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# Expression, Purification, and Properties of Recombinant Encephalomyocarditis Virus RNA-Dependent RNA Polymerase

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**Encephalomyocarditis (EMC) virus RNA-dependent RNA polymerase was expressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferase (GST), which allowed easy purification of the fusion protein by affinity chromatography on immobilized glutathione. Inclusion of a thrombin cleavage site between the GST carrier and the viral enzyme facilitated the release of purified mature EMC virus RNA polymerase from the GST carrier by proteolysis with thrombin. The purified recombinant enzyme has a molecular mass of about 52 kDa and is recognized by polyclonal immune serum raised against a peptide sequence corresponding to the C-terminal region of the protein. The recombinant enzyme comigrates with immunoprecipitated EMC virus RNA polymerase from infected mouse L929 cell extracts when run in parallel lanes on a sodium dodecyl sulfate-polyacrylamide gel. The enzyme exhibits rifampin-resistant, poly(A)-dependent poly(U) polymerase activity and RNA polymerase activity, which are both oligo(U) dependent. Template-size products are synthesized in in vitro reactions with EMC virus genomic RNA or globin mRNA. The availability of recombinant EMC virus RNA polymerase in a purified form will allow biochemical analysis of its role in the replication of the virus as well as structure-function studies of this unique class of enzyme.**

Encephalomyocarditis (EMC) virus, a cardiovirus in the *Picornaviridae* family, consists of a single plus-stranded RNA genome of about 7.8 kb in size (29). The picornavirus RNA is polyadenylated at its 3′ end (49) and has a virus-encoded protein, VPg, covalently attached to its 5′ end (1, 21, 48). During infection, the viral RNA is translated into a polyprotein that is subsequently cleaved by mainly virus-encoded protease 3C to give rise to mature structural and nonstructural proteins (19, 26, 28, 30–32). One of the viral nonstructural proteins, the RNA-dependent RNA polymerase ($3D^{pol}$), replicates the single-stranded RNA genome, giving rise to both plus- and minus-strand RNAs (5). Currently, there is very little detailed knowledge about the biochemistry of replication of EMC virus. On the other hand, the replication of poliovirus, an enterovirus in the picornavirus family, has been studied extensively. In vitro studies of poliovirus RNA replication have shed some light on the replication process, yet fundamental questions regarding the mechanism of initiation and the specificity of template selection are yet to be answered.

Studies with crude replication complexes isolated from infected HeLa cells, in which mainly plus-strand RNA is synthesized, provided evidence that VPg protein or one of its precursors may serve as a primer for the initiation of RNA synthesis by $3D^{pol}$ (39–42, 44). Based on these observations, a model was proposed in which both plus- and minus-strand RNA synthesis is primed by VPg or its precursors (39). Attempts to reconstruct the replication reaction in vitro by using purified components led to the discovery that highly purified preparations of poliovirus $3D^{pol}$ are incapable of initiating RNA synthesis in vitro with poliovirus plus-strand RNA as the template (7, 14, 47). Initiation of RNA synthesis required the addition of a primer in the form of oligo(U) or host factors isolated from uninfected cells (4, 6, 9, 11, 24, 50). Mostly minus-strand RNA was synthesized in these in vitro reactions. The nature of the host factor and its role in the replication of the virus in vivo are not well understood. It has been suggested to be a protein kinase or a terminal uridylyl transferase capable of adding UMP residues to the poly(A) tail of the viral genome (2, 3, 24). The resultant oligo(U)-poly(A) would form a hairpin structure that may serve as a primer for RNA synthesis. However, in vitro replication assays with different preparations of $3D^{pol}$ and host factor isolated from cells have yielded different products and given rise to different models for the replication of poliovirus (6, 9, 17, 18, 22, 50).

To overcome these problems, researchers have attempted to reconstruct authentic virus replication complexes by using recombinant viral proteins expressed in nonmammalian hosts such as *Escherichia coli*. The expression of enzymatically active poliovirus $3D^{pol}$ in *E. coli* has been reported (25, 35). The recombinant enzyme was found to possess properties similar to the enzyme obtained from virus-infected cells. However, more recently, mature poliovirus $3D^{pol}$ has again been expressed in *E. coli* and surprisingly found to synthesize dimer-sized products in the absence of a primer or host factor (33). Because the recombinant poliovirus enzyme from different laboratories has yielded different results, we have decided to use a virus in another genus of the picornavirus family (EMC virus) to determine which of the current models of replication proposed for poliovirus hold true for other picornaviruses. As a first step, we required a purified preparation of EMC virus $3D^{pol}$. Initial attempts to isolate the RNA polymerase of EMC virus from infected cells resulted in partially purified preparations of the enzyme which exhibited poly(C)-dependent RNA polymerase activity (34, 45). Further characterization of the enzyme could not be carried out because of the instability of the enzyme. To overcome this problem, we have produced the mature EMC virus $3D^{pol}$ in *E. coli* with an expression system which permits extremely rapid purification of a highly active enzyme under nondenaturing conditions.

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## MATERIALS AND METHODS

**Construction of plasmid pT12.** The plasmid pEM3, containing a cDNA copy of 7,200 nucleotides from the 3′ end of the EMC virus genome, was obtained from Ann C. Palmenberg (29). The expression vector, pGEX-1, was purchased from AMRAD Corporation, Victoria, Australia. The vector contains the glutathione *S*-transferase (GST) gene from *Schistosoma japonicum* under the control of the *tac* promoter (37, 38). Downstream from this gene is a multiple cloning site containing restriction enzyme sites for *Bam*HI, *Sma*I, and *Eco*RI. Inserts cloned into these sites may be expressed as fusion proteins with GST. A recognition sequence for cleavage by the site-specific protease thrombin was inserted between the genes for GST and $3D^{pol}$ to allow cleavage of the expressed $3D^{pol}$ from GST following purification of the fusion protein by affinity chromatography. In order to express $3D^{pol}$ as a fusion protein, a 1.904-kb *Xho*I-*Bam*HI fragment (nucleotides 8204 to 375 of pEM3), containing most of the $3D^{pol}$ gene, which starts at nucleotide 8197, was isolated from pEM3 and ligated to a synthetic oligodeoxynucleotide linker to rebuild the 5′ end of the gene and add a cleavage recognition site for thrombin just upstream of the $3D^{pol}$ gene. The linker contained a *Bam*HI site at the 5′ end and an *Xho*I site at its 3′ end. The reconstructed gene was inserted into alkaline phosphatase-treated, *Bam*HI-digested pGEX-1 to generate the expression plasmid pT12 (Fig. 1). Both pGEX-1 and pT12 were subsequently transformed into *E. coli* HB101.

