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Replication of Poliovirus RNA and Subgenomic RNA Transcripts in Transfected Cells

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Full-length and subgenomic poliovirus RNAs were transcribed in vitro and transfected into HeLa cells to study viral RNA replication in vivo. RNAs with deletion mutations were analyzed for the ability to replicate in either the absence or the presence of helper RNA by using a cotransfection procedure and Northern (RNA) blot analysis. An advantage of this approach was that viral RNA replication and genetic complementation could be characterized without first isolating conditional-lethal mutants. A subgenomic RNA with a large in-frame deletion in the capsid coding region (P1) replicated more efficiently than full-length viral RNA transcripts. In cotransfection experiments, both the full-length and subgenomic RNAs replicated at slightly reduced levels and appeared to interfere with each other's replication. In contrast, a subgenomic RNA with a similarly sized out-of-frame deletion in P1 did not replicate in transfected cells, either alone or in the presence of helper RNA. Similar results were observed with an RNA transcript containing a large in-frame deletion spanning the P1, P2, and P3 coding regions. A mutant RNA with an in-frame deletion in the P1-2A coding sequence was self-replicating but at a significantly reduced level. The replication of this RNA was fully complemented after cotransfection with a helper RNA that provided 2A in *trans*. A P1-2A-2B in-frame deletion, however, totally blocked RNA replication and was not complemented. Control experiments showed that all of the expected viral proteins were both synthesized and processed when the RNA transcripts were translated in vitro. Thus, our results indicated that 2A was a *trans*-acting protein and that 2B and perhaps other viral proteins were *cis* acting during poliovirus RNA replication in vivo. Our data support a model for poliovirus RNA replication which directly links the translation of a molecule of plus-strand RNA with the formation of a replication complex for minus-strand RNA synthesis.

Poliovirus is a small RNA virus which is a member of the *Picornaviridae* family. It contains a single-stranded polyadenylated RNA genome of positive polarity approximately 7,500 nucleotides long. A small viral protein, Vpg, is covalently linked to the 5′ end of virion RNA and all newly synthesized viral RNA (10, 17, 22). Virion RNA contains a large 5′-terminal noncoding sequence (742 nucleotides) followed by a large open reading frame which encodes the viral polyprotein. This polyprotein includes three domains (P1, P2, and P3) and is cleaved by three viral proteases ($2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$) to form the viral proteins found in infected cells. The 3′ end of virion RNA contains a noncoding sequence (71 nucleotides) and a heterogeneous poly(A) sequence that is required for the infectivity of virion RNA (28) and RNA transcribed in vitro from plasmid DNA (26).

Understanding the molecular mechanisms involved in the replication of poliovirus and other plus-stranded RNA viruses remains of fundamental importance. Although many aspects of poliovirus RNA replication have been studied in detail, little is known about the RNA sequences and structures required for replication and only limited information is available regarding the specific roles of the viral proteins during replication. Previous studies have shown that poliovirus defective interfering particles contain in-frame deletions in the capsid coding region (P1) of the genome (7, 12) and that they replicate their RNA efficiently, either alone or in the presence of wild-type virus. The isolation of an infectious cDNA clone (23) made it possible to generate, isolate, and characterize specific poliovirus mutants. Various (temperature-sensitive, host range, and plaque size) conditional mutants have been generated by using this method. Reports from a number of laboratories suggest that most nonstructural proteins, as well as the 5′ and 3′ noncoding sequences, play a direct role in viral RNA replication in vivo (1–3, 6, 11, 13, 18, 24, 25, 27).

It has been demonstrated that infectious viral RNA transcripts can be synthesized in vitro and that the specific infectivity of the synthesized RNA is within an order of magnitude of the infectivity of poliovirion RNA (26, 30). RNA transcripts with poly(A) sequences that are similar in length to virion RNA also have infectivities equivalent to that of virion RNA (26). By preparing mutant RNAs with in-frame deletions in the P1 region of the genome, Kaplan and Racaniello (14) showed that most of the sequence that encodes the capsid proteins was not required for RNA replication or translation in cultured cells. Hagino-Yamagishi and Nomoto (12) found that mutant RNAs with in-frame deletions of limited size in the P1 region would replicate and would form defective interfering particles in the presence of a helper virus. In contrast, defective interfering particles were not formed with RNAs that contained an out-of-frame deletion, presumably because this blocked RNA replication.

