JOURNAL OF VIROLOGY, June 1986, p. 715–723
0022-538X/86/060715-09$02.00/0

Vol. 58, No. 3

Anti-VPg Antibody Precipitation of Product RNA Synthesized In Vitro by the Poliovirus Polymerase and Host Factor Is Mediated by VPg on the Poliovirion RNA Template

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Received 18 November 1985/Accepted 12 February 1986

Antibody to the poliovirus genome-linked protein, VPg, specifically immunoprecipitated the product RNA synthesized in vitro by the poliovirus RNA polymerase and HeLa cell host factor when VPg-linked poliovirion RNA was used as a template. The largest product RNA that was immunoprecipitated was twice the size of the template RNA. The complete denaturation of the product RNA with CH_3HgOH had no effect on the immunoprecipitation reaction. In contrast, CH_3HgOH denaturation prevented the immunoprecipitation of the oligo(U)-primed product RNA. Immunoprecipitation of the product RNA synthesized in the host-factor-dependent reaction was prevented if VPg was removed from the template RNA by pretreatment with proteinase K or if an RNA template without VPg was used in the reaction. The results support our previous evidence that a covalent linkage exists between the labeled negative-strand product RNA and the VPg-linked template RNA and suggest that the purified polymerase and host factor initiated RNA synthesis in vitro in the absence of VPg or a VPg-precursor protein.

Poliovirus has a single-stranded RNA genome of positive polarity that is characterized by a 5′ covalently linked protein VPg (3B in L434 nomenclature [31] (20, 25, 29) and a 3′-terminal poly(A) sequence (2, 34, 40). Poliovirus RNA replicates in the cytoplasm of infected cells by using a virus-specific RNA-dependent RNA polymerase. The polymerase has been purified to a soluble and template-dependent form from the cytoplasm of infected cells (14, 18, 19, 21). A single virus-specific protein, designated $3D^{pol}$ (31) (also P3-4b, p63, and NCVP4 in previous publications [31]) has been shown to be responsible for this polymerase activity (5, 36, 37).

Highly purified forms of the poliovirus polymerase require an oligo(U) primer (8, 38) or a host factor (9, 13, 15, 41) to initiate RNA synthesis in vitro. In the presence of oligo(U), the polymerase synthesizes full-length template-sized copies of poliovirion RNA ($M_r = 2.5 \times 10^6$) and other polyadenylated RNAs (8, 38). In the presence of the host factor, we have observed that the largest product RNA synthesized by the polymerase is twice the size ($M_r = 5 \times 10^6$) of the poliovirion RNA template (41). The labeled product RNA is complementary to and covalently linked to the template RNA (41). In addition, the polymerase and host factor synthesize dimer-sized product RNA on nonpolyadenylated template RNAs (41). On the basis of these and other results, we proposed that in vitro the polymerase can initiate RNA synthesis in the presence of the host factor by using the 3′ end of the template RNA as a primer (see reference 41 for an additional discussion of this model). Andrews et al. (1) have proposed a similar model based on their observation that terminal uridylyl transferase from reticulocytes acts as a host factor for the polymerase.

Based on indirect evidence, it was previously suggested that a VPg-related protein functions as a primer in vitro in reactions containing purified poliovirus polymerase and host factor. It was reported that anti-VPg antibody can inhibit RNA synthesis in vitro (7, 26) and that anti-VPg antibody immunoprecipitates some product RNA synthesized in vitro on a poliovirion RNA template by the polymerase and host factor (7, 27, 28). These investigators suggested that a VPg-related protein present in the purified polymerase preparation was functioning as a primer for RNA synthesis. The only evidence for VPg-related proteins being present in the purified polymerase is preliminary data from a solid-phase radioimmunoassay (27). VPg or a VPg-related protein, however, could not be immunoprecipitated from the polymerase preparations used in these studies (27). In addition, efforts by N. Crawford (Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1984) to detect VPg-related proteins in purified polymerase preparations yielded negative results. Recently, it was shown that VPg-pUpU can be purified from infected cells (12) and synthesized in infected-cell extracts (35). It was hypothesized that this may be the form in which VPg acts as a primer for the polymerase (12, 35).

Our finding that a covalent linkage exists between the template RNA and the product RNA synthesized in reactions containing polymerase and host factor suggests that the immunoprecipitation of the product RNA might be mediated by VPg on the template RNA. In this study we show that anti-VPg antibody immunoprecipitated dimer-sized product RNA synthesized in vitro. The immunoprecipitation of the product RNA was prevented if VPg was removed from the poliovirion RNA template by pretreatment with proteinase K. Thus, the results show that the purified polymerase and host factor can synthesize product RNA in vitro that is not directly linked to VPg or a VPg-precursor protein and are consistent with a template-priming model for the initiation of RNA synthesis.

MATERIALS AND METHODS

Virus and cell cultures. Suspension cultures of HeLa S3 cells in Joklik modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 7% fetal calf serum

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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 (39). Infected cells and uninfected cells were labeled with [^{35}S]methionine as described previously (37).

Polymerase purification. The poliovirus RNA polymerase was purified from HeLa cells at 5 h postinfection by a modification of our previously published procedure (37). A high-speed supernatant (200,000 × *g*) was prepared from a cytoplasmic extract of the infected cells (fraction 1), and the polymerase was precipitated with 35% ammonium sulfate (fraction 2). After chromatography on phosphocellulose (fraction 3), the fractions containing the polymerase activity were loaded directly on a hydroxylapatite column (fraction 4-HA). The polymerase was eluted from the hydroxylapatite column with a 60-ml, 0 to 0.3 M potassium phosphate (pH 7.5) gradient. The peak fractions of polymerase activity that eluted from the hydroxylapatite column were pooled, concentrated against solid sucrose, dialyzed against 50 mM Tris hydrochloride (pH 8.0)–50% glycerol–0.1 M KCl–2 mM dithiothreitol, and divided into small portions that were stored at −70°C. Polymerase activity was measured at each purification step with a poly(A) template and an oligo(U) primer as previously described (37). The purified (fraction 4-HA) polymerase was dependent on oligo(U) or host factor for activity on poliovirion RNA. Chromatography on Sephacryl S-200 (37) was eliminated from the purification protocol since most of the endogenous host factor was separated from the polymerase by the gradient elution step on hydroxylapatite (C. A. Clifford and J. B. Flanegan, unpublished data). For some experiments, the fraction 3 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 fractions that were stored at −70°C. Both the fraction 3 (0.3 μg/μl) and fraction 4-HA (0.1 μg/μl) 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 under standard conditions.