**Expression and purification of EMC virus RNA polymerase.** Overnight cultures of *E. coli* transformed with pGEX-1 or pT12 were diluted 1:50 in 1 liter of fresh 2× LB medium (23) containing ampicillin (100 μg/ml) and grown at 30°C to an $OD_{600}$ of 0.45, when IPTG (isopropyl-1-thio-β-D-galactoside) was added to 0.3 mM. After a further 4 h of growth at 30°C, the cells were harvested by centrifugation, and the pellet was stored at −20°C or resuspended in 60 ml of 50 mM Tris (pH 8.0)–150 mM NaCl–0.25 mM EDTA (buffer 1).

All subsequent purification steps were performed at 4°C. The suspension was sonicated in 10-ml aliquots in three 20-s bursts with a 0.5-in. (ca. 13 mm) probe on a 20Kc sonicator (Heat Systems–Ultrasonics, Plainview, N.Y.). The lysate was then centrifuged at 12,000 rpm for 1 h in a Sorvall SS34 rotor. The supernatant was loaded under gravity onto a 2-ml glutathione-Sepharose 4B column (Pharmacia LKB Biotechnology, Inc.) which had been previously washed with 3 M NaCl and equilibrated in buffer 1. The eluate was retained and reapplied to the column. Following absorption of the fusion protein to the glutathione affinity matrix, the column was washed eight times with 10 ml of buffer 1. After the final wash, the gel slurry containing the absorbed fusion protein was transferred to two 2-ml Eppendorf tubes and washed twice with 700 μl of buffer 1. The gel slurry, resuspended in 400 μl of buffer 1, was then incubated with 480 ng of thrombin (Sigma Chemical Co.) for 3 to 4 h. The slurry was then centrifuged, and the supernatant containing the released purified EMC virus $3D^{pol}$ was saved. The slurry was washed three more times with 400 μl of buffer, and the washes were combined and sterile filtered. An aliquot (~150 μl) was kept aside for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Concentrated glycerol and $MgCl_2$ were added to the remainder of the purified EMC virus $3D^{pol}$ preparation to give a final composition of 46 mM Tris (pH 8.0), 138 mM NaCl, 0.23 mM EDTA, 10 mM $MgCl_2$, and 5% glycerol.

**Antibody production, immunoprecipitation, and immunoblot analysis.** Polyclonal antisera were raised against a synthetic peptide corresponding to a predicted hydrophilic surface region of $3D^{pol}$ near the C-terminus. The peptide sequence chosen was Cys-Gly-Gly-Gly-Leu-Lys-Arg-Lys-Phe-Lys-Lys-Glu-Gly-Pro-Leu-Tyr. A nonnatural cysteine and three glycine spacers were added to the N-terminus of the peptide to enable linkage to the carrier protein, keyhole limpet hemocyanin (Sigma Chemical Co.). Linkage was carried out with the bifunctional reagent *m*-maleimidobenzoyl-*N*-hydroxy succinimide ester (27). The conjugated peptide preparations were prepared in complete Freund's adju-

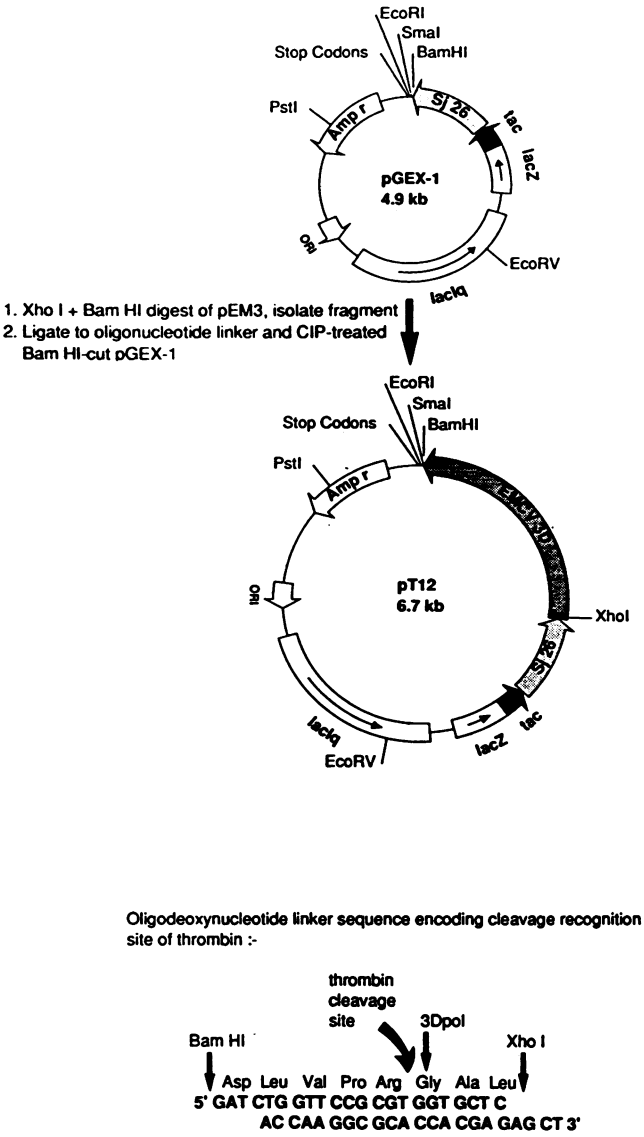


FIG. 1. Schematic representation of the cloning strategy used to generate EMC virus $3D^{pol}$ as a fusion protein with GST. In pGEX-1, the unique *Bam*HI cloning site is shown downstream of Sj26, which denotes the cDNA coding for the C-terminus of the GST gene from *Schistosoma japonicum*. EMCV 3D represents the cDNA encoding the EMC virus $3D^{pol}$. The oligonucleotide linker sequence used to regenerate the 5′ end of the cDNA of EMC virus $3D^{pol}$ is shown. The linker includes a thrombin recognition site to facilitate cleavage of the recombinant protein from the GST carrier. CIP, calf intestinal phosphatase.

vant and used to immunize rabbits. In subsequent injections, incomplete Freund's adjuvant was used.

