JOURNAL OF VIROLOGY, Oct. 1986, p. 43–53
0022-538X/86/100043-11$02.00/0

Vol. 60, No. 1

Initiation of Poliovirus Plus-Strand RNA Synthesis in a Membrane Complex of Infected HeLa Cells

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Received 14 April 1986/Accepted 16 June 1986

An in vitro poliovirus RNA-synthesizing system derived from a crude membrane fraction of infected HeLa cells was used to analyze the mechanism of initiation of poliovirus plus-strand RNA synthesis. This system contains an activity that synthesizes the nucleotidyl proteins VPg-pU and VPg-pUpU. These molecules represent the 5′-terminal structure of nascent RNA molecules and of virion RNA. The membranous replication complex is also capable of synthesizing nucleotidyl proteins containing nine or more of the poliovirus 5′-proximal nucleotides as assayed by the formation of the RNase T_1-resistant oligonucleotide VPg-pUUAAAACAGp or by fingerprint analysis of the in vitro-synthesized RNA. Incubation of preformed VPg-pUpU with unlabeled nucleoside triphosphates resulted in the formation of VPg-pUUAAAACAGp. This reaction, which appeared to be an elongation of VPg-pUpU, was stimulated by the addition of a soluble fraction (S-10) obtained from uninfected HeLa cells. Preformed VPg-pU could be chased into VPg-pUpU in the presence of UTP. Our data are consistent with a model that VPg-pU can function as a primer for poliovirus plus-strand RNA synthesis in the membranous replication complex and that the elongation reaction may be stimulated by a host cellular factor.

The mechanism of replication of poliovirus genomic RNA has been enigmatic, despite numerous efforts to purify various viral and cellular components that may participate in viral RNA synthesis. To date, a cell-free system capable of replicating poliovirus RNA, i.e., the efficient synthesis of a replica of exogenously added template RNA, has not been developed. Such a system would be immensely useful in deciphering different steps in replication and in elucidating the functions of polypeptides and cofactors involved in the process.

RNA replication of poliovirus genomic RNA is complicated for two reasons. First, all newly synthesized RNA isolated from infected cells is covalently bound at the 5′ end to a small, 22-amino-acid, protein called VPg (16, 23, 32, 34). VPg is linked to the RNA via an ester bond between the 5′-terminal phosphate of the RNA and the O^4-hydroxy group of the tyrosine residue in VPg (1, 37). Like all poliovirus proteins, VPg is the product of proteolytic processing of precursor proteins (19, 39, 40) but, unlike other proteins, it does not appear to accumulate in cells during the infectious cycle. The mechanism by which a precursor(s), or VPg itself, participates in RNA synthesis and by which VPg is linked to the RNA is unknown. Second, the 3′ terminus of plus-sense virion RNA is polyadenylated, whereas the 3′ end of the minus-strand RNA is heteropolymeric (22, 35, 50, 51). During replication, the 3′-terminal poly(A) of plus strands is transcribed into 5′-terminal poly(U) of minus strands (50), which, in turn, serves as a template for polyadenylation of plus strands (13). It follows that initiation of synthesis of minus and plus strands occurs on template RNAs whose 3′-terminal nucleotide sequences are different. Therefore, the possibility cannot be excluded that the initiation of RNA synthesis on plus- and minus-stranded templates occurs by different mechanisms.

Two different strategies have been followed in attempts to elucidate the mechanism of poliovirus RNA replication. The first involves a soluble, membrane-free system with partially or highly purified polypeptides and virion RNA as template (4, 10, 15, 17, 46). In this system, initiation and elongation of only minus-stranded RNA has been studied, since the products of the reaction are transcripts of the plus-stranded template. The most important result of this work is the characterization of the virus-encoded RNA polymerase (polypeptide 3D; formerly known as NCVP4 or P3-4b [for a new nomenclature, see reference 38]) that was found to be template and primer dependent (15). Oligo(U) can serve as a primer on any poly(A)$^+$ RNA, but a cellular protein, termed host factor, appears to allow the initation of transcription in the absence of oligo(U) (10). The function of the host factor is unknown. Some evidence has been presented suggesting that some of the product RNA is linked to a viral protein of about 48 kilodaltons (see reference 29 and references therein), but VPg-linked RNA has not been synthesized in any membrane-free system in vitro. More recently, it has been suggested that host factor is a uridylylate transferase and that the mechanism of initiation involves snap-back structures (2, 3, 52).

The second strategy involves the isolation of membranous fractions, termed crude replication complexes (7, 27, 43, 44) from infected host cells that are capable of synthesizing viral RNA. This strategy is based on the observation that poliovirus RNA replication occurs on membranes (5–9, 30). The RNA synthesized by the membranous replication complex is predominantly of plus-strand polarity (9, 14). Hence, the template for the reaction is minus-stranded RNA, and

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initiation of the nascent strands must occur on the 3'-terminal sequence . . . $CUGUUUUAA_{OH}$ (21).

We have previously analyzed endogenous replication complexes and have shown that the crude replication complex can synthesize VPg-pU and VPg-pUpU, the 5'-terminal moiety of both plus-and minus-stranded RNA (43). A similar result was obtained by Vartapetian et al. (47) with membranous complexes isolated from encephalomyocarditis virus-infected cells. In these previous studies, we were unable to demonstrate that full-length viral RNA linked to VPg was synthesized in the crude membrane complex. In this paper we report evidence for de novo initiation of RNA synthesis in vitro, a reaction yielding VPg-linked plus-strand RNA. We show also that preformed VPg-pU can be elongated to VPg-pUpU. Moreover, our data suggest that preformed VPg-pUpU may be used as a primer in the in vitro system.

MATERIALS AND METHODS

Virus infection and preparation of the crude membrane fraction. Suspension cultures of S3 HeLa cells (5×10^6 cells per ml) were infected with poliovirus type 1 (Mahoney) at a multiplicity of infection of 500 PFU per cell, incubated at 37°C, and harvested at various times postinfection. Approximately 5×10^8 cells were suspended in 10 ml of hypotonic cold buffer (10 mM Tris hydrochloride [pH 7.5], 10 mM NaCl, 1.5 mM $MgCl_2$) to be swollen for 10 min on ice and were ruptured by 20 strokes in a tightly fitted glass Dounce homogenizer. The unbroken cells and the nuclei were removed by centrifugation at 800 × *g* for 5 min, and the supernatant fraction was centrifuged at 30,000 × *g* for 20 min (27, 49). The pellet was suspended in 1 ml of buffer (10 mM Tris hydrochloride [pH 8.0], 10 mM NaCl, 15% glycerol) and stored at −70°C. This crude membrane fraction contained ca. 4 mg of protein per ml when determined by the method of Lowry et al. (25). The polymerase activity of the preparation was unchanged after prolonged storage at −70°C. Further fractionation of this membrane fraction was carried out by discontinuous sucrose density gradient centrifugation as described previously (8, 44). The crude membrane fraction was also treated with DEAE-cellulose as follows. A 10-μl portion of the crude membrane fraction was diluted with 80 μl of buffer (10 mM Tris hydrochloride [pH 8.0], 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol) and 10 μl of DEAE-Sephacel (50% suspension in 10 mM Tris hydrochloride [pH 8.0]–10 mM NaCl; Pharmacia Fine Chemicals) was added. The suspension was kept on ice for 15 min to sediment the cellulose beads, and the supernatant was carefully removed. The membrane fraction was recovered by centrifugation and suspended in 10 μl of buffer (10 mM Tris hydrochloride [pH 8.0], 10 mM NaCl, 15% glycerol). In some experiments the crude membrane fraction was treated with DEAE-cellulose twice in the same manner.