In this study, we evaluated the replication of mutant poliovirus RNAs in the presence and absence of helper RNA by transfecting or cotransfecting mutant and helper RNAs into cultured cells. An RNA transcript with an in-frame deletion in the P1 coding region replicated efficiently, both alone and in the presence of helper RNA. An RNA which contained an out-of-frame deletion in P1 did not replicate

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and was not complemented by helper RNA. Our results showed that poliovirus RNA with a P1-2A in-frame deletion replicated at a significantly reduced level and was fully complemented in the presence of helper RNA. In contrast, RNAs with deletions that extended into the 2B coding region and beyond did not replicate and were not complemented in *trans* with helper RNA. Translation of these RNAs in vitro confirmed that the predicted proteins encoded by these deletion constructs were expressed. Thus, the transfection procedure described in this report can be used to characterize the unique genetic strategies utilized by poliovirus in replicating its genomic RNA.

MATERIALS AND METHODS

Cells and virus. Suspension cultures of HeLa S3 cells were maintained at 2×10^5 to 4×10^5 cells per ml in Joklik's modified Eagle medium (ICN Flow, Costa Mesa, Calif.) supplemented with 5% calf serum and 2% fetal calf serum. Cells were infected with poliovirus type 1 (Mahoney strain) as previously described (31).

DNA manipulation and mutant construction. pT7D-polio DNA was generously supplied by Peter Sarnow (University of Colorado, Denver) and was used as the parental clone for all of the mutants constructed (26). Digestion of plasmid DNA with restriction enzymes, filling of recessed 3′ ends or polishing of 3′ extensions by using Klenow polymerase, and ligation reactions were all done in accordance with the specification sheets provided by the manufacturers. *Nru*I, *Bst*EII, *Sal*I, and the DNA polymerase I large fragment (Klenow) were purchased from Bethesda Research Laboratories, Gaithersburg, Md.; *Ava*I, *Sna*BI, and *Ban*II were purchased from New England BioLabs, Beverly, Mass.; *Asu*II and the synthetic *Sal*I linker (pGGTCGACC) were purchased from Promega Corporation, Madison, Wis.; T4 DNA ligase was purchased from International Biotechnologies, Inc., New Haven, Conn.

RNA 1 was constructed by digesting pT7D-polio DNA with both *Ava*I and *Nru*I, filling the recessed 3′ end of the *Ava*I site with Klenow polymerase, and blunt end ligating the resulting fragment with T4 DNA ligase. RNA 2 was constructed by digestion of pT7D-polio DNA with *Nru*I and *Sna*BI and blunt-end ligation of the resulting fragment with T4 DNA ligase. RNA 3 was constructed by digestion of pT7D-polio DNA with *Asu*II and ligation of the resulting fragment with T4 DNA ligase. RNA 4 was constructed by digesting pT7D-polio DNA with *Ban*II, polishing the 3′ extensions with Klenow polymerase, and ligating synthetic oligonucleotides (8-mer) containing a unique *Sal*I site onto the blunt ends. This construct was digested with *Sal*I, and the resulting fragment was ligated with T4 DNA ligase. RNA 5 was constructed by digestion of pT7D-polio with *Nru*I and *Bst*EII, filling of the recessed 3′ ends of the *Bst*EII site with Klenow polymerase, and blunt-end ligation of the resulting fragment with T4 DNA ligase.

Bacterial transformation and growth. Competent *Escherichia coli* HB101 cells (Bethesda Research Laboratories) were transformed with plasmid DNA in accordance with the specification sheet provided by the manufacturer for small-scale transformation. Resulting colonies were inoculated into 5 ml of L broth (GIBCO-Bethesda Research Laboratories L Broth Base) starter cultures for miniprep analysis. Minipreps of plasmid DNA were prepared as previously described (35). Large preparations of plasmid DNA for the putative mutant constructs were isolated as recommended by the manufacturer for p-GEM plasmids (Promega Corporation).