To determine whether the antiserum was specific against EMC virus $3D^{pol}$, immunoprecipitation experiments were carried out with [$^{35}$S]methionine-labeled EMC virus-specified proteins from infected mouse L929 cell extracts, which were prepared as described by Sankar et al. (36). Either the immune sera or preimmune sera (20 μl) were incubated with 5 μl of [$^{35}$S]methionine-labeled EMC virus-infected cell extract in 300 μl of immunoprecipitation buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 2% Triton X-100) at 22°C for 16 h with gentle agitation. Then, 100 μl of protein A-Sepharose CL-4B was added, and the mixture was incubated at 22°C for 1 h and centrifuged. The pellet was washed three times with immunoprecipitation buffer and 10 mM Tris (pH 7.5), resuspended in 70 μl of lysis buffer (25 mM Tris [pH 6.8], 3% SDS, 25% glycerol, 7.5% 2-mercaptoethanol), boiled for 10 min, and analyzed by SDS-PAGE. For immunoblot analysis of extracts of *E. coli* carrying pGEX-1 and pT12, protein preparations from various stages of purification of EMC virus $3D^{pol}$ were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose as described by Towbin et al. (43) and Hawkes et al. (16). After transfer, the blots were soaked in 5% nonfat skimmed milk in phosphate-buffered saline for 1 h to saturate the additional binding sites. The blots were then treated sequentially with biotinylated goat anti-rabbit immunoglobulin antisera followed by streptavidin-horseradish peroxidase conjugate and reacted with 4-chloro-1-naphthol as the chromogenic substrate.

**Preparation of viral genomic RNA and oligo(U) primer.** EMC virus RNA was prepared by the procedure described by Sankar et al. (36). Briefly, infected mouse L929 cells were treated to two freeze-thaw cycles, and the virions released into the supernatant were precipitated with an equal volume of 14% (wt/vol) polyethylene glycol–4.6% (wt/vol) NaCl and centrifuged. The virion pellet, resuspended in 5 ml of buffer (50 mM Tris [pH 7.8], 50 mM NaCl), was layered onto a sucrose cushion (30% sucrose, 1 M NaCl, 20 mM Tris-acetate [pH 7.5], 0.1% bovine serum albumin) and centrifuged (38,000 rpm, 4°C, 5 h) with an SW41 Ti rotor. The virion pellet was resuspended in 400 μl of 10 mM Tris (pH 8.0)–50 mM NaCl–1 mM EDTA, treated with SDS and 2-mercaptoethanol, and heated at 60°C (90 s). The virus preparation was extracted three times with a phenol-chloroform mixture, and the RNA was ethanol precipitated. The purified EMC virus RNA was routinely analyzed on denaturing formaldehyde-agarose gels (23) and found to consist of a single RNA band of 7.8 kb.

Oligo(U) was prepared by alkali hydrolysis of poly(U) as described by Plotch et al. (33). The size of the resultant oligo(U) was checked by end-labeling with [γ-$^{32}$P]ATP and electrophoresis on a 20% polyacrylamide gel. Autoradiography of the gel indicated an average length of 15 to 30 nucleotides (data not shown). Globin mRNA used in the in vitro assays was obtained commercially from Bethesda Research Laboratories.

**Enzymatic assays.** The poly(A)-dependent oligo(U)-primed poly(U) polymerase assay was performed as described by Flanegan and Baltimore (12) except that rifampin at a concentration of 20 μg/ml was used instead of dactinomycin. The reactions were carried out at 30°C for 60 min in 50 μl containing 5 μl (~600 ng) of the purified enzyme, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 8.0), 10 μM UTP, 4 mM dithiothreitol, 3 mM magnesium acetate, 6 μM zinc chloride, 20 μg of rifampin per ml, 1.0 μg of oligo(U), 2.5 μg of poly(A), and 5 μCi of [α-$^{32}$P]UTP (Amersham, 400 Ci/mmol). The in vitro-synthesized product was precipitated by 10% trichloracetic acid with 100 μg of carrier tRNA in 0.2 M sodium pyrophosphate, collected on 0.45-μm Whatman GF/C filters, vacuum dried, and solubilized in scintillation fluid. The radioactivity was determined in a scintillation counter. RNA polymerase activity was measured in 50 μl containing 5 μl of the purified enzyme, 50 mM HEPES (pH 8.0), 0.4 mM each ATP, CTP, and GTP, 5 μM UTP, 4 mM dithiothreitol, 3 mM magnesium acetate, 6 μM zinc chloride, 1 μg of EMC virus RNA, 50 U of human placental RNase inhibitor (Amersham), 50 ng of oligo(U), and 10 μCi of [α-$^{32}$P]UTP. For reactions with globin mRNA as the template, 0.25 μg of globin mRNA and 100 ng of oligo(U) primer were used. After incubation at 30°C for 60 min, the reaction mixture was phenol-chloroform extracted and the RNA was ethanol precipitated in the presence of 0.3 M sodium acetate (pH 5.2) and 20 μg of carrier tRNA.

**Gel electrophoresis and protein concentration.** RNA was analyzed on 1% formaldehyde–agarose gels (23). SDS-PAGE was performed as described by Laemmli (20). The protein concentration was determined by the Bio-Rad protein assay.

**Materials.** Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and rifampin were obtained from Boehringer Mannheim. *m*-Maleimidobenzoyl-*N*-hydroxy succinimide ester was purchased from Pierce Chemical Co. Protein A-Sepharose CL-4B, poly(U), poly(A), ribonucleoside triphosphates, and *E. coli* DNA-dependent RNA polymerase were purchased from Pharmacia LKB Biotechnology, Inc. Goat anti-rabbit immunoglobulin antisera and horseradish peroxidase conjugate were obtained from Bethesda Research Laboratories. 4-Chloro-1-naphthol was obtained from Sigma Chemical Co. Human placental RNase inhibitor was purchased from Amersham.

## RESULTS

**Construction of plasmid for expression of EMC virus $3D^{pol}$.** To obtain large quantities of recombinant EMC virus $3D^{pol}$ in a form that can be easily purified, we used the expression vector pGEX-1, which directs the synthesis of foreign polypeptides in *E. coli* as fusions with a 26-kDa GST encoded by the parasitic helminth *Schistosoma japonicum* (38). This expression system, based on the *E. coli tac* promoter, provides a convenient means for the expression of fusion proteins in *E. coli* and their subsequent purification by affinity chromatography on immobilized glutathione under nondenaturing conditions. The cloning strategy used to generate the expression vector pT12 expressing EMC virus $3D^{pol}$ as a fusion protein is shown in Fig. 1 (see also Materials and Methods). A cleavage site for the protease thrombin was engineered between the GST gene and the $3D^{pol}$ gene so that proteolysis of the fusion protein would release $3D^{pol}$ with the authentic N-terminal amino acid, glycine.

