ATP Is Required for Initiation of Poliovirus RNA Synthesis In Vitro: Demonstration of Tyrosine-Phosphate Linkage Between In Vitro-Synthesized RNA and Genome-Linked Protein

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Poliovirus replicase- and host factor-catalyzed copying of 3'-terminal polyadenylic acid [poly(A)] of poliovirion RNA was studied. Host factor-stimulated synthesis of polyuridylic acid [poly(U)] by the replicase required ATP in addition to UTP. ATP was not required for the oligouridylic acid-primed copying of 3'-terminal poly(A) of virion RNA. GTP, CTP, and AMP-PCP (5'-adenylyl β - γ methylenediphosphate, an ATP analog) could not replace ATP in host factor-stimulated synthesis of poly(U). Antibodies to poliovirus genome-linked protein (VPg) specifically precipitated in vitro-synthesized poly(U) from a host factor-stimulated reaction. The poly(U) synthesized in a host factor-stimulated reaction was shown to be attached to VPg precursor polypeptide(s) via a tyrosine-phosphate bond as found in poliovirion VPg-RNA.

The RNA genome of poliovirus is 7,433 nucleotides long (26, 36), polyadenylated at the 3'-terminus (3, 52), and covalently linked to a small, virus-specific protein (VPg) at the 5'-terminus (20, 23, 28, 29, 33). The amino acid sequence of VPg is encoded in viral RNA within the portion of the genome that encodes the precursor for the RNA polymerase (25, 26, 34, 36). Poliovirus VPg is 22 amino acids long (1, 25, 26, 36, 39) and contains only one tyrosine residue, which forms the bridge via its O⁴ hydroxyl group to the 5'-terminal phosphate of the nucleotide chain (VPg-Tyr-O⁴-pUU AAAAC.....) (2, 37). VPg has also been found in preparations of replicative intermediate RNA, at the 5'-termini of the nascent chains of plus-strand RNA, and covalently attached to the polyuridylic acid [poly(U)] tract found at the 5'-end of negative strands of both replicative intermediate and double-stranded replicative-form RNA (20, 27, 33, 35, 50). Based on these findings, it has been proposed that VPg may act as a primer for initiating the synthesis of poliovirus plus and minus RNA. Since no free VPg has been detected in poliovirus-infected cells, it has been suggested that VPg enters the RNA replication complex in the form of a precursor polypeptide. Recently, polypeptide precursors to VPg have been identified by immunoprecipitation with anti-VPg antibodies prepared against synthetic oligopeptides to all or part of the 22-amino acid sequence of VPg (7, 32, 38).

The RNA genome of poliovirus is replicated by an RNA-dependent RNA polymerase (replicase) found in cells infected with poliovirus (4). A template-dependent form of the enzyme (14) was first isolated as a polyadenylic acid [poly(A)] · oligouridylic acid [oligo(U)]-dependent poly(U) polymerase (18). The virus-specific poly(U) polymerase activity copurifies with template-dependent replicase activity (14). A single viral protein called p63 (NCVP4, P3-4b) is believed to be responsible for poly(U) polymerase activity in vitro as well as for replicase activity in poliovirus-infected HeLa cells (19, 21, 45). Recently, antibodies specific to P63 have been prepared and shown to inhibit poly(U) polymer-

ase activity as well as poliovirus template-dependent replicase activity (9, 40). Highly purified template-dependent poliovirus replicase has been shown to copy an entire virion RNA molecule in the presence of an oligo(U) primer (6, 12, 46). A host cell protein (host factor) (15) isolated from uninfected HeLa cells can substitute for oligo(U) in poliovirus replicase-catalyzed in vitro synthesis of full-length (35S) minus-strand RNA (6, 12), suggesting a role for host factor in initiation of RNA synthesis. Host factor, a 67,000-dalton protein, has recently been purified and shown to physically interact with poly(U) polymerase both in vitro and in poliovirus-infected HeLa cells (5, 12, 13).

Two laboratories (8, 32) have recently shown that anti-VPg antibodies specifically inhibit host factor-stimulated transcription of poliovirion RNA by the viral replicase, whereas the oligo(U)-primed copying of viral RNA is not affected by the antibody. Anti-VPg antibodies have also been shown to specifically precipitate in vitro-synthesized RNA covalently linked to VPg precursor polypeptides from host factor-stimulated replicase reactions (8). Therefore, VPg precursor(s) and host factor both appear necessary for de novo synthesis of complementary RNA by the viral replicase.

Since the first event during the replication of the genomic RNA should be the copying of 3'-terminal poly(A), it is expected that if a protein becomes attached to the 5'-end of the complementary RNA it should be linked to the 5'-terminal poly(U) sequence. We have, therefore, investigated the requirements for the host factor-catalyzed, poliovirion RNA-dependent synthesis of anti-VPg-immunoprecipitable poly(U) by the viral replicase. We report here that the formation of anti-VPg-immunoprecipitable poly(U) by the poliovirus replicase in the presence of [α -³²P]UTP is greatly stimulated by ATP. GTP, CTP, and 5'-adenylyl β - γ methylenediphosphate (AMP-PCP; an ATP analog) cannot substitute for ATP in this reaction. Oligo(U)-primed copying of virion RNA in the presence of [α -³²P]UTP does not require ATP. The poly(U) synthesized in a host factor-stimulated reaction is attached to VPg precursor(s) via a tyrosine-phosphate bond as found in poliovirion RNA (2, 37).