Synthesis of the nucleotidyl protein and RNA in vitro. The synthesis of the nucleotidyl protein was carried out in a reaction mixture (40 μl containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH (pH 8.0), 3.5 mM magnesium acetate, 10 mM dithiothreitol, 50 mM KCl, 10 μg of actinomycin D (Calbiochem-Behring) per ml, 2.5 mM phosphoenol pyruvate (Calbiochem-Behring), 2 μg of pyruvate kinase (Boehringer Mannheim Biochemicals), 30 μCi of [α-^{32}P]UTP (3,000 Ci/mmol [1 Ci = 3.7×10^{10} Bq]; New Englannd Nuclear Corp.), and 40 μg of the crude or DEAE–cellulose-treated membrane fraction at 30°C for various periods.

In vitro RNA synthesis was performed in the same reaction mixture in the presence of 1 mM ATP, 1 mM GTP, and 1 mM CTP.

Fingerprinting analysis of RNase T_1-resistant oligonucleotides. The procedure used for fingerprinting analysis of RNase T_1-resistant oligonucleotides was similar to that described by Lee and Wimmer (24), except Tris-borate buffer was used for the second dimension of a two-dimensional gel electrophoresis (22, 26, 33). Purified [^{32}P]-labeled virion RNA or [α-^{32}P]UMP-labeled in vitro product RNA were extensively digested with 25 U of RNase T_1 (P-L Biochemicals, Inc.) in the presence of 10 μg of transfer RNA in 20 μl of buffer (10 mM Tris hydrochloride [pH 7.5], 2 mM EDTA) for 30 min at 37°C. A 30-μl portion of sample buffer (6 M urea, 50 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol FF) was added. The sample was incubated for 3 min at 50°C and mixed with 50 μl of melted (50°C) 1% agarose (Bethesda Research Laboratories, Inc.). The sample was immediately loaded onto a slab gel. Electrophoresis in the first dimension was carried out at 4°C in a polyacrylamide gel (8% [wt/vol] containing 6 M urea at pH 3.5 with citric acid) (80 by 270 by 1.3 mm) until the bromophenol blue dye migrated 15 cm at constant voltage (1,000 V). The gel strip containing radioactivity as visualized by autoradiography was cut out and embedded into a second slab gel. Electrophoresis in the second dimension was performed at room temperature in a polyacrylamide gel (22% [wt/vol] 50 mM Tris-borate [pH 8.3]) (375 by 450 by 1.0 mm) until the bromophenol blue dye reached 15 cm from the first-dimensional gel strip. The gel was exposed to Kodak SB-5 X-ray film with an intensifying screen at 4°C.

Immunoprecipitation of VPg-containing nucleotidyl proteins by anti-VPg antibody. Antibody raised against a synthetic peptide containing the carboxy-terminal seven amino acids of VPg was prepared as described previously (39). Nuclease-free anti-VPg rabbit immunoglobulin G (IgG) was obtained by passing the serum through a column of DEAE-Affi-gel Blue (Bio-Rad Laboratories) as described previously (18). The protein concentration of the IgG fraction was determined by the method of Lowry et al. (25) and was adjusted to 5 mg/ml.

Immunoprecipitation of [α-^{32}P]UMP-labeled VPg-pU, VPg-pUpU, and intermediate molecules (VPg containing oligonucleotide longer than VPg-pUpU but shorter than 5' T_1-oligonucleotide) synthesized in the isolated membrane fraction was performed as follows. The reaction mixture was diluted with a 10× volume of phosphate-buffered saline containing 1% Triton X-100, 0.5% Nonidet P-40, 0.5% sodium dodecyl sulfate (SDS), and 1% Trasylol (Calbiochem-Behring) and boiled for 3 min. Purified IgG was added to the solution, which was then incubated for 30 min on ice. Then 50 μl of a 10% suspension of *Staphylococcus aureus* cells (Calbiochem-Behring) was added, and this mixture was incubated on ice for an additional 10 min. After centrifugation, the immunoprecipitates were washed twice with the detergent containing phosphate-buffered saline and once with phosphate-buffered saline, suspended in 30 μl of gel sample buffer, boiled for 3 min, and separated from *S. aureus* cells by centrifugation. Samples were then subjected to electrophoresis on a 13.5% SDS-polyacrylamide gel (20).

To immunoprecipitate the 5' T_1-oligo, [α-^{32}P]UMP-labeled RNA was phenol extracted either from the reaction mixture after addition of SDS to 1% and EDTA to 5 mM or from the supernatant after the nucleotidyl protein-antibody-*S. aureus* complex had been sedimented by centrifugation. The RNA was digested in 20 μl of buffer (10 mM Tris hydrochloride [pH 7.5], 2 mM EDTA) with 25 U of RNase T_1 in the

presence of 10 μg of tRNA for 30 min at 37°C. The reaction mixture was diluted with the detergent containing phosphate-buffered saline and then immunoprecipitated as described above.

RESULTS

General properties of the in vitro system. The crude membrane fraction used in the experiments described in this paper was a preparation obtained by sedimenting the postnuclear supernatant of poliovirus-infected HeLa cells at 30,000 × *g* for 20 min. This membrane fraction contains endogenous RNA template molecules and promotes the synthesis of three species of virus-specific RNA. As can be observed by agarose gel electrophoresis (Fig. 1B; lane C), the in vitro RNA products are single-stranded genome-length RNA and double-stranded RNA (replicative form [RF]). The material migrating above the RF can be considered to be partially double-stranded replicative-intermediate (RI) RNA on the basis of its molecular weight and the fact that it disappears when the components are chased with unlabeled nucleoside triphosphates (data not shown). The in vitro synthesis of these products has also been reported previously (14, 27). Fingerprint analysis showed that most of the newly synthesized RNA molecules are plus strands (14; see below).

Isopycnic centrifugation of the crude replication complex in a stepwise sucrose gradient resulted in the fractionation of the membranous material (9, 13). It has been shown previously that the membranous material in each of the fractions contains virtually all the known poliovirus-specific polypeptides (44). When assayed for the ability to synthesize viral RNAs, the fraction containing smooth membranes was found to have the highest activity (Fig. 1, lane 1). Membrane fractions with higher densities (lanes 2 to 5) were also able to synthesize viral RNA, albeit to a lesser extent. (The absence of single-stranded RNA synthesis in the bottom fraction 5 of Fig. 1B cannot be explained at present.)

We previously reported that the crude replication complex contains an activity which can synthesize the nucleotidyl proteins VPg-pU and VPg-pUpU (43), two structures that represent the 5′ ends of both plus- and minus-strand RNA (32). Since poliovirus RNA synthesis occurs on membranes in vivo, we suggested that the formation of VPg-pUpU is a step in the initiation of poliovirus RNA replication and that initiation requires a membranous environment (43). This synthesis of VPg-pUpU in the crude replication complex is shown in Fig. 1A, lane C. When the individual fractions of the sucrose gradient were assayed, it was found that the extent of synthesis of VPg-pUpU was very similar to that of viral RNA (Fig. 1A, lanes 1 to 5). Again, synthesis of VPg-pUpU is highest in the smooth-membrane fraction (lane 1). We conclude that the formation of these 5′-terminal, virus-specific structures occurs in a similar environment as RNA synthesis and is not confined to a particular fraction of the crude membrane preparation. So far, we have made no attempts to further characterize the nature of the cellular membranous components banding in any of the five fractions.

