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Poliovirus RNA-Dependent RNA Polymerase and Host Cell Protein Synthesize Product RNA Twice the Size of Poliovirion RNA In Vitro

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The poliovirus RNA-dependent RNA polymerase required an oligouridylate primer or a HeLa cell protein (host factor) to initiate RNA synthesis on poliovirion RNA in vitro. The polymerase synthesized template-sized product RNA in the oligouridylate-primed reaction. In the host factor-dependent reaction, the largest product RNA synthesized by the polymerase was twice the size of the template RNA. About half of the product RNA recovered from this reaction was shown to exist in the form of a snapback sequence. Time-course reactions and pulse-chase experiments showed that the product RNA was only slightly larger than the template RNA at early reaction times and that with time it increased in size to form the dimer-sized product RNA. Inhibition of the elongation reaction by adding only [α-^{32}P]UTP and ATP resulted in the formation of template-sized product RNA. The dimer-sized product RNA was unaffected by phenol extraction or proteinase K treatment but was converted to template-sized molecules by S1 nuclease. Dimer-sized poliovirus RNA that was sensitive to S1 nuclease was also isolated from poliovirus-infected cells. The results from this study indicate that the labeled negative-strand product RNA synthesized in vitro was covalently linked to the positive-strand template RNA. Thus, in vitro, the primer-dependent poliovirus RNA polymerase may initiate RNA synthesis in the presence of the host factor by using the 3′ end of the template RNA as a primer.

Poliovirus has a single-stranded RNA genome of positive polarity that has a polyadenylic acid sequence at its 3′ end (1, 28, 37) and a small viral protein (VPg) covalently linked to its 5′ end (15, 18, 23). Poliovirus RNA replicates in the cytoplasm of infected cells and requires a virus-specific RNA-dependent RNA polymerase. A soluble and template-dependent form of the poliovirus RNA polymerase has been purified from cytoplasmic extracts of infected cells (10, 13, 14, 16). A single-virus-specific protein, designated as P3-4b (3D^{pol} in L434 nomenclature [25]; also p63 and NCVP4 in previous publications), copurifies with polymerase activity (14, 33). Highly purified forms of the polymerase contain only trace amounts of proteins other than P3-4b (32, 33). Baron and Baltimore (3) confirmed the identity of P3-4b as an RNA-dependent RNA polymerase by showing that an affinity purified antibody specific for a synthetic peptide of P3-4b completely inhibits the in vitro activity of the viral polymerase.

Highly purified forms of the viral polymerase synthesize full-sized copies of poliovirion RNA and various other polyadenylated RNAs, but only when an oligouridylate [oligo(U)] primer is added to the in vitro reactions (6, 32, 34). Thus, unlike many other RNA polymerases, the poliovirus RNA polymerase is a primer-dependent enzyme. The requirement for the oligo(U) primer, however, can be eliminated by adding a cellular protein component or "host factor" to the in vitro reaction (5, 12). Partially purified forms of the polymerase are active on poliovirion RNA (10, 12) and several other RNAs (12, 32) without adding either oligo(U) or the host factor. Because this activity is inhibited by anti-host factor antibody (11), it appears that partially purified forms of the polymerase contain sufficient amounts of the host factor to mediate the initiation of RNA synthesis. Thus, it is clear that the host factor stimulates the initiation activity of the viral polymerase, but its mechanism of action is not understood.

To further understand the molecular mechanisms involved in the initiation of RNA synthesis on poliovirion RNA in vitro, we characterized the product RNA synthesized by the polymerase in the presence of the host factor. In this study, we show that the largest product RNA synthesized in these reactions was twice the size of poliovirion RNA and that a significant amount of this product RNA would rapidly snap back into a double-stranded structure after complete denaturation. This and other evidence indicated that the product RNA was complementary to and covalently linked to the virion RNA template. Thus, in the presence of the host factor, the polymerase can use a template-priming mechanism to initiate RNA synthesis in vitro.

MATERIALS AND METHODS

Virus and cell cultures. Suspension cultures of HeLa S3 cells in Joklik modified Eagle medium (GIBCO Laboratories) supplemented with 7% fetal calf serum were maintained at 3 × 10^5 to 6 × 10^5 cells per ml. Cells were infected with poliovirus type 1 (Mahoney strain) as previously described (35).

Polymerase purification. The poliovirus RNA polymerase was purified from HeLa cells at 5 h postinfection as previously described (33). This procedure involved the preparation of a high-speed (200,000 × *g*) supernatant from a cytoplasmic extract of the infected cells (fraction I), precipitation of the polymerase with ammonium sulfate (fraction II), and chromatography of the polymerase on phosphocellulose (fraction III), Sephacryl S-200 (fraction IV), and hydroxylapatite (fraction V). The purified polymerase was dialyzed against 50 mM Tris-hydrochloride (pH 8.0)–50% glycerol–0.1 M KCl–2 mM dithiothreitol (DTT) and then

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divided into small portions that were stored at −70°C. Polymerase activity was measured at each purification step with a polyadenylic acid template and an oligo(U) primer as described previously (33). The protein composition of the purified polymerase was analyzed by electrophoresis in a 10% polyacrylamide gel containing sodium dodecyl sulfate (17). Proteins in the gel were detected by a silver staining technique (19). The polymerase protein, P3-4b, was the major protein present and represented about 80% of the total protein detected in the gel.

In several experiments, the fraction III polymerase was used without additional purification. The peak fractions containing the largest amounts of polymerase activity that eluted from the phosphocellulose column were pooled, concentrated against solid sucrose, and divided into small portions that were stored at −70°C. Both the fraction III and fraction V polymerase preparations were free of RNase activity. No degradation of single-stranded poliovirion RNA was detected by CH_3HgOH-agarose gel electrophoresis after a 1-h reaction at standard conditions.

