIG. 1. Cloning strategy used to generate pT7-POL. The (−2) denotes the transcription starting point of the T7 RNA polymerase two G residues upstream of the first poliovirus RNA residue (a U residue), which is denoted by (+1). The POL gene (▩) starts at poliovirus nucleotide 5987. The first nucleotide of the recognition sequence of each of the restriction enzymes is indicated by a number in parentheses. P_L represents the lambda promoter region in pRC-POL. The oligonucleotide linker sequence is as follows:

AATTCATATGGGTGAAATCCAGTGGATGAGACCTT
GTATACCCACTTTAGGTCACCTACTCTGGAAGC

The ATG and TAGTAA sequences in parentheses indicate the starting and terminating codons, respectively. The region marked SD is the Shine-Dalgarno sequence between the promoter and the initiating codon in pT7-POL. Δtet indicates a deletion in the *tet* gene that occurred during the preparation of the plasmid pT7K. The relative sizes of the various regions within the plasmids are not necessarily drawn to scale.

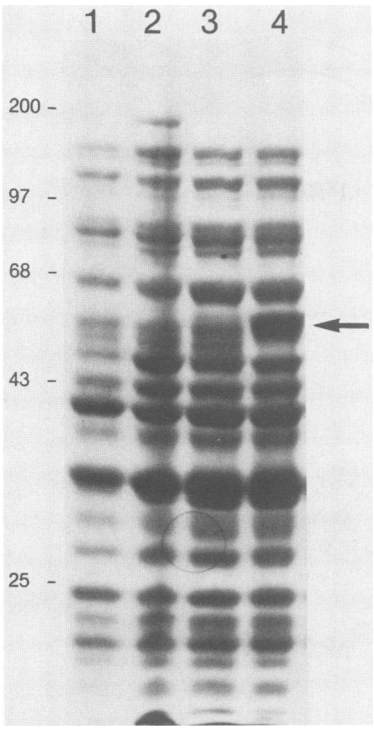

FIG. 2. Expression of poliovirus RNA polymerase in cells transformed with pT7-POL. BL21(DE3)(LysS) cells transformed with either pT7-POL-Nde/rev (lanes 1 and 2) or pT7-POL (lanes 3 and 4) were grown to an optical density at 600 nm of 1.0 in LB broth at 37°C. IPTG to 1.0 mM was then added to half of each culture. After another 3 h at 37°C, 0.5-ml aliquots of cells incubated either with (lanes 2 and 4) or without (lanes 1 and 3) IPTG were pelleted and boiled in Laemmli disruption buffer, and the proteins were resolved by SDS-PAGE. Gels were stained with Coomassie blue. The arrow indicates the protein band corresponding to the RNA polymerase. Migration position of molecular mass marker proteins (in kilodaltons) are indicated.

virtually all of the polymerase binds to the column under these conditions and is recovered following gradient elution. We invariably detect an increase in total enzymatic units recovered from the gradient-eluted fractions compared with the units detected in the undiluted crude extract (Table 1). This presumably is due to inhibitors, possibly RNA and/or nucleases present in the extract, which are removed by phosphocellulose chromatography. Others have also observed this phenomenon (35). Dilution of the crude extract also results in significant increases in enzymatic activity; this result is consistent with the diluting out of an inhibitor (data not shown).

During the course of cloning pT7-POL, a mutant was generated as a result of the deletion of a trinucleotide in the N-terminal portion of the coding region [pT7-POL(TRP$^-$)]. The protein expressed from this mutant lacked a tryptophan residue normally present at the fifth amino acid from the N-terminal glycine. This protein had no detectable enzymatic activity either in crude extracts or after elution from a phosphocellulose column, although the protein band was clearly visible on stained SDS-gels (data not shown). Another mutant, pT7-POL(AvrII), which lacked the C-terminal 53 amino acids of the normal protein, was also inactive.