Host-factor purification. Host factor was partially purified as previously described (41, protocol II). The host-factor preparation (0.25 μg/μl) 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 $MgCl_2$, 8 mM dithiothreitol, 10 to 30 μCi of [α-^{32}P]GTP (or [α-^{32}P]UTP) (410 Ci/mmol), 500 μM ATP, 500 μM CTP, 500 μM UTP (or GTP when [α-^{32}P]UTP was used), 1 μg of poliovirion RNA, and 1 to 3 μl of purified polymerase. When [α-^{32}P]UTP was used as the labeled substrate, it was necessary to use 3 to 5 μM UTP (total concentration) to ensure efficient elongation of the product RNA. Oligo(U) (0.025 μg) or host factor (2 to 3 μl) was added to reaction mixtures as indicated. Contaminating RNase was avoided by treatment of all solutions and reaction tubes with diethyl pyrocarbonate as described previously (38). The reactions were run for 1 h at 30°C. To determine the amount of labeled ribonucleotide that was incorporated into product RNA, a sample of the reaction mixture was precipitated with trichloroacetic acid, collected on membrane filters, and counted in 5 ml of Aquasol 2 scintillation fluid (New England Nuclear Corp., Boston, Mass.) with a Beckman LS7500 liquid scintillation counter. Oligo(U) was prepared from poly(U) (Miles Laboratories, Inc., Elkhart, Ind.) following a procedure described by Bock (10). Poly(U) (1 to 2 mg) was incubated at 90°C for 40 min in 0.5 ml of 0.1 M NH_4HCO_3-NH_4OH (pH 10.0). The pH was adjusted to pH 1.0 by adding an equal volume of 1 M HCl. After incubation at 20°C for 20 min, the solution was neutralized with an equal volume of 1 M Tris hydrochloride (pH 8.0). The oligo(U) was ethanol precipitated and resuspended in 50 μl of 10 mM Tris hydrochloride (pH 8.0) containing 18 U of bacterial alkaline phosphatase. After incubation at 37°C for 1 h, the oligo(U) was phenol extracted, ethanol precipitated, suspended in water, and stored at −20°C. The [α-^{32}P]GTP (PB 161) and [α-^{32}P]UTP (PB 163) were obtained from Amersham Corp. (Arlington Heights, Ill.) in 50% ethanol. The ethanol was removed from the isotope solution by evaporation under reduced pressure with a Buchler Rotary Evapo-Mix.

RNA preparation. Poliovirion RNA was extracted from virions banded in a CsCl density gradient by phenol extraction and ethanol precipitation 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 disodium EDTA. Proteinase K-treated RNA was prepared by digesting purified virion RNA (50 μg) for 1 h at 37°C in a 100-μl solution of 0.5% sodium dodecyl sulfate (SDS)–100 mM NaCl–10 mM Tris hydrochloride (pH 7.5)–1 mM disodium EDTA–proteinase K (200 μg/ml) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The RNA was then phenol-chloroform extracted and precipitated with ethanol.

Peptide synthesis. The 14-amino acid carboxyl-terminal peptide of VPg [VPg (14/22)] and the full-length VPg peptide [VPg (22/22)] were synthesized by solid-phase methods (4) essentially as previously described (17). N^{α}-tert-butyloxycarbonyl (N^{α}-tBOC)-protected amino acids were obtained from Chemalog, Peninsula Laboratories, Inc., and U.S. Biochemical Corp. The cesium salt of N^{α}-tBoc-glutamine was esterified to 10 g of chloromethylated resin obtained from Bio-Rad Laboratories (Bio-Beads, 1% cross-linked polystyrene with 1.25 mmol of Cl^- per g of dry resin) according to the procedure of Gisin (22). Approximately 6 g of the resulting N^{α}-tBoc-glutamine-resin (0.17 mmol of glutamine per g) was used for the synthesis of the peptides. A Vega model 50 peptide synthesizer was used to automate the reagent delivery steps, mixing, and solvent washing. Sequential synthesis was performed with 50% (vol/vol) trifluoroacetic acid (Fisher Certified) in CH_2Cl_2 (Fisher high-pressure liquid chromatography grade) for deprotection and 10% diethylamine in CH_2Cl_2 for neutralization. The protected amino acid to be added (three- to fivefold excess over resin amino groups) and an equivalent amount of 1-hydroxybenzotriazole hydrate (Pierce Chemical Co., Rockford, Ill.) were dissolved in 30 ml of CH_2Cl_2. In some cases a small volume of dimethylformamide (Fisher Scientific Co., Pittsburgh, Pa.) was added to dissolve the protected amino acid before the CH_2Cl_2 was added. An equimolar amount of dicyclohexylcarbodiimide (Aldrich Chemical Co., Inc., Milwaukee, Wis.) dissolved in CH_2Cl_2 was added. The coupling reaction time was 90 min. The progress of synthesis was monitored by ninhydrin analysis (32) of small samples of peptide-resin after each round of coupling and after amino-terminal deprotection with trifluoroacetic acid. Two to four coupling cycles were performed for each amino acid to obtain maximum coupling. After coupling of the carboxy-terminal 14 amino acids of VPg, the resin was dried and weighed, and one half (3.8 g) was removed for hydrogen fluoride cleavage. The remaining

3.8 g of resin was used for the synthesis of the complete VPg peptide. A ^{3}H-labeled amino acid was incorporated into the growing peptide chain. ^{3}H-N$^\alpha$-tBoc-L-alanine was prepared from 1 mCi of L-[2,3-^{3}H]alanine (56 Ci/mmol; Amersham) and 178 mg of L-alanine by the procedure of Itoh et al. (23). BOC-ON (2-[tert-butyloxycarbonyloxyimino]-2-phenylacetonitrile) was obtained from Aldrich. A twofold molar excess of ^{3}H-N$^\alpha$-tBoc-L-alanine was used during the first coupling cycle, and a fourfold excess of unlabeled N$^\alpha$-tBoc-L-alanine was used during the second coupling cycle. The specific activity of the peptides was approximately 250 cpm/nmol.