RNA transcription and RNA preparation. RNA transcripts were synthesized in 100-μl reaction mixtures containing 5 μg of *Mlu*I-cut plasmid DNA, 40 mM Tris (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 40 U of RNasin (Promega Corporation), 1 mM ATP, 1 mM UTP, 1 mM GTP, 1 mM CTP, 10 μCi of [^{3}H]UTP (40 Ci/mmol; Amersham Corporation, Arlington Heights, Ill.), and 2,000 U of T7 RNA polymerase. Reaction mixtures were incubated for 4 h at 37°C. Five microliters was precipitated in trichloroacetic acid and counted to determine the efficiency of each reaction. For transfections, the reaction mixture was used directly without further processing. A portion of each reaction mixture was saved for subsequent gel fractionation and Northern (RNA) blot analysis. For the RNA probe, 50 μCi of [^{32}P]UTP was substituted for [^{3}H]UTP, the cold UTP was omitted, and the products were phenol extracted. The probe was a minus-strand poliovirus RNA transcript of pOF1265 (20) which was complementary to bases 5240 to 6775 in the viral genome. For in vitro translation reactions, transcript RNAs were phenol and chloroform extracted in the presence of sodium dodecyl sulfate (SDS), ethanol precipitated, purified by chromatography on a Sephedex G-50 column to remove unincorporated nucleotides, and ethanol precipitated. Unincorporated nucleotides interfered with the translation reactions if not removed from the transcript RNAs.

Virion RNA was prepared from CsCl-banded virus by phenol-chloroform extraction and ethanol precipitation. Virion RNA was quantitated by determination of its A_{260}.

RNA sequencing. All mutant constructs were analyzed to confirm that they contained the correct junction sequence. RNA transcripts were sequenced by using a GemSeq sequencing kit (Promega Corporation) in accordance with the manufacturer's directions. Briefly, 1 μg of an RNA transcript was annealed with 5 ng of the appropriate DNA primer. All primers were synthetic DNA oligonucleotides (21-mers) that were minus-strand sequences approximately 50 bases 5′ of the junction sequence. RNA transcripts were synthesized by using avian myeloblastosis virus reverse transcriptase in the presence of dideoxynucleoside triphosphates and [^{35}S]dATP (Amersham Corporation). The resulting transcripts were fractionated on 6% polyacrylamide gels with 7 M urea by using a buffer consisting of 50 mM Tris base, 41.5 mM boric acid, and 0.5 mM EDTA. The gels were dried and autoradiographed.

RNA transfection. Transfection reactions utilized HeLa spinner cells placed in a monolayer 24 h prior to use. Cells (3×10^6) were plated onto 6-cm-diameter dishes in Eagle's minimum essential medium with 10% fetal calf serum, resulting in 60 to 70% confluence. For transfection, the medium was removed and the cells were rinsed once for 1 min with 1 ml of phosphate-buffered saline supplemented with magnesium and calcium. Cells were transfected for 60 min at 37°C with 0.5 ml of phosphate-buffered saline supplemented with magnesium and calcium and containing 500 μg of DEAE dextran (Pharmacia, Piscataway, N.J.) per ml and 1 to 5 μg of the RNA transcript in transcription buffer. Following the 60-min incubation, the DEAE dextran mixture was removed, the cells were rinsed once for 1 min with 1 ml of phosphate-buffered saline supplemented with magnesium and calcium, and 5.0 ml of Eagle's minimum essential medium with 10% fetal calf serum was added. Transfected cells were then incubated for 8 h or the times indicated in the figures prior to harvesting. To label viral RNA in vivo,

transfected cells were rinsed three times with 2 ml of phosphate-free Eagle's minimum essential medium and then exposed to 5 μg of actinomycin D per ml for 15 min in 2 ml of phosphate-free Eagle's minimum essential medium with 10% dialyzed fetal calf serum. Following this incubation, 1 mCi of $^{32}P_i$ (Amersham Corporation) was added and the cells were incubated as usual.