Antibody preparation. The purified protein was used to prepare polyclonal antibodies in rabbits. To test the specificity of the antibodies, we incubated immune and nonimmune sera with lysates from IPTG-induced cells harboring either pT7-POL or pT7-POL-Nde/rev and labeled with [^{35}S]methionine. An antibody made against poliovirus RNA polymerase purified from virus-infected HeLa cells was included as a positive control. After addition of protein A-Sepharose, the labeled proteins bound to antibody were collected by centrifugation and analyzed by SDS-PAGE and autoradiography. The immune sera detected the recombinant RNA polymerase in the lysates (Fig. 4, lanes 6 and 7), as did the antibody made against the polymerase from infected HeLa cells (lane 5) (the signal was weaker in the latter because of a lower immunoglobulin G titer in this antibody preparation). Neither antibody immunoprecipitated any labeled protein from lysates of cells containing pT7-POL-Nde/rev (lanes 2 and 3). In addition, nonimmune sera did not immunoprecipitate any labeled material from any of the lysates (lanes 1 and 4).

RNA synthesis. To determine whether the recombinant enzyme could synthesize a full-length copy of a large RNA template, plus-strand poliovirus RNA, prepared by transcription of a full-length cDNA copy of the viral genome by T7 RNA polymerase, was incubated with the purified enzyme in the presence of oligo(U_{15-30}) and all four ribonucleoside triphosphates. The poliovirus RNA transcribed by the T7 enzyme differed somewhat from authentic poliovirion RNA in that it lacked VPg at its 5′ end, containing instead two additional G residues, and its 3′ end contained several extra nucleotides beyond the poly(A) tail whose sequence was determined by runoff transcription of an *Eco*RI restriction site (57) (see below). RNA molecules over a wide size range were synthesized by the recombinant poliovirus enzyme; the most prominent of these was a band migrating with the same mobility as the template RNA, 7.5 kilobases in length (Fig. 5, lane 4). In addition, a small amount of RNA migrated more slowly than template RNA, suggesting that RNA up to twice the size of the template had also been synthesized. When the enzyme was incubated with template RNA in the absence of an oligo(U) primer (lane 3), RNA synthesis over a wide size range again occurred, with the largest species being twice the length of the template. This time, the prominent band at 7.5 kilobases was absent. Although the efficiency of total RNA synthesis was several

TABLE 1. Poly(A)-dependent oligo(U)-primed poly(U) polymerase activity of sonic extracts of pT7-POL-containing cells

Fraction	Vol (ml)[a]	Concn (mg/ml)	Amt of protein (mg)	Units[b]	Units/mg[b]	Yield (%)[c]	Fold purification
Supernatant	28	33.4	934	2.8×10^6	3×10^3	(100)	(1)
Phosphocellulose	18	3.0	54	6.1×10^6	1.1×10^5	218	37
Mono Q	1.7	3.7	6.3	4.1×10^6	7.7×10^5	147	257
Poly(U)-Sepharose	8.8	0.16	1.4	2.3×10^6	1.7×10^6	84	560

[a] Yield from 1.8 liters of bacteria.
[b] One unit is defined as the number of picomoles of UMP incorporated into trichloroacetic acid-precipitable poly(U) in 30 min at 30°C.
[c] The value of 100% yield in parentheses denotes the activity obtained when using undiluted supernatant.