Newly synthesized virus-specific RNA and the nucleotidyl proteins have been found to be tightly associated with cellular membrane fractions. Moreover, some viral proteins, including VPg-containing precursors, display properties characteristic of integral membrane proteins rather than of peripheral proteins, since the addition of high salt concentrations (e.g., 2 M NaCl) fails to release them (45; our own

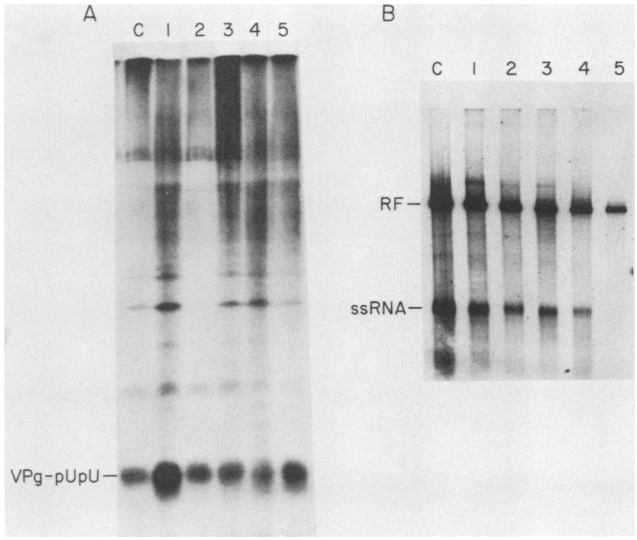

FIG. 1. RNA synthesis and VPg-pUpU formation in the crude replication and in membrane fractions after separation of the crude membrane complex on a discontinuous sucrose gradient (8, 13, 44). The crude membrane complex and partially purified membrane fractions, designated fractions 1 through 5, were incubated with [α-^{32}P]UTP and other precursors for the synthesis of VPg-pUpU (A) and RNA (B) as described in Materials and Methods, except that the reaction mixture used in panel A contained 1 mM ATP. None of the fractions were DEAE-cellulose treated or supplemented with an ATP regenerating system as described in later experiments. VPg-pUpU was immunoprecipitated with anti-VPg antiserum and analyzed by 13.5% SDS-polyacrylamide gel electrophoresis. The labeled in vitro RNA products were recovered by phenol-chloroform extraction and analyzed by 0.8% agarose gel electrophoresis. Unlabeled poliovirus RF and virion RNA (single-stranded RNA) were coelectrophoresed as markers and visualized by ethidium bromide staining prior to autoradiography. Lanes: C, crude membrane fraction; 1 to 5, fractions 1 to 5, smooth to rough endoplasmic reticulum, respectively. The protein content in each fraction was 70 (lane C), 2 (lane 1), 7 (lane 2), 12 (lane 3), 10 (lane 4), and 17 μg (lane 5), respectively.

unpublished data). In addition, treatment of the isolated membranes with trypsin results in the protection of specific regions of certain polypeptides, an observation suggesting a specific association between the membrane and polypeptides (39, 44, 45). Despite endogenous nuclease activities in the membrane preparation, the newly synthesized viral RNAs copied from the endogenous template are quite stable for several hours if the membrane complex remains unperturbed. However, treatment of the complex with RNase T_1 destroys the newly synthesized single-stranded RNAs, an observation indicating that viral RNA synthesis occurs on the outside of the membranous complexes and not in the luminus vesicles (data not shown).

A low concentration of nonionic detergent (0.1% Nonidet P-40) completely abolishes the VPg-pU- and VPg-pUpU-forming activity (43; see below). We have found that, concomitant with the loss of VPg-pUpU synthesis, the partially solubilized complex is also unable to synthesize RI RNA and to release single-stranded RNA from the RI (data not shown). This observation was also made by Etchison and Ehrenfeld (14).

Synthesis and detection of the 5′-terminal oligonucleotide VPg-pUUAAAACAGp. Our previous results with the crude membrane fraction suggested that the nucleotidyl proteins

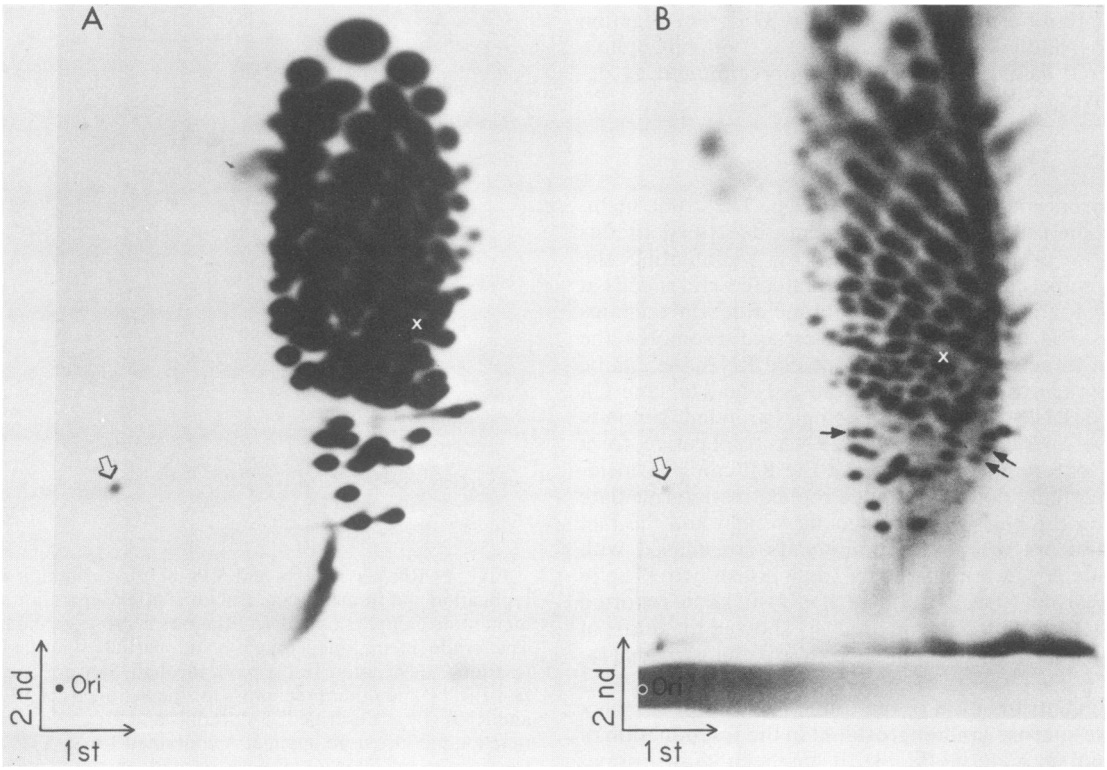

FIG. 2. Identification of the 5′ oligonucleotides (VPg-pUUAAAACAGp) in poliovirus RNA synthesized (A) in vivo and (B) in vitro. (A) A 2-μg amount of ^{32}P-labeled purified virion RNA (2×10^6 cpm) was treated with RNase T_1, and the digest was analyzed by two-dimensional RNA gel electrophoresis as described in Materials and Methods. (B) A 400-μg amount of the crude membrane fraction was incubated in a standard reaction mixture with 300 μCi of [α-^{32}P]UTP for 60 min at 30°C. RNA contained in the complex was extracted with phenol-chloroform, digested with RNase T_1, and fingerprinted as described for panel A. The origin (Ori) is at the bottom left. The VPg-containing RNase T_1-resistant oligonucleotide (VPg-pUUAAAACAGp) migrates slowly in both dimensions, as indicated by the open arrows. The closed arrows indicate oligonucleotides that are likely to have originated from minus-stranded RNA. The positions of the bromophenol blue dye marker are indicated by crosses.