**Expression of EMC virus $3D^{pol}$ in *E. coli* and its purification.** Induction of the *tac* promoter with IPTG in cells transformed with pGEX-1 resulted in the synthesis of a 27.5-kDa polypeptide consisting of GST with an additional 10 amino acid residues at its C terminus (Fig. 2, lane 1). This band was absent in cell extracts obtained from IPTG-induced *E. coli* HB101 cells harboring pT12; instead, a new, higher-molecular-mass protein band of about 78 kDa was observed (Fig. 2, lane 2). The molecular mass correlated with the predicted mass of the fusion protein of GST and

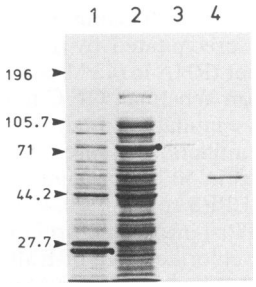


FIG. 2. Expression and purification of recombinant EMC virus $3D^{pol}$. *E. coli* HB101 strains harboring pT12 and the parental expression vector pGEX-1 were induced with IPTG and grown for 4 h, after which total-cell extracts were prepared and analyzed in a 0.1% SDS–10% polyacrylamide gel, which was stained with Coomassie brilliant blue. Lane 1, polypeptide profile obtained from pGEX-1. The dot indicates the 27.5-kDa GST protein specified by pGEX-1. Lane 2, polypeptides synthesized by *E. coli* harboring pT12. The dot denotes the fusion protein of GST and EMC virus $3D^{pol}$. Lane 3, glutathione affinity-purified fusion protein obtained from *E. coli* harboring pT12. Lane 4, purified recombinant EMC virus $3D^{pol}$ obtained by thrombin cleavage of the fusion protein while immobilized on glutathione. The positions of the molecular mass markers (in kilodaltons) are indicated by arrowheads on the left side.

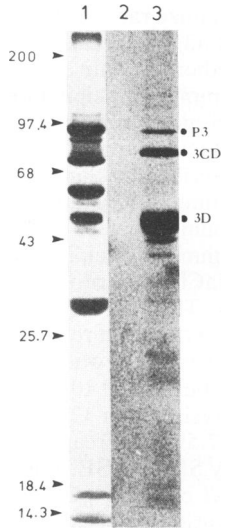


FIG. 3. Immunoprecipitation of EMC virus $3D^{pol}$ by peptide-specific antiserum raised against a C-terminal region of the protein. Autoradiograph of a 0.1% SDS–10% polyacrylamide gel showing [$^{35}$S]methionine-labeled proteins: lane 1, proteins synthesized in EMC virus-infected mouse L929 cells; lanes 2 and 3, immunoprecipitates of the infected-cell lysates incubated with preimmune and immune sera, respectively. Immunoprecipitated proteins (P3, 3CD, and 3D) are indicated by dots. The arrowheads on the left indicate the positions of molecular mass standards (in kilodaltons).

EMC virus $3D^{pol}$. To show that this band was indeed the fusion protein, extracts prepared from sonicated IPTG-induced cells harboring pT12 were passed through a glutathione-Sepharose column which had been equilibrated with buffer 1. The bound protein was eluted with buffer 1 containing 5 mM reduced glutathione and shown to be approximately 78 kDa in size (Fig. 2, lane 3).

Preliminary experiments with purified fusion protein indicated that the $3D^{pol}$ could be released from the carrier protein by thrombin cleavage (data not shown), but the released recombinant protein had to be separated from GST by rechromatography on glutathione-Sepharose. Previous data (34, 45) indicated that partially purified EMC virus $3D^{pol}$ isolated from infected cells is labile. Therefore, to further reduce the number of steps required for purification, we developed an essentially one-step purification of EMC virus $3D^{pol}$ by in situ proteolysis of the fusion protein immobilized on the glutathione-Sepharose column (15). In addition, all steps, including thrombin cleavage, were performed at 4°C. In the modified procedure, the fusion protein was not eluted with glutathione. Instead, the gel slurry containing bound fusion protein was incubated with thrombin for several hours at 4°C to allow proteolytic digestion of the fusion protein. The released recombinant $3D^{pol}$ was washed off the gel slurry with buffer 1 and sterile filtered (see Materials and Methods). Figure 2 (lane 4) shows the resultant purified EMC virus $3D^{pol}$, which has the expected molecular mass of 52 kDa. N-terminal sequencing of this purified protein revealed that the first 20 amino acids of the recombinant EMC virus $3D^{pol}$ matched the N-terminal amino acid sequence predicted from the cDNA sequence (29).

**Generation of antibodies to EMC virus $3D^{pol}$.** To aid in the detection of EMC virus $3D^{pol}$ in *E. coli*, a peptide corresponding to a hydrophilic region within the C-terminal region of the protein was used to generate polyclonal antibodies in rabbits. To test the specificity of the antibodies, we incubated the immune and preimmune sera with EMC virus-infected mouse L929 cell extracts which had been labeled with [$^{35}$S]methionine. After addition of protein A-Sepharose, the labeled proteins bound to antibody were collected by centrifugation and analyzed by SDS-PAGE and autoradiography. Figure 3 (lane 1) shows the protein profile obtained from EMC virus-infected mouse L929 cell extracts. The bands correspond to [$^{35}$S]methionine-labeled virus-specified proteins. The immune sera detected the EMC virus $3D^{pol}$ as well as its precursors 3CD and P3 (Fig. 3, lane 3), whereas the preimmune sera did not immunoprecipitate any labeled material from the infected cell extract (Fig. 3, lane 2).

The immune serum generated against EMC virus $3D^{pol}$ was subsequently used in an immunoblot analysis to detect the expression of EMC virus $3D^{pol}$ in extracts prepared from induced *E. coli* containing the vector alone or the recombinant (pT12). The immune serum did not react with control extracts prepared from *E. coli* harboring the vector alone (Fig. 4B, lane 1). However, a protein band of about 78 kDa, corresponding to the size of the fusion protein of GST and EMC virus $3D^{pol}$, was detected by the immune serum in both the crude soluble extracts (Fig. 4B, lane 2) and to a greater extent in the material eluted from the glutathione-Sepharose column (Fig. 4B, lane 3). Finally, the 52-kDa $3D^{pol}$ released by thrombin cleavage of the fusion protein also reacted with the immune serum (Fig. 4B, lane 4). The purified recombinant EMC virus $3D^{pol}$ migrated with the same mobility as immunoprecipitated [$^{35}$S]methionine-labeled EMC virus $3D^{pol}$ isolated from EMC virus-infected mouse L929 cells when run in parallel lanes on an SDS-polyacrylamide gel (data not shown).