Cleavage and deprotection. The completed peptides were removed from the polystyrene resin, and all side chain-protecting groups were removed, by treatment with anhydrous hydrogen fluoride (HF) in a Toho Kasei liquid HF apparatus as previously described (17). Anisole and free amino acid side chain-protecting groups were removed from the peptide-resin mixture by several ethyl acetate washes, and the peptide was extracted from the resin with three portions of 50% (vol/vol) glacial acetic acid. The acetic acid was removed by rotary evaporation, water was added, and the peptide was lyophilized and stored at 4°C. Recovery of both peptides from the resin was essentially 100%.

Amino acid and peptide analysis. Amino acid analysis was performed at several points during the synthesis of the peptides and after each purification step with a Beckman 120C amino acid analyzer as previously described (16). The analyses confirmed that both peptides were of the correct composition. At several points during the synthesis, and at the end of the synthesis, solid-phase peptide sequencing was performed on 10-mg samples of peptidyl-resin with a Sequemat Mini-15 solid-phase peptide sequencer and by high-pressure liquid chromatographic analysis as previously described (17). In each case the results confirmed the correctness of the synthetic procedure.

Purification of peptides. Approximately 100 mg of crude VPg (14/22) (one-fourth of the total crude peptide after hydrogen fluoride cleavage) was dissolved in 0.02 M ammonium acetate buffer (pH 5.5). The peptide solution was diluted until the conductivity was less than that of the starting buffer (2.2 mS), and it was loaded on a column (0.9 by 40 cm) of CM-Sepharose equilibrated with 0.02 M ammonium acetate buffer (pH 5.5). After the sample was applied and the column was washed with 100 ml of starting buffer, a linear gradient of 0.02 to 1.0 M ammonium acetate was used to elute the peptide. A 50-μl portion of each 8.4-ml fraction was assayed for radioactivity. The peak fractions were pooled, lyophilized three times, dissolved in 1 ml of 0.1 M acetic acid, and loaded on a Sephadex G-10 column (0.9 by 30 cm) in 0.1 M acetic acid. Eighty 1.5-ml fractions were collected and assayed for radioactivity, and the peak fractions were pooled, lyophilized, and stored at 4°C. The final recovery after purification was 50 mg. Purification was confirmed by high-pressure liquid chromatography.

The crude VPg (22/22) peptide (about 750 mg after cleavage from the resin) was dissolved in 0.1 M acetic acid and divided into several aliquots which were chromatographed individually on a column (0.9 by 30 cm) of Sephadex G-15 in 0.1 M acetic acid. Fractions of 5 ml were collected, and the peak fractions were pooled, lyophilized, and stored at 4°C. The final recovery after purification was about 700 mg.

Immunizations. VPg (14/22) was coupled to bovine serum albumin (BSA) (Miles) with glutaraldehyde (Sigma Chemical Co., St. Louis, Mo.) following published procedures (6, 24). The efficiency of coupling was monitored by chromatography of a small sample of ^{3}H-labeled peptide-BSA conjugate on a column of Sephadex G-50 in phosphate-buffered saline (PBS). The column was calibrated with ^{3}H-labeled peptide and ^{14}C-labeled BSA. The percentage of ^{3}H-labeled peptide that chromatographed with BSA was determined by liquid scintillation counting of a portion of each fraction. The reaction was allowed to continue for 3.5 h when the efficiency of coupling was 42%. This resulted in approximately 17 mol of peptide per mol of BSA. The peptide-BSA conjugate was then dialyzed for 40 h against PBS with three changes of buffer.

Two-month-old New Zealand White rabbits were immunized with 300 μg of VPg (14/22) coupled to BSA emulsified in complete Freund adjuvant by intradermal injections at multiple sites. The rabbits were boosted subcutaneously with 200 μg of VPg (14/22) coupled to BSA in incomplete Freund adjuvant 1 and 3 months after the primary immunization. Antisera were drawn 3 to 6 weeks after immunization, allowed to coagulate, clarified by centrifugation, and stored in aliquots at −70°C.

Preparation of affinity-purified anti-VPg antibody. Anti-VPg antibody was purified from sera by affinity chromatography on VPg (14/22) peptide-agarose (Affigel 10; Bio-Rad Laboratories, Richmond, Calif.). For coupling of the peptide to Affigel 10, 8 ml of wet gel was incubated for 20 h at room temperature with 10 mg of crude VPg (14/22) in 4 ml of 0.5 M HEPES buffer (pH 7.5). The ligand solution was removed, and the gel was washed with 50 ml of 0.5 M HEPES buffer (pH 7.5). Approximately 5.5 mg of peptide was coupled to the resin. Unreacted groups on the resin were blocked by incubation overnight at room temperature with 10 ml of 1 M ethanolamine (pH 8), and the gel was washed twice with 100 ml of 0.2 M glycine hydrochloride (pH 2.5) and twice with 100 ml of PBS. A column (1 by 2 cm) was poured in PBS, washed with 100 ml of 0.1 M glycine hydrochloride (pH 2.5), and equilibrated with 100 ml of PBS. A 12-ml portion of antiserum was applied to the column. The column was washed with 200 ml of PBS, and the anti-VPg antibody was reverse eluted from the column with 0.1 M glycine hydrochloride (pH 2.5). Fractions (1 ml) were collected, immediately neutralized to pH 7.0 with 1 M Tris base, and assayed for protein concentration (11). The peak fractions were pooled (7 ml), lyophilized, suspended in 1 ml of PBS, dialyzed against PBS, and frozen at −70°C. The concentration of anti-VPg antibody was 1.8 mg/ml. The affinity-purified antibody was shown to specifically immunoprecipitate known VPg precursor proteins from an infected-cell extract and to give a strong positive reaction with synthetic VPg (22/22) and a negative reaction with BSA and ovalbumin in Immun-Blot assays (Bio-Rad).