RNA isolation. Whole-cell RNA was harvested by using a guanidinium isothiocyanate procedure. Cells were scraped from the dishes with a sterile rubber policeman and collected by centrifugation at 1,000 × *g* in a clinical centrifuge. The supernatant was removed, the tube was dried by blotting, and the pellet was resuspended in 0.25 ml of lysis buffer (4 M guanidinium isothiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1 M β-mercaptoethanol). The pellet was resuspended by repeated micropipetting and placed into a sterile microcentrifuge tube. A 30-μl volume of 2 M sodium acetate (pH 4.0) was added, and the solution was vortexed for 5 s. A 300-μl volume of phenol saturated with H_2O was added, and the mixture was vortexed for 5 s. Following addition of 60 μl of chloroform-isoamyl alcohol (24:1), the mixture was vortexed three times for 5 s each time and placed on ice for 10 min. The solution was then centrifuged for 10 min at 4°C, and the supernatant was transferred to a clean microcentrifuge tube. A 300-μl volume of isopropanol was added, the mixture was vortexed for 5 s, and the RNA was precipitated overnight at −20°C.

Gel fractionation, Northern blotting, and probe hybridization. Gel fractionation, Northern blotting, and probe hybridization were performed in accordance with the recommendation of the nitrocellulose manufacturer (Schleicher & Schuell, Keene, N.H.). Briefly, the RNA was fractionated on a 2.2 M formaldehyde–1% agarose gel containing 0.5× MOPS buffer (20 mM 4-morpholinepropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA). Fractions (15 μg) of the whole-cell RNA preparation were added to each lane and electrophoresed for 4 h at 150 V on a 15-cm-long gel. Ten nanograms of original transcript was run on the same gel as a marker to ensure that the transcripts were full length and that equimolar amounts of each transcript were used in each experiment. Following electrophoresis, gels containing radiolabeled RNA were dried directly. For Northern blot analysis, the gels were rinsed twice, for 5 min each time, with H_2O and then capillary blotted onto 0.45-μm-pore-size nitrocellulose paper overnight by using 20× SSC (3.0 M NaCl, 0.3 M sodium citrate [pH 7.0]). Following this step, the nitrocellulose was dried for 5 min under a heat lamp and baked for 30 min at 80°C in vacuo. The nitrocellulose was then prehybridized for 20 min at 42°C in 20 ml of 50% formamide–5× Denhardt solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin)–0.3% SDS–5× SSPE (0.9 M NaCl, 0.05 M $NaPO_4$ [pH 7.7], 5 mM EDTA)–100 μg of fragmented salmon sperm DNA. The nitrocellulose was then hybridized overnight at 42°C in 20 ml of fresh prehybridization solution to which 5 × 10^6 cpm of the probe and 2.0 ml of 50% dextran sulfate were added. Following hybridization, the nitrocellulose was rinsed twice, for 10 min each time, at 22°C in 100 ml of 2× SSPE with 0.1% SDS, once for 10 min at 22°C in 100 ml of 0.2× SSPE with 0.1% SDS, and once for 60 min at 60°C in 200 ml of 0.2× SSPE with 0.1% SDS. The hybridized probe was detected by autoradiography.

In vitro translation reactions. HeLa S10 extracts and HeLa cell initiation factors were prepared essentially as described by Brown and Ehrenfeld (5). Translations were performed in 20-μl reaction mixtures which contained 50% HeLa S10 extracts and 20% HeLa initiation factors by volume in the presence of 1 mM ATP–0.2 mM GTP–25 mM creatine phosphate–400 μg of creatine kinase (Boehringer Mannheim) per ml–200 μg of rabbit liver tRNA (Sigma) per ml–120 mM KCH_3CO_2–35 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4)–3 mM dithiothreitol–2.75 mM $Mg(CH_3CO_2)_2$–20 μM amino acids (minus methionine) (Promega)–20 μCi of [^{35}S]methionine (1,000 Ci/mmol; Amersham Corporation). Reactions were run with or without 50 μg of virion RNA or T7 transcript RNA per ml, as indicated. Reaction mixtures were incubated at 30°C for 22 h, and reactions were terminated by addition of 10 volumes of Laemmli sample buffer (2% SDS [Sigma]), 62.5 mM Tris HCl [pH 6.8], 0.5% 2-mercaptoethanol, 0.1% bromophenol blue, 20% glycerol). Samples (20 μl; 2 μl of translation products) were separated by gel electrophoresis at 30 mA of constant current in a 10% polyacrylamide gel (29:1 acrylamide to bisacrylamide) and a 10 to 20% gradient polyacrylamide gel containing 0.1% SDS and 187.5 mM Tris HCl (pH 8.8). Gels were fixed in 50% trichloroacetic acid, fluorographed by using 22% PPO (2,5-diphenyloxazole) in dimethyl sulfoxide, dried, and exposed to XAR-5 film (Kodak) at −70°C for 3 to 4 h.