**Characterization of enzymatic activity of recombinant EMC virus $3D^{pol}$.** As a first step towards establishing an assay system for the $3D^{pol}$, we used the poly(A)-dependent oligo(U)-primed poly(U) polymerase assay as described by Flanegan and Baltimore (12, 13). Very low concentrations of the purified recombinant $3D^{pol}$ exhibited significant poly(U) polymerase activity that was resistant to rifampin (Table 1).

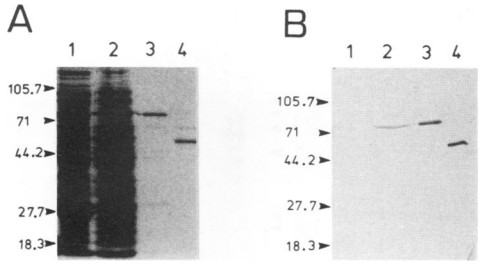


FIG. 4. Immunoblot analysis of recombinant EMC virus 3D$^{pol}$ preparations. Proteins from IPTG-induced *E. coli* harboring pGEX-1 or pT12 as well as purified forms of the GST fusion protein and the cleaved recombinant protein were separated in a 0.1% SDS–10% polyacrylamide gel. (A) Coomassie blue stain. Lane 1, soluble proteins from pGEX-1-transformed cells; lane 2, soluble proteins from pT12-transformed cells; lane 3, purified GST-3D$^{pol}$ fusion protein; lane 4, purified recombinant EMC virus 3D$^{pol}$. (B) Identical loadings were made on the same 0.1% SDS–10% polyacrylamide gel as shown in panel A, except that the proteins were electrophoretically transferred to nitrocellulose. Western immunoblot analysis was carried out with the antiserum raised against a peptide from near the C terminus of 3D$^{pol}$. Lanes 1 to 4 in panel B therefore show the immunoblots corresponding to the Coomassie blue stain in lanes 1 to 4 of panel A. The arrowheads on the left-hand side of panels A and B represent the positions of molecular mass standards (in kilodaltons).

Rifampin, an antibiotic which is known to block the reinitiation of RNA chains by the *E. coli* DNA-dependent RNA polymerase (8), was included in the assays to determine the contribution, if any, of the *E. coli* DNA-dependent RNA polymerase to the poly(U) polymerase activity detected in our assays. As indicated in Table 1, rifampin at 20 μg/ml had virtually no effect on the poly(U) polymerase activity of recombinant EMC virus 3D$^{pol}$. In contrast, purified *E. coli* DNA-dependent RNA polymerase exhibited significant poly(U) polymerase activity, which was almost completely inhibited in the presence of rifampin at 20 μg/ml (Table 1).

The poly(U) polymerase activity exhibited by 3D$^{pol}$ was next characterized with respect to template and primer requirements for activity. The poly(U) polymerase activity was completely dependent on the addition of both poly(A) and oligo(U) (Table 1). Oligo(dT) was as efficient as oligo(U)

TABLE 1. Characterization of poly(U) polymerase activity exhibited by recombinant EMC virus 3D$^{pol}$

| Enzyme[a] | Template/primer (other additions) | [α-$^{32}$P]UMP incorporated ($10^3$ cpm) |
|---|---|---|
| EMC virus 3D$^{pol}$ | Poly(A), oligo(U) | 765 |
| | Poly(A), oligo(U) (rifampin) | 680 |
| | Oligo(U) (rifampin) | 0 |
| | Poly(A) (rifampin) | 0 |
| | Poly(A), oligo(U) (rifampin, 100 mM KCl) | 26 |
| | Poly(A), oligo(U) (rifampin, 5 mM EDTA) | 13 |
| *E. coli* RNA polymerase | Poly(A), oligo(U) | 750 |
| | Poly(A), oligo(U) (rifampin) | 30 |

[a] Either 600 ng of the purified EMC virus RNA polymerase or 1 μl of *E. coli* RNA polymerase was used in the standard poly(A)-dependent oligo(U)-primed poly(U) polymerase assay.

as a primer in the poly(U) polymerase assay when added at the same concentration (data not shown). The enzyme activity was inhibited by KCl and EDTA, as for poliovirus 3D$^{pol}$ (7, 10).

Further biochemical characterization of the recombinant 3D$^{pol}$ was carried out to determine the kinetics of poly(U) polymerase activity as well as the temperature optimum for the reaction. The poly(U) polymerase activity was linear for 60 min (Fig. 5A), and the temperature optimum for the reaction was 30°C (Fig. 5B). The amount of product synthesized in a 60-min reaction by the purified recombinant enzyme was found to be a linear function of enzyme concentration up to 1.2 μg of protein (Fig. 5C). At higher concentrations, a drop in activity was noted. This can be explained by the presence of glycerol in the enzyme storage buffer, which at high concentration could inhibit the poly(U) polymerase activity. Finally, the purified recombinant EMC virus 3D$^{pol}$ was calculated to have a specific activity of 27 pmol of UMP incorporated per μg of protein in a 60-min reaction at 30°C. This value is 2 orders of magnitude lower than the value reported by Plotch and coworkers (33) for the recombinant poliovirus 3D$^{pol}$ but is similar to the value reported by Morrow et al. (25) for their preparation of recombinant poliovirus 3D$^{pol}$.

To determine whether the recombinant enzyme exhibits any RNA polymerase activity, plus-strand EMC virus RNA was incubated with the purified enzyme in the presence of oligo(U) and all four ribonucleoside triphosphates. RNA molecules over a wide range of sizes were synthesized by the recombinant EMC virus 3D$^{pol}$, the most prominent being about 7.8 kb in length, which corresponds to full-length EMC virus RNA (Fig. 6, lane 1). To further confirm that template-size products can be synthesized in an in vitro reaction, the enzyme was incubated with purified globin mRNA, and template-size copies of globin mRNA of ~600 nucleotides were synthesized (Fig. 6, lane 2). The presence of a smear of products rather than a clean single band (Fig. 6, lanes 1 and 2) is probably due to premature termination of the enzyme activity rather than nucleolytic degradation, since the assays were carried out in the presence of human placental RNase inhibitor. When the enzyme was incubated with EMC virus template RNA in the absence of an oligo(U) primer, no products were detected (data not shown). These results differ from the observations made by Plotch and coworkers (33), who reported that their recombinant poliovirus 3D$^{pol}$ exhibits oligo(U)-independent RNA polymerase activity, resulting in the synthesis of products up to twice the size of the template.