VPg-pU and VPg-pUpU are part of the initiation complex of plus-strand RNA synthesis (43). We were unable, however, to show that preformed VPg-pUpU can be elongated. Our assay for initiation and elongation reported here is the formation of ^{32}P-labeled 5′-terminal VPg-pUUAAAACAGp, an oligonucleotide that would be produced after labeling with [α-^{32}P]UTP and digestion of the products with RNase T_1.

Experiments designed to synthesize full-length viral RNA were performed with the replication complex that had been supplemented with an ATP regenerating system (see below). Agarose gel electrophoresis (Fig. 1B) showed that the predominant products of this reaction were authentic RI, RF, and single-stranded RNA. The entire RNA synthesized in vitro was analyzed by fingerprinting under conditions that would allow the detection of the 5′-terminal RNase T_1-resistant oligonucleotide. In a control experiment, a fingerprint was prepared first with ^{32}P-labeled virion RNA (22), in which the 5′ oligonucleotide was identified as an aberrantly migrating spot (Fig. 2A, open arrow). The slow migration of the 5′ oligonucleotide, particularly in the first dimension, is presumably due to the positively charged VPg, since the same oligonucleotide lacking VPg (pUUAAAACAGp) migrates in the upper right portion of a similar fingerprint (32). The identity of the marked spot in Fig. 2A was proven by sequence analysis after proteinase K digestion and labeling at the 3′ end with [5′-^{32}P]pCp (11, 21). The results (not shown) confirmed that the material was the expected 5′-terminal oligonucleotide. When in vitro-synthesized [α-^{32}P]UMP-labeled RNA was isolated and analyzed in the same fashion, the 5′-terminal oligonucleotide was also observed (Fig. 2B, open arrow). Treatment of the in vitro-synthesized RNA with proteinase K prior to fingerprint analysis abolished this spot (not shown).

The data shown in Fig. 2B document for the first time de novo synthesis of VPg-linked poliovirus plus-strand RNA in vitro. Since the label in the 5′-terminal oligonucleotide originates from UTP precursors, we conclude that the crude membrane system can initiate RNA synthesis de novo. It is therefore likely that the mechanism of initiation functioning in this case is the same as that functioning in vivo. The fingerprint shown in Fig. 2B also confirms that the RNA synthesized by the membranous replication complex is of plus-strand polarity (14). Careful analysis of the pattern, however, revealed three distinct spots (Fig. 2B, closed arrows) that, by comparison with fingerprints of in vivo-labeled minus-stranded poliovirus RNA (our own unpublished results), are diagnostic for poliovirus minus-stranded RNA. It is not unlikely that, under the conditions described here, the crude replication complex is also synthesizing minus strands.

Rabbit antisera prepared from VPg-specific oligopeptides (39, 43, 44) were then used to assay for the formation of the 5′ oligonucleotide. These rabbit antisera will precipitate VPg and VPg-containing polypeptides, particularly 3AB (formerly called P3-9), a membrane-bound polypeptide thought

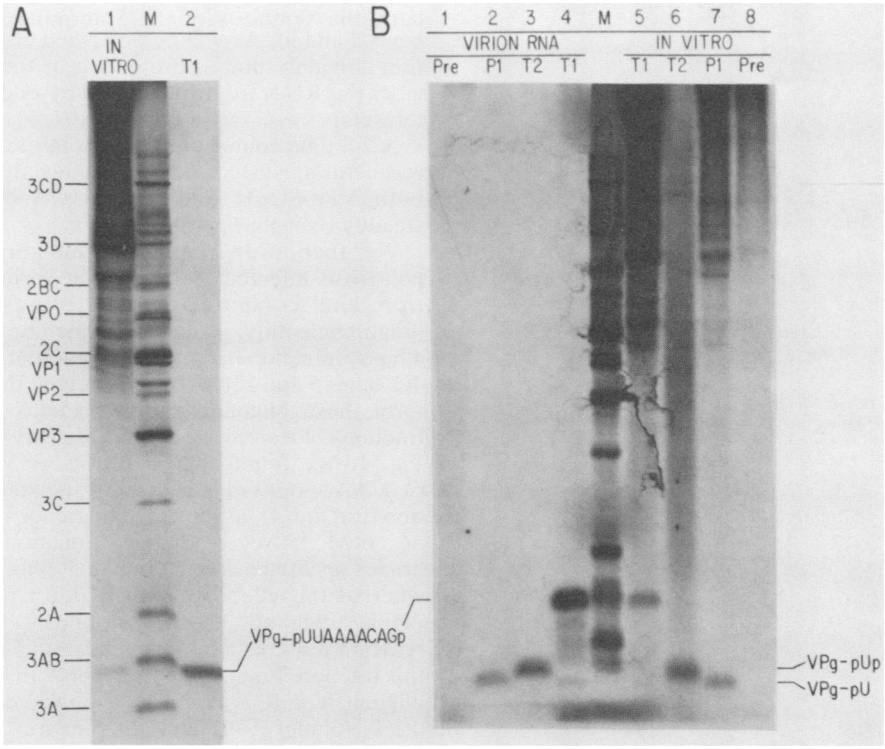

FIG. 3. SDS-polyacrylamide gel electrophoresis of immunoprecipitates of oligonucleotidyl-VPg and nucleotidyl-VPg with anti-VPg antibodies. (A) Detection of the 5′-terminal RNase T_1-resistant oligonucleotide of in vitro-synthesized RNA (lane 1) in comparison with the same VPg-linked oligonucleotide isolated from ^{32}P-labeled virion RNA (lane 2); lane M, [^{35}S]methionine-labeled proteins in a lysate of poliovirus-infected HeLa cells. (B) Analysis of the VPg-containing RNA fragments after digestion of in vivo- and in vitro-synthesized RNAs with RNase T_1, RNase T_2, or nuclease P1. ^{32}P-labeled virion RNA (7×10^6 cpm) (lanes 1 to 4) or [α-^{32}P]UMP RNA extracted from a reaction mixture containing 40 μg of the crude membrane fraction (lanes 5 to 8) was digested with nucleases in the presence of 10 μg of tRNA, and the products were incubated with preimmune serum (lanes 1 and 8) or anti-VPg immune serum (lanes 2 to 7). Lanes: 1 and 8, RNAs were treated with 25 U of RNase T_1 in 10 mM Tris hydrochloride (pH 7.5)–2 mM EDTA for 30 min at 37°C; 2 and 7, RNAs were treated with 6 U (10 μg) of nuclease P1 (Pharmacia) in 50 mM sodium acetate (pH 6.0) for 60 min at 37°C and immunoprecipitated with anti-VPg rabbit IgG; 3 and 6, RNAs were treated with 5 U of RNase T_2 (Pharmacia) in 50 mM ammonium-acetate (pH 4.5)–1 mM EDTA for 60 min at 37°C and immunoprecipitated with anti-VPg rabbit IgG; 4 and 5, same treatment as lane 1, but anti-VPg rabbit IgG was used for immunoprecipitation; M, as for panel A.

to be a precursor to VPg (3AB → 3A + VPg) (39, 43, 44). An analysis of the 5′ end of in vivo- and in vitro-synthesized poliovirus RNAs by immunoprecipitation of nuclease digests is shown in Fig. 3. The 5′ oligonucleotide of virion RNA (Fig. 3A, lane 2) migrates slightly faster than polypeptide 3AB (lane M). A band comigrating with the virion RNA-derived 5′ oligonucleotide can be seen also in an RNase T_1 digest of in vitro-labeled RNA (lane 1) and is thus the 5′ end of newly synthesized RNA. Note that a considerable amount of labeled material appears in the upper portion of lane 1. We believe that this material represents RNA fragments that nonspecifically coprecipitated from the bulk of the RNase digest with the antibodies. This conclusion is supported by the following considerations: (i) treatment with either nuclease P1 or RNase T_2 prior to immunoprecipitation greatly reduced the amount of these bands (Fig. 3B, lanes 6 and 7); (ii) the bands were absent when the replication complex was incubated only with [α-^{32}P]UTP (see Fig. 9); (iii) the bands could not be immunoprecipitated again after they were eluted from the gel (data not shown); (iv) material similar in size was immunoprecipitated also by preimmune serum (Fig. 3B, lane 8); (v) the appearance of the bands was not reproducible.