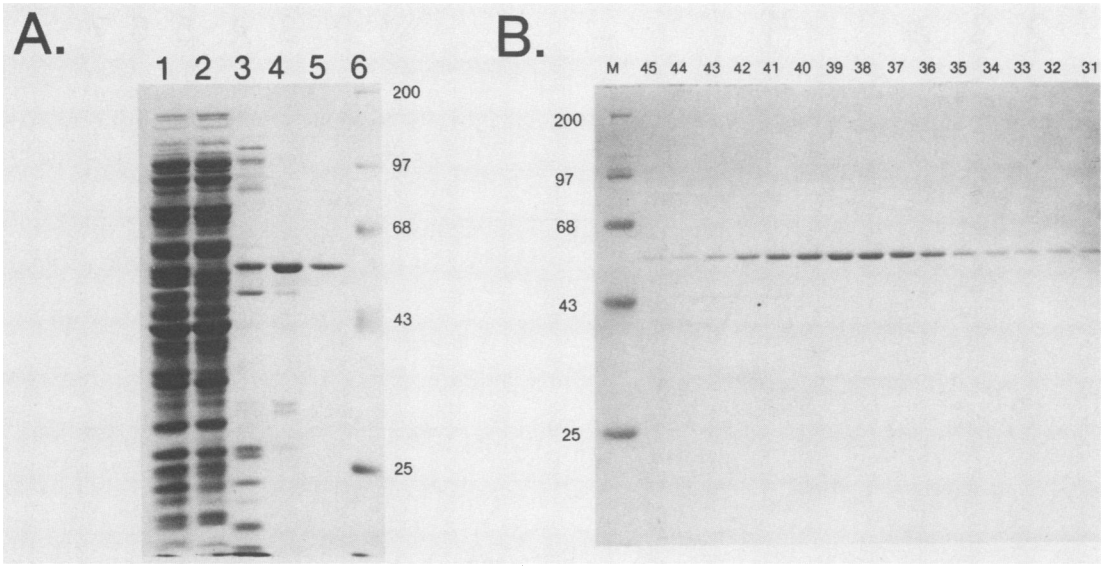

FIG. 3. Purification of poliovirus RNA polymerase from IPTG-induced cells transformed with pT7-POL. (A) SDS-PAGE of pooled fractions from each step of the purification protocol. Lanes: 1, pooled high- and low-salt extracts of sonicated cells; 2, phosphocellulose column flowthrough; 3, pooled active fractions eluted from phosphocellulose column; 4, pooled active fractions eluted from Mono Q column; 5, pooled fractions 33 to 39 eluted from the poly(U)-Sepharose column (from panel B); 6, molecular mass marker proteins (in kilodaltons). (B) Individual fractions (numbers above lanes) containing poly(U) polymerase activity eluted from the poly(U)-Sepharose column. The salt gradient increases from right to left. Lane M, molecular mass marker proteins (in kilodaltons).

times greater in the presence of oligo(U) than in its absence, synthesis of double-length RNA was more efficient in the absence of the oligo(U) primer. These results suggested that the purified recombinant enzyme not only was capable of synthesizing a full-length copy of the template RNA by using an oligo(U) primer but also could utilize the template RNA as primer to generate a double-length product RNA in the absence of any added eucaryotic host factor. Previous investigators, using enzyme obtained from infected eucaryotic cells or expressed in *E. coli*, have not detected the synthesis of double-length RNA without incubation in the presence of a host protein, either added exogenously or already present in the polymerase preparation. We do not know whether the oligo(U)-independent double-length product observed here was due to extension of the poly(A) tail by a TUTase-like activity of the enzyme followed by snap-back hairpin priming of covalently linked minus-strand synthesis (4, 5) or was merely the result of a nuclease activity in the reaction that nicks the template just upstream of the poly(A) tail, thereby creating a template primer for the synthesis of a near-double-length product (22). We have not detected any significant nuclease activity in our purified enzyme preparation, as determined by denaturing agarose or 7 M urea–acrylamide gel analysis of labeled poliovirus RNA incubated in the presence of enzyme and in the absence of nucleoside triphosphates (unpublished experiments). This, however, does not rule out a low level of nicking during the reaction in conjunction with a very efficient concurrent elongation reaction. The small amount of double-length RNA made in the presence of oligo(U) may be the result of the same, although somewhat suppressed, mechanisms operating in the absence of oligo(U) described above. Alternatively, these molecules may have resulted from oligo(U)-primed minus-strand synthesis followed by plus-strand synthesis originating from the 3′ end of the minus strands that have snapped back to form a hairpin (28). We have not as yet determined whether both plus- and minus-strand RNA is synthesized in our reactions. Our results differ from those in