Immunoprecipitation of native proteins and product RNA. Labeled cell pellets (4×10^8 cells) were lysed in 18 ml of PLB (phospholysis buffer: 10 mM sodium phosphate (pH 7.5), 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing 1 mM phenylmethylsulfonyl fluoride (Sigma). The nuclei and cell debris were removed by centrifugation at 900 × *g* for 5 min. The cytoplasmic extract was clarified by centrifugation at 200,000 × *g* for 1 h at 4°C. The supernatant was divided into small portions that were stored at −70°C. Aliquots of the cleared lysate were recleared at 12,000 × *g* for 5 min in an Eppendorf microcentrifuge immediately before use. The aliquots were diluted to 100 μl with PLB and incubated with 5 μl of anti-VPg serum or 5 μl of preimmune serum for 2 h at room temperature. A 50-μl sample of a 10% (vol/vol) suspension of *Staphylococcus aureus* cells containing 10 mg of ovalbumin per ml was

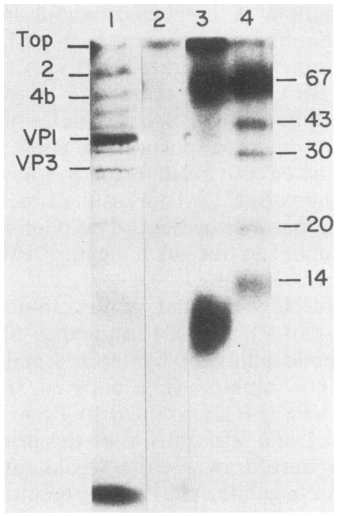

FIG. 1. Immunoprecipitation of VPg-pUp by VPg-specific antiserum. VPg on virion RNA was iodinated with Bolton-Hunter reagent. VPg-pUp was released from the virion RNA by digestion with RNases T_1, T_2, and A (150, 5, and 200 U/ml, respectively) in 40 μl of 10 mM Tris hydrochloride (pH 7.5) for 1.5 h at 37°C. The labeled VPg-pUp was incubated with 5 μl of preimmune serum or 5 μl of VPg-specific antiserum. The immunoprecipitates were collected and electrophoresed in a 15% SDS-polyacrylamide gel. The figure shows an autoradiogram of the gel containing a poliovirus-infected cell lysate labeled with [^{35}S]methionine (lane 1), the immunoprecipitate of VPg-pUp with preimmune serum (lane 2), the immunoprecipitate of VPg-pUp with VPg-specific antiserum (lane 3), and iodinated protein markers (lane 4). The numbers on the right indicate the molecular weights ($\times 10^3$) of the marker proteins. Poliovirus proteins are indicated on the left. Proteins 2, 4b, VP1, and VP3 are 3CD, 3D, 1D, and 1C, respectively, using the L434 nomenclature.

added, and incubation was continued for 60 min on ice. The bacteria were collected at 12,000 × *g* for 30 s and washed three times with 0.5 ml of PLB. The bacterial pellet was suspended in 50 μl of protein electrophoresis sample buffer (62.5 mM Tris hydrochloride [pH 6.8], 2% SDS, 20% glycerol, 5% β-mercaptoethanol, 0.001% bromphenol blue) and heated for 30 min at 68°C. The bacteria were removed by centrifugation at 12,000 × *g* for 3 min, and the supernatant containing the immune precipitates was analyzed by SDS-polyacrylamide gel electrophoresis as described previously (19).

Labeled product RNA was isolated from the in vitro polymerase reactions by ethanol precipitation, dried in vacuo, and suspended in a small volume of 0.1 mM disodium EDTA. A portion (5 to 10%) of the labeled product RNA was removed and placed on ice for nonimmunoprecipitated controls. The remaining product RNA was divided into aliquots which were diluted to 100 μl with PLB and incubated with 1 to 2 μl of affinity-purified anti-VPg antibody or 5 μl of preimmune serum for 1 h on ice. A 10% suspension of *S. aureus* cells containing 1 mg of ovalbumin per ml (50 to 100 μl) was added to the samples. After incubation for 60 min on ice, the bacteria were collected and washed as described above. The bacterial pellets were suspended in 50 μl of protein electrophoresis sample buffer (see above) without β-mercaptoethanol or dye and incubated for 30 min at 68°C. The bacteria were removed by centrifugation, and the labeled product RNA was precipitated from the sample buffer with ethanol and washed once with 70% ethanol containing 0.1 M sodium acetate. The untreated product RNA control and the immunoprecipitated product RNA were dried in vacuo, denatured with 50 mM CH_3HgOH (Alfa Products), and electrophoresed in 1% agarose gels containing 5 mM CH_3HgOH as described previously (3, 41).

RESULTS

Immunoprecipitation of native VPg and VPg precursor proteins by antipeptide antibody. To investigate the synthesis of VPg-linked product RNA in vitro, large quantities of highly purified VPg-specific antibody were needed. Following the general outline described by Baron and Baltimore (6), we synthesized the complete VPg peptide, VPg (22/22), and the carboxy-terminal peptide of VPg, VPg (14/22). Amino acid analysis and solid-phase peptide sequencing at several points during the synthesis of the peptide confirmed the correctness of the procedure. VPg (14/22) was purified by chromatography on CM-Sepharose and Sephadex G-10, coupled to BSA with glutaraldehyde, and used to immunize rabbits.

The ability of the immune serum to react with native VPg was tested by immunoprecipitation analysis with ^{125}I-labeled VPg-pUp isolated from poliovirion RNA treated with Bolton-Hunter reagent. The immune serum, but not the preimmune serum, was able to immunoprecipitate labeled VPg-pUp (Fig. 1, lanes 2 and 3). The labeled VPg-pUp migrated to a size position similar to that observed in

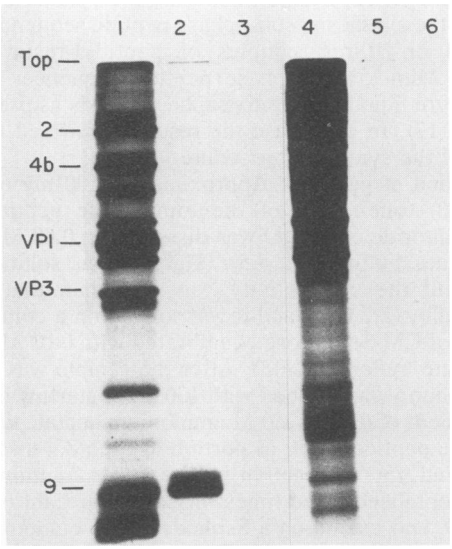

FIG. 2. Immunoprecipitation of virus-specific proteins by VPg-specific antiserum. Cell lysates were prepared from poliovirus-infected and uninfected cells labeled with [^{35}S]methionine. The lysates were incubated with VPg-specific antiserum or preimmune serum as indicated below, and the immunoprecipitates were processed as described in Materials and Methods and electrophoresed in a 12.5% SDS-polyacrylamide gel. The figure shows a fluorogram of the gel containing the poliovirus-infected cell lysate (lane 1), the immunoprecipitate of the infected-cell lysate with VPg-specific antiserum (lane 2) or preimmune serum (lane 3), the uninfected-cell lysate (lane 4), and the immunoprecipitate of the uninfected-cell lysate with VPg-specific antiserum (lane 5) or preimmune serum (lane 6). Lane 1 represents one-third of the material used for incubation with antisera in lanes 2 and 3. Lane 4 represents one-fifth of the material used in lanes 5 and 6. Poliovirus proteins are indicated on the left. Proteins 2, 4b, VP1, VP3, and 9 are 3CD, 3D, 1D, 1C, and 3AB, respectively, using the L434 nomenclature.