To prove that both uridine phosphates adjacent to VPg in the 5′ oligonucleotide of in vitro RNA were labeled, we performed a series of nuclease digestions of phenol-extracted RNA. This was followed by immunoprecipitation with anti-VPg sera and analysis of the precipitates by SDS-polyacrylamide gel electrophoresis (Fig. 3B). As a control, we treated virion RNA, labeled with ^{32}P-phosphate in vivo, with nuclease P1, RNase T_2, or RNase T_1 and obtained VPg-pU, VPg-pUp, or VPg-pUUAAAACAGp, respectively (Fig. 3B, lanes 2 to 4) (31, 32). The same products were found with RNA labeled in vitro with [α-^{32}P]UTP (Fig. 3B, lanes 5 to 7). In this case, the intensity of the bands corresponding to the 5′ oligonucleotide (lane 5) and to VPg-pUp (lane 6) was, as expected, roughly the same, whereas that of VPg-pU (lane 7) was decreased. We conclude that the uridylylation of VPg in the crude membrane complex must have occurred de novo.

Synthesis of the 5′ oligonucleotide in comparison with VPg-pUpU. To gain insight into the relationship between the synthesis of the 5′ oligonucleotide and VPg-pUpU, we compared different parameters of their formation. Initially, the yield of VPg-pUpU from incubations of the crude membrane fraction was low and synthesis of the 5′ oligonucleotide was not observed (43). We found, however, that the addition of an ATP regenerating system remarkably stimu-

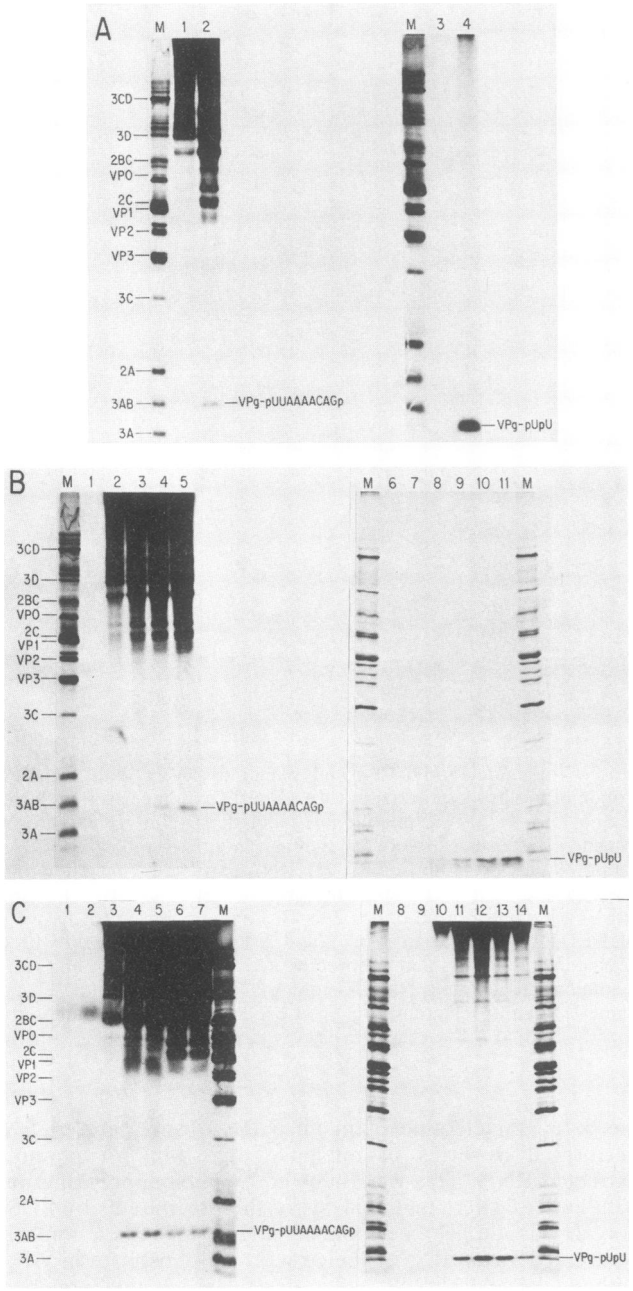

FIG. 4. Parameters of in vitro synthesis of the 5′ oligonucleotide and of VPg-pUpU in membranous replication complexes. (A) Stimulation of VPg-pUUAAAACAGp and VPg-pUpU synthesis by pyruvate kinase and phosphoenol pyruvate (lanes 2 and 4, respectively) compared with identical reaction conditions, but without the ATP generating system (lanes 1 and 3). For conditions of synthesis and immunoprecipitations, see Materials and Methods. Lane M: see Fig. 3A. (B) Time course of 5′ oligonucleotide and VPg-pUpU synthesis by the crude replication complex. Lanes: 1 to 5, synthesis of VPg-pUUAAAACAGp, recovered from [α-^{32}P]UMP-labeled RNA by immunoprecipitation, after an incubation of the replication complex for 0, 30, 60, 90, and 120 min, respectively; 6 to 11, synthesis of VPg-pUpU from the same reaction mixture after an incubation of 0, 30, 60, 90, 120, and 180 min, respectively; M, see Fig. 3A. (C) Activity of the crude replication complex isolated from poliovirus-infected HeLa cells at 0, 1, 2, 3, 4, 5, and 6 h postinfection (lanes 1 to 7 and 8 to 14, respectively). The replication complex was incubated for 2 h at 30°C in the presence of an ATP generating system, and the 5′ oligonucleotide and VPg-pUpU were isolated by immunoprecipitation as described in Materials and Methods. Lane M: see Fig. 3A.

lated the synthesis of the 5′-terminal structures (Fig. 4A, lanes 2 and 4). As will be published elsewhere, this stimulation is mainly due to protection of the [α-^{32}P]UTP used to label the RNA from hydrolysis by endogenous phosphoesterase(s).

A 2-h time course of synthesis in the presence of the ATP regenerating system (Fig. 4B) showed that the formation of both VPg-pUpU and VPg-pUUAAAACAGp increased steadily over that period.

We then prepared crude membrane fractions from poliovirus-infected HeLa cells at various times after infection and compared the activities that formed the (oligo)nucleotidyl protein. The membrane fraction prepared 4 h postinfection had the highest activity in both assays (Fig. 4C, lanes 5 and 12). We then assayed the activity required to form these oligonucleotidyl proteins in each of the five fractions of a stepwise sucrose density gradient described in Fig. 1. We found that synthesis of VPg-pUpU and VPg-pUUAAAACAGp is highest in the smooth-membrane fraction (fraction 1), as expected (data not shown). It should also be noted, however, that fractionation of the crude membranes resulted in a reduction of 5′ oligonucleotide systhesis, whereas this effect was not seen for the synthesis of VPg-pUpU (data not shown). It appears that the elongation reaction requires a factor present only in small quantities in this fraction. Finally, synthesis of both VPg-pUpU and the 5′ oligonucleotide is completely abolished in the presence of 0.1% Nonidet P-40 (data not shown).

