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Synthesis of Plus- and Minus-Strand RNA from Poliovirion RNA Template In Vitro

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The poliovirus RNA polymerase, $3D^{pol}$, was used to synthesize RNA in vitro in the presence of a host factor preparation from uninfected HeLa cells and poliovirion RNA as the template. The transcription products included molecules approximately twice the length of the template, apparently resulting from hairpin formation and template-directed elongation, as previously reported (D. C. Young, D. M. Tuschall, and J. B. Flanegan, J. Virol. 54:256–264, 1985). Other polyadenylated template RNAs also yielded products that were twice the length of the template. The polarity of the products synthesized from plus-strand poliovirus RNA template was analyzed by Southern blotting using labeled product RNA to probe single-stranded poliovirus DNAs cloned into M13 vectors. The results demonstrated that host factor-mediated polymerase products contain newly synthesized plus-strand sequences as well as the expected minus-strand sequences. Polymerase products primed with oligo(U) were all of minus-strand polarity.

RNA-dependent RNA synthesis is a reaction required only of RNA-containing viruses. The biochemistry of this reaction is not well understood for any virus, although it is central to the process of genome replication. Poliovirus contains a single-stranded RNA genome of positive polarity that replicates in the cytoplasm of infected cells (21). It is 7,433 nucleotides long and is polyadenylated on its 3′ end (19, 30). A small protein called VPg is covalently attached to the 5′ end of the RNA; however, it is not known whether VPg serves as a primer for RNA synthesis or is added after synthesis has begun (17, 20).

In infected HeLa cells, poliovirus RNA synthesis occurs in a replication complex associated with membranous structures (7, 14, 34). A single viral polypeptide, $3D^{pol}$ (formerly NCVP4, 4b, or p63), was shown to be purified with the polymerase activity (15, 22). Efforts to develop a soluble in vitro poliovirus RNA replication system that was dependent on exogenous template resulted in the purification of $3D^{pol}$ (11, 16, 18, 36). This viral polypeptide catalyzed the synthesis of RNA complementary to poliovirus RNA template, but it required the addition of an oligo(U) primer (5, 18, 37). Synthetic oligo(U) can also serve as a primer for other polyadenylated RNA templates (35, 36). A host factor (HF) has been purified from uninfected HeLa cells or from rabbit reticulocytes, which substitutes for the oligo(U) primer, thereby allowing $3D^{pol}$ to initiate RNA synthesis directed by poliovirus RNA (4, 10–13).

Several reports describe the products of the HF-polymerase reaction as complementary (minus) strands, the largest of which are equal in length to the plus-strand virion RNA used as the template (5, 10). At least some of these products appear to be covalently linked to a polypeptide that is antigenically related to VPg (28, 29), and anti-VPg antibodies have been shown to inhibit the HF-polymerase reaction, but not the oligo(U)-primed reaction (3, 26). The source of the VPg donor in these reactions is thought to be a contaminant in the preparation of $3D^{pol}$ but has not been identified.

More recently, Young et al. (38) described the products of their HF-polymerase reaction as minus-strand sequences covalently linked to the template RNA, presumably synthesized by a self-priming hairpin mechanism induced by HF. The resulting products are up to twice as long as the template. The reasons for the differences in the products synthesized in different laboratories are as yet unclear, but they may be attributable to the purities of both the polymerase and HF preparations and the undefined biochemical roles of all of the components in the reaction.

Morrow et al. (27) reported that HF is copurified with a protein kinase activity, but the mechanism of involvement of protein phosphorylation with RNA synthesis is unclear. Andrews et al. (1) have described a uridylyl transferase activity in highly purified preparations of HF and suggested that the addition of a small number of uridylate residues to the polyadenylated 3′ end of the poliovirus RNA template would permit formation of a hairpin sufficient to allow self-priming and production of the dimer-length products.

In this report, we confirm the synthesis of poliovirus RNA products larger than the template that appear to result from a template self-priming mechanism as previously described (38). A highly purified viral $3D^{pol}$ and a relatively crude HF preparation were used. Globin mRNA, when used as the template, also yielded products approximately twice the length of the template. In addition, the polarity of the products was analyzed by hybridization to single-stranded M13 DNA containing poliovirus DNA inserts. These experiments showed that HF-primed products contain newly synthesized plus-strand sequences as well as minus strands. Conversely, oligo(U)-primed products are all of minus-strand polarity, as predicted.

MATERIALS AND METHODS

Materials. Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Agarose, globin mRNA, and restriction enzymes were from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Oligo(U) was from Collaborative Research, Inc., Waltham, Mass. DEAE-Sephacel, Sephacryl S200, and Sephadex G25

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were from Pharmacia Fine Chemicals, Piscataway, N.J. Phosphocellulose was from Whatman, Inc., Clifton, N.J. All radioisotopes were from New England Nuclear Corp., Boston, Mass. Methyl mercury hydroxide was from Alpha Products, Danvers, Mass. Nitrocellulose BA85 was from Schleicher & Schuell, Inc., Keene, N.H. Seal-A-Meal bags were from Dazey, Industrial Airport, Kans.