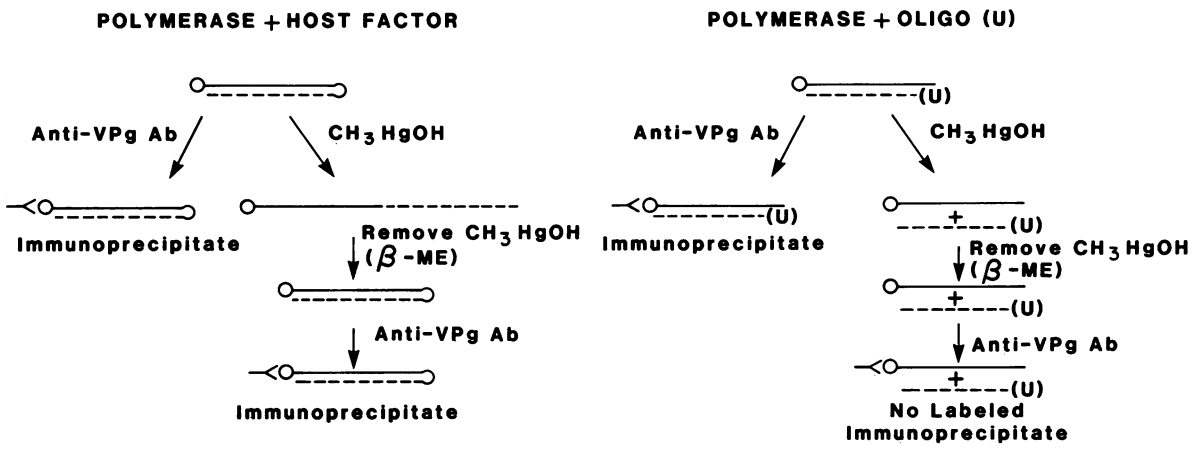

FIG. 3. Model predicting the immunoprecipitation by anti-VPg antibody of the product RNA synthesized by the polymerase in the presence of either the host factor or oligo(U). Product RNA from the host-factor-dependent reaction would immunoprecipitate before and after complete denaturation with CH_3HgOH because of the covalent linkage between the product RNA and the VPg-linked template RNA. Product RNA from the oligo(U)-primed reaction would immunoprecipitate before denaturation because the product RNA remains base paired to the template RNA. Product RNA from the oligo(U)-primed reaction would not immunoprecipitate after denaturation because the product RNA is no longer associated with the template RNA. Symbols: O, VPg; —<, anti-VPg antibody; ——, template RNA; -----, product RNA.

previous studies (30). The labeled material running near the top of the gel that was immunoprecipitated by the immune serum (Fig. 1, lane 3) was not characterized but may be due to incomplete digestion of the virion RNA. The immune serum was also tested for its ability to immunoprecipitate native virus-specific proteins containing VPg-related sequences. Poliovirus-infected cells were labeled with [^{35}S]methionine, and the cytoplasmic extracts of labeled cells were analyzed by immunoprecipitation with the immune or preimmune serum (Fig. 2, lanes 1 to 3). The immune serum precipitated large amounts of protein 9 (3AB) from the infected-cell extracts (Fig. 2, lane 2). Anti-VPg antiserum prepared by Baron and Baltimore (6) also precipitated large amounts of protein 9 (data not shown). A longer exposure of the gel showed immunoprecipitation of small amounts of the larger precursor proteins of VPg observed in previous studies (6, 33). No labeled cellular proteins from uninfected-cell lysates were precipitated with either the immune or preimmune serum (Fig. 2, lanes 4 to 6).

Immunoprecipitation of product RNA. On the basis of our previous finding of a covalent linkage between the poliovirion template RNA and the product RNA synthesized in vitro by the polymerase and host factor (41), we predicted that anti-VPg antibody would immunoprecipitate labeled product RNA because of the VPg linked to the template RNA (Fig. 3). To avoid degradation of the product RNA it was necessary to affinity purify the VPg-specific antibody on a VPg (14/22)-Affigel 10 column. The affinity-purified antibody was found to be free of contaminating RNase activity and was used to immunoprecipitate VPg-linked product RNAs from our in vitro reactions. The immunoprecipitated product RNAs were electrophoresed in CH_3HgOH-agarose gels.

The affinity-purified anti-VPg antibody was able to specifically immunoprecipitate product RNA when either oligo(U) or host factor was used to initiate RNA synthesis (Fig. 3, model, and Fig. 4A and B, lanes 3). Most of the product RNA was immunoprecipitated from the host-factor-dependent reaction even after complete denaturation with CH_3HgOH (Fig. 3 and 4A, lane 4). No product RNA, however, was immunoprecipitated from the oligo(U)-primed reaction when the product RNA was first denatured from the template with CH_3HgOH (Fig. 3 and 4B, lane 4). When affinity-purified anti-BSA antibody was used as a control instead of preimmune serum, no product RNA was precipitated (data not shown). We found that boiling the product RNA was not sufficient to denature the oligo(U)-primed product RNA from the template RNA. After the product RNA was boiled in low salt (Fig. 4C) or PLB (data not shown), we observed the immunoprecipitation of oligo(U)-primed product RNA.

The size distribution of the product RNA that was immunoprecipitated from the reaction containing host factor was very similar to the size distribution of the total product RNA (Fig. 4A). A small, but significant, amount of dimer-sized product RNA ($M_r = 5 \times 10^6$) was immunoprecipitated from the host-factor-dependent reactions both before and after denaturation with CH_3HgOH (Fig. 4A, lanes 3 and 4). Product RNA smaller than genome length was also immunoprecipitated (Fig. 4A, lanes 3 and 4).