Pulse-chase experiments with VPg-pUpU. The characteristics of VPg-linked nucleotide and 5′ oligonucleotide synthesis may suggest that the formation of the 5′ oligonucleotide is coupled to the synthesis of VPg-pUpU, e.g., that VPg-pUpU is the primer for virus-specific RNA synthesis. We therefore attempted to chase preformed VPg-pUpU into heteropolymeric RNA strands. However, the low yields of the VPg-linked RNA and a high degree of variability in yield, particularly of the 5′ oligonucleotide, made pulse-chase experiments impossible. Moreover, in the presence of an ATP generating system, synthesis of some 5′ oligonucleotide in the crude membrane fraction was occasionally seen even if only [α-^{32}P]UTP was added, an observation suggesting the presence of endogeneous nucleoside triphosphate pools. These obstacles were overcome when the membrane fraction was incubated with granular DEAE-cellulose. Following the removal of the ion exchanger, RNA synthesis was carried out in the presence of exogeneously added nucleoside triphosphates and the ATP regenerating system.

The assay for the nucleotidyl protein products was carried out as follows. After incubation, the membranous material was solubilized with detergent, and VPg-pUpU was precipitated with anti-VPg antibodies and protein A. The supernatant from that immunoprecipitation was extracted with phenol; RNA was recovered by ethanol precipitation and digested with RNase T_1, and the 5′ oligonucleotide was immunoprecipitated with anti-VPg antibodies and protein A (for details, see Materials and Methods). It should be stressed that our anti-VPg antibodies do not precipitate VPg-linked polynucleotides under the immunoprecipitation conditions that we used. Thus, this procedure allows the differentiation between VPg-pUpU synthesis and VPg-linked RNA synthesis in the same reaction sample.

Figure 5A and B show the synthesis of the 5′ oligonucleotide and VPg-pUpU, respectively; Fig. 5C is a composite of the two upper panels. The membrane complex shows active synthesis of VPg-pUpU (Fig. 5B, lane Pre).

If the preformed VPg-pUpU was chased overnight (12 h) with an excess of unlabeled UTP, material migrating between VPg-pUpU and the 5′ oligonucleotide appeared (Fig. 5B, lane 2), but very little, if any, 5′ oligonucleotide was found (Fig. 5A, lane 2). We have not sequenced the material migrating slightly slower than VPg-pUpU, but its formation required more than 2 h (see below). It is VPg linked because it was precipitated with anti-VPg antisera but not with preimmune serum. It was also formed if UTP and ATP are added (Fig. 5A, lane 3). The nature of these lower-migrating species is unknown. Surprisingly, the 5′ oligonucleotide was formed in the presence of UTP, ATP, and CTP, but without the addition of GTP (Fig. 5A, lane 4). The synthesis of the 5′ oligonucleotide in the absence of added GTP can only be explained if one assumes that the treatment of the replication complex did not sufficiently remove GTP or its precursors. Nevertheless, synthesis of the 5′ oligonucleotide was enhanced if all four nucleoside triphosphates are added to the chase mixture (Fig. 5A, lane 5). As the yield of the 5′ oligonucleotide increased, the amount of preformed VPg-pUpU decreased. Indeed, all radioactivity of the preformed VPg-pUpU can be accounted for in products formed during the chase (Table 1). This observation suggests a precursor-product relationship, although other possibilities for the 5′ oligonucleotide synthesis cannot be excluded (see Discussion).

A time course for the formation of the 5′ oligonucleotide and other products from the [α-^{32}P]UTP-prelabeled replication complex showed that the products of the elongation reaction were evident after 1 h (data not shown). However, the products migrating between VPg-pUpU and the 5′ oligonucleotide were formed much more slowly than the 5′ oligonucleotide. This observation suggests that these products are not intermediates in the synthesis of the 5′ oligonucleotide.

Treatment of the replication complex with DEAE-cellulose could have led to the reduction of components involved in the formation of the VPg-linked nucleotides. Indeed, when the DEAE-treated complex was supplemented with a small amount of a cell extract of uninfected HeLa cells (S-10), the yield of the 5′ oligonucleotide increased twofold (Fig. 6, lane 3), whereas the synthesis of VPg-pUpU remained unaffected (Fig. 6, lane 2). This stimulating effect of the S-10, although small, can be seen very reproducibly; it is not further enhanced if an S-10 of infected cells is used (not shown).

Is VPg-pU the precursor to VPg-pUpU? Incubation of the crude membrane fraction, treated twice with DEAE-cellulose to remove endogenous nucleoside triphosphates and with [α-^{32}P]UTP for 30 min at 30°C yielded VPg-pU and VPg-pUpU at a ratio of approximately 1:1 (Fig. 7, lane 1). Subsequent addition of an excess of unlabeled UTP to the preformed products and incubation of the complex for 30, 60, or 180 min (lanes 2, 3, and 4, respectively) resulted in the loss of VPg-pU. The disappearance of VPg-pU was accompanied by an increase of radioactivity in VPg-pUpU (as determined by densitometer tracing of the autoradiogram; see legend to Fig. 6), i.e., the overall radioactivity in each of the lanes remained roughly constant. This was expected, since unlabeled UTP was used in the chase. These data are consistent with a precursor-product relationship between VPg-pU and VPg-pUpU and support a mechanism by which

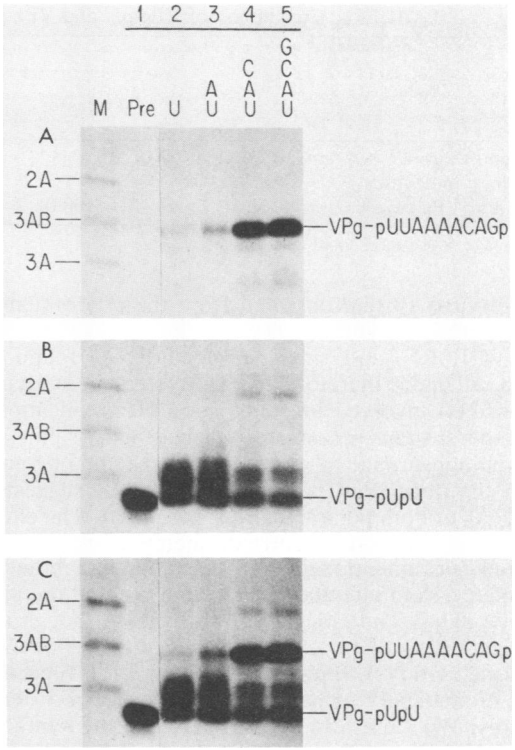

FIG. 5. Formation of VPg-pUUAAAACAGp from VPg-pUpU in a pulse-chase experiment. VPg-pUpU was synthesized with 40 μg of DEAE-cellulose-treated replication complex in 40 μl of a reaction mixture containing 30 μCi of [α-^{32}P]UTP for 120 min at 30°C. The reaction mixture was then incubated for 12 h at 30°C in the presence of cold nucleoside triphosphates. VPg-pUpU, the products of intermediate size migrating between VPg-pUpU, and the 5′ oligonucleotide were immunoprecipitated from the reaction mixture. RNA remaining in the supernatant was recovered by phenol-chloroform extraction and digested with RNase T_1. The 5′ oligonucleotide was subsequently isolated by immunoprecipitation. Immunoprecipitates were analyzed in 13.5% SDS-polyacrylamide gels. Panels A and B show the results of immunoprecipitation of VPg-pUUAAAACAGp and VPg-pUpU, respectively. Panel C was made by overlaying panels A and B. Note that only the lower portions of the autoradiograms are shown. The chase was performed in the presence of 10 mM UTP (lane 2), 10 mM UTP, and 1 mM ATP (lane 3); 10 mM UTP, 1 mM ATP, and 1 mM CTP (lane 3); 10 mM UTP, 1mM ATP, 1 mM CTP, and 1 mM GTP (lane 4). Lane 1: prechase sample. Lane M: see Fig. 3A.