Cells and virus. Growth of HeLa S3 cells and purification of the Mahoney strain of poliovirus type I has been previously described (8).

Preparation of RNA. Poliovirus RNA was prepared from virions dialyzed against TNE (10 mM Tris hydrochloride [pH 8.3], 0.15 M NaCl, 5 mM EDTA). The suspension was extracted twice with TNE-saturated phenol at room temperature. The aqueous phase was collected and adjusted to 0.2 M sodium acetate, and poliovirus RNA was precipitated with 2 volumes of ethanol at −20°C overnight. After collection by centrifugation at 16,000 × *g* at 0°C for 30 min, RNA was dissolved in sterile water and stored at a concentration of 1 mg/ml. The RNA was used directly or adjusted to 10 mM Tris hydrochloride (pH 7.4)–0.1% sodium dodecyl sulfate (SDS)–200 μg of pronase per ml and incubated at 37°C for 1 h. The pronase-treated RNA was again extracted with phenol and precipitated with ethanol as described above. Both pronase-treated and untreated RNAs behaved identically for the experiments described below.

Purification of poliovirus polymerase. The polymerase preparation was as previously described (36) with the omission of the final hydroxylapatite column. Briefly, 1.5×10^9 HeLa cells were infected with the Mahoney strain of poliovirus type 1 at a multiplicity of infection of 100 PFU/cell for 5 h. The cells were collected, washed, suspended in 20 ml of RSB (10 mM Tris hydrochloride [pH 7.4], 10 mM NaCl, 1.5 mM $MgCl_2$), and disrupted by Dounce homogenization. Nuclei were removed by centrifugation at 9,000 × *g* for 5 min. A high-speed supernatant was prepared from the cytoplasmic extract by centrifugation at 200,000 × *g* for 2 h at 4°C. A solution of saturated ammonium sulfate was added to achieve 35% saturation, and the mixture was incubated on ice for 20 min with stirring followed by 20 min without stirring. The precipitate was collected by centrifugation at 10,000 × *g* for 20 min and then dissolved in 20 ml of TB-1 buffer (50 mM Tris hydrochloride [pH 8.0], 20% glycerol, 0.1% Nonidet P-40, 2 mM dithiothreitol, 10 μg of bovine serum albumin per ml) prior to application to a phosphocellulose column (1 by 6 cm) as prepared by Burgess (6). The column was previously equilibrated with TB-1 buffer at a flow rate of 20 ml/h. After loading, the column was washed with 40 ml of TB-1 and eluted with a 40 ml of 0 to 0.5 M KCl gradient in TB-1. Fractions were assayed for poly(A)–oligo(U)-dependent poly(U) polymerase activity as described below. Active fractions (8 to 10 ml) were pooled and dialyzed against 4 liters of TB-1 overnight at 4°C and then concentrated three- to fourfold against solid sucrose and applied to a Sephacryl S-200 column (1 by 80 cm) equilibrated in TB-1 with 0.2 M KCl. The column was eluted with the same buffer, and active fractions were pooled and dialyzed against TB-2 buffer (50 mM Tris hydrochloride [pH 8.0], 50% glycerol, 2 mM dithiothreitol, 0.1 M KCl). The enzyme was stored in aliquots at −70°C and showed little loss in activity even after 1 year of storage.

Purification of HF. The purification of HF was adapted from Baron and Baltimore (4). Uninfected HeLa cells (4×10^9) were washed in phosphate-buffered saline and suspended in 60 ml of buffer I (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 8.0], 15 mM NaCl, 1.5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 5 mM phenylmethylsulfonyl fluoride). The cells were allowed to swell at 0°C for 10 min before disruption by Dounce homogenization. Nuclei and membranes were removed by centrifugation at 27,000 × *g* for 3 min. A high-speed cytoplasmic supernatant (S130) was prepared by centrifugation at 130,000 × *g* for 2 h at 4°C. Pulverized ammonium sulfate was added to the S130 with stirring for 30 min, and the 40 to 60% saturated fractional precipitate was allowed to stir for an additional 30 min at 4°C. The precipitate was collected by centrifugation at 20,000 × *g* for 15 min, suspended in buffer A (20 mM HEPES [pH 8.0], 50 mM KCl, 5% glycerol, 0.1 mM EDTA, 5 mM 2-mercaptoethanol) and dialyzed overnight against 4 liters of buffer A with one change. The material was chromatographed on a DEAE-Sephacel column (1.5 by 20 cm) equilibrated in buffer A. The column was washed with 140 ml of buffer A before elution with a 100-ml 0 to 0.5 M KCl gradient in buffer A. The flow rate was maintained at 20 ml/h. Fractions were assayed for HF activity as described below, and active fractions were pooled and dialyzed against 4 liters of buffer A. The HF was stored frozen at −70°C and was stable for at least 6 months.