Host factor purification. The host factor was partially purified from uninfected HeLa cells by a modification of published procedures (5, 9, 12). For protocol I a supernatant (20,000 × *g*) was prepared from a cytoplasmic extract of uninfected HeLa cells (10^9) by centrifugation for 30 min at 4°C. The supernatant (20,000 × *g*) was adjusted to 0.3 M KCl, incubated for 1 h on ice, and subjected to high-speed centrifugation at 200,000 × *g* for 2 h at 4°C. The host factor was precipitated from the high-speed supernatant by adding solid ammonium sulfate with constant stirring at 0°C until the solution was 80% saturated. The precipitate that formed was collected by centrifugation at 10,000 × *g* for 10 min at 4°C and resuspended in 1 ml of 10 mM Tris-hydrochloride (pH 8.0)–10 mM NaCl (TN buffer) containing 20% glycerol. The solution was diluted with 4 ml of 50 mM Tris-hydrochloride (pH 8.0)–20% glycerol–0.1% Nonidet P-40–2 mM DTT–10 μg of ovalbumin per ml and chromatographed on phosphocellulose with a 0 to 1 M KCl elution gradient. A 2-μl sample of each fraction was assayed for host factor activity by using the polymerase assay conditions described below and the fraction V polymerase. The peak of host factor activity eluted at about 0.13 M KCl. The fractions comprising the peak of host factor activity were pooled, dialyzed against 50 mM Tris-hydrochloride (pH 8.0)–20% glycerol–2 mM DTT, concentrated against solid sucrose, and divided into small samples that were stored at −70°C. For protocol II the above procedure was slightly modified as follows. A supernatant (200,000 × *g*) was prepared from a cytoplasmic extract of uninfected HeLa cells (4 × 10^9 cells), precipitated with a 40 to 60% ammonium sulfate cut, and chromatographed on phosphocellulose. The peak fraction was used directly. The host factor isolated by either of the above two protocols was free of detectable amounts of RNase activity.

Polymerase reaction conditions. Unless otherwise indicated, the reaction mixture (final volume, 30 μl) contained 42 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 8.0], 3 mM magnesium acetate, 8 mM DTT, 10 μCi of [α-^{32}P]UTP (or [α-^{32}P]GTP) (410 Ci/mmol), 10 μM ATP, 10 μM CTP, 10 μM GTP (or UTP when [α-^{32}P]GTP was used), 2 μg of poliovirion RNA, and 4 μl of purified polymerase (about 0.8 μg of total protein of fraction III polymerase and about 35 ng of total protein of fraction V polymerase). Oligo(U) (0.05 μg) or host factor (2 to 4 μl) (0.2 to 0.4 μg of total protein) was added to some reaction mixtures, as indicated. The reactions were normally run for 1 h at 30°C. The oligo(U) (Calbiochem-Behring) was treated with bacterial alkaline phosphatase, phenol extracted, and ethanol precipitated before being used in these reactions. Contaminating RNase was avoided by treatment of all solutions and reaction tubes with diethyl pyrocarbonate as described previously (34). The integrity of the template RNA recovered from each reaction was examined by CH_3HgOH-agarose gel electrophoresis as described below. In all experiments shown, there was no detectable degradation of the template RNA. The amount of labeled ribonucleotide that was incorporated into product RNA was measured by collecting the labeled product RNA on membrane filters (GN-6, 0.45 μm; Gelman Sciences, Inc.) after precipitation with 7% trichloroacetic acid (TCA)–2% sodium pyrophosphate–100 μg of carrier RNA. The filters were counted in 5 ml of Aquasol-2 scintillation fluid (New England Nuclear Corp.) with a Beckman LS7500 liquid scintillation counter.

Isolation of labeled product RNA. At the times indicated, the polymerase reaction mixtures were adjusted to 0.4 M sodium acetate and the product RNA was precipitated by adding 2.5 volumes of cold 95% ethanol. The labeled product RNA was collected by centrifugation at 12,000 × *g* for 5 min. The ethanol was removed, and the RNA precipitate was dried in vacuo.

Agarose gel electrophoresis. The size of the labeled product RNA was determined by electrophoresis in 1% agarose (high gelling-temperature agarose; Miles Laboratories, Inc.) gels containing 5 mM CH_3HgOH (Alpha Products) as described by Bailey and Davidson (2). The product RNA recovered from the in vitro reactions was suspended in 15 μl of electrophoresis buffer containing 50 mM H_3BO_3, 5 mM $Na_2B_4O_7 \cdot H_2O$, 10 mM Na_2SO_4, 1 mM disodium EDTA, and 50 mM CH_3HgOH. The RNA suspension was incubated for 20 min at room temperature and then mixed with 15 μl of a 1% bromphenol blue dye solution containing 10% glycerol and 50 mM CH_3HgOH. The sample was layered on a vertical 1% agarose slab gel containing 5 mM CH_3HgOH in electrophoresis buffer and electrophoresed at 100 V for 4 h. After electrophoresis, the gel was soaked for 30 min in 0.5 M ammonium acetate containing 2 μg of ethidium bromide per ml, photographed, and dried. Autoradiography of the dried gel was carried out at −70°C with Kodak XAR-5 X-ray film and a Cronex Lightning-Plus enhancing screen (E. I. du Pont de Nemours & Co.). When ^{3}H-labeled RNA was electrophoresed, the gel was washed twice with methanol for 30 min, soaked for 4 h in methanol containing 10% 2,5-diphenyloxazole, and soaked for 1 h in water. The gel was dried and exposed to Kodak XAR-5 X-ray film at −70°C.

RNA preparation. Poliovirion RNA was extracted from purified virions as described previously (16) and stored at −20°C in 70% ethanol. Immediately before use, the RNA was collected by centrifugation at 12,000 × *g* for 10 min, dried in vacuo, and suspended at 1 μg/μl in 0.1 mM EDTA. Brome mosaic virus (BMV) RNA 3, squash mosaic virus middle-component RNA, and tobacco etch virus RNA were a generous gift of Ernest Hiebert, University of Florida, Gainesville, and were purified as described previously (32).

^{3}H-labeled poliovirus single-stranded RNA and ^{3}H-labeled double-stranded RNA were prepared by infecting HeLa cells (1 liter, 6 × 10^8 cells) in the presence of 1 mCi of [5,6-^{3}H]uridine (52 Ci/mmol). ^{3}H-labeled single-stranded RNA was extracted from purified virions, precipitated with 70% ethanol, and stored at −20°C as described above. ^{3}H-labeled double-stranded RNA was prepared by a modification of the procedure of Spector and Baltimore (29). A cytoplasmic extract was prepared at 6 h postinfection by resuspending the infected cells in TN buffer–1% Nonidet

P-40. After the nuclei were removed by centrifugation at 900 × *g* for 5 min, the extract was adjusted to 1% sodium dodecyl sulfate–0.1 M NaCl, and the proteins were removed from the sample by phenol extraction. The RNA was precipitated in 70% ethanol, collected by centrifugation at 12,000 × *g* for 10 min, dried in vacuo, and suspended in TN buffer–2 M LiCl–1% sodium dodecyl sulfate. The sample was incubated at 0°C overnight and then centrifuged at 10,000 × *g* for 15 min to remove the single-stranded RNA. The double-stranded RNA was precipitated from the supernatant with 70% ethanol, suspended in 10 mM Tris-hydrochloride (pH 7.5)–100 mM NaCl–1 mM disodium EDTA–0.5% sodium dodecyl sulfate, layered on a 15 to 30% sucrose gradient in the same buffer, and centrifuged at 21,000 rpm in a Beckman SW27 rotor for 16 h at 22°C. The double-stranded RNA in the 20S region of the gradient was precipitated with 70% ethanol and stored at −20°C.