The immunoprecipitation of product RNA synthesized in the host-factor-dependent reactions was inhibited by adding increasing amounts of VPg (22/22) during the incubation of the product RNA with anti-VPg antibody (Fig. 5). Immunoprecipitation of product RNA was significantly inhibited by 1 μg of VPg and was completely inhibited by 50 μg of VPg (Fig. 5). Thus, the affinity-purified anti-VPg antibody specifically precipitated product RNA synthesized in our in vitro reactions containing the polymerase and host factor. Dimer-sized product RNA was precipitated from the host-factor-dependent reaction even after complete denaturation with CH_3HgOH.

Effect of removing VPg from template RNA on immunoprecipitation reaction. We predicted that the immunoprecipitation of the product RNA synthesized by the polymerase and host factor would be prevented if VPg was removed from the poliovirion RNA that was added as a template to the in vitro reaction (Fig. 6). To test this, poliovirion RNA was pretreated with proteinase K to remove VPg and then used as a template. The amount and size of the product RNA that was synthesized on the proteinase K-treated virion RNA was essentially the same as that synthesized on untreated virion

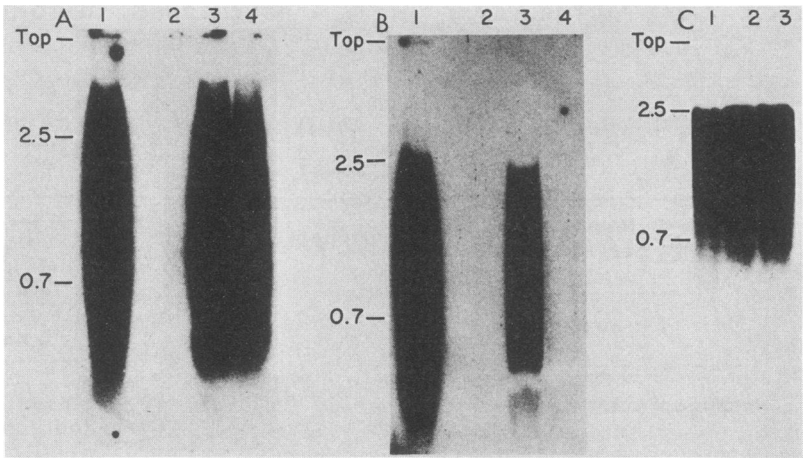

FIG. 4. Immunoprecipitation of product RNA with affinity-purified anti-VPg antibody. (A) Product RNA was synthesized in a standard reaction containing poliovirion RNA, [α-^{32}P]GTP, fraction 4-HA polymerase, and host factor. The product RNA was ethanol precipitated from the reaction mixture, dried in vacuo, and suspended in a small volume of 50 mM H_3BO_3–5 mM $Na_2B_4O_7 \cdot H_2O$–10 mM Na_2SO_4–1 mM disodium EDTA. A sample was removed for a nonimmunoprecipitated control (about 5% of the total product RNA), and the remaining product RNA was divided into three aliquots which were diluted to 15 μl with the buffer described above. The buffer also contained 50 mM CH_3HgOH if the product was to be denatured. After incubation for 20 min at room temperature, 2 μl of 1.25 M β-mercaptoethanol was added if the sample contained CH_3HgOH. The samples were incubated with affinity-purified anti-VPg antibody or preimmune serum, and the immunoprecipitates were electrophoresed in a 1% CH_3HgOH-agarose gel. For this experiment, the preimmune sample was treated with CH_3HgOH and β-mercaptoethanol to demonstrate that the denaturation treatment did not promote nonspecific precipitation. The figure shows an autoradiogram of the gel containing the nonimmunoprecipitated control (lane 1), the immunoprecipitate of product RNA with preimmune serum (lane 2), the immunoprecipitate of product RNA with anti-VPg antibody before denaturation (lane 3), and the immunoprecipitate of product RNA with anti-VPg antibody after denaturation (lane 4). (B) Product RNA was synthesized as described in panel A, but oligo(U) was used in place of host factor. Lanes 1 to 4, Same as in panel A. (C) Product RNA was synthesized as described in panel B, a sample was removed for a nonimmunoprecipitated control (lane 1), and the remaining product RNA was divided into two aliquots. One of the samples was boiled for 3 min in 50 μl of 0.1 mM disodium EDTA to attempt to denature the product from the template, and both samples were immunoprecpitated with anti-VPg antibody. Lane 2, Not boiled; lane 3, boiled. The numbers indicate the molecular weights ($\times 10^6$) of the marker nucleic acids.

RNA (Fig. 7, lanes 1 and 2). None of the product RNA synthesized on the proteinase K-treated virion RNA, however, was immunoprecipitated with anti-VPg antibody (Fig. 7, lane 4). Thus, the removal of VPg from the template RNA before it was added to the in vitro reaction did not affect the ability of the polymerase to copy the template RNA but prevented the immunoprecipitation of the product RNA.

We also predicted that product RNA synthesized by the polymerase and host factor on a cellular mRNA template that does not contain VPg (e.g., globin mRNA, $M_r = 2 \times 10^5$) would be twice the size of the template RNA and would not immunoprecipitate with anti-VPg antibody (Fig. 6). When globin mRNA was used as a template, the majority of the labeled product RNA synthesized by the polymerase and host factor was found to migrate as a band in a CH_3HgOH-agarose gel at a position that indicated that it was twice the size ($M_r = 4 \times 10^5$) of the globin mRNA template (Fig. 8A, lane 2, and B, lanes 3 and 3a). The small product RNA that migrated faster than globin mRNA in Fig. 8A, lane 2, was not characterized, but may represent product RNA synthesized on degradation fragments of the globin mRNA template. In oligo(U)-primed reactions, the majority of the labeled product RNA was the same size as the globin mRNA template (Fig. 8A, lane 1, and B, lane 2). The polymerase preparation used in Fig. 8B appears to still contain a small amount of endogenous host-factor activity since some dimer-sized product RNA was synthesized by the polymerase alone and in the oligo(U)-primed reaction (Fig. 8B, lanes 1 and 2). Although large amounts of labeled product RNA were synthesized by the polymerase and host factor, none of this labeled RNA was immunoprecipitated by anti-VPg antibody (Fig. 8B, lane 4). In a control reaction run at the same time with poliovirion RNA as a template, a large amount of the labeled product RNA was immunoprecipitated as expected. Thus, the polymerase and host factor can efficiently initiate RNA synthesis on a globin mRNA template to yield a dimer-sized product RNA that does not immunoprecipitate with anti-VPg antibody.