Polymerase assay conditions. The polymerase was assayed under three different conditions, either as a poly(U) polymerase, an oligo(U)-dependent RNA transcriptase, or an HF-dependent RNA transcriptase. Reaction conditions were identical in all three cases and varied only in the added template and primer. A total of 5 μl of polymerase (approximately 50 ng of protein) was assayed in a 50-μl reaction mixture containing 50 mM HEPES (pH 8.0); 500 μM each ATP, CTP, and GTP; 4 mM dithiothreitol; 3 mM $Mg(CH_3COO)_2$; and 60 μM $ZnCl_2$. UTP was used as the labeling nucleotide and was present at 11.4 μM. The specific activity of [^{3}H]UTP was 8×10^4 dpm/pmol, and that of [^{32}P]UTP was 3×10^4 dpm/pmol. The template used for the poly(U) polymerase assay was 1 μg of poly(A) in the presence of 0.34 μg of oligo(U). Oligo(U)-dependent RNA transcriptase activity was monitored with 1 μg of poliovirus RNA as the template in the presence of 0.034 μg of oligo(U). HF-dependent RNA transcription was measured with 1 μg of poliovirus RNA as the template and 5 μl of HF (approximately 5 μg of protein). Incubation was for 60 min at 30°C.

Construction of pPOV-1, pPOV-2, and pPOV-3. pPOV-1 contains a 1.4 kilobases of poliovirus cDNA *Bam*HI fragment from the 5′ third of the poliovirus genome (see Fig. 4) inserted into the *Bam*HI site of pBR322. pPOV-2 contains a 2.5-kilobase poliovirus cDNA *Bam*HI fragment from the middle third of the poliovirus genome also inserted into pBR322 at its *Bam*HI site. These cDNAs were synthesized by reverse transcription of poliovirus RNA primed with oligo(dT) (31). After hydrolysis of the RNA template in 0.1 M NaOH and second-strand synthesis with reverse transcriptase, product DNA was cut with *Bam*HI and ligated to pBR322 linearized with *Bam*HI. pPOV-3 contains a 2.9-kilobase poliovirus cDNA from the 3′-terminal sequence of the poliovirus genome inserted into the *Bam*HI-*Sph*I gap of pBR322. pBR322 was cut with *Sph*I and tailed with deoxythymidylate by using terminal transferase. The tailed plasmid was used to prime cDNA synthesis directly on poliovirus RNA. RNA in the RNA-DNA hybrids was hydrolyzed in alkali, and second-strand synthesis was accomplished with reverse transcriptase. The DNA was then cut with *Bam*HI and ligated. All DNAs were used to transform *Escherichia coli* HB101, ampicillin-resistant colonies were screened for the presence of poliovirus cDNA by colony blot hybridization (24), and plasmid preparations grown from

isolated colonies were analyzed by restriction enzyme digestion to identify cDNA inserts.

Preparation of M13mp18-1809(+) and M13mp18-1809(−) strands. The pSV-2 plasmid used in the construction of M13mp18-1809 was a gift from D. Baltimore. pSV-2 contains DNA representing the poliovirus sequence from nucleotides 1 to 1809 inserted into the *Pst*I site of pBR322.

M13mp18 was a gift from J. Messing. Phage growth and purification of both replicative-form and single-stranded DNAs were as previously described (25). Both pSV-2 and M13mp18 replicative-form DNAs were cut with *Pst*I, phenol extracted, and ethanol precipitated. The two DNAs were then mixed, ligated, and used to transform *E. coli* JM101. Phage were screened on B plates in the presence of X-Gal and IPTG (5-bromo-4-chloro-3-indolyl-D-galactopyranoside and isopropyl-β-D-thiogalactopyranoside, respectively) (25). Clear plaques were picked and used to grow 2-ml phage stocks. The stocks were spotted onto nitrocellulose paper as previously described (25) and probed separately with both labeled poliovirion RNA (plus strand) and poliovirus cDNA synthesized by calf thymus DNA-primed reverse transcription (minus strand). This screen allowed the detection of phage stocks with either the plus or minus polarity of the poliovirus sequence. Additionally, replicative-form DNA was purified from stocks containing poliovirus sequences, and restriction analysis was done to ensure the presence of the entire 1,809-nucleotide poliovirus DNA. M13mp18-1809(+) contains the poliovirus plus-strand DNA insert; i.e., it is identical to poliovirion RNA. M13mp18-1809(−) contains the poliovirus minus-strand DNA insert; i.e., it is complementary to poliovirion RNA.

Denaturing agarose electrophoresis. Denaturing agarose electrophoresis was done as described by Bailey and Davidson (2) with the following modifications. All equipment and solutions, except methyl mercury hydroxide, were either autoclaved or treated with 0.1% diethylpyrocarbonate to inactivate contaminating ribonucleases. Samples were denatured for 15 min at room temperature in sample buffer containing 15 mM methyl mercury hydroxide, 0.05 M boric acid, 0.005 M sodium borate, 0.001 M EDTA, 0.01 M sodium sulfate, and 10% glycerol. Electrophoresis was through either 0.7 or 1% agarose with a Bethesda Research Laboratories horizontal minigel apparatus. Staining with ethidium bromide was done in the presence of 0.5 M $NH_4C_2H_3O_2$ as described by Bailey and Davidson (2). After examination of the ethidium bromide staining material, the gel was fixed with 10% acetic acid for 15 min and soaked for several hours in methanol. The methanol was removed under vacuum, and the gel was exposed to Kodak XAR film overnight at −70°C with a DuPont Cronex Lightning-Plus intensifying screen.