Materials. The [α-^{32}P]GTP (PB 161) and [α-^{32}P]UTP (PB 163) were obtained from Amersham Corp. in 50% ethanol. The ethanol was removed from the isotope solution by evaporation under reduced pressure with a Buchler-Rotary Evapo-Mix. The [5,6-^{3}H]uridine (TRK 410) was obtained from Amersham Corp. in aqueous solution. All unlabeled ribonucleotides were obtained from Calbiochem-Behring.

RESULTS

Primer-independent polymerase activity. To study the initiation of RNA synthesis in vitro, we characterized the product RNA synthesized by a primer-independent form of the poliovirus RNA polymerase. The partially purified fraction III polymerase was active on poliovirus RNA in the presence or absence of oligo(U) (Table 1; Fig. 1A). The addition of oligo(U) increased the activity of the fraction III polymerase about sevenfold (Table 1). Additional purification of the polymerase by chromatography on Sephacryl S200 and hydroxylapatite (see above) resulted in a loss of primer-independent activity (Table 1). Most of this activity was lost at the hydroxylapatite step which separated the endogenous host factor from the polymerase (C. Clifford and J. B. Flanegan, unpublished data). The initiating activity of the fraction V polymerase was restored by adding host factor purified from uninfected cells (Table 1; Fig. 1B, lane 1 and 3). The level of activity obtained by adding the host factor was about the same as that obtained by adding oligo(U) (Table 1). No activity was detected with the host factor alone (Fig. 1B, lane 4).

TABLE 1. Activity of poliovirus RNA polymerase on poliovirion RNA in vitro[a]

Polymerase fraction	Oligo(U)	Host factor	cpm (10^5) of [^{32}P]NMP incorporated (pmol)
III	−	−	2.0 (0.2)
III	+	−	14.0 (1.6)
V	−	−	0.0 (0.0)
V	+	−	13.0 (1.4)
V	−	+	12.0 (1.3)

[a] The reaction mixtures contained 2 μg of poliovirion RNA, 4 μl of fraction III polymerase or 2 μl of fraction V polymerase, and 0.05 μg of oligo(U) or 2 μl of host factor (purified by protocol I), where indicated. Either [α-^{32}P]GTP (fraction V) or [α-^{32}P]UTP (fraction III) was used as the labeled substrate. The reaction mixtures were incubated at 30°C for 1 h. The labeled product RNA was then precipitated with 7% TCA, collected on a membrane filter, and counted.

[b] NMP, Nucleoside 5′-monophosphate.

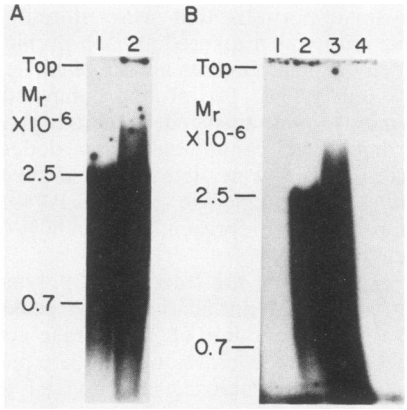

FIG. 1. Electrophoresis of labeled product RNA on a CH_3HgOH-agarose gel. Product RNA was synthesized on poliovirion RNA in reactions that contained fraction III or fraction V polymerase, and oligo(U) or host factor where indicated. (A) Autoradiogram of a gel containing product RNA synthesized by the fraction III polymerase in the presence (lane 1) or absence (lane 2) of oligo(U) in reactions containing 3 mM $MgCl_2$, 10 μCi of [α-^{32}P]UTP, 10 μM UTP, and 500 μM each of ATP, GTP, and CTP. (B) Autoradiogram of a gel containing product RNA synthesized by the fraction V polymerase in the absence (lane 1) or presence (lane 2) of oligo(U) and in the presence of host factor (lane 3). In lane 4 the reaction contained host factor but no polymerase. The host factor was purified by protocol II as described in the text. The reactions contained 3 mM $MgCl_2$, 10 μCi of [α-^{32}P]GTP, and 500 μM each of ATP, UTP, and CTP.

The composition of the labeled product RNA synthesized in vitro by the fraction III polymerase in the absence of oligo(U) was characterized by conducting a nearest-neighbor analysis and by determining the resistance of the product RNA to digestion by single-strand specific RNase before and after annealing with excess poliovirion RNA. Using procedures similar to those described elsewhere (9, 34), we showed that the labeled component of the product RNA was both heteropolymeric (data not shown) and complementary to poliovirion RNA (Table 2).

Size of the product RNA. The size of the labeled product RNA synthesized on a poliovirion RNA template was analyzed by agarose gel electrophoresis in the presence of a

TABLE 2. Resistance of product RNA to RNase digestion before and after annealing with excess poliovirion RNA[a]

Treatment	% RNase resistance
None	37
Boiled and quickly chilled	14
Boiled and annealed with no RNA	41
Boiled and annealed with HeLa cell RNA	42
Boiled and annealed with poliovirus RNA	95

[a] ^{32}P-labeled product RNA from a reaction containing fraction III polymerase, 2 μg of poliovirion RNA, 12 μM [α-^{32}P]GTP, 3 mM $MgCl_2$, and 500 μM each of ATP, UTP, and CTP was TCA and ethanol precipitated. The RNA pellet was dissolved in 10 mM Tris-hydrochloride (pH 7.5) and divided into five 0.4-ml samples, each containing 35,000 cpm and 0.4 μg of template RNA. Either 15 μg of poliovirion RNA or 15 μg of HeLa cytoplasmic RNA was added to specified tubes. Certain samples were boiled for 10 min and then quick-chilled or reannealed for 60 min at 50°C after the addition of NaCl to a final concentration of 0.15 M. All samples were chilled and divided into two equal portions, one of which was treated with RNases T_1, T_2, and A (0.02, 0.005, and 0.015 U/ml, respectively) for 30 min at 20°C in 0.25 M NaCl. Samples were TCA precipitated and counted. The results are expressed as the percentage of RNase-resistant RNA in each sample.