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MATERIALS AND METHODS

All chemicals unless specifically stated were purchased from Sigma Chemical Co., St. Louis, Mo. Unlabeled nucleotides were obtained from Calbiochem-Behring, La Jolla, Calif.; poly(A) was purchased from Miles Laboratories, Inc., Elkhart, Ind. Oligo (U)₁₀₋₂₀ was purchased from Collaborative Research, Inc., Waltham, Mass. Poly(U) Sepharose 4B was obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Phosphocellulose was purchased from Whatman Inc., Clifton, N.J. All radioisotopes were purchased from New England Nuclear, Boston, Mass. The nonapeptide Gly-Ala-Tyr-Thr-Gly-Leu-Pro-Asn-Lys (VPg-N₉) corresponding to the N-terminal of VPg was made to order by Penninsula Laboratories, San Carlos, Calif.

Cell culture. HeLa cells were grown in Joklik modified medium supplemented with 5 to 8% calf serum (14, 16).

Poliovirus infection. Suspension cultures of HeLa cells were infected with poliovirus type 1 (Mahoney strain) as previously described (14, 16). Fifteen minutes after infection, cells were treated with actinomycin D (5 µg/ml). At 5 to 6.5 h after infection, cells were collected by centrifugation, washed once in phosphate-buffered saline, and kept frozen (−70°C) until use.

Purification of poliovirus replicase [poly(U) polymerase] and host factor. The purification of poliovirus replicase through phosphocellulose (fraction II) and poly(U) Sepharose 4B (fraction IV) have been described (14, 16). Host factor was purified as previously described (12).

Poly(U) polymerase, replicase, and host factor assays. The poly(A) · oligo(U)-dependent poly(U) polymerase activity was assayed for 30 min at 30°C (18). The standard reaction mixture for poliovirus RNA-dependent replicase activity contained (in 50 µl): 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 8.0), 5 mM magnesium acetate, 4 mM dithiothreitol, 10 µg of actinomycin D per ml, 0.2 mM each of three other unlabeled nucleoside triphosphates, 1 µM α-³²P-labeled nucleoside triphosphate (specific activity, 50 to 100,000 cpm/pmol), and 1 µg of poliovirus RNA. Fraction IV replicase (1 to 2 µg; gradient eluted) along with 0.03 µg of host factor were used in the reaction mixtures. RNA synthesis in the absence of host factor served as a control. Incubation was for 1 h at 30°C. The RNA products were either precipitated with trichloroacetic acid and counted or used in the immunoprecipitation assays.

Poliovirion RNA. Unlabeled poliovirion RNA was prepared by the method of Spector and Baltimore (41). HeLa mRNA was purified through oligodeoxythymidylate-cellulose by a previously described method (31).

Generation and purification of anti-VPg antibody. The anti-VPg antibodies were prepared as previously described (32; C. D. Morrow, M. Navab, C. Peterson, J. Hocko, and A. Dasgupta, *Virus Res.*, in press). Briefly, the peptide was coupled to bovine serum albumin (BSA) by using glutaraldehyde, emulsified in complete Freund adjuvant, and injected into New Zealand White rabbits. The rabbits were boosted with 200 µg of the peptide-BSA conjugate in Freund incomplete adjuvant at 4, 6, and 8 weeks after the primary injection. Blood was drawn at various times after immunization, allowed to coagulate, and clarified by centrifugation. Anti-BSA antibodies were removed by chromatography on BSA agarose. The rabbit immunoglobulin G (IgG) was then purified from the serum by using protein A-agarose chromatography as previously described (32). The final concentration of IgG (both immune and preimmune) was adjusted to 10 mg/ml, and the antibody was stored at −20°C until use. The

anti-VPg antibodies were characterized by their capacity to specifically precipitate the nonapeptide and native VPg obtained by enzymatic digestion of poliovirion RNA (32). The specificity of the anti-VPg antibodies was confirmed by the ability of unlabeled nonapeptide to compete with the precipitation of labeled VPg.

Immunoprecipitation. All immunoprecipitations were carried out in phosphate-buffered saline (treated with diethylpyrocarbonate) containing 1% Triton X-100, 0.5% Nonidet P-40, 0.5% sodium dodecyl-sulfate (SDS), and 2 mM phenylmethylsulfonyl fluoride (IP buffer). The incubation conditions for the antigen-antibody binding are described in the figure legends. In vitro-synthesized, labeled RNA products were precipitated by ethanol, using 5 to 10 µg of yeast tRNA as a carrier. Precipitated RNA was resuspended in 100 µl of IP buffer, incubated at 95°C for 5 min, chilled quickly, and precipitated with anti-VPg antibody in the presence of 0.1 M unlabeled UTP to inhibit nonspecific uridylylation of IgG. After 1 h of incubation at room temperature, protein A-agarose was added to 5 mg per reaction to bind the IgG. After further incubation of the reaction at 4°C for 60 min, the unbound IgG and antigen were removed from protein A-agarose by centrifugation. The pellets were washed three or four times with IP buffer and resuspended in 30 to 40 µl of electrophoresis sample buffer and boiled for 5 min. The supernatants were then analyzed by SDS-polyacrylamide gel electrophoresis.