Hybridizations. Gel electrophoresis of DNA to be blotted was done in 0.7% agarose in Tris-acetate buffer as described by Maniatis et al. (23). Transfer of DNA from the agarose gel to BA85 nitrocellulose was done as described by Southern (32). Labeled product RNA was used as a probe and prepared as follows. In vitro transcription reaction mixtures of 100 μl were run under standard reaction conditions with [^{32}P]UTP as the label. The reaction mixture was extracted with phenol, the aqueous phase was collected and adjusted to 0.3 M sodium acetate (pH 5.5), and the nucleic acid was precipitated with 3 volumes of 95% ethanol at −20°C overnight. The precipitate was collected and suspended in 200 μl of 10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA and loaded onto a G-25 Sephadex column (1 by 40 cm) equilibrated in 0.03 M Tris hydrochloride (pH 7.4)–0.2 M LiCl–0.002 M EDTA–0.1% SDS. The flow rate of the column was 20 ml/h, and 1-ml fractions were collected. The excluded peak of radioactivity was pooled and ethanol precipitated. The final precipitate was suspended in water and stored at −20°C. Routinely, 100,000 cpm Cerenkov was used to probe blots. The probe was first fragmented in 0.05 M NaOH at 20°C for 30 min. This treatment left the probe in fragments less than 2,000 bases long. The nitrocellulose was transferred to a Seal-A-Meal bag, and a hybridization solution of 50% formamide–0.4 M NaCl–0.1 M HEPES (pH 8.0)–5 mM EDTA–0.2% SDS–50 μg of yeast RNA per ml was added. Hybridization occurred at 42°C with rocking for 24 h. After hybridization, the blots were washed at room temperature with two changes, 15 min each, of 0.3 M NaCl–0.03 M sodium citrate–0.1% SDS. The blots were then washed at 52°C with two changes of 0.015 M NaCl–1.5 mM sodium citrate–0.1% SDS. The blots were dried and then exposed to Kodak XAR film with a DuPont Cronex Lighting-Plus intensifying screen at −70°C overnight.

TABLE 1. Activity of poliovirus RNA polymerase

Reaction conditions[a]	[^{3}H]UMP incorporated (pmol)
Complete + oligo(U)	5.7
Complete + HF	1.25
Minus primer	<0.05
Minus template	<0.05
Minus enzyme	<0.05

[a] The reaction conditions were as described in Materials and Methods. Labeled product RNA was precipitated with 7% trichloroacetic acid, collected on glass fiber filters, and analyzed for radioactivity.

RESULTS

The RNA polymerase activities of the 3D^{pol} preparations used in this study were completely dependent on the addition of a template RNA and either an oligo(U) primer or an HF preparation from uninfected HeLa cells. (Table 1; Fig. 1). The reason for the lag in the appearance of HF products in Fig. 1B is unclear, although a similar lag was noted by Morrow et al. (27). Analysis of the enzyme preparation by SDS-polyacrylamide gel electrophoresis revealed four polypeptides by silver staining, the most prominent of which migrated with an apparent molecular weight of 53,000 and which we assume is 3D^{pol} of approximately 80% purity (data not shown).

The products of both the oligo(U)-primed and HF-mediated reactions were examined by electrophoresis through methyl mercury hydroxide-containing agarose gels. When oligo(U) was used to prime reactions directed by poliovirus RNA, the products were a population of RNAs up to the size of the template (7,500 nucleotides) in length (Fig. 2, lane 1). The reaction products synthesized in the presence of HF (lane 2), however, included RNAs of twice the length of the template. These results are in agreement with data presented by Young et al. (38) and are consistent with a template priming reaction, mediated by HF, occurring in vitro.

Figure 3 shows the products of reactions in which globin mRNA (lanes 1 and 2) or poliovirus RNA (lanes 3 and 4) was used as the template. The autoradiogram was exposed for a shorter period to detect relative intensities of products. When oligo(U) was used to prime the reaction (lanes 1 and 3), intense bands comigrating with the template were observed, indicating that an abundance of full-length products

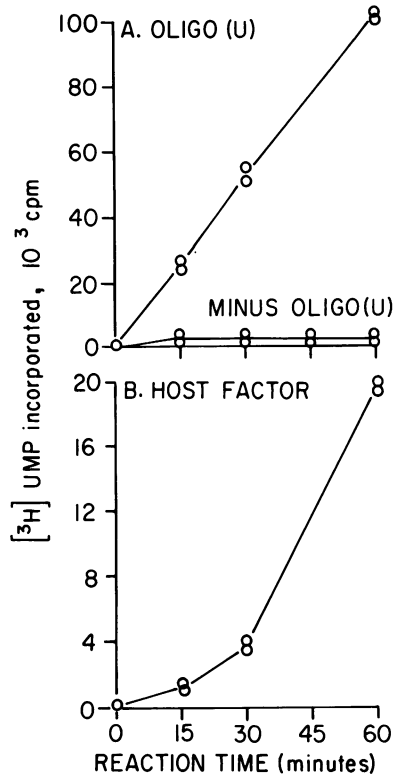

FIG. 1. Time course of RNA synthesis by poliovirus RNA polymerase. Reaction conditions were as described in Materials and Methods except that the reaction volume was 150 μl. Each reaction contained 15 μl of polymerase and 3 μg of poliovirion RNA as the template. (A) Reaction carried out in the presence of 0.1 μg of oligo(U). (B) Reaction carried out in the presence of 15 μl of HF. Samples (20 μl) were withdrawn at the indicated times, precipitated in 7% trichloroacetic acid, filtered, and counted.