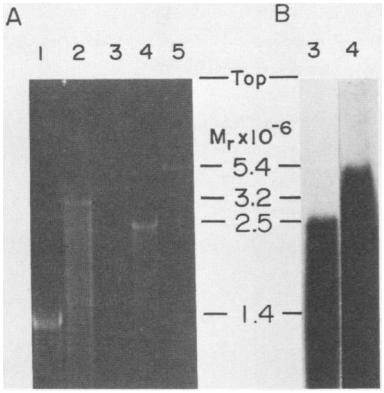

FIG. 2. Size analysis of labeled product RNA. Product RNA was synthesized on poliovirion RNA in a reaction that contained [α-^{32}P]UTP, fraction III polymerase, and oligo(U) as indicated. The product RNA was analyzed on a CH_3HgOH-agarose gel containing several marker nucleic acids. (A) Photograph of the gel stained with ethidium bromide. Lane 1, squash mosaic virus middle-component RNA; lane 2, tobacco etch virus RNA; lane 3, poliovirion RNA recovered from a reaction containing fraction III polymerase and oligo(U); lane 4, poliovirion RNA recovered from a reaction containing fraction III polymerase and no oligo(U); lane 5, pSM32 (36) DNA, digested with *Hin*dIII. The numbers indicate the molecular weights (10^{-6}) of the marker nucleic acids. (B) Autoradiogram of lanes 3 and 4. Only a small portion of the product RNA from the oligo(U)-primed reaction was electrophoresed, and therefore the template RNA is not visible in lane 3 of panel A.

strong denaturing agent, CH_3HgOH (2). In the presence of oligo(U), the largest product RNA that was synthesized by the fraction III and fraction V polymerase was equivalent in size to the template RNA (Fig. 1A, lane 1; Fig. 1B, lane 2). The largest product RNA that was synthesized by the fraction III polymerase in the absence of oligo(U), however, was significantly larger than the template RNA (Fig. 1A, lane 2). The same result was obtained with the fraction V polymerase plus host factor (Fig. 1B, lane 3). Note that no detectable product RNA was synthesized by the fraction V polymerase in the absence of the host factor or oligo(U) (Fig. 1B, lane 1). Electrophoresis in the presence of various size markers showed that the largest RNA synthesized by the fraction III polymerase was twice the size of poliovirion RNA (Fig. 2, lanes 4).

The size of this product was not affected by phenol extraction (data not shown) or by treatment with proteinase K (Fig. 3A, lane 2). Inhibiting the elongation reaction by deleting GTP and CTP, however, blocked the formation of the dimer-sized product RNA. When only [α-^{32}P]UTP and ATP were added to the reactions, the largest product RNA recovered was the same size as the template RNA (Fig. 3A, lane 3). The addition of ATP increased the amount of product RNA synthesized in this reaction, in agreement with the findings of Morrow et al. (21). In the absence of ATP, we found that a small but detectable amount of template-sized product RNA was also synthesized (data not shown). In a control reaction, we showed that dimer-sized product RNA was again synthesized in the presence of all four ribonucleoside triphosphates (Fig. 3A, lane 2). The product RNA synthesized in an oligo(U)-primed reaction that contained only [α-^{32}P]UTP and ATP was very small (Fig. 3A, lane 4). We showed that template-sized product RNA was synthesized in an identical reaction containing oligo(U) and all four ribonucleoside triphosphates (Fig. 3A, lane 1).

An experiment was done to determine whether pulse-labeled product RNA could be chased into dimer-sized product RNA. After a 1-h chase, some labeled dimer-sized product RNA was observed (Fig. 3B, lane 2), indicating that the polymerase was able to elongate some of the strands initiated during the pulse. Most of the product RNA, however, did not chase into dimer-sized RNA. The polymerase may have terminated synthesis on some strands initiated during the pulse and been unable to reinitiate synthesis on these strands during the chase. Similar results were previously observed in oligo(U)-primed reactions (34). In addition, the polymerase may have initiated synthesis on some subgenomic-sized RNA molecules that were present in small amounts in our preparations of poliovirion RNA (see light smear of RNA below band of full-length virion RNA in a gel stained with ethidium bromide; Fig. 2A, lane 4).

Activity on BMV RNA 3. In a previous study, it was determined that the fraction III polymerase synthesized dimer-sized product RNA on a nonpolyadenylated plant virus RNA, BMV RNA 3 (32). The fraction V polymerase, however, was not active on this RNA (Fig. 4B, lane 1; 32). In the presence of the host factor, however, the fraction V polymerase synthesized the dimer-sized product RNA (Fig. 4B, lane 3). Electrophoresis in the CH_3 HgOH-agarose gel gave a large separation between the dimer-sized product RNA (calculated M_r, 1.4×10^6) and the BMV RNA 3 template (M_r, 0.7×10^6).

Time course of product RNA synthesis. A time course experiment was used to compare the size of the product

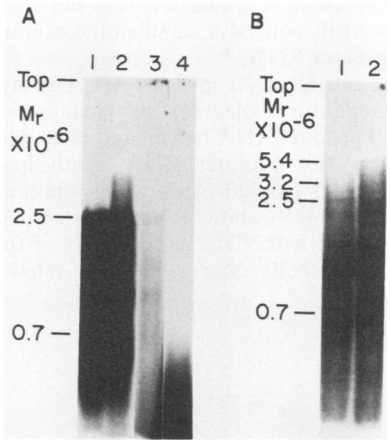

FIG. 3. Electrophoresis of product RNA synthesized in a reaction with [α-^{32}P]UTP and ATP only and in a pulse-chase experiment. (A) Autoradiogram of CH_3HgOH-agarose gel containing product RNA synthesized by the fraction III polymerase in the presence (lanes 1 and 4) or absence (lanes 2 and 3) of oligo(U). The reactions in lanes 1 and 2 contained 3 mM $MgCl_2$, 10 μCi of [α-^{32}P]UTP, 10 μM UTP, and 500 μM each of ATP, GTP, and CTP. The reactions in lanes 3 and 4 contained 3 mM $MgCl_2$, 10 μCi of [α-^{32}P]UTP, and 250 μM ATP but no GTP or CTP. The product RNAs were precipitated from the reaction mixtures with ethanol and digested with proteinase K as described previously (15). The product RNAs were then ethanol precipitated, washed once with ethanol, and electrophoresed as described in the text. (B) Autoradiogram of a CH_3HgOH-agarose gel containing the product RNA synthesized in a pulse-chase experiment by the fraction III polymerase. Lane 1 contains the product RNA synthesized during a 45-min pulse in a complete reaction containing 10 μCi of [α-^{32}P]UTP (410 Ci/mmol), 3 mM magnesium acetate, and 10 μM each of ATP, GTP, and CTP. Lane 2 contains the product RNA recovered after a 1-h chase with 3 mM UTP–6 mM magnesium acetate.