Enzymatic digestion. For RNase A digestion, the RNA pellet was resuspended in 15 µl of sterile water containing 5 µg of RNase A. The mixture was incubated at 95 to 98°C for 5 min and quickly chilled on ice; an additional 5 µg of RNase A was then added, and incubation was continued for 1 h. For proteinase K digestion, precipitated product was suspended in 20 µl of proteinase K (200 µg/ml), 10 mM Tris-hydrochloride (pH 7.5), 1 mM EDTA, and 0.5% SDS and digested for 2 h at 37°C.

Alkali hydrolysis of RNA. Ethanol-precipitated RNA was resuspended in 15 µl of 0.3 M KOH and was incubated for 15 h at room temperature (or 5 h at 37°C). The reaction mixture was then neutralized by addition of an equimolar amount of perchloric acid. The precipitate was removed by centrifugation, and the supernatant was analyzed by high-voltage paper electrophoresis.

Acid hydrolysis of VPg precursor(s). Immunoprecipitated RNA [poly(U)] was first digested with RNase A. RNase A-digested material was further digested with micrococcal nuclease to generate protein phosphate (2). Phosphorylated VPg precursor(s) was passed through a Sephadex G-25 column. Radioactive material in the void volume was pooled and precipitated with acetone in the presence of 20 µg of BSA. Precipitated proteins were hydrolyzed in sealed glass ampoules under nitrogen in 200 µl of 2 M HCl at 110°C for 15 h. The hydrolysate was freeze-dried and dissolved in water.

High-voltage paper electrophoresis. Alkali-hydrolyzed immunoprecipitated RNA and acid-hydrolyzed VPg precursor were analyzed by ionophoresis at pH 3.5 on Whatman 3 MM paper (20). Electrophoresis was at 30 V/cm until the marker dye, xylene cyanol, had moved about 10 cm. The labeled products were located by subsequent autoradiography. The amino acid phosphate markers were detected by staining with ninhydrin.

SDS-polyacrylamide gel electrophoresis. Immunoprecipitated RNA (³²P labeled) and proteins were analyzed on 15% SDS-polyacrylamide gels containing 0.37 M Tris-hydrochloride (pH 8.8), 0.1% SDS, and 0.1% *N,N'*-methylenebisacrylamide. The stacking gel contained 4% acrylamide, 0.1%

FIG. 1. Effect of ATP on host factor- and oligo(U)-stimulated copying of 3'-terminal poly(A) of poliovirus RNA. Poliovirus template-dependent replicase (1 μ g, fraction IV) (16) was incubated with various amounts of purified, fraction VII (15) host factor (A) or oligo(U) (B) in the presence of 1 μ g of poliovirus RNA with (●) or without (○) added ATP (250 μ M). [α - 32 P]UTP (specific activity, 5,000 cpm/pmol) was used as the labeled nucleoside triphosphate. Assay conditions (total volume, 50 μ l) were identical to those described previously (12, 14, 16). Incubation was for 30 min at 30°C. Labeled products were collected on membrane filters after precipitation with 7% trichloroacetic acid in the presence of 100 μ g of carrier yeast tRNA, and the filters were counted in a scintillation counter with 5 ml of Bray solution.

methylelene acrylamide, 0.125 M Tris-hydrochloride (pH 6.8), and 0.1% SDS. Electrophoresis was carried out in 0.05 M Tris–0.384 M glycine–0.1% SDS at 120 V for 5 to 6 h. The gel was fixed for 15 min in 10% ethanol and 10% acetic acid and dried, and the labeled products were visualized by autoradiography.

RESULTS

Requirement of ATP in initiation of RNA synthesis. Poliovirus replicase can be isolated in a form that depends on either oligo(U) or a host cell protein (host factor) for the initiation of copying of poliovirus (plus) RNA (5, 6, 12, 16, 47). The most prominent product of either reaction is full-length (35S) minus-strain RNA (6, 12). Proper initiation by the poliovirus replicase for synthesis of minus RNA should start at or near the 3'-terminal poly(A) of the virion RNA. After initiation has taken place, viral poly(U) polymerase should be able to copy the poly(A) tail of virion RNA in the presence of UTP, and therefore, the initial product of the reaction in the absence of other nucleoside triphosphates should be mainly poly(U). This reasoning led us to examine poliovirus RNA-dependent synthesis of poly(U) by the viral replicase–host factor combination.