was formed. A variable and minor amount of product larger than the template was sometimes detectable in oligo(U)-primed reactions, resulting from contamination of the polymerase preparation with small amounts of endogenous HF. Template-length products were also obtained with vesicular stomatitis virus mRNAs in the presence of oligo(U) primer (data not shown). The reaction products synthesized from poliovirion RNA template include a reproducible set of subgenomic RNAs that appear to be strong stop products of various lengths (Fig. 3, lane 3, arrows). The lengths of these products are consistent with stops occurring at regions of computer-predicted secondary structures in the template strand, as described by Curry et al. (9a). Since all of the products probably start by extension of the oligo(U) primer, it seems likely that, under in vitro transcription conditions, the poliovirus polymerase prematurely terminates when attempting to traverse regions of tightly folded templates.

In the presence of HF, globin mRNA template yielded a major product migrating at exactly twice the length of the template (Fig. 3, lane 2). The same result was also obtained with vesicular stomatitis virus mRNAs (data not shown). Poliovirus RNA template (Fig. 3, lane 4) showed a population of products, the majority of which ranged in size from approximately 1,000 nucleotides to twice the length of the template.

The polarity of the product RNAs was tested by hybridization to cloned poliovirus DNA sequences that were blotted onto nitrocellulose sheets after electrophoresis through agarose gels. pPOV-1, pPOV-2, and pPOV-3 are double-stranded cDNAs in pBR322 representing nearly the entire poliovirus genome (Fig. 4). M13mp18-1809(+) and M13mp18-1809(−) are single-stranded DNAs corresponding to the 5′ end of the plus strand and the 3′ end of the minus strand of poliovirus, respectively (Fig. 4). Poliovirus plus-strand RNA was provided as a template, and products from oligo(U)-primed or HF-mediated reactions were fragmented to an average size of about 1,000 nucleotides and used as probes; the results of the hybridizations are shown in Fig. 5. The oligo(U)-primed products hybridized strongly to pPOV-1, pPOV-2, and pPOV-3 (Fig. 5A), indicating the presence of product sequences from the entire poliovirus genome. This was expected, since a large proportion of the product was of genome length. The relatively reduced hybridization to pPOV-1 may be indicative of the increased probability of the polymerase to terminate as it traverses the template and therefore represents a deficiency of 3′ minus-strand sequences in the shorter, strong stop RNAs seen in Fig. 3. There was efficient hybridization to M13mp18-1809(+), indicating the presence of a minus-strand product complementary to the 5′ end of the plus-strand template. No hybridization to M13mp18-1809(−) was evident, demonstrating the absence of plus-strand sequences in the oligo(U)-primed product.

Products synthesized in an HF-polymerase reaction also hybridized to pPOV-1, pPOV-2, and pPOV-3 (Fig. 5B). In this case, hybridization to pPOV-3 was less intense than that which occurred with the oligo(U)-primed product. We be-

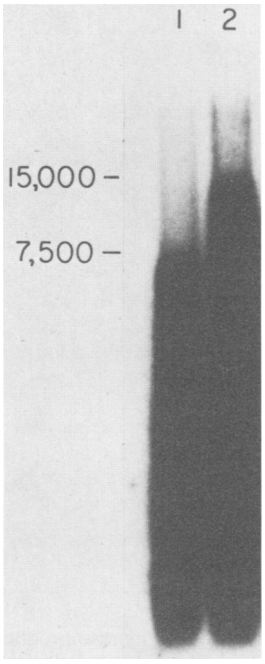

FIG. 2. Analysis of labeled product RNA by CH_3HgOH agarose gel electrophoresis. Product RNA labeled with [^{32}P]UTP was synthesized under standard reaction conditions, extracted with phenol, and precipitated with ethanol prior to analysis by denaturing agarose gel electrophoresis. The autoradiogram was overexposed to emphasize the size difference of product RNA transcribed from a poliovirion RNA template in the presence of oligo(U) (lane 1) or HF (lane 2). Molecular weight estimates were made by ethidium bromide staining *Eco*RI digestion products of P22 DNA analyzed in an adjacent lane.