RNA synthesized on poliovirion RNA by the primer-independent fraction III polymerase and by the fraction V polymerase in the presence of oligo(U). In the oligo(U)-primed reaction, only subgenomic-sized product RNA was detected at early reaction times (Fig. 5A). About 20 min was required for the synthesis of full-sized product RNA (Fig. 5A). In the primer-independent reaction with the fraction III polymerase, however, the largest labeled product RNA synthesized at early reaction times was about the same size as or slightly larger than the template RNA (Fig. 5B). The elongation of this RNA to form the dimer-sized product RNA required about 20 min (Fig. 5B). Thus, the largest product RNA synthesized by the fraction III polymerase was about the same size as the template RNA at early reaction times and twice the size of the template RNA at late reaction times.

Presence of snapback sequences. The observation that the largest product RNA was twice the size of the template RNA suggested that a covalent linkage existed between the product RNA and the template RNA. This would occur if the polymerase used the 3′ end of the template RNA as a primer to initiate RNA synthesis. Such a mechanism would result in the formation of a snapback sequence.

To examine the product RNA for the presence of a snapback sequence, the RNA was denatured with CH_3HgOH and then treated with DTT. The DTT tightly binds to the CH_3HgOH (log K_a, 11.6) and thereby reverses its binding to the RNA (log K_a, 4.3 for uridine and 3.4 for guanosine) (2). Thus, after adding DTT to inactivate the CH_3HgOH any snapback sequences should rapidly reanneal to form a duplex structure. Heat denaturation was not used for this experiment since this caused a significant amount of degradation of the product RNA.

Equilibrium centrifugation in a Cs_2SO_4 density gradient was used to separate molecules of single-stranded and double-stranded product RNA before and after denaturation with CH_3HgOH. The product RNA synthesized in the presence of oligo(U) banded at densities characteristic of both single- and double-stranded RNA (Fig. 6A). After being reversibly denatured with CH_3HgOH, most of the product RNA banded at a density characteristic of single-stranded

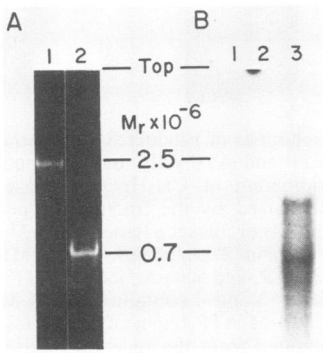

FIG. 4. Electrophoresis of labeled product RNA synthesized by the fraction V polymerase on BMV RNA 3. Product RNA was synthesized in the presence of [α-^{32}P]GTP and host factor, as indicated, and was analyzed by CH_3HgOH-agarose gel electrophoresis. (A) Photograph of the gel stained with ethidium bromide. Lane 1, poliovirion RNA marker; lane 2, template RNA recovered from the reaction with BMV RNA 3 shown in panel B, lane 3. (B) Autoradiogram of the gel showing the labeled product RNAs synthesized on BMV RNA 3 in the absence (lane 1) or presence (lane 3) of the host factor. Lane 2 is blank.

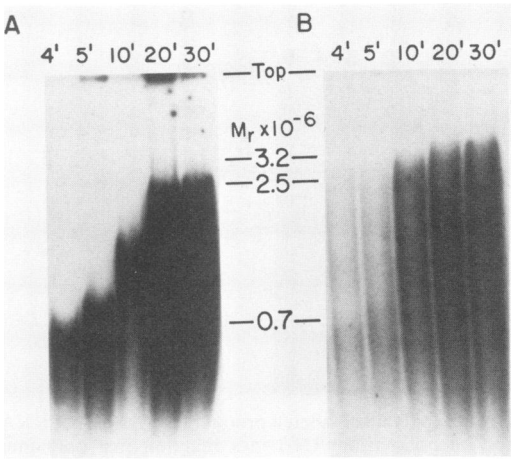

FIG. 5. Effect of reaction time on the size of the product RNAs synthesized in the oligo(U)-dependent and in the host factor-dependent reactions. (A) Product RNA synthesized on poliovirion RNA in a reaction that contained [α-^{32}P]GTP, oligo(U), and the fraction V polymerase. At the indicated times, portions of the reaction mixture were removed, and the product RNAs were electrophoresed on a CH_3HgOH-agarose gel. (B) Product RNA synthesized in a reaction that contained the fraction III polymerase but no oligo(U).

RNA (Fig. 6B). The product RNA synthesized in the absence of oligo(U) also banded at densities characteristic of both single- and double-stranded RNA (Fig. 6C). In other experiments, more of the labeled product was found in the double-stranded region of the gradient, and the overall distribution was similar to that shown in Fig. 6A (data not shown). Reversibly denaturing the product RNA with CH_3HgOH had little effect on its distribution in the gradient (Fig. 6D). About the same amount of double-stranded RNA was recovered in the gradient before and after the reversible denaturation step. The areas under the peaks in Fig. 6D suggested that about 50% of the product RNA was double stranded and apparently existed in the form of a snapback sequence.