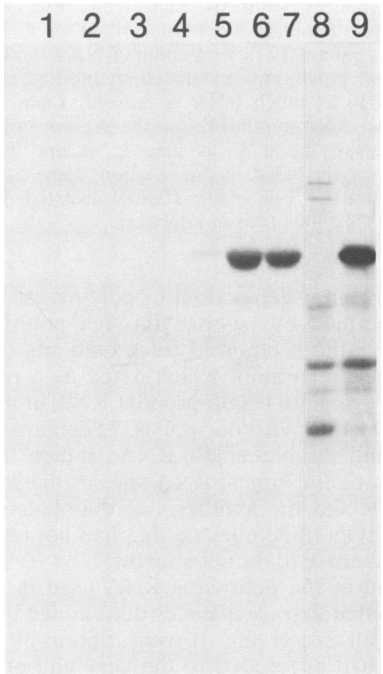

FIG. 4. Immunoprecipitation of recombinant poliovirus RNA polymerase by antipolymerase antibody. SDS-PAGE and autoradiography of immunoprecipitates of [^{35}S]methionine-labeled proteins from lysates of cells transformed with pT7-POL-Nde/rev (lanes 1 to 3) or pT7-POL (lanes 4 to 7) were performed following IPTG induction. Lane 8, total lysate from pT7-POL-Nde/rev-transformed cells; lane 9, total lysate from pT7-POL-transformed cells; lanes 1 and 4, incubation with nonimmune serum; lanes 2 and 5, incubation with antibody directed against poliovirus RNA polymerase from infected eucaryotic cells; lanes 3 and 6, incubation with immune serum obtained from rabbit 1; lane 7, incubation with immune serum obtained from rabbit 2.

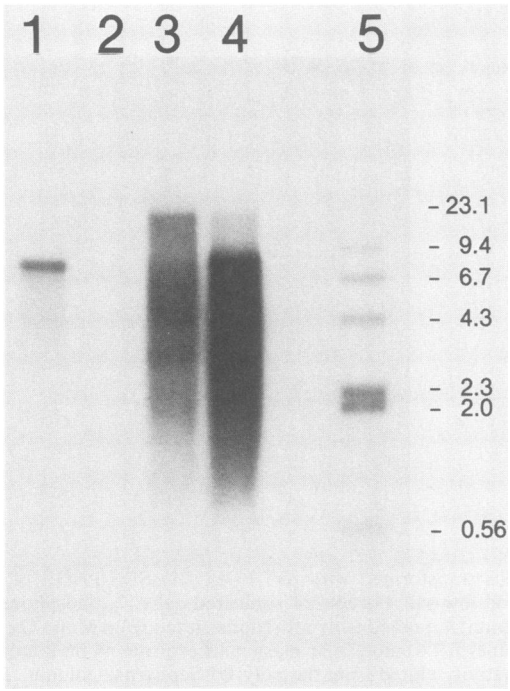

FIG. 5. Agarose gel electrophoresis of [^{32}P]-labeled RNA synthesized by recombinant poliovirus RNA polymerase with poliovirus plus-strand RNA as the template. The RNA was denatured with glyoxal prior to electrophoresis. Lane 1, [α-^{32}P]UTP-labeled poliovirus plus-strand template RNA transcribed by T7 RNA polymerase; this lane contains 10 times as much RNA as was present in the poliovirus RNA polymerase-catalyzed reactions. Lane 2, as lane 1, except that 1/10 as much RNA is present. Lane 3, [α-^{32}P]UTP-labeled product RNA synthesized in the presence of the poliovirus RNA polymerase. Lane 4, as lane 3, except that 12.5 ng of oligo(U_{15-30}) was included in the reaction. Lane 5, marker DNA: glyoxal-denatured, ^{32}P-labeled, *Hin*dIII-digested lambda DNA. Sizes of bands are indicated in kilobases.