lieve that this result was due to snapback hybridization of the hairpin fragments in the probe that contain the 3′ end of the template and its covalently attached complementary product RNA. Self-annealing of these fragments would effectively prevent them from hybridizing to pPOV-3. The HF products hybridized, as expected, to M13mp18-1809(+) (Fig. 5B). Surprisingly, however, there was also efficient hybridization to M13mp18-1809(−), indicating the presence of plus-strand sequences in the product RNA. These hybridization results could not be due to covalent linkage of minus-strand product to plus-strand template RNA because the use of M13mp18-1809(−) selects for sequences at the 5′ end of the plus strand, over 6,000 nucleotides away from the 3′ end. The product fragments used as probes in hybridization experiments were all smaller than 2,000 bases. Finally, we considered the possibility that short snapback products (<2,000 bases) may have formed on 5′-end fragments of template molecules and that these would hybridize to M13mp18-1809(−) by virtue of the covalent linkage of the labeled minus-strand sequences to the plus-strand template fragments. To eliminate this possibility, labeled product RNA was denatured and resolved on a preparative agarose gel, and gel fragments containing only molecules larger than 7,500 nucleotides were excised and phenol extracted to prepare a single-stranded probe. This single-stranded material was then fragmented and used to probe the same DNAs shown in Fig. 5. The results were identical to those shown in Fig. 5B, indicating that the plus-strand sequences were not derived from short template fragments attached to a labeled snapback product.

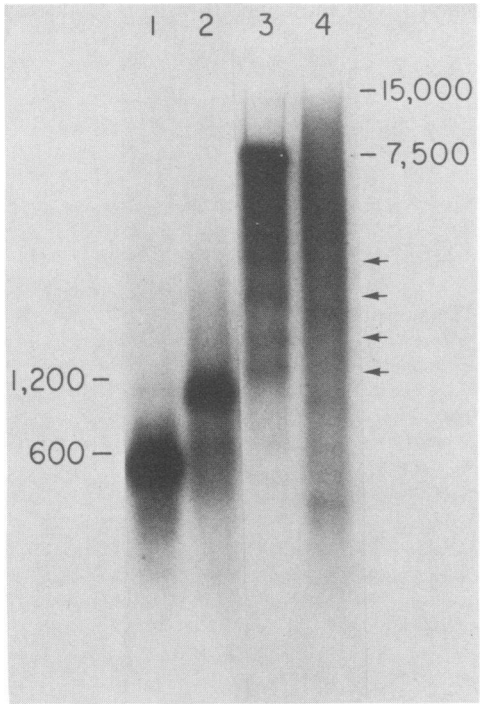

FIG. 3. Poliovirus RNA polymerase products synthesized from different templates. Product RNA labeled with [^{32}P]UTP was synthesized under standard reaction conditions using either globin mRNA (lanes 1 and 2) or poliovirion RNA (lanes 3 and 4) as the template. The autoradiogram was made following CH_3HgOH agarose gel electrophoresis of product RNA transcribed from globin mRNA template in the presence of oligo(U) (lane 1) or HF (lane 2) and from poliovirion RNA template in the presence of oligo(U) (lane 3) or HF (lane 4). Arrows show strong stop bands in lane 3. Molecular weight determinations were made by ethidium bromide staining markers in adjacent lanes. Globin mRNA is approximately 600 nucleotides; poliovirion RNA is approximately 7,500 bases.

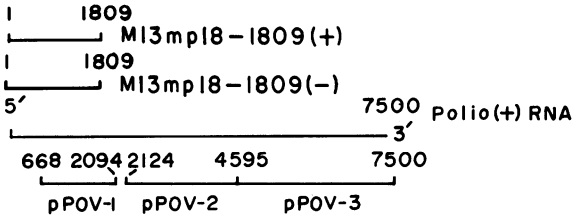

FIG. 4. Map of poliovirus DNA inserts in cloned plasmids and phage. The plasmids pPOV-1, pPOV-2, and pPOV-3 (below line) represent double-stranded poliovirus DNAs cloned into the *Bam*HI site of pBR322. M13mp18-1809(+) and M13mp18-1809(−) represent single-stranded DNAs cloned into the *Pst*I site of M13mp18. M13mp18-1809(+) contains sequences identical in polarity to the poliovirion RNA 5′ end, while M13mp18-1809(−) represents the complement of the poliovirion RNA 5′ end.

HF-mediated reaction products were prepared as probes for blot hybridization at early (7.5 min), intermediate (15 min), and late (30 min) times during the course of the in vitro polymerase reaction. The results suggested that plus-strand sequences did not appear in the product until after minus-strand sequences were synthesized (data not shown). Thus, taken together, these data suggest that minus-strand product RNA generated in vitro is utilized as the template for