When poliovirus replicase and host factor were incubated with virion RNA and [α - 32 P]UTP, no RNA synthesis as measured by trichloroacetic acid-insoluble radioactivity was detected (Fig. 1A). When unlabeled ATP was included in the reaction, synthesis of [α - 32 P]UTP-labeled product was evident (Fig. 1A). Since [α - 32 P]UTP was used as the labeled nucleoside triphosphate, we will refer to the products synthesized as poly(U). Later we will show that the product actually is poly(U). In the presence of a constant amount of viral replicase, addition of increasing amounts of host factor resulted in linear increase of UMP incorporation in acid-insoluble products. At higher concentration of host factor, however, poly(U) synthesis reached a plateau. When host

factor was replaced with oligo(U) in the reaction, synthesis of poly(U) was clearly evident in the absence of ATP (Fig. 1B). In this particular experiment, oligo(U)-primed reactions resulted in a five- to sixfold increase in UTP incorporation over the host factor-stimulated reaction. However, addition of unlabeled ATP did not affect oligo(U)-primed synthesis of poly(U). The results suggested that ATP was important for the host factor-stimulated, replicase-catalyzed poly(U) synthesis in response to virion RNA but not for the oligo(U)-stimulated reaction.

In the presence of a constant amount of host factor (optimal concentration), addition of increasing concentrations of viral replicase resulted in increased synthesis of poly(U), and the reaction was completely dependent on added ATP (Fig. 2A). When ATP was replaced with either GTP or CTP, virtually no poly(U) synthesis was observed (Fig. 2B). At the highest concentration tested, CTP showed slight stimulation, which could have been due to contamination of this particular CTP preparation with ATP. Poly(U) synthesis was stimulated at ATP concentrations as low as 50 μ M (Fig. 2B).

Immunoprecipitation of in vitro-synthesized poly(U). To examine the possibility that poly(U) synthesized in vitro by the poliovirus replicase is linked to a VPg-related protein(s), we used anti-VPg antibodies to immunoprecipitate the labeled material. When in vitro-synthesized [α - 32 P]UMP-labeled material was immunoprecipitated with anti-VPg antibodies and the immunoprecipitates were analyzed on a 10% SDS-polyacrylamide gel, anti-VPg immunoprecipitable poly(U) was evident in reactions containing ATP (Fig. 3). Although individual addition of CTP and GTP to the reaction did not support the synthesis of anti-VPg-immunoprecipitable poly(U), in the presence of ATP, addition of CTP or GTP resulted in increased synthesis of poly(U). This stimulation varied in different experiments. Quantitation of immunoprecipitated material indicated that approximately 20 to 40%

FIG. 2. Effects of increasing concentrations of replicase and different nucleoside triphosphates on poliovirus RNA-dependent synthesis of poly(U). (A) Various amounts of poliovirus replicase (0.3 mg/ml, fraction IV) (16) was incubated with a constant amount of host factor (0.03 μg , fraction VII) (12) in the presence of 1 μg of poliovirus RNA with (●) or without (○) added ATP under standard RNA synthesis assay conditions as described in the legend to Fig. 1. (B) Poliovirus replicase (1 μg) and 0.03 μg of host factor were incubated with various concentrations of ATP (●), CTP (▲), GTP (△), or no added nucleotide (○). All reactions contained $[\alpha - ^{32}P] \text{ UTP}$. Labeled products were collected on membrane filters and counted.

FIG. 3. Immunoprecipitation of in vitro-synthesized poly(U) by anti-VPg IgG. $[\alpha - ^{32}P] \text{ UMP}$ -labeled RNA was synthesized in replicase-host factor reactions as described in the legend to Fig. 1. $[^{32}P] \text{ UMP}$ -labeled RNA was phenol extracted and precipitated from the aqueous phase by ethanol. Precipitated RNA was resuspended in IP buffer, incubated at 95°C for 5 min, chilled quickly, and precipitated with 1 μl of anti-VPg IgG in the presence of 0.1 M unlabeled UTP. Incubation was for 1 h at room temperature. Antigen-antibody was recovered by binding to protein A-agarose. The protein A-agarose pellet was washed three or four times with IP buffer, resuspended in 30 μl of electrophoresis sample buffer, and boiled for 5 min. The supernatant was phenol extracted, and the aqueous phases were analyzed on 10% SDS-polyacrylamide gels. Radiolabeled material was localized by autoradiography at -70°C with an intensifying screen. Each lane represents immunoprecipitation of pooled material equivalent to six individual 50- μl reactions. The labeled nucleoside triphosphate used was $[\alpha - ^{32}P] \text{ UTP}$. Unlabeled nucleotides were used at 500 μM . Lane 1, $[^{32}P] \text{ UTP}$ plus

more ATP-stimulated poly(U) was synthesized in the presence of CTP or GTP compared to the amount synthesized in an ATP-stimulated reaction. At present, we do not have an explanation for this observation. Immunoprecipitated material was completely resistant toward digestion by RNase T1 (data not shown). Precipitation of poly(U) was specific to immune IgG. Preimmune IgG did not precipitate any labeled material. When immunoprecipitation was performed from a reaction containing all four nucleoside triphosphates, higher-molecular-weight material was found to be precipitated by the immune IgG, indicating synthesis of heteropolymeric RNA in the presence of all four nucleoside triphosphates. The yield of immunoprecipitated, heteropolymeric RNA synthesized in the presence of all four nucleoside triphosphates was somewhat less than that of poly(U). This is most probably due to the inability of higher-molecular-weight heteropolymeric RNAs to enter a 10% gel.