VPg-pU is uridylylated to yield VPg-pUpU. Note that in this experiment very little radioactive material appeared in the upper portion of the autoradiogram. This we consistently observe when a more extensive treatment of the complex with DEAE-cellulose is performed prior to incubations.

DISCUSSION

The ability of a membrane-bound replication complex to synthesize 3′-polyadenylated poliovirus RNA was demonstrated several years ago (15, 51). The data presented in this paper provide the first evidence that this complex allows de novo initiation of RNA synthesis in vitro and that the 5′ ends of the newly synthesized RNA strands are identical to the VPg-linked terminus of poliovirus RNA. An important question has therefore been answered: this in vitro system is capable of initiating the synthesis of authentic virion RNA.

TABLE 1. Conversion of VPg-pU and VPg-pUpU to the VPg-containing molecules longer than VPg-pUpU

Product	Radioactivity (cpm) found in product[a]				
	Prechase	Chased in the presence of:			
		U	U, A	U, A, C	U, A, C, G
5′ T_1-oligo (VPg-pUUAAAACAGp)	0	28 (3.9)	64 (8.8)	276 (35.5)	312 (42.4)
Intermediate molecules	0	418 (57.9)	430 (59.2)	272 (35.0)	230 (31.3)
VPg-pU and VPg-pUpU	721 (100.0)	276 (38.2)	232 (32.0)	230 (29.5)	194 (26.3)

[a] Percentage of the total count.

The in vitro system obtained from the crude membrane fraction faithfully synthesizes all of the virus-induced RNA species (RI, RF, and single-stranded RNA) found in an infected cell and is therefore an ideal system for studying the mechanism(s) involved in poliovirus RNA replication. Although the system appears to produce mainly plus-strand RNA, the appearance of minus strand-specific oligonucleotides in the fingerprint presented in Fig. 2B suggests that viral RNAs of both polarities are synthesized. The extent of minus-strand synthesis is currently being investigated.

A number of manipulations of the crude membrane complex isolated from infected cells have increased the yield of both VPg-pUpU and elongation products. Although a variety of inhibitors of phosphodiesterase had no effect (our unpublished results), the inclusion of an ATP regenerating system dramatically enhanced the synthesis of 5′-terminal structures. We were also able to separate the synthesis of VPg-pUpU from the formation of the 5′ oligonucleotide by treating the complex with DEAE-cellulose. It appears that the treatment with DEAE-cellulose, and possibly also the fractionation on a sucrose gradient, of the crude membrane complex resulted in the partial depletion of a host factor required for the formation of the 5′ oligonucleotide. In any event, the stimulation of the elongation of VPg-pUpU to the 5′ oligonucleotide reproducibly observed with an S-10 extract of uninfected HeLa cells will be the basis for the search of host cellular factors(s). Whether or not the host factor previously described (28) is involved in any of the reactions described here remains to be seen. It should be pointed out that Morrow et al. (28) used a partially purified system void of membranous components; the RNAs they synthesized are of minus-strand polarity only and the newly synthesized RNA is not linked to the naturally occurring VPg. Thus, their data cannot be compared with ours at this time. The same argument holds for experiments with another host factor (terminal uridylylate transferase) reported recently by Andrews and colleagues (2, 3).

The pulse-chase experiment shown in Fig. 5 suggests, but does not prove, that preformed VPg-pUpU is the precursor to elongation. Two major obstacles prevent us from drawing firmer conclusions. First, the kinetics of appearance of the components of intermediate size suggest that these nucleotidyl peptides are not intermediates in elongation. Their identity remains to be solved. Perhaps they are the result of nuclease activity. Second, although the treatment of the replication complex with DEAE-cellulose prior to incubation with [α-^{32}P]UTP greatly reduced the background of labeled material that could be immunoprecipitated with anti-VPg antibodies (Fig. 7), other larger RNA molecules are labeled under these conditions (but do not immunoprecipitate). These larger molecules could serve as precursors for the 5′ oligonucleotide. This problem could be solved if it were possible to remove endogeneous viral template RNA and replace it by exogenously added plus- or minus-stranded RNA. However, we have found that the template RNA within the replication complex is unusually resistant to degradation with micrococcal nuclease (unpublished results). Moreover, the ability to synthesize VPg-pUpU remains unchanged if the complex is extracted with 2 M KCl prior to incubation (unpublished results). Since nearly all viral proteins are found in the membranous replication complex (44), the nature and significance of specific interac-

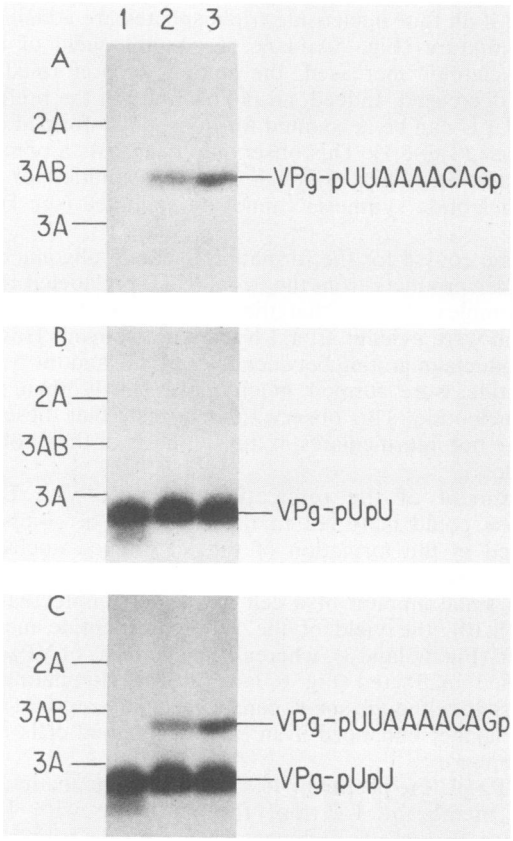

FIG. 6. Effect of a soluble fraction from uninfected HeLa cells (S-10) on the formation of the 5′ oligonucleotide. [α-^{32}P]UMP-labeled VPg-pUpU was synthesized in a 40-μl reaction mixture containing 30 μCi of [α-^{32}P]UTP and 40 μg of crude membrane complex that was treated twice with DEAE-cellulose. The samples in lanes 2 and 3 were further incubated under chase conditions for 1 h at 30°C. VPg-pUpU and VPg-pUUAAAACAGp were recovered as described in the legend to Fig. 5. Lanes: 1, prechase sample. 2, incubation in the presence of 10 mM UTP, 1 mM ATP, 1 mM CTP, and 1 mM GTP in a total volume of 60 μl; 3, as in lane 2, except that 5 μl of an S-10 fraction prepared from uninfected HeLa cells (optical density per ml at 260 nm, 100) was included. Densitometer tracings indicated that the yield of the 5′ oligonucleotide in lane 3 was 1.9 times that seen in lane 2.

tions between template RNA and various viral gene products are difficult to assess.