RESULTS

We developed an assay to evaluate poliovirus RNA replication following transfection or cotransfection of mutant and helper RNAs into mammalian cell cultures. RNA replication was evaluated by isolating whole-cell RNA, fractionating the RNA by gel electrophoresis, Northern blotting, and probing with radiolabeled minus-strand RNA. Since both the mutant and helper RNAs were made by transcription of cDNA clones in vitro, there was no need to isolate and grow virus stocks of conditional mutants or to require that the mutant RNA be packaged. By using this approach, it was possible to evaluate even lethal mutations, a significant improvement over previous methods of analysis. In this study, we characterized a number of poliovirus deletion mutants for the ability of their RNA genomes to replicate in transfected cells.

The parental clone, pT7D-polio, contained a complete cDNA copy of the poliovirus genome (26). By using pT7D-polio DNA, we constructed a number of deletion subclones (Fig. 1). These included both out-of-frame (RNA 1) and in-frame (RNA 2) deletion mutations in the capsid coding region (P1), a large in-frame deletion spanning the P1, P2, and P3 coding regions (RNA 3), an in-frame deletion encompassing the P1 region and some of the 2A coding sequence (RNA 4), and an in-frame deletion encompassing the P1-2A region and extending into the 2B coding region (RNA 5). These constructs were verified by direct sequencing of the RNA and by in vitro translation.

Replication of T7D-polio RNA in transfected cells. Following synthesis, RNA transcripts were transfected into cell cultures to determine their infectivity. T7D-polio RNA transcripts were infectious when transfected into HeLa cells as previously reported (26). Conversely, none of the RNAs transcribed from the deletion clones resulted in a measurable cytopathic effect on the transfected cells (data not shown). We examined the replication of T7D-polio RNA in transfected cells both by using a Northern blot hybridization technique and by $^{32}P_i$ labeling poliovirus RNA in vivo. Transfection of T7D-polio RNA into HeLa cells in the presence of actinomycin D and $^{32}P_i$ resulted in the synthesis of ^{32}P-labeled poliovirus RNA (Fig. 2). A band of labeled