DISCUSSION

To study the initiation of poliovirus RNA synthesis, we used anti-VPg antibody to immunoprecipitate product RNAs synthesized on poliovirion RNA by the poliovirus RNA polymerase and the HeLa cell host factor. The results indicated that the immunoprecipitation of the product RNA was mediated by the VPg linked to the template RNA. This was consistent with template-primer-initiated RNA synthesis and indicated that the polymerase and host factor can initiate RNA synthesis in vitro without using VPg or a VPg-precursor protein as a primer.

Anti-VPg antibody was prepared with a synthetic peptide of VPg and was affinity purified to remove contaminating RNase. The purified antibody was specific for VPg and VPg precursor proteins and was used to immunoprecipitate intact labeled product RNAs from our in vitro reactions. An important objective of this study was to determine whether the immunoprecipitation of product RNAs resulted from VPg linked directly to the labeled product RNA or from VPg linked to the template RNA. We were not able to immunoprecipitate any labeled product RNA from our in vitro reactions containing polymerase and host factor when the poliovirion RNA template was pretreated with proteinase K

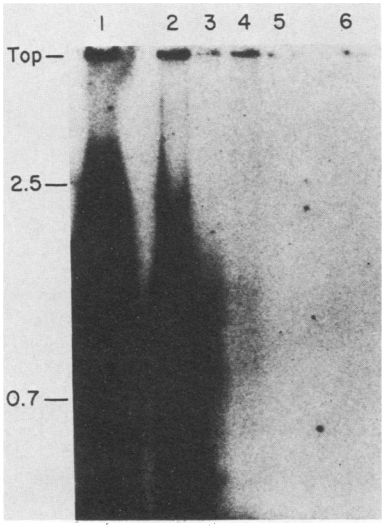

FIG. 5. Inhibition of immunoprecipitation of product RNA by synthetic VPg. Product RNA was synthesized in a standard reaction containing poliovirion RNA, [α-^{32}P]GTP, fraction 3 polymerase, and host factor. A sample was removed for a nonimmunoprecipitated control, and the remaining product RNA was divided into aliquots which were incubated with anti-VPg antibody in the presence or absence of synthetic VPg (22/22) as indicated below. The immunoprecipitates were electrophoresed in a 1% CH_3HgOH-agarose gel. The figure shows an autoradiogram of the gel containing the nonimmunoprecipitated control (lane 1), and the immunoprecipitate of product RNA with anti-VPg antibody (lane 2), anti-VPg antibody plus 1 μg of VPg (lane 3), anti-VPg antibody plus 10 μg of VPg (lane 4), anti-VPg antibody plus 50 μg of VPg (lane 5), and 5 μl of preimmune serum (lane 6). Lane 1 represents about one-fifth of the product RNA used for incubation with antibody in lanes 2 to 6. The numbers are as described in the legend to Fig. 4.

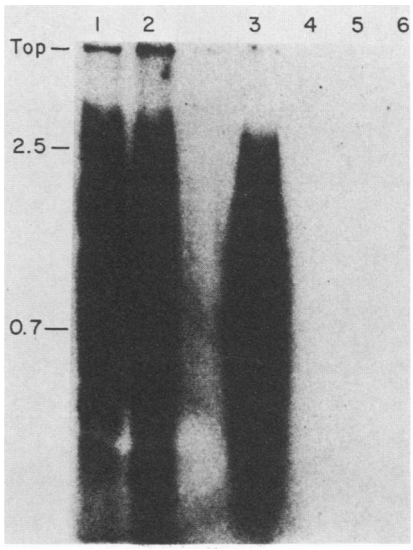

FIG. 7. Effect of proteinase K treatment of the poliovirion template RNA on the immunoprecipitation reaction. Product RNA was synthesized in a standard reaction containing fraction 4-HA polymerase, host factor, [α-^{32}P]GTP, and poliovirion RNA or proteinase K-treated poliovirion RNA. Samples were removed for nonimmunoprecipitated controls, and aliquots of the remaining product RNA were incubated with anti-VPg antibody or preimmune serum. The immunoprecipitates were electrophoresed in a 1% CH_3HgOH-agarose gel. The figure shows an autoradiogram of the gel containing nonimmunoprecipitated product RNA synthesized on virion RNA (lane 1) and proteinase K-treated RNA (lane 2), the immunoprecipitate with anti-VPg antibody (lane 3) or preimmune serum (lane 5) of product RNA synthesized on virion RNA, and the immunoprecipitate with anti-VPg antibody (lane 4) or preimmune serum (lane 6) of product RNA synthesized on proteinase K-treated RNA. Lanes 1 and 2 represent about one-half of the product RNA used for incubation with antibody in lanes 3 to 6. Numbers are as described in the legend to Fig. 4.

to remove VPg. This result indicates that the immunoprecipitation of the product RNA was entirely due to the VPg on the template RNA and suggests that VPg-related proteins were not directly linked to the newly synthesized product RNA.

The affinity-purified anti-VPg antibody immunoprecipitated genome-length product RNA from the oligo(U)-primed reaction and dimer-sized product RNA from the host-factor-dependent reaction. After complete denaturation with CH_3HgOH, however, only the product RNA synthesized in the host-factor-dependent reaction was immunoprecipitated. This result is consistent with our previous evidence that a covalent linkage exists between the template RNA and the product RNA synthesized in the host-factor-dependent reaction (41). We would expect the template-linked product RNA to immunoprecipitate because of the VPg on the template RNA.

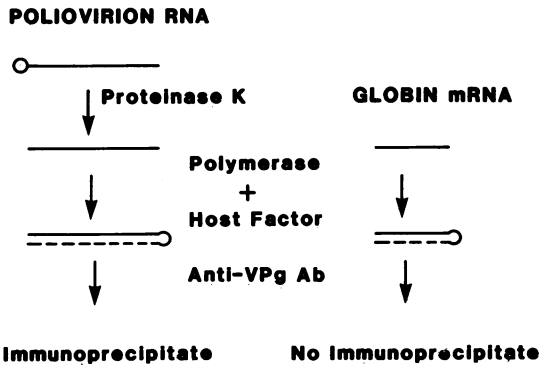

FIG. 6. Model predicting the effect of RNA templates without VPg on the immunoprecipitation of product RNA by anti-VPg antibody. The pretreatment of the poliovirion template RNA with proteinase K would inhibit the immunoprecipitation of the product RNA if the immunoprecipitation is mediated by the VPg on the template. Similarly, if an RNA template without VPg, globin mRNA, is used, product RNA would not immunoprecipitate. Symbols: ○, VPg; ——, template RNA; -----, product RNA.