Kinetics of RNA synthesis and characterization of immunoprecipitated product. Kinetics of poly(U) synthesis by the replicase-host factor combination in the presence of ATP and poliovirus RNA showed that the reaction was linear with respect to time for the first 30 to 60 min (Fig. 4). AMP-

immune IgG; lane 2, $[^{32}P] \text{ UTP}$, unlabeled CTP plus immune IgG; lane 3, $[^{32}P] \text{ UTP}$, unlabeled ATP plus immune IgG; lane 4, $[^{32}P] \text{ UTP}$, unlabeled GTP plus immune IgG; lane 5, $[^{32}P] \text{ UTP}$, unlabeled ATP plus preimmune IgG; lane 6, $[^{32}P] \text{ UTP}$, unlabeled CTP and ATP plus immune IgG; lane 7, $[^{32}P] \text{ UTP}$, unlabeled GTP and ATP plus immune IgG; lane 8, $[^{32}P] \text{ UTP}$, unlabeled GTP and CTP plus immune IgG; lane 9, $[^{32}P] \text{ UTP}$, unlabeled ATP, GTP, CTP plus preimmune IgG; lane 10, same as lane 9 except immune IgG was used. Approximately half of the labeled material entered the 10% gel in lane 10. Poliovirus-specific proteins were analyzed on the same gel.

PCP, an analog of ATP, could not substitute for ATP in this reaction. Immunoprecipitation of poly(U) synthesized at early stages of the reaction with anti-VPg antibodies and subsequent analysis of the immunoprecipitates on a 20% SDS-polyacrylamide gel showed the appearance of a radioactive broad band of approximate molecular weight 49,000 (49K) (Fig. 5, lane 6). As the reaction proceeded, synthesis of higher-molecular-weight material was evident (Fig. 5, lane 7). Omission of Mg^{2+} (lane 1), ATP (lane 2), and virion RNA (lane 3) from the reaction resulted in complete loss of synthesis of immunoprecipitable material. Addition of unlabeled nonapeptide (50 μ g) (against which anti-VPg antibody was prepared [31]) during immunoprecipitation significantly inhibited precipitation of the 49K band and higher-molecular-weight materials (lane 8). In the presence of 100 μ g of peptide, immunoprecipitation was completely inhibited (data not shown). RNase A digestion of the immunoprecipitate removed the majority of radiolabeled material. After RNase A digestion of the immunoprecipitate, a radiolabeled broad band migrating at ca. 49K was evident (Fig. 5, lane 9). This 49K band comigrated with the band that appeared early in the poly(U) synthesis reaction. In other experiments, an additional RNase A-resistant, [α - 32 P]UMP-labeled band (14K) was observed after nuclease digestion of the immunoprecipitate (Fig. 5, lane 10). Identical [32 P]UMP-labeled bands (49K and 14K) were previously found in the RNase A digest of [32 P]UMP-labeled, anti-VPg-immunoprecipitable material recovered from replicase reactions containing all four ribonucleoside triphosphates (Morrow et al., in press). These bands were proteinase-sensitive and phenol extractable, indicating the protein nature of these residues (8;

FIG. 4. Kinetics of replicase-host factor-catalyzed synthesis of poly(U). Fraction IV replicase (1 μ g) (16) and 0.05 μ g of fraction VII host factor (12) were incubated with 1 μ g of poliovirus RNA under standard RNA synthesis assay conditions in the absence (○) and presence (●) of 250 μ M unlabeled ATP or AMP-PCP (△). Labeled RNA synthesized was assayed as described previously (12).

FIG. 5. Immunoprecipitation by anti-VPg of in vitro synthesized, [α - 32 P]UMP-labeled material at early stages of RNA synthesis and RNase A digestion of the immunoprecipitated material. RNA synthesis was carried out as described in the legend to Fig. 4. Reactions were stopped at various times during RNA synthesis by adding 5 mM EDTA. Immunoprecipitations by anti-VPg were carried out as described in the legend to Fig. 3, except for the following changes: first, immunoprecipitation was performed directly from the reactions (without prior phenol extraction of the reaction mixture), and second, the supernatant recovered after boiling the protein A-agarose pellet with gel sample buffer was directly analyzed on the gel. Each lane represents immunoprecipitation of pooled material equivalent to 10 individual 50- μ l reactions. Lane 1, After 20 min of synthesis, reaction lacking Mg^{2+} ; lane 2, after 20 min of synthesis, reaction lacking ATP; lane 3, after 20 min of synthesis, reaction lacking poliovirus RNA; lane 4, complete reaction, stopped at 0 min; lane 5, complete reaction, stopped at 3 min; lane 6, complete reaction, stopped at 10 min; lane 7, complete reaction, stopped at 20 min; lane 8, complete reaction, stopped at 20 min and with 50 μ g of VPg-peptide added during immunoprecipitation; lane 9, RNase A digestion of the material in lane 7; lane 10, RNase A digestion of a sample similar to that in lane 7 except that a different batch of replicase was used in the reaction. [35 S]methionine-labeled viral proteins were analyzed on a parallel lane.