The product RNA was also characterized before and after CH_3HgOH denaturation by measuring its sensitivity to a single-strand specific RNase (pancreatic RNase). Before denaturation, most of the product RNA that was synthesized in either the presence or absence of oligo(U) was resistant to RNase digestion (Table 3). After the reversible denaturation step with CH_3HgOH, about 20% of the oligo(U)-primed product RNA was RNase resistant (Table 3). This result was consistent with previous studies with the oligo(U)-primed product RNA (34) and may result from the presence of some secondary structure in the single-stranded product RNA. In contrast, about 60% of the product RNA synthesized in the absence of oligo(U) was RNase resistant after the denaturation step (Table 3). After subtracting the 20% value obtained in the oligo(U)-primed reactions, it appeared that about 40% of this product RNA was present in the form of a snapback sequence. This was consistent with the results from the Cs_2SO_4 density gradients which indicated that about half of the product RNA was present in a snapback sequence. Since a single nick would destroy all or part of a snapback sequence, some snapback sequences originally present may have been lost during the isolation and characterization of the product RNA.

Isolation of dimer-sized RNA from infected cells. Poliovirus RNA was isolated from infected cells to determine whether

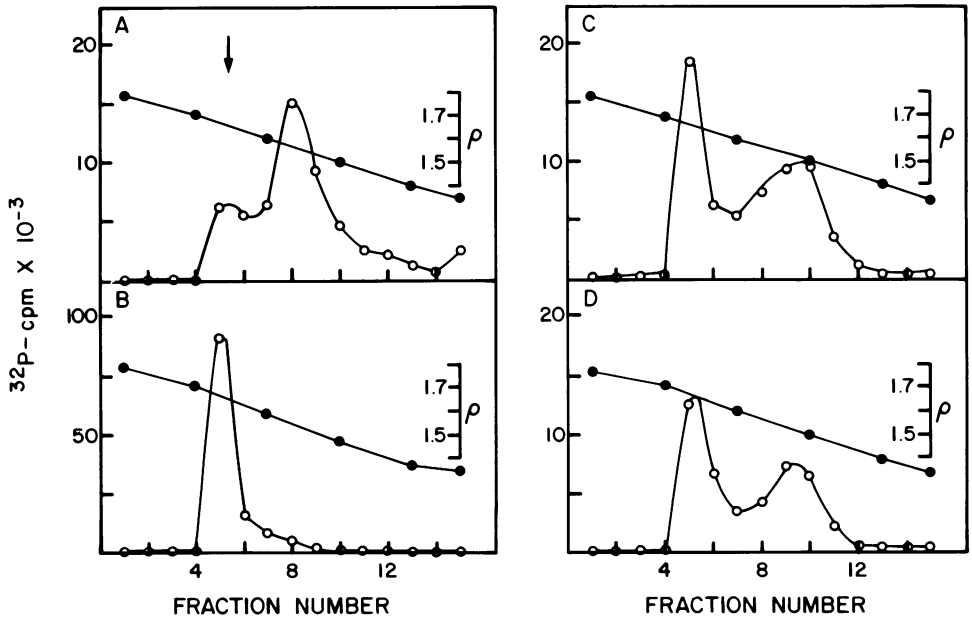

FIG. 6. Equilibrium centrifugation in Cs_2SO_4 density gradients of labeled product RNAs before and after denaturation with CH_3HgOH. Product RNA was synthesized on poliovirion RNA in the presence or absence of oligo(U) in reactions containing [α-^{32}P]UTP, 1 mM magnesium acetate, and the fraction III polymerase. The labeled product RNA from each reaction was divided in half, ethanol precipitated, and suspended in 15 μl of 50 mM H_3BO_3–5 mM $Na_2B_4O_7 \cdot H_2O$–10 mM Na_2SO_4–1 mM disodium EDTA. The buffer also contained 50 mM CH_3HgOH if the product RNA was to be denatured. After incubation at room temperature for 20 min, 15 μl of 100 mM DTT and 200 μl of 10 mM Tris-hydrochloride (pH 7.5) were added, and each sample was mixed throughout a 4.5-ml solution of 48% (wt/wt) Cs_2SO_4 in 10 mM Tris-hydrochloride (pH 7.5)–20 mM NaCl–1mM disodium EDTA–10 μg of 28S HeLa carrier RNA per ml. The denatured and nondenatured samples were centrifuged in siliconized polyallomer tubes at 35,000 rpm in an SW50.1 rotor for 64 h at 22°C. Fifteen 300-μl fractions were collected, and their buoyant densities were determined by measuring the refractive index of each fraction. The labeled product RNA was precipitated from the fractions with 7% TCA, collected on membrane filters, and counted. (A) Density gradient centrifugation of product RNA synthesized by the fraction III polymerase in the presence of oligo(U). (B) Same as A but treated with CH_3HgOH and DTT. (C) Density gradient centrifugation of product RNA synthesized by the fraction III polymerase in the absence of oligo(U). (D) Same as C but treated with CH_3HgOH and DTT. In Cs_2SO_4, single-stranded poliovirus RNA has a buoyant density of about 1.65 g/cm^3 and double-stranded RNA has a buoyant density of about 1.55 g/cm^3.

molecules of dimer-sized RNA existed in vivo. The viral RNA was labeled by adding [^{3}H]uridine to infected cells in the presence of actinomycin D. Poliovirus double-stranded RNA was isolated by procedures similar to those described by Spector and Baltimore (29). As expected, the double-stranded RNA migrated slower than single-stranded virion RNA on a nondenaturing agarose gel (Fig. 7A). After being treated with CH_3HgOH, however, most of the denatured double-stranded RNA comigrated with denatured poliovirion RNA (Fig. 7B). This autoradiogram was overexposed to detect minor RNA species and showed that some labeled RNA larger than virion RNA was present in the poliovirus double-stranded RNA isolated from infected cells (Fig. 7B, lane 1). The largest RNA detected was twice the size of virion RNA and comigrated with the dimer-sized product RNA synthesized in vitro (Fig. 7C, lanes 3 and 4).

TABLE 3. Resistance of product RNA to RNase digestion

Oligo(U)	Treatment with CH_3HgOH	% RNase resistance		
		Expt 1	Expt 2	Avg
+	–	97	97	97.0
+	+	22	21	21.5
–	–	92	90	91.0
–	+	57	64	60.5

[a] A reaction mixture containing the fraction III polymerase, [α-^{32}P]UTP, 1 mM magnesium acetate, and poliovirion RNA was incubated for 60 min at 30°C in the presence or absence of added oligo(U). The labeled product RNA in each reaction was divided in half, ethanol precipitated, dried, and suspended in 15 μl of buffer containing 50 mM H_3BO_3, 5mM $Na_2B_4O_7 \cdot H_2O$, 10 mM Na_2SO_4, and 1 mM disodium EDTA. Samples to be denatured also contained 50 mM CH_3HgOH and were incubated at room temperature for 20 min. After the addition of 200 μl of 10 mM Tris-hydrochloride (pH 7.5)–1 mM disodium EDTA–300 mM NaCl–15 μl of 100 mM DTT, the samples were divided into two equal portions, one of which was treated with pancreatic RNase (50 μg/ml) for 30 min at 20°C. The labeled RNA in each sample was then precipitated with 7% TCA, collected on a membrane filter, and counted. The results are expressed as the percentage of the labeled RNA in each sample that was resistant to RNase digestion.