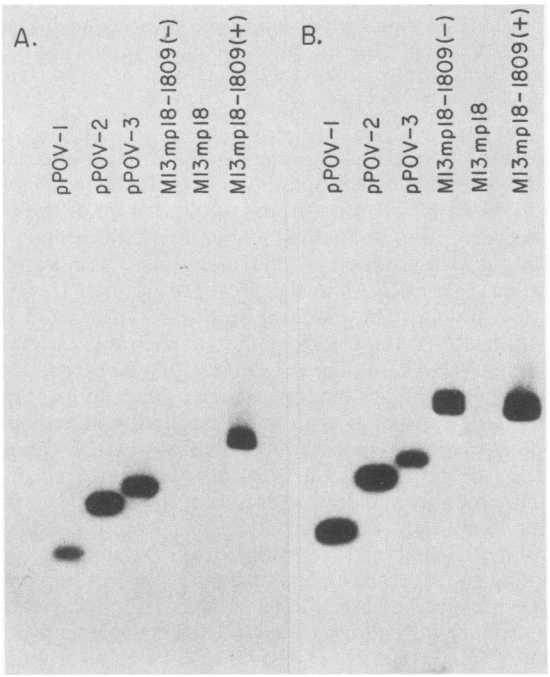

FIG. 5. Southern blots of poliovirus DNAs made by using in vitro transcription products as probes. Plasmids pPOV-1 and pPOV-2 were cut with *Bam*HI prior to electrophoresis through agarose gels. Plasmid pPOV-3 was cut with both *Bam*HI and *Sal*I. Equal amounts of poliovirus DNA were applied to each gel lane. (A) The blot was probed with oligo(U)-primed transcription products from a poliovirion RNA template. (B) The blot was probed with HF-mediated transcription products from a poliovirion RNA template.

synthesis of new plus-strand sequences by poliovirus polymerase and HF.

DISCUSSION

We studied poliovirus RNA synthesis in an in vitro transcription system consisting of a highly purified poliovirus polymerase preparation and a relatively crude HF fraction. Transcription of poliovirion RNA, as well as globin or vesicular stomatitis virus mRNAs, yielded products approximately twice the length of the templates. These results agree with those of Young et al. (38), who reported covalent addition of product RNA to poliovirus RNA template in a similar system. The polarity of the products synthesized from a plus-strand poliovirus RNA template was analyzed by Southern blotting experiments by using hybridization of labeled product RNA to probe single-stranded poliovirus DNAs cloned into M13 vectors. The results demonstrated that the HF-mediated polymerase reaction produces plus-strand products, presumably arising from transcription of newly synthesized minus-strand products.

The mechanism that produces a plus-strand product is not known at this time. If HF functions to induce or stabilize a terminal hairpin so that templates can self-prime, then it is possible that the same reaction occurs to produce a self-priming hairpin on the newly synthesized minus strand, allowing secondary hairpins and synthesis of new plus-strand sequences. Alternative models include nicking activity in the HF preparation that nicks plus-strand template which is hybridized to newly synthesized minus-strand product. Peeling off of the downstream template and elongation of the 3′ end at the nick site would result in synthesis of plus-strand sequences. Clarification of the biochemical roles of HF is essential to the interpretation of these results.

The production of dimer-length products in vitro raises the question of whether such a structure represents an intermediate in the synthesis of monomer-length single strands or whether it results from a reaction that occurs when authentic initiation events are prevented owing to the absence of a required component such as a VPg donor or another unidentified factor. In the former case, VPg addition would be coupled to subsequent endonucleolytic cleavage at the hairpin, as suggested by Young et al. (38). Significant concentrations of snapback dimers are found in infected cells (38; unpublished data), but these could similarly represent either replication intermediates or dead-end products formed when a component required for single-strand initiation is limiting. In this case, hairpin formation would be an aberrant reaction, and the initiation event that generates single-stranded RNA products would require priming by a VPg donor or another unidentified component. VPg-pUpU has been detected in vivo (9) and can be synthesized in vitro by crude membrane-associated replication complexes from infected cells (33). Evidence that it or any other form of VPg sequences can be incorporated into newly made RNA, however, is still lacking.

Whether or not the snapback dimer is an intermediate in the reaction, reports of different reaction products obtained by different laboratories (5, 10, 38) remain to be reconciled. One suggestion is that monomer-length products might result when the polymerase preparation is contaminated with a functional VPg donor. Such a donor could either prime the reaction or clip a hairpin, leaving monomer-length products attached to VPg, as previously reported (5, 10, 29). Conversely, a polymerase preparation free of VPg donor molecules could synthesize complementary product RNAs added directly to the template by an HF-induced hairpin formation. The enzyme preparation used in the studies reported here was highly purified and similar to the polymerase preparation used by others to form snapback products. Furthermore, we were unable to detect VPg sequences in our enzyme preparations by immunoblotting with anti-VPg serum (data not shown). Consistent with this hypothesis is the observation that anti-VPg antibodies immunoprecipitate product RNAs synthesized by the monomer-producing enzyme-HF preparation, and the VPg sequences appear to be linked to the product by the naturally occurring uridylphosphotyrosine bond (28). Products synthesized from the dimer-producing enzyme preparation are not immunoprecipitated by anti-VPg antibodies if VPg sequences have been removed from the template by proteinase K treatment (38).

Although much progress has been made in the study of poliovirus RNA polymerase reactions in vitro, caution still needs to be exercised in applying these results to our knowledge of the RNA replication reaction in vivo.