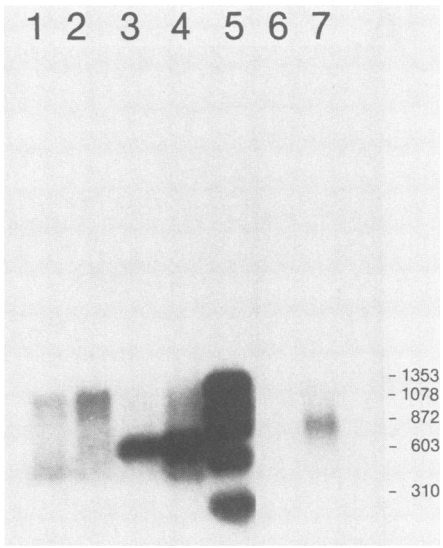

FIG. 6. Agarose gel electrophoresis of ^{32}P-labeled RNA synthesized by recombinant poliovirus RNA polymerase with globin mRNA as the template. The RNA was denatured by glyoxal prior to electrophoresis. Lanes 1 to 4, [α-^{32}P]UTP-labeled RNA synthesized in the presence of the poliovirus RNA polymerase and four ribonucleoside triphosphates; lane 7, [α-^{32}P]UTP-labeled RNA generated in the presence of the poliovirus RNA polymerase and UTP as the only nucleoside triphosphate. Amount of globin mRNA: lanes 1 and 3, 0.065 μg; lanes 2 and 4, 0.13 μg; lane 7, 0.26 μg. Reaction products shown in lanes 3 and 4 were made in the presence of 12.5 ng of oligo(U_{15-30}). Lane 5 contains marker DNA: glyoxal-denatured, ^{32}P-labeled, *Hae*III-digested ϕX174 DNA. Lane 6 is an empty lane. Sizes of bands are indicated in bases.

a recent report in which poliovirus polymerase from infected cells was purified by passage through poly(U)-Sepharose (63). Those authors reported trace amounts of oligo(U) in their enzyme preparation which acted as a primer for the synthesis of template length product RNA in the absence of added host factor. With our poly(U)-Sepharose-purified enzyme we find double-length RNA, rather than template length RNA, as the largest RNA species made. In addition, we have detected the synthesis of double-length RNA by using partially purified enzyme that had not been applied to poly(U)-Sepharose (data not shown).

The 3′ end of the poliovirus RNA used in these experiments contained extra sequences determined by runoff transcription of an *Eco*RI site. If transcription by the T7 RNA polymerase had proceeded to the very end of the digested DNA template, the RNA synthesized would contain several additional nucleotides beyond the poly(A) tail, ending in GAAUU (57). It might then be possible to explain the synthesis of double-length RNA by the poliovirus polymerase by assuming that the two 3′-terminal U residues could snap back on the poly(A) tail, creating a weak but nevertheless sufficient template primer for the synthesis of minus-strand RNA. To address this possibility, we used a different RNA, globin mRNA obtained from reticulocytes, as the template in poliovirus polymerase-catalyzed reactions. This RNA does not contain any U residues at its 3′ end. Mostly template length RNA was synthesized in the presence of the oligo(U_{15-30}) primer (Fig. 6, lanes 3 and 4), and double-length RNA was made in the absence of primer (lanes 1 and 2); these results are identical to those seen with the synthetic poliovirus RNA. It would appear, therefore, that preformed U residues at the 3′ end of the template are not required for double-length RNA synthesis to occur.