Morrow et al., in press). The 49K and 14K proteins were previously shown to comigrate with known VPg-precursor polypeptides (8; Morrow, et al., in press).

The anti-VPg-immunoprecipitable poly(U) synthesized in the presence of [α - 32 P]UTP and unlabeled ATP appeared to be of fairly long size since most of the labeled products sedimented at ca. 25 to 45 when analyzed by denaturing sucrose density gradient centrifugation (data not shown). This value is consistent with the finding that poly(U) in the replicative intermediate and replicative-form RNAs ranges from 50 to well over 200 nucleotides (42).

To confirm that the in vitro-synthesized labeled material was actually poly(U), the immunoprecipitated material was first digested extensively with proteinase and then hydrolyzed with alkali followed by high-voltage paper ionophoresis of the hydrolysate at pH 3.5. Only radiolabeled spots comigrating with marker UMP was observed (Fig. 6). In the absence of ATP in the reaction, no UMP was detected (Fig. 6, lane 2). Addition of increasing concentrations of ATP (50 and 500 μ M ATP in lanes 3 and 4, respectively) resulted in higher yields of UMP, indicating increased synthesis of poly(U) at higher ATP concentrations as found earlier (Fig. 2B). No radiolabeled spots migrating with marker AMP were observed, indicating that ATP was not physically incorporated into the material synthesized in vitro. When [α - 32 P]ATP was used in the presence of unlabeled UTP, no radiolabeled anti-VPg-immunoprecipitable material was recovered (data not shown).

FIG. 6. High-voltage paper ionophoresis of alkali-hydrolyzed [^{32}P]UMP-labeled immunoprecipitated material. [$\alpha\text{-}^{32}\text{P}$]UMP-labeled immunoprecipitated material was prepared as described in the legend to Fig. 3. Labeled RNA was extracted twice with phenol and precipitated from aqueous phase in the presence of 20 μg of yeast tRNA. Labeled RNA was digested with proteinase K and phenol extracted, and the RNA was hydrolyzed with alkali as described in the text. Hydrolyzed RNA was analyzed by high-voltage paper ionophoresis at pH 3.5 as described in the text. Lane 1, Nucleoside monophosphate markers; lane 2, reaction lacking ATP; lane 3, reaction including 50 μM unlabeled ATP; lane 4, reaction including 500 μM unlabeled ATP. XC, Position of xylene cyanol marker dye.

Tyrosine-phosphate linkage between in vitro-synthesized poly(U) and VPg-related proteins. To determine whether VPg-sequences are attached to the UMP residues through a tyrosine-phosphate bond as found in poliovirus VPg-RNA (2, 37), we digested RNase A-resistant material (Fig. 5, lanes 9 and 10) with micrococcal nuclease to generate VPg precursors linked to phosphates (2). Phosphoproteins were then acid hydrolyzed, and the hydrolysate was analyzed by paper ionophoresis at pH 3.5. Only one radiolabeled spot (other than the P_i spot) comigrating with unlabeled tyrosine-phosphate marker was found in the acid hydrolysate (Fig. 7A, lane 3). When ^{32}P -VPg-p isolated from ^{32}P -labeled poliovirus RNA was subjected to the same analysis, a radioactive spot comigrating with unlabeled tyrosine-phosphate marker was evident (Fig. 7A, lane 2). Tyrosine-phosphates recov-

ered from both ^{32}P -labeled poliovirus RNA and anti-VPg-immunoprecipitated poly(U) synthesized in the in vitro reaction comigrated with ^{32}P -labeled tyrosine-phosphate marker (Fig. 7A, lane 1).

Template specificity of poliovirus replicase. Previous results have shown that purified poliovirus replicase can copy a variety of poly(A)-containing RNAs in the presence of host factor or oligo(U) (5, 16, 44). However, with host factor, the efficiency of copying of poliovirus RNA was two- to three-fold more than that of other poly(A)-containing RNAs (5,

FIG. 7. High-voltage paper ionophoresis of the acid hydrolysate of RNase A-digested anti-VPg immunoprecipitable material. RNase A-digested material was prepared as described in the legend to Fig. 5. Radiolabeled material was pooled from 10 RNase A digests. Pooled material was digested with micrococcal nuclease to generate protein-phosphate as previously described (2). Labeled material was passed through a Sephadex G-25 column. Radioactive material eluting at the void volume was pooled and precipitated with acetone in the presence of 20 μg of BSA. Precipitated material was lyophilized and the proteins were hydrolyzed in 2 M HCl as described in the text. Hydrolyzed material was spotted on 3MM paper and subjected to ionophoresis at pH 3.5. Lane 1, ^{32}P -labeled tyrosine phosphate marker; lane 2, poliovirus RNA-derived tyrosine phosphate (from ^{32}P -labeled VPg-P); lane 3, material obtained after acid hydrolysis of RNase A and micrococcal nuclease-digested [^{32}P]UMP-labeled immunoprecipitated material. Migration of unlabeled phosphoamino acids are shown by dashed circles. XC, Position of xylene cyanol.