Sensitivity of dimer-sized RNA to S1 nuclease. The covalent linkage of two complementary strands of poliovirus RNA would result in the formation of a small single-stranded hairpin loop at the junction between the strands. Treatment with single-strand specific S1 nuclease should nick the linkage between the strands and generate monomer-length molecules. Poliovirus double-stranded RNA purified from infected cells was treated with S1 nuclease and electrophoresed on a denaturing agarose gel (Fig. 8A). After treatment with S1, no dimer-sized RNA remained (Fig. 8A, lanes 2 and 3) and the denatured RNA migrated with the single-stranded RNA marker (Fig. 8A, lane 4). S1 nuclease completely digested single-stranded poliovirion RNA (Fig. 8A, lane 5).

The dimer-sized product RNA synthesized in vitro by the fraction III polymerase was also sensitive to S1 nuclease (Fig. 8B). After treatment with S1 nuclease and denaturation with CH_3HgOH, the largest product RNA was the same size as virion RNA (Fig. 8B, lane 2).

DISCUSSION

To study the initiation of poliovirus RNA synthesis, we characterized the product RNA synthesized in vitro on poliovirion RNA by the poliovirus RNA polymerase in the presence of the host factor. Characterization of the product RNA indicated that the labeled negative-strand product RNA was covalently linked to the positive-strand template RNA. This suggests that the polymerase can use the 3′ end of the template RNA as a primer to initiate RNA synthesis in the reactions containing the host factor.

The size of the product RNA synthesized by the poliovirus RNA polymerase was characterized by CH_3HgOH-agarose gel electrophoresis. Two forms of the polymerase were used in this study. The highly purified fraction V polymerase required the addition of a host factor or oligo(U) to initiate RNA synthesis. In contrast, the fraction III polymerase initiated RNA synthesis without added host factor or oligo(U). This activity apparently resulted from the copurification of some host factor with the fraction III polymerase. The largest product RNA synthesized by the fraction V polymerase plus host factor or by the fraction III polymerase was twice the size of the poliovirion RNA template. Dimer-sized product RNA was also synthesized on a nonpolyadenylated plant virus RNA, BMV RNA 3. Thus, we repeatedly observed the synthesis of dimer-sized product RNA in the host factor-dependent reactions. Other investigators have reported the synthesis of template-sized product RNA in similar reactions (6, 9). We cannot explain the difference between these findings and our own, but we are currently investigating what effect the different protocols used in the purification of the polymerase have on the size of the product RNAs synthesized in vitro.

The mechanism involved in the synthesis of the dimer-sized product RNA was investigated. The polymerase was allowed to initiate RNA synthesis but not elongate the product RNA by adding only labeled UTP and unlabeled ATP to the in vitro reaction. Some template-sized product RNA was always recovered from this reaction. The addition of ATP stimulated the amount of product RNA synthesized in this reaction as previously reported (21). Another experimental approach used was to characterize the size of the product RNA as a function of time. At early reaction times the largest product RNA was slightly larger than the template RNA. As the reaction progressed, the product RNA increased in size to form the dimer-sized product. These results were consistent with the template-priming model and showed that the dimer-sized RNA was not synthesized by the polymerase copying the full length of the template RNA and then switching strands and copying back on the negative-strand product RNA.

The RNA synthesized in the host factor-dependent reaction would contain a snapback sequence and a terminal hairpin loop if the 5′ end of the negative-strand product RNA was covalently linked to the 3′ end of the positive-strand template RNA. About half of the product RNA recovered from the reactions of the polymerase plus host factor was found to exist in the form of a snapback sequence. As expected, none of the oligo(U)-primed product RNA was found to contain a snapback sequence. Direct treatment of the product RNA with the single-strand specific S1 nuclease was shown to digest the dimer-sized product RNA. The largest labeled RNA recovered after S1 treatment was the same size as the template RNA. Thus, it appeared that a significant fraction of the product RNA contained a terminal hairpin loop and existed in the form of a snapback sequence.

The results suggest that the host factor may stimulate the initiation of RNA synthesis in vitro by using the 3′-terminal nucleotide in the template RNA as a primer. The host factor may stabilize a small hairpin structure at the 3′ end of the template RNA or hold the 3′ end of a second molecule of

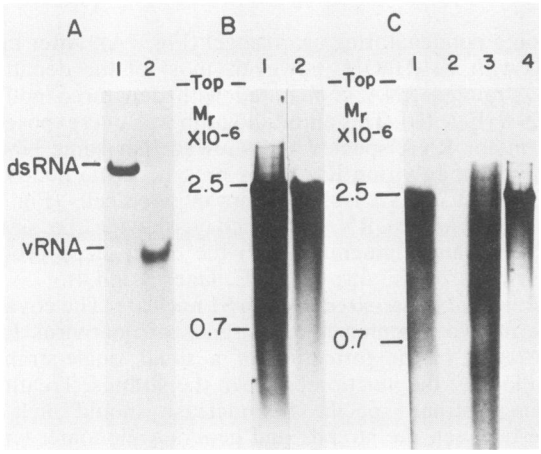

FIG. 7. Agarose gel electrophoresis of ^3H-labeled double-stranded and single-stranded poliovirus RNA isolated from infected cells. (A) Electrophoresis of double-stranded RNA (lane 1) and virion RNA (lane 2) in a nondenaturing agarose gel. (B) CH_3HgOH-agarose gel electrophoresis of double-stranded RNA (lane 1) and virion RNA (lane 2). Each sample was denatured with 50 mM CH_3HgOH for 20 min before electrophoresis. (C) CH_3HgOH-agarose gel electrophoresis of product RNA synthesized in vitro by the fraction III polymerase with [α-^{32}P]GTP and 1 mM magnesium acetate in the presence (lane 1) and absence (lane 3) of oligo(U). Poliovirus double-stranded RNA isolated from infected cells is shown in lane 4. Lane 2 is blank.