As mentioned above, one mechanism whereby double-length molecules could be generated would be if the recombinant enzyme itself contained TUTase-like activity. To test for this possibility, we incubated globin mRNA with enzyme and [α-^{32}P]UTP at high specific activity in the absence of the other three ribonucleoside triphosphates. A diffuse band migrating somewhat more slowly than oligo(U)-primed minus-strand globin RNA (template length) was observed (Fig. 6, lane 7), suggesting that a stretch of U residues was added to the template globin mRNA by the enzyme. If this addition occurred at the 3′ end of the RNA, it could result in the formation of a poly(A)-poly(U) hairpin structure which could serve as a template primer for the synthesis of double-length RNA in the presence of all four nucleoside triphosphates. U addition to synthetic poliovirus RNA has also been observed (data not shown). In other experiments in which enzyme and globin mRNA were incubated with either [α-^{32}P]ATP, [α-^{32}P]CTP, or [α-^{32}P]GTP as the only nucleoside triphosphate present in the reaction mixture, significant incorporation of label into template RNA also occurred in the presence of [α-^{32}P]ATP (data not shown). The mechanisms of these addition reactions are under investigation.

DISCUSSION

We have inserted the gene for poliovirus RNA polymerase into a high-efficiency bacterial expression vector and have purified milligram quantities of the expressed protein to near homogeneity. The enzyme, which was engineered to contain only a single non-virus-encoded amino acid, the N-terminal methionine, demonstrates both oligo(U)-primed poly(A)-dependent poly(U) polymerase and RNA polymerase activity. The fact that enzymatic activity is present in a polypeptide not generated as the result of cleavage of a precursor suggests that the enzyme need not be generated in precursor form to assume an active conformation. Rather, the opposite is suggested, i.e., that the protease sequences in the precursor block rather than contribute to the formation of an active polymerase conformation. The enzyme synthesizes mostly template length product RNA when incubated with either polioviral or globin RNA as the template in the presence of an oligo(U) primer, whereas in the absence of a primer or eucaryotic host factor, dimer-sized product is generated. We do not as yet know the mechanism by which the dimer-sized RNA is synthesized, nor have we directly demonstrated that the product RNA is covalently linked to the template, although this most probably is the case. If dimer synthesis is the result of a nicking reaction in double-stranded stem regions just upstream of the poly(A) tail of the template followed by extension from the free 3′-OH of the nicked nucleotide, our results simply confirm previously reported similar observations (22). It will be important to rigorously rule out nuclease activation of template RNA as the source of double-length RNA synthesis. However, if dimer synthesis is the result of copying back from the 3′-OH of the poly(A) tail of the template, our results indicate a previously undetected capability of the poliovirus polymerase. In support of this possibility, we have shown that our enzyme preparation is capable of catalyzing the incorporation of UMP residues into polyadenylated (globin) RNA in the absence of the other three ribonucleoside triphosphates. If this incorporation occurs by UMP addition to the end of the poly(A) tail, it could result in the subsequent synthesis of double-length RNA in the presence of all four ribonucleoside triphosphates. The source of this UMP-adding activity is puzzling. Others have not reported such an activity in purified preparations of the enzyme obtained from infected cells. In fact, the proposed host factor TUTase fulfills the lack of this very function in the polymerase (4, 5). One possible explanation is that enzyme preparations obtained from infected cells also contain this activity, but it is masked by the presence of some inhibitor, which is absent from our highly purified recombinant enzyme. Another possibility is that the presence of an N-terminal methionine in our enzyme imparts this additional capability to the RNA polymerase (although we have not determined whether the N-terminal methionine remains associated with the mature protein or is cleaved posttranslationally). A third possibility is that this activity is derived from some very-low-level bacterial protein contaminant that copurifies with the recombinant polymerase. In preliminary experiments, however, we have not detected any UMP (or AMP)-adding activity in extracts of untransformed bacteria that had been subjected to the same protocol of phosphocellulose and Mono Q chromatography used to purify the polymerase (data not shown). Further investigation is required to determine whether the U residues are added to the end of the poly(A) tail and, equally importantly, whether this activity is catalyzed solely by the viral polymerase.

Notwithstanding its demonstrated enzymatic capabilities, it is clear that the $3D^{pol}$ polymerase is only one of several proteins involved in the replication of the poliovirus genome, and further insight into the mechanism of polioviral RNA replication in vitro will require the expression and purification of other poliovirus proteins, e.g., VPg precursor protein(s), in addition to any required host cell proteins.

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