16). Poly(A)-minus RNAs, including depolyadenylated poliovirus RNA, were almost completely inactive as templates (16). Similar results were obtained when various poly(A)-containing templates were tested for their ability to support poly(U) synthesis by the poliovirus replicase in the presence of purified host factor (data not shown).

We further examined the template specificity of poliovirus replicase by determining whether anti-VPg antibodies would inhibit transcription of other poly(A)-containing RNAs compared with that of poliovirus RNA. We chose HeLa mRNA as the heterologous RNA to be tested because the poliovirus replicase appears to specifically copy viral RNA in the cytoplasm of infected HeLa cells and therefore should discriminate between viral and cellular poly(A)-containing RNAs. Poliovirus RNA-dependent synthesis of poly(U) was progressively inhibited by increasing concentrations of immune IgG (Fig. 8). At the highest concentration of anti-VPg, almost 90% inhibition of poly(U) synthesis was observed. However, poly(U) synthesis in response to HeLa mRNA was not significantly inhibited by the antibody. Slight inhibition of copying of HeLa mRNA at higher concentrations of immune IgG was also noted with the preimmune IgG (data not shown). The results suggested that poly(U) synthesis by the viral replicase-host factor combination in response to HeLa mRNA did not utilize VPg precursor(s). This conclusion was also supported by the inability of anti-VPg IgG to immunoprecipitate any labeled material from a reaction programmed with HeLa mRNA (data not shown).

DISCUSSION

The poly(U) stretch found at the 5'-terminus of poliovirus minus-strand RNA arises by copying of the 3'-terminal poly(A) of poliovirus (plus) RNA (42, 51, 53). We have shown that the synthesis of poly(U) by the poliovirus replicase in response to poliovirus RNA is greatly stimulated by ATP. Oligo(U)-primed copying of 3'-terminal poly(A) of virus RNA is not stimulated by ATP, indicating that ATP is not involved in the elongation step of poly(U) synthesis. Poly(U) synthesized by the replicase-host factor combination appears to be attached to VPg precursor polypeptides through tyrosine-phosphate bonds, indicating specific initiation of poliovirus minus-strand RNA synthesis in the *in vitro* system.

Since GTP and CTP (singly or together) cannot replace ATP in replicase-host factor-catalyzed *in vitro* synthesis of poly(U), the possibility that the ATP stimulation is due to the presence of a nonspecific nucleoside triphosphatase in the enzyme preparation that degrades labeled UTP seems unlikely. Lack of stimulation of oligo(U)-primed poly(U) synthesis by ATP also argues against this possibility.

The facts that the replicase-host factor-catalyzed RNA synthesis in response to poliovirus RNA is almost completely inhibited by anti-VPg antibody and that the anti-VPg antibodies specifically immunoprecipitate *in vitro*-synthesized poly(U) directly implicate VPg in this reaction. In fact, RNase A digestion of the *in vitro*-synthesized material shows the presence of UMP-linked VPg precursors. Identical VPg precursors have previously been shown to be covalently attached to poly(U) linked to heteropolymeric sequences synthesized *in vitro* by the replicase-host factor combination in the presence of [α - 32 P]UTP and three other unlabeled nucleoside triphosphates (8; Morrow et al.).

Kinetics of [α - 32 P]UMP-labeled poly(U) synthesis in the presence of unlabeled ATP reveals the formation of a radiolabeled band with an approximate molecular weight of

FIG. 8. Effects of anti-VPg antibody on poliovirus replicase-catalyzed synthesis of poly(U) in response to poliovirus RNA and HeLa mRNA. Portions of poliovirus replicase (1 μ g, fraction IV) (16) were preincubated with various amounts of affinity-purified anti-VPg IgG for 12 h in ice. The portions of replicase were then assayed for host factor-stimulated synthesis of poly(U) by using poliovirus RNA (●) or oligodeoxythymidylate-purified HeLa mRNA (○) as templates in the presence of 2 μ Ci of [α - 32 P]UTP (specific activity, 10,000 cpm/pmol) and 250 μ M unlabeled ATP. Reaction conditions are described in the text. 32 P-labeled RNAs synthesized in the reactions were precipitated with trichloroacetic acid and collected on membrane filters, and the filters were counted with 5 ml of Bray solution. RNA syntheses (100%) with poliovirus RNA and HeLa mRNA as templates were approximately 80,000 and 33,000 cpm, respectively.