Incubation of the replication complex with UTP leads to synthesis of VPg-pU and VPg-pUpU in which all of the former can be chased into the latter (Fig. 7). The mechanism of the uridylylation reaction of VPg remains obscure. According to a more recent model, linking of VPg to the 5′ end of newly formed RNA may be the result of cleavage of an oligo(U)-loop at the 3′ end of template RNA (2, 3, 52). This model suggests that a terminal uridylylate transferase activity can participate in poliovirus RNA synthesis in vitro by adding a few U residues to the 3′ end of the template RNA. The newly synthesized oligo(U) tail is thought to snap back and form a hairpin, thus providing a free 3′ hydroxyl group that can function as a primer for the polymerase 3D. The product of such a reaction in vitro is double-stranded RNA covalently linked at one end. Indeed, covalently linked RF molecules have been observed in cells infected with poliovirus or encephalomyocarditis virus (36, 41, 52).

There is no evidence that links the hairpin model derived from in vitro studies to poliovirus RNA replication in vivo. The model predicts that minus strands of RI molecules should carry additional 3′-terminal U residues. This has not been studied; instead, the nucleotide sequences of the heteropolymeric ends of RF molecules have been found to

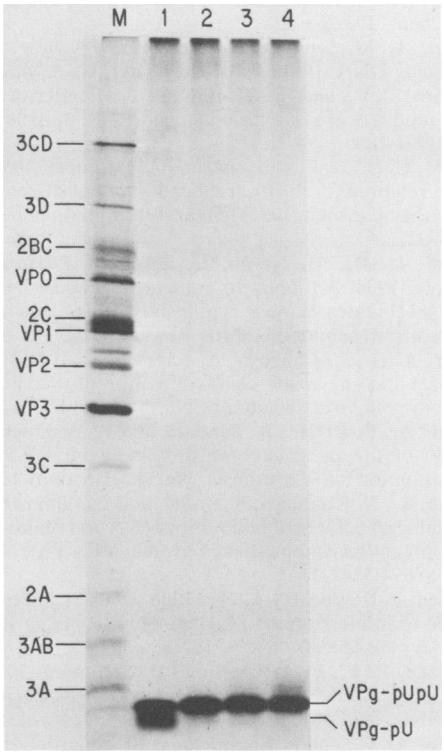

FIG. 7. Pulse-chase analysis in the formation of VPg-pUpU from VPg-pU. [α-^{32}P]UMP-labeled VPg-pU and VPg-pUpU were formed in 40 μl of a reaction mixture containing 30 μCi of [α-^{32}P]UTP and 40 μg of crude membrane complex that was treated twice with DEAE-cellulose. Incubation was for 30 min at 30°C. The chase experiment was carried out in the presence of 10 mM cold UTP for 0, 30, 60, and 180 min (lanes 1 to 4) at 30°C. VPg-pU and VPg-pUpU were recovered from the reaction mixture by immunoprecipitation and analyzed by 13.5% SDS-polyacrylamide gel electrophoresis. The relative ratios (determined by densitometer tracing) of the bands were, for lane 1 (VPg-pU and VPg-pUpU combined), 1.0; for lane 2, 1.05; for lane 3, 0.93; for lane 4, 0.93. Lane M: see Fig. 3A.

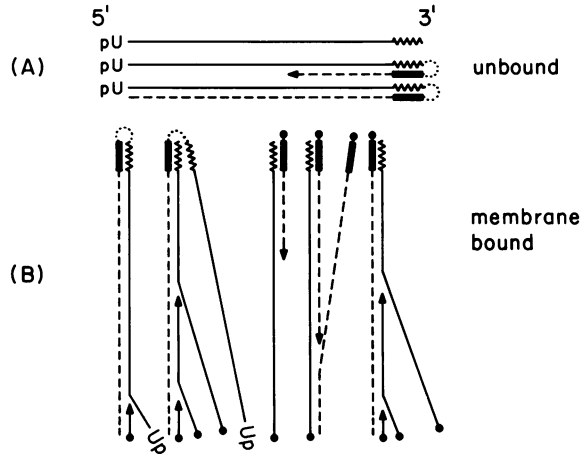

FIG. 8. Model of RNA replication in poliovirus-infected cells. (A) Very early in infection, viral mRNA may be modified at the 3′ end by a terminal uridylyl transferase (3) that leads to the priming of viral RNA polymerase 3D via a hairpin. The resulting RF molecules will be homolinked (double-stranded RNA linked at the poly(A)-poly(U) homopolymeric duplex). Attachment of virus-encoded protein(s) may transport this molecule to the membrane. Although proposed here to occur early in infection, homo-linked RF molecules may be formed to some extent throughout the infectious cycle. (B) Initiation of VPg-pUpU-primed synthesis of plus strands of homo-linked RF molecules followed by strand replacement during the elongation reaction. Termination of transcription of the minus strands occurs at the end of the homopolymeric region should readthrough occur, the resulting structures would be twice the length of genome RNA and would consist of inverted repeats of two end-to-end-linked RF molecules. Such structures have been found in picornavirus-infected cells (41). Minus-strand synthesis in the membrane-bound complex commences by a VPg-pUpU-primed reaction. Note that this model does not require a nicking-linking activity. It also predicts that hetero-linked RF molecules may not be formed. It should be stressed that the majority of RF molecules found in HeLa cells are unlinked at either end; instead, the 5′ termini of their RNA strands are VPg linked (31).

be mostly blunt ended (21). Only a small fraction of the minus-stranded RNA in RF carries extra A, but not U residues (21, 35). Moreover, it is difficult to explain how VPg-pU can be formed by a snap-back model because the loop to be cleaved by VPg (or its precursor polypeptides) would be stabilized only by a single U · A base pair. Nevertheless, the possibility of an aberrant nicking-linking reaction to yield VPg-pU cannot be excluded until the activity responsible for uridylylation has been found. If such aberrant nicking-linking occurred, however, it would be difficult to explain how the preformed VPg-pU could be chased to VPg-pUpU (Fig. 7).

A model of poliovirus RNA replication that involves hairpin priming as well as VPg-pU priming, but without the need for a nicking-linking activity, is shown in Fig. 8. Formation of homo-linked RF molecules may occur in the cytoplasm, and this could be the mechanism of early minus-strand RNA synthesis (Fig. 8A). Proteins synthesized prior to the formation of this structure may position the homo-linked RF in membranes. Initiation of plus-strand RNA synthesis then occurs by VPg-pU(pU) priming (see reference 48 and references therein), as will all subsequent synthesis of minus strands. Should read-through occur in transcription reactions of homo-linked RF molecules (e.g., if polymerase 3D fails to terminate transcription at the hairpin), RF molecules twice the length of the genome would be formed. Such

linear RF dimers have been observed in cells infected with another picornavirus, encephalomyocarditis virus (41). The model predicts that no hetero-linked RF molecules occur in the infected cell. It also predicts that the majority of RNA replication does not involve cellular factors that contain a terminal uridylylate transferase activity. Clearly, these predictions can be tested.

ACKNOWLEDGMENTS

We are grateful to Aniko Paul, Sylvie van der Werf, Haruka Toyoda, Martin Nicklin, and Steven Pincus for stimulating discussions and to Lynn Zawacki for preparation of the manuscript. We thank Bert L. Semler for suggestions and for the critique of the manuscript.

This work was supported by Public Health Service grants AI 15122 and CA 28146 from the National Institutes of Health. R.J.K. is a member of the Molecular Biology Graduate Training Program of the State University of New York at Stony Brook.

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