The size distribution of product RNA that was immunoprecipitated from the host-factor-dependent reaction after CH_3HgOH denaturation was similar to the size distribution of the total product RNA before immunoprecipitation. Thus, it appears that the product RNA was not extensively degraded during the immunoprecipitation procedure. This, in turn, suggests that the smaller immunoprecipitated product RNAs were not the result of the degradation of larger product RNAs. We have previously suggested that the small product RNAs are synthesized by initiation of RNA synthesis on subgenomic-sized fragments of virion RNA that are present in our RNA preparations (41). Initiation on small subgenomic molecules containing VPg would result in small product RNAs that would immunoprecipitate with anti-VPg antibody even after complete denaturation. This appears to be the most reasonable explanation for the small RNAs that

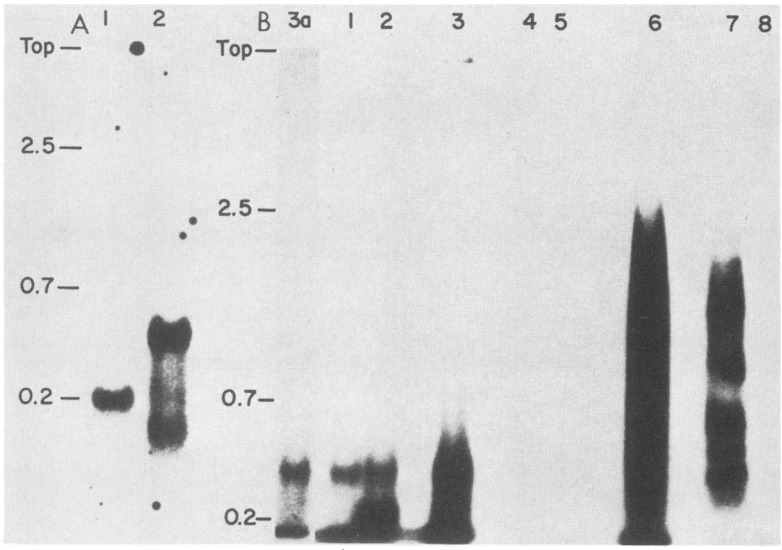

FIG. 8. Immunoprecipitation analysis of product RNA synthesized on globin mRNA. (A) Autoradiogram of a 1.2% CH_3HgOH-agarose gel containing product RNA synthesized on globin mRNA in a standard reaction containing [α-^{32}P]GTP, fraction 4-HA polymerase, and oligo(U) (lane 1) or host factor (lane 2). (B) Autoradiogram of a 1% CH_3HgOH-agarose gel containing product RNA synthesized on globin mRNA in a reaction containing [α-^{32}P]GTP, fraction 4-HA polymerase only (lane 1), and oligo(U) (lane 2) or host factor (lane 3). Aliquots of the host-factor-dependent reaction were incubated with anti-VPg antibody or preimmune serum, and the immunoprecipitates are shown in lanes 4 and 5, respectively. Lane 3 represents about one-fifth of the material used for incubation with antibody in lanes 4 and 5. Lane 3a is a lighter exposure of lane 3. Lanes 6 to 8 are the same lanes as 3 to 5, except that poliovirion RNA was used as a template. Numbers to the left of the gels are described in the legend to Fig. 4.

we found in our immunoprecipitates. Although these results suggest that the in vitro reaction is not specific for full-length poliovirion RNA templates, it is assumed that virion RNA templates are intact in vivo and that these small product RNAs are not synthesized.

Dimer-sized product RNA was synthesized when a cellular RNA, globin mRNA, was used as template in reactions containing polymerase and host factor, In the case of this small mRNA molecule, the majority of the product RNA was dimer sized. We were not able to immunoprecipitate any product RNA from the host-factor-dependent reaction when globin mRNA was used as template. 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 (27). These results again support a template-priming mechanism for initiating RNA synthesis in vitro.

Other investigators used different protocols to purify the poliovirus polymerase that was used in their immunoprecipitation studies (9, 14, 15). These investigators immunoprecipitated product RNA from their host-factor-dependent reactions with anti-VPg antibody and suggested that the product RNA was directly linked to a VPg-specific protein (7, 27, 28). We purified the poliovirus polymerase by their published protocols and were not able to immunoprecipitate any VPg-linked product RNA from reactions containing polymerase and host factor when the template RNA was first pretreated with proteinase K to remove VPg (data not shown). This indicates that VPg-related proteins were not directly linked to the product RNAs synthesized in these reactions.

The results from this study indicate that the immunoprecipitation of product RNA from reactions containing the poliovirus polymerase and host factor was mediated by the VPg on the template RNA. We were unable to immunoprecipitate any product RNA if the poliovirion template RNA was pretreated with proteinase K or if an RNA without VPg was used as a template. In addition, we did not detect VPg-related proteins in any of the polymerase preparations used in this study or reproducibly inhibit host-factor-dependent polymerase activity with affinity-purified anti-VPg antibody (data not shown). Thus, VPg-related proteins do not appear to be involved in the initiation of RNA synthesis in these reactions. The results are consistent with our template-priming model for the initiation of RNA synthesis in vitro (41). In this model, the polymerase in the presence of the host factor uses the 3′ end of the template RNA as a primer. This results in a covalent linkage between the template RNA and the product RNA.

Because VPg is found covalently linked to the 5′ end of poliovirus negative-strand RNA, we postulate that a mechanism exists for separating the product RNA from the template RNA and for linking VPg to the 5′ end of the negative-strand product RNA. Studies are now under way to determine whether such a mechanism exists for the synthesis of VPg-linked product RNA in vitro. Establishing that VPg-linked product RNA can be synthesized by this mechanism in vitro would support a similar model for the replication of poliovirus RNA in vivo.

ACKNOWLEDGMENTS

We thank Melba Jimenez, Benne Parten, Concepta Clifford, Joan Morasco, and Mike Duke for excellent technical assistance.

This work was supported by Public Health Service grant AI15539 from the National Institute of Allergy and Infectious Diseases. D.C.Y. was supported by American Cancer Society postdoctoral fellowship PF2090 and Public Health Service training grant AI07110 from the National Institute of Allergy and Infectious Diseases. J.B.F. is the recipient of Research Career Development Award AI00523 from the National Institutes of Health.

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