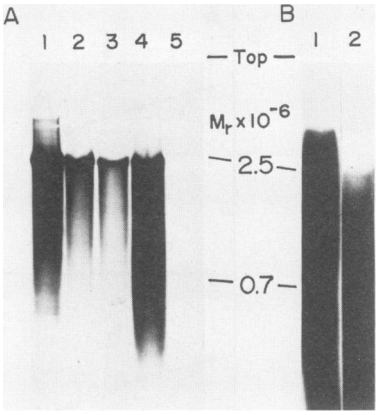

FIG. 8. Effect of S1 nuclease digestion on dimer-sized RNA synthesized in vitro and isolated from infected cells. Double-stranded poliovirus RNA isolated from infected cells and product RNA synthesized in vitro by the fraction III polymerase were treated with S1 nuclease and characterized by CH_3HgOH-agarose gel electrophoresis. (A) Gel containing ^3H-labeled poliovirus double-stranded RNA before (lane 1) and after (lane 2) treatment with S1 nuclease at 7 U/ml and at 70 U/ml (lane 3). ^3H-labeled single-stranded virion RNA before (lane 4) and after (lane 5) treatment with S1 nuclease at 7 U/ml. The ^3H-labeled single-stranded virion RNA used in this experiment was several months old and partially degraded. (B) Autoradiogram of gel containing product RNA synthesized by the fraction III polymerase with [α-^{32}P]GTP, before (lane 1) and after (lane 2) treatment with S1 nuclease at 70 U/ml.

RNA in position so that it can function as a primer. In this model, it is assumed that the primer nucleotide is held in position by the host factor itself and not by conventional base pairing. This model explains why the largest product RNA is twice the size of the template RNA and contains a snapback sequence. In addition to the dimer-sized product RNA, a significant amount of product RNA smaller than the template RNA was recovered. Some of this small product RNA may be generated by a specific processing reaction which cleaves the linkage between the template RNA and the newly synthesized product RNA. Another possibility is that the polymerase and host factor can initiate RNA synthesis on subgenomic-sized fragments of the template RNA. Additional studies are required to distinguish between these possibilities and to fully characterize the molecular mechanisms involved in the synthesis of the product RNAs recovered from reactions containing the host factor.

Because VPg is covalently linked to the 5′ ends of both plus- and minus-strand RNA and to the nascent chains in the replicative intermediate (15, 18, 23, 24), it was proposed that VPg may act as a protein primer to initiate poliovirus RNA synthesis. Recently it was shown that VPgpUpU can be recovered from infected cells (8) and synthesized in infected cell extracts (30). The reaction mechanism for the synthesis of VPgpUpU and its role in poliovirus RNA replication have not yet been established, but it was hypothesized that VPgpUpU may be the form in which VPg functions as a primer to initiate viral RNA replication (8, 30). Two types of experimental evidence suggest that VPg in some form may function as a primer in vitro. First, it was reported that anti-VPg antibody immunoprecipitates labeled product RNA synthesized on poliovirion RNA by the polymerase and host factor (4, 22). Our finding that a covalent linkage exists between the template and the product RNAs suggests an alternate interpretation of this result. The immunoprecipitation of the product RNA may result from the VPg that is linked to the 5′ end of the template RNA. We recently have shown that the immunoprecipitation of the product RNA with anti-VPg antibody was totally inhibited by removing VPg from the poliovirion RNA by pretreatment with proteinase K (J. B. Flanegan, C. A. Clifford, and D. C. Young, unpublished data). This is consistent with the reported inability of anti-VPg antibody to immunoprecipitate labeled product RNA synthesized by the polymerase and host factor on HeLa mRNA (21). A second type of evidence for VPg priming in vitro is the observed inhibition by anti-VPg antibody of host-factor-dependent polymerase activity on poliovirion RNA (4, 20). We are currently investigating what effect affinity-purified anti-VPg antibody has on our host-factor-dependent polymerase reactions that contain either virion RNA or proteinase K-treated virion RNA. It is interesting to note that anti-VPg antibody does not inhibit the activity of the polymerase plus host factor in reactions containing HeLa mRNA (21).

If poliovirus RNA synthesized in vivo by the polymerase is covalently linked to the template RNA or to another RNA that was used as a primer, then a mechanism must exist for specifically processing the linkage between the product RNA and the priming RNA. This would involve a cleavage of the phosphodiester linkage between the 5′-terminal phosphate in the product RNA and the 3′ OH in the terminal nucleotide in the priming RNA. Because VPg is found covalently linked to the 5′ end of poliovirus RNA, the second step in this reaction would be the formation of a phosphodiester linkage between the 5′-terminal phosphate in the product RNA and the single tyrosine residue in VPg. Studies are now underway to identify virus or host-coded proteins that may have these activities. One possibility is that VPg or one of its precursors is involved in both steps of the reaction. A related type of cleavage activity is observed with the bacteriophage φX174 A* protein (26) and with some topoisomerases (7, 31) which form covalent intermediates with DNA via phosphotyrosine bonds.

In summary, the results from this study indicate that the polymerase in the presence of the host factor can use the 3′-terminal nucleotide in the template RNA as a primer. It is not known whether a similar mechanism functions in vivo, but some dimer-sized poliovirus RNA was isolated from infected cells. Palindrome-like dimers of encephalomyocarditis virus double-stranded RNA were found by Senkevich et al. (27) in virus-infected cells. Their results indicate that this RNA is composed of two complementary single-stranded RNA dimers, each containing a positive and a negative RNA strand covalently linked. Thus, it appears that some mechanism does exist whereby covalently linked dimers of positive- and negative-strand RNA can be synthesized in picornavirus-infected cells. It remains to be determined whether this RNA has any role in the normal replication of picornavirus RNA.

Determining that covalently linked dimers of poliovirus RNA can be synthesized both in vitro and in vivo does not rule out the possibility that VPg in some form may function as a primer to initiate viral RNA synthesis in vivo. These results do suggest, however, an alternate mechanism for initiating RNA synthesis and an activity for VPg that should be considered in future studies. Additional work is required to further characterize the structure of the covalently linked dimers synthesized in vitro and isolated from infected cells. It must also be determined whether covalently linked dimers are synthesized on negative-strand RNA templates. This should help us understand the molecular mechanisms involved in the replication of poliovirus RNA and the function of the viral and cellular proteins required for this process.

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