49,000 during early stages of synthesis. Synthesis of most of the higher-molecular weight, anti-VPg-immunoprecipitable material follows the formation of 49K radiolabeled band. After RNase A digestion of the labeled material synthesized later in the reaction, the residue left is the same 49K band that is recognized by anti-VPg IgG. The result suggests that uridylation of this 49K protein may be necessary before poly(U) synthesis occurs. However, direct proof of this suggestion must come from purification of this VPg precursor and *in vitro* experiments demonstrating uridylation of the precursor polypeptide and the ability of the uridylylated precursor to prime minus RNA synthesis. Another VPg precursor (14K) (7, 38; Morrow et al.) is occasionally found in the RNase A digest of the *in vitro*-synthesized poly(U). Although we previously detected a virus-specific protein (49K) in our replicase preparation which comigrated with [32 P]UMP-labeled, RNase A-digested immunoprecipitates recovered from replicase reactions (8, 32; unpublished data, we had not previously seen the 14K band in replicase preparations. This VPg precursor (14K) has been shown to be mainly associated with the membranes prepared from poliovirus-infected cells (38). Since the replicase used in this study was purified from the soluble phase of infected cells, it is unlikely that this protein was present in replicase preparation. Whether the 14K protein is a breakdown product of the 49K protein is not known at present.

It is clear from the result presented in Fig. 7 that the linkage of VPg precursor(s) with UMP residue is through a tyrosine-phosphate bond. To our knowledge, this is the first

demonstration of a tyrosine-phosphate linkage in poliovirus complementary RNA sequences synthesized *in vitro* by the viral replicase. Since tyrosine-phosphate cannot be recovered from acid hydrolysates of anti-VPg-immunoprecipitable products synthesized in the presence of α -³²P-labeled ATP, GTP, or CTP (data not shown) but only from products labeled with [α -³²P]UTP, the proteins with VPg sequence must be attached to UMP residues. The data actually strengthen the notion that *de novo* initiation of poliovirus minus RNA synthesis occurs by forming a bond between a tyrosine residue in the VPg-precursor(s) and the first nucleotide (U) of the polyribonucleotide chain.

Other poly(A)-containing RNAs including HeLa mRNA are copied by the replicase to a lesser extent compared with that of poliovirus RNA. It is interesting to note that the copying of HeLa mRNA by the poliovirus replicase is not affected by anti-VPg, whereas poly(U) synthesis in response to poliovirus RNA is almost completely inhibited by the antibody. The implication of this result is that VPg precursor(s) is not involved in copying of HeLa mRNA. It appears then that initiation of RNA synthesis on a nonpolio template by the viral replicase may occur via a different mechanism than that responsible for poliovirus complementary RNA synthesis. Whether the copying of HeLa mRNA by the poliovirus replicase is simply due to nonspecific initiation on this template is not known at present.

How ATP stimulates synthesis of anti-VPg-immunoprecipitable poly(U) in response to poliovirus RNA is not clear at present. Since AMP-PCP, an ATP analog possessing a nonhydrolyzable linkage between the β and γ phosphates, cannot substitute for ATP in poly(U) synthesis, it seems likely that the cleavage of the β - γ bond of ATP is important for this reaction. The cleavage of the β - γ bond of ATP also appears necessary for the synthesis of complete minus strand RNA since substitution of ATP by AMP-PCP in a host factor-stimulated reaction containing all four ribonucleoside triphosphates does not support the formation of 35S RNA in response to virion RNA (data not shown). An ATP requirement for initiation of RNA synthesis in different DNA- and RNA-containing viruses as well as in eucaryotic cells has already been suggested (10, 22, 43, 47). Also known is the ATP requirement for the synthesis of single-stranded RNA of encephalomyocarditis virus, a member of the picornavirus family (17).

Whether ATP is required for the formation of the linkage between VPg precursor(s) and UMP has yet to be determined. ATP has been shown to play a role in the initiation of adenoviral and bacteriophage ϕ 29 DNA synthesis, where virus-coded polypeptides react with dCTP and dATP to form protein-dCMP and protein-dAMP covalent complexes, respectively (11, 24, 30, 47). However, in adenoviral DNA synthesis, ATP is only required when a double-stranded DNA is used as a template (24). Initiation of DNA synthesis on a single-stranded DNA template does not require ATP. With a double-stranded adenoviral DNA template, ATP is believed to be involved in unwinding of some regions of the DNA to facilitate proper initiation. The possibility that unwinding of secondary structures within the poliovirus RNA molecule may be important for initiation of RNA synthesis cannot be ruled out.

The results presented in this paper are also compatible with the assumption that the ATP-requiring function in question is a protein kinase or some other enzyme which cleaves the γ -phosphate from ATP. Phosphorylation of host or viral proteins may play an important role in initiation of viral RNA synthesis (48, 49). Our preliminary experiments indi-

cate phosphorylation of a protein with an approximate molecular weight of 64,000 to 65,000 present in the replicase preparations. The origin of this protein (viral or host) is not known at present, nor do we know whether phosphorylation is really required for RNA transcription. Further studies are in progress to answer this question.

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