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LITERATURE CITED

1. **Andrews, N. C., D. Levin, and D. Baltimore.** 1985. Poliovirus replicase stimulation by terminal uridylyl transferase. J. Biol. Chem. **260**:7628–7635.
2. **Bailey, J. M., and N. Davidson.** 1976. Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. Anal. Biochem. **70**:75–85.
3. **Baron, M. H., and D. Baltimore.** 1982. Anti-VPg antibody inhibition of the poliovirus replicase reaction and production of covalent complexes of VPg-related proteins and RNA. Cell **30**:745–752.
4. **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.
5. **Baron, M. H., and D. Baltimore.** 1982. *In vitro* copying of viral positive strand RNA by poliovirus replicase. J. Biol. Chem. **257**:12359–12366.
6. **Burgess, R. R.** 1969. A new method for large scale purification of *Escherichia coli* deoxyribonucleic acid-dependent ribonucleic acid polymerase. J. Biol. Chem. **244**:6160–6167.
7. **Caliguiri, L. A., and I. Tamm.** 1970. Characterization of poliovirus-specific structures associated with cytoplasmic membranes. Virology **42**:112–122.
8. **Celma, M. L., and E. Ehrenfeld.** 1974. Effect of poliovirus double stranded RNA on viral and host-cell protein synthesis. Proc. Natl. Acad. Sci. USA **71**:2440–2444.
9. **Crawford, N. M., and D. Baltimore.** 1983. Genome-linked protein VPg of poliovirus is present as free VPg and VPgpUpU in poliovirus-infected cells. Proc. Natl. Acad. Sci. USA **80**:7452–7455.

9a. **Currey, K. M., B. M. Peterlin, and J. Maizel.** 1986. Secondary structure of poliovirus RNA: correlation of computer predicted with electron microscopically observed structure. Virology **148**:33–46.

10. **Dasgupta, A.** 1983. Purification of host factor required for *in vitro* transcription of poliovirus RNA. Virology **128**:245–251.
11. **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.
12. **Dasgupta, A., P. Hollingshead, and D. Baltimore.** 1982. Antibody to a host protein prevents initiation by the poliovirus replicase. J. Virol. **42**:1114–1117.
13. **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.

14. **Etchison, D., and E. Ehrenfeld.** 1981. Comparison of replication complexes synthesizing poliovirus RNA. Virology **111**:33–46.
15. **Flanegan, J. B., and D. Baltimore.** 1979. Poliovirus polyuridylic acid polymerase and RNA replicase have the same viral polypeptide. J. Virol. **29**:352–360.
16. **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.
17. **Flanegan, J. B., R. F. Pettersson, V. Ambros, M. J. Hewlett, and D. Baltimore.** 1977. Covalent linkage of a protein to a defined nucleotide sequence at the 5′-terminus of virion and replicative intermediate RNAs of poliovirus. Proc. Natl. Acad. Sci. USA **74**:961–965.
18. **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.
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. vanderWerf, C. W. Anderson, and E. Wimmer.** 1981. Primary structure, gene organization and polypeptide expression of poliovirus RNA. Nature (London) **291**:547–553.
20. **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.
21. **Levintow, L.** 1974. The reproduction of picornaviruses, p. 109. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology 2. Plenum Publishing Corp., New York.
22. **Lundquist, R. E., E. Ehrenfeld, and J. V. Maizel.** 1974. Isolation of a viral polypeptide associated with poliovirus RNA polymerase. Proc. Natl. Acad. Sci. USA **71**:4773–4777.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 150–185. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 309–360. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
26. **Morrow, C. D., and A. Dasgupta.** 1983. Antibody to a synthetic nonapeptide corresponding to the NH_2 terminus of poliovirus genome-linked protein VPg reacts with native VPg and inhibits in vitro replication of poliovirus RNA. J. Virol. **48**:429–439.
27. **Morrow, C. C., 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.
28. **Morrow, C. D., J. Hocko, M. Navab, and A. Dasgupta.** 1984. ATP is required for initiation of poliovirus RNA synthesis in vitro: demonstration of tyrosine-phosphate linkage between in vitro-synthesized RNA and genome-linked protein. J. Virol. **50**:515–523.
29. **Morrow, C. D., M. Navab, C. Peterson, J. Hocko, and A. Dasgupta.** 1984. Antibody to poliovirus genome-linked protein (VPg) precipitates *in vitro* synthesized RNA attached to VPg-precursor polypeptide(s). Virus Res. **1**:89–100.
30. **Racaniello, V. R., and D. Baltimore.** 1981. Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proc. Natl. Acad. Sci. USA **78**:4887–4891.
31. **Rice, C. M., and J. H. Strauss.** 1981. Synthesis, cleavage and sequence analysis of DNA complementary to the 26S messenger RNA of sindbis virus. J. Mol. Biol. **150**:315–340.
32. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
33. **Takegami, T., R. J. Kuhn, C. W. Anderson, and E. Wimmer.** 1983. Membrane-dependent uridylylation of the genome-linked protein of poliovirus. Proc. Natl. Acad. Sci. USA **80**:7447–7451.
34. **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.
35. **Tuschall, D. M., E. Heibert, and J. B. Flanegan.** 1982. Poliovirus RNA-dependent RNA polymerase synthesizes full-length copies of poliovirion RNA, cellular mRNA, and several plant virus RNAs in vitro. J. Virol. **44**:209–216.
36. **Van Dyke, T. A., and J. B. Flanegan.** 1980. Identification of poliovirus polypeptide P63 as a soluble RNA-dependent RNA polymerase. J. Virol. **35**:732–740.
37. **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.
38. **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.