## DISCUSSION

We have subcloned the cDNA containing the putative RNA-dependent RNA polymerase gene of EMC virus and showed that it encodes an enzymatically active protein. Using the GST expression system, we have achieved high-level expression of the EMC virus 3D$^{pol}$ as a fusion protein with GST and purified the enzyme to near homogeneity by thrombin cleavage of the fusion protein bound to glutathione-Sepharose. The enzyme was engineered to be identical to the EMC virus 3D$^{pol}$ holoenzyme from infected eukaryotic cells. The fact that we obtained enzymatically active protein without coexpression of the protease 3C supports the findings of Plotch et al. (33) that this class of polymerase need not be expressed as a fusion with protease 3C in order to become active.

Previous attempts to characterize the enzyme from in-

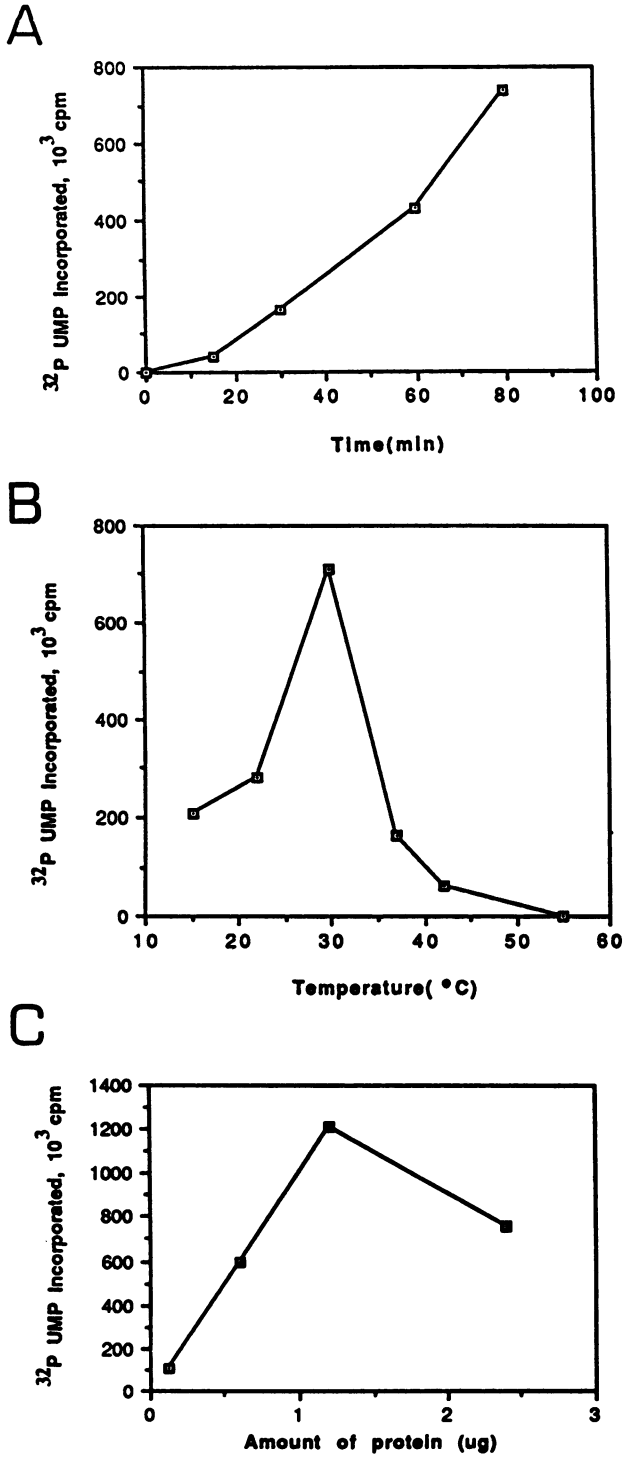


FIG. 5. Poly(U) polymerase activity of recombinant EMC virus $3D^{pol}$ as a function of time, temperature, and enzyme concentration. The recombinant EMC virus $3D^{pol}$ was purified as described in Materials and Methods. (A) 1.2 μg of the enzyme was assayed for poly(A)-dependent oligo(U)-primed poly(U) polymerase activity as a function of time at 30°C. (B) The amount of labeled [α-$^{32}$P]UMP incorporated in a 60-min assay with 600 ng of the purified enzyme was measured as a function of temperature. (C) Quantitative analysis of the poly(U) polymerase activity was carried out to determine the optimum concentration of the purified EMC virus $3D^{pol}$ in a 60-min assay at 30°C.

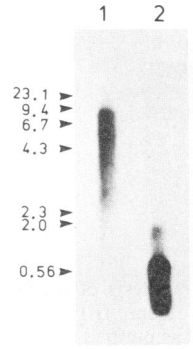


FIG. 6. Autoradiograph of an agarose gel showing $^{32}$P-labeled RNA synthesized by recombinant EMC virus $3D^{pol}$. The RNA was denatured by formaldehyde prior to electrophoresis. Lane 1, [α-$^{32}$P]UMP-labeled product RNA synthesized with EMC virus RNA as the template and oligo(U) as the primer. Lane 2, [α-$^{32}$P]UMP-labeled product RNA synthesized with globin mRNA as the template and oligo(U) as the primer. Arrowheads on the left side of the figure represent $^{32}$P-labeled *Hin*dIII-digested lambda DNA size markers (in kilobases).

fected eukaryotic cells met with difficulties owing to the low content of the enzyme, the association of the enzyme with smooth cytoplasmic membranes, requiring the use of detergents to separate the enzyme from the membrane, and the instability of partially purified preparations of the enzyme. Nevertheless, Traub et al. (45) reported poly(C)-dependent polymerase activity of partially purified preparations of authentic EMC virus $3D^{pol}$. Our recombinant enzyme, on the other hand, shows poly(A)-dependent poly(U) polymerase activity, which was absent from their partially purified extracts of EMC virus $3D^{pol}$. However, several lines of evidence support the conclusion that the recombinant EMC virus $3D^{pol}$ is indeed the viral RNA polymerase holoenzyme. First, the size of the recombinant protein is 52 kDa, which correlates with the size predicted from the cDNA (29). In addition, when run in parallel lanes on an SDS-polyacrylamide gel, the recombinant enzyme migrates with the same mobility as the immunoprecipitated [$^{35}$S]methionine-labeled EMC virus $3D^{pol}$ obtained from infected mouse L929 cell extracts. Second, the protein is recognized by specific immune serum raised against a peptide sequence corresponding to the C-terminal region of EMC virus $3D^{pol}$. Third, the amino acid sequence we derived for the N-terminal 20 amino acids of the purified protein matches exactly the sequence predicted from the cDNA (29). Fourth, the recombinant enzyme exhibits several enzymatic properties analogous to those observed for poliovirus $3D^{pol}$ isolated from infected HeLa cells (7, 10, 12, 13, 46), i.e., poly(U) polymerase activity that is template and primer dependent and is sensitive to KCl and EDTA. Finally, the enzyme exhibits RNA polymerase activity that is primer dependent. Template-size products are synthesized from both viral genomic RNA and globin mRNA. These results agree with those found by Rothstein et al. (35) for recombinant poliovirus $3D^{pol}$ but disagree with those of Plotch and coworkers (33), who found that their preparation of poliovirus $3D^{pol}$ is primer and host factor independent and synthesizes RNA molecules up to twice the size of the template viral RNA. However, Plotch et al. (33) used transcripts from a cDNA clone rather than purified viral RNA as the template, and they have not completely ruled out the possibility of a contaminant contributing to the primer-independent activity. In addition,

their enzyme may have an N-terminal methionine that imparts this additional ability to the recombinant $3D^{pol}$.

The expression of enzymatically active EMC virus $3D^{pol}$ in *E. coli* provides a starting point for further experiments to understand the mechanism of replication of EMC virus. In addition, the availability of purified EMC virus RNA polymerase will also allow us to map the functional domains of this unique enzyme and determine its relatedness to the DNA-dependent RNA polymerases and reverse transcriptases. Because the GST expression system has allowed the purification of milligram quantities of EMC virus $3D^{pol}$, attempts to crystallize this enzyme and solve its structure are now in progress.

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## REFERENCES

1. **Ambros, V., and D. Baltimore.** 1978. Protein is linked to the 5'-end of poliovirus RNA by a phosphodiester linkage to tyrosine. J. Biol. Chem. **253**:5263–5266.
2. **Andrews, N. C., and D. Baltimore.** 1985. Poliovirus replicase stimulation by terminal uridylyltransferase. J. Biol. Chem. **260**:7628–7635.
3. **Andrews, N. C., and D. Baltimore.** 1986. Purification of a terminal uridylyltransferase that acts as host factor in the *in vitro* poliovirus replicase reaction. Proc. Natl. Acad. Sci. USA **83**:221–225.
4. **Andrews, N. C., and D. Baltimore.** 1986. Lack of evidence for VPg priming of poliovirus RNA synthesis in the host factor-dependent in vitro replicase reaction. J. Virol. **58**:212–215.
5. **Baltimore, D.** 1969. The replication of picornaviruses, p. 101–176. *In* H. B. Levy (ed.), The biochemistry of viruses. Marcel Dekker, Inc., New York.
6. **Baron, M. H., and D. Baltimore.** 1982. Purification and properties of a host cell protein required for poliovirus RNA replication *in vitro*. J. Biol. Chem. **257**:12351–12358.
7. **Baron, M. H., and D. Baltimore.** 1982. *In vitro* copying of viral positive strand RNA by poliovirus replicase. J. Biol. Chem. **257**:12359–12366.
8. **Chamberlin, M., R. Kingston, M. Gilman, J. Wiggs, and A. de Vera.** 1983. Isolation of bacterial and bacteriophage RNA polymerases and their use in synthesis of RNA *in vitro*. Methods Enzymol. **101**:540–568.
9. **Dasgupta, A.** 1983. Purification of host factor required for *in vitro* transcription of poliovirus RNA. Virology **128**:245–251.
10. **Dasgupta, A., M. H. Baron, and D. Baltimore.** 1979. Poliovirus replicase: a soluble enzyme able to initiate copying of poliovirus RNA. Proc. Natl. Acad. Sci. USA **76**:2679–2683.
11. **Dasgupta, A., P. Zabel, and D. Baltimore.** 1980. Dependence of the activity of the poliovirus replicase on a host cell protein. Cell **19**:423–429.
12. **Flanegan, J. B., and D. Baltimore.** 1977. Poliovirus-specific primer-dependent RNA polymerase able to copy poly(A). Proc. Natl. Acad. Sci. USA **74**:3677–3680.
13. **Flanegan, J. B., and D. Baltimore.** 1979. Poliovirus polyuridylic acid polymerase and RNA replicase have the same viral polypeptide. J. Virol. **29**:352–360.
14. **Flanegan, J. B., and T. A. Van Dyke.** 1979. Isolation of a soluble and template-dependent poliovirus RNA polymerase that copies virion RNA in vitro. J. Virol. **32**:155–161.
15. **Gearing, D. P., N. A. Nicola, D. Metcalf, S. Foote, T. A. Willson, N. M. Gough, and R. L. Williams.** 1989. Production of leukemia inhibitory factor in *Escherichia coli* by a novel procedure and its use in maintaining embryonic stem cells in culture. Biotechnology **7**:1157–1161.
16. **Hawkes, R., E. Niday, and J. Gordon.** 1982. A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. **119**:142–147.
17. **Hey, T. D., O. C. Richards, and E. Ehrenfeld.** 1986. Synthesis of plus- and minus-strand RNA from poliovirion RNA template in vitro. J. Virol. **58**:790–796.
18. **Hey, T. D., O. C. Richards, and E. Ehrenfeld.** 1987. Host factor-induced template modification during synthesis of poliovirus RNA in vitro. J. Virol. **61**:802–811.
19. **Kitamura, N., B. L. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J. Dorner, E. A. Emini, R. Hanecak, J. J. Lee, S. van der Werf, C. W. Anderson, and E. Wimmer.** 1981. Primary structure, gene organisation and polypeptide expression of poliovirus RNA. Nature (London) **291**:547–553.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Lee, Y. F., A. Nomoto, B. M. Detjen, and E. Wimmer.** 1977. A protein covalently linked to poliovirus genome RNA. Proc. Natl. Acad. Sci. USA **74**:59–63.
22. **Lubinski, J. M., L. R. Ramone, and A. Dasgupta.** 1987. Primer-dependent synthesis of covalently linked dimeric RNA molecules by poliovirus replicase. J. Virol. **61**:2997–3003.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Morrow, C. D., G. F. Gibbons, and A. Dasgupta.** 1985. The host protein required for *in vitro* replication of poliovirus is a protein kinase that phosphorylates eukaryotic initiation factor-2. Cell **40**:913–921.
25. **Morrow, C. D., B. Warren, and M. R. Lentz.** 1987. Expression of enzymatically active poliovirus RNA-dependent RNA polymerase in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **84**: 6050–6054.
26. **Nicklin, M. J. H., H. Toyoda, M. G. Murray, and E. Wimmer.** 1986. Proteolytic processing in the replication of polio and related viruses. Bio/Technology **4**:33–42.
27. **Palfreyman, J. W., T. C. Aitcheson, and P. Taylor.** 1984. Guidelines for the production of polypeptide specific antisera using small synthetic oligopeptides as immunogens. J. Immunol. Methods **75**:383–393.
28. **Pallansch, M. A., O. M. Kew, B. L. Semler, D. R. Omilianowski, C. W. Anderson, E. Wimmer, and R. R. Rueckert.** 1984. The protein processing map of poliovirus. J. Virol. **49**:873–880.
29. **Palmenberg, A. C., E. M. Kirby, M. R. Janda, N. L. Drake, G. M. Duke, K. F. Potraz, and M. S. Collett.** 1984. The nucleotide and deduced amino acid sequences of the encephalomyocarditis viral polyprotein coding region. Nucleic Acids Res. **12**:2969–2985.
30. **Palmenberg, A. C., M. A. Pallansch, and R. R. Rueckert.** 1979. Protease required for processing picornaviral coat protein resides in the viral replicase gene. J. Virol. **32**:770–778.
31. **Palmenberg, A. C., and R. R. Rueckert.** 1982. Evidence for intramolecular self-cleavage of picornaviral replicase precursors. J. Virol. **41**:244–249.
32. **Pelham, H. R. B.** 1978. Translation of encephalomyocarditis virus RNA *in vitro* yields an active proteolytic processing enzyme. Eur. J. Biochem. **85**:457–462.
33. **Plotch, S. J., O. Palant, and Y. Gluzman.** 1989. Purification and properties of poliovirus RNA polymerase expressed in *Escherichia coli*. J. Virol. **63**:216–225.
34. **Rosenberg, H., B. Diskin, L. Oron, and A. Traub.** 1972. Isolation and subunit structure of polycytidylate-dependent RNA polymerase of encephalomyocarditis virus. Proc. Natl. Acad. Sci. USA **69**:3815–3819.
35. **Rothstein, M. A., O. C. Richards, C. Amin, and E. Ehrenfeld.** 1988. Enzymatic activity of poliovirus RNA polymerase synthesized in *Escherichia coli* from viral cDNA. Virology **164**:301–308.
36. **Sankar, S., K.-C. Cheah, and A. G. Porter.** 1989. Antisense oligonucleotide inhibition of encephalomyocarditis virus RNA translation. Eur. J. Biochem. **184**:39–45.
37. **Smith, D. B., K. M. Davern, P. G. Board, W. U. Tiu, E. G. Garcia, and G. F. Mitchell.** 1986. $M_r$ 26,000 antigen of *Schisto-*

*soma japonicum* recognized by resistant WEHI 129/J mice is a parasite glutathione *S*-transferase. Proc. Natl. Acad. Sci. USA **83**:8703–8707.

38. **Smith, D. B., and K. S. Johnson.** 1989. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. Gene **67**:31–40.
39. **Takeda, N., R. J. Kuhn, C.-F. Yang, T. Takegami, and E. Wimmer.** 1986. Initiation of poliovirus plus-strand RNA synthesis in a membrane complex of infected HeLa cells. J. Virol. **60**:43–53.
40. **Takeda, N., C. F. Yang, R. J. Kuhn, and E. Wimmer.** 1987. Uridylation of the genome-linked protein of poliovirus *in vitro* is dependent upon an endogenous RNA template. Virus Res. **8**:193–204.
41. **Takegami, T., R. J. Kuhn, C. W. Anderson, and E. Wimmer.** 1983. Membrane-dependent uridylation of the genome-linked protein VPg of poliovirus. Proc. Natl. Acad. Sci. USA **80**:7447–7451.
42. **Takegami, T., B. L. Semler, C. W. Anderson, and E. Wimmer.** 1983. Membrane fractions active in poliovirus RNA replication contain VPg precursor polypeptides. Virology **128**:33–47.
43. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
44. **Toyoda, H., C.-F. Yang, N. Takeda, A. Nomoto, and E. Wimmer.** 1987. Analysis of RNA synthesis of type 1 poliovirus by using an in vitro molecular genetic approach. J. Virol. **61**:2816–2822.
45. **Traub, A., B. Diskin, H. Rosenberg, and E. Kalmar.** 1976. Isolation and properties of the replicase of encephalomyocarditis virus. J. Virol. **18**:375–382.
46. **Tuschall, D. M., E. Hiebert, and J. B. Flanegan.** 1982. Poliovirus RNA-dependent RNA polymerase synthesizes full-length copies of poliovirus RNA, cellular mRNA, and several plant virus RNAs in vitro. J. Virol. **44**:209–216.
47. **Van Dyke, T. A., R. J. Rickles, and J. B. Flanegan.** 1982. Genome-length copies of poliovirion RNA are synthesized *in vitro* by the poliovirus RNA-dependent RNA polymerase. J. Biol. Chem. **257**:4610–4617.
48. **Vartapetian, A., Y. Drygin, K. Chumakov, and A. Bogdanova.** 1980. The structure of the covalent linkage between proteins and RNA in encephalomyocarditis virus. Nucleic Acids Res. **8**:3729–3742.
49. **Yogo, Y., and E. Wimmer.** 1972. Polyadenylic acid at the 3′-terminus of poliovirus RNA. Proc. Natl. Acad. Sci. USA **69**:1877–1882.
50. **Young, D. C., D. M. Tuschall, and J. B. Flanegan.** 1985. Poliovirus RNA-dependent RNA polymerase and host cell protein synthesize product RNA twice the size of poliovirion RNA in vitro. J. Virol. **54**:256–264.