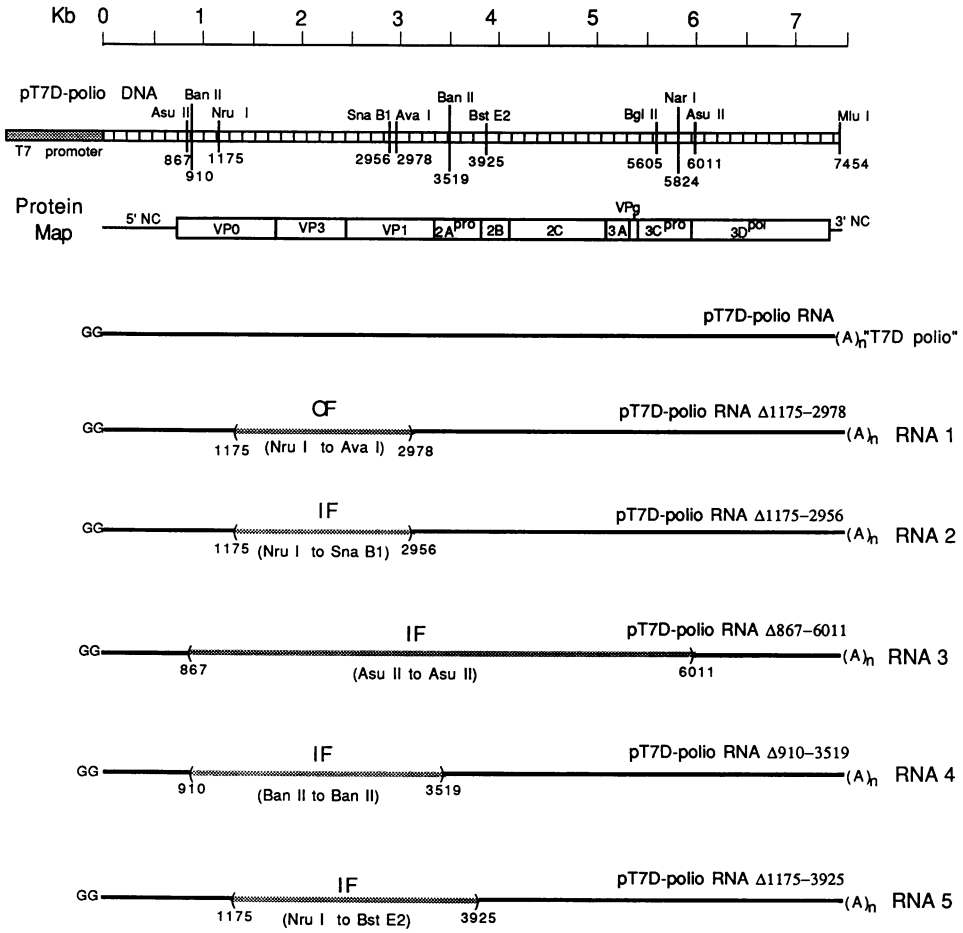

FIG. 1. RNA transcripts synthesized by bacteriophage T7 RNA polymerase on pT7D-polio DNA and the various deletion subclones generated from it. All DNA was linearized with *Mlu*I. Protein and restriction site maps are shown for reference. IF and OF are in-frame and out-of-frame deletions, respectively. The restriction sites used to create the deletion in each subclone are shown. Additional details concerning the construction of the subclones are described in Materials and Methods. NC, noncoding.

viral RNA was clearly present in the preparation of total cellular RNA from the transfected cells that was not present in the mock-transfected control (Fig. 2, middle and left lanes). The viral RNA was the only labeled polyadenylated RNA that was recovered in this experiment (Fig. 2, right lane). This result indicated that the T7D-polio RNA efficiently replicated in the transfected cells. Our ability to select the labeled viral RNA on an oligo(dT)-cellulose column indicated that the poly(A) tail on this RNA was elongated in vivo and was significantly longer than the poly(A) sequence on the input T7D-polio RNA which did not bind to an oligo(dT) column (data not shown).

Northern blot analysis of whole-cell RNA isolated from cells transfected with T7D-polio RNA confirmed that poliovirus-specific RNA of the expected size was synthesized (Fig. 3). Analyses at 2 and 8 h posttransfection showed that no viral RNA was detected at 2 h but that a significant amount of viral RNA was present by 8 h (Fig. 3). This also indicated that the full-length viral RNA transcript replicated in the transfected cells. Similar results were obtained with a subgenomic transcript, RNA 2, which contained an in-frame deletion in the P1 coding region (Fig. 3).

Deletions in the P1 coding sequence. We evaluated the replication of two subgenomic RNAs which contained either an out-of-frame deletion (RNA 1) or an in-frame deletion (RNA 2) in the P1 coding sequence (Fig. 1). Northern blot analysis of whole-cell RNA isolated at 8 h posttransfection showed that RNA 1 did not replicate either alone or when cotransfected with T7D-polio RNA (Fig. 4). Conversely, RNA 2 replicated when transfected alone and in the presence of T7D-polio RNA (Fig. 4). On average, RNA 2 replicated approximately five times more efficiently than the full-length transcript (Fig. 3 and 4; see also Fig. 7). One final observation was that during cotransfection experiments, both T7D-polio RNA and RNA 2 replicated at a slightly reduced level relative to the replication observed when either RNA was transfected alone (Fig. 4).

Large in-frame deletion in the P1-P2-P3 coding sequence. Since it was not possible to complement the out-of-frame deletion in the P1 region (i.e., RNA 1), we restricted further investigation to in-frame deletions. We constructed a large in-frame deletion (RNA 3) that spanned the P1, P2, and P3 coding sequences to determine whether the 5′- and 3′-terminal sequences of poliovirus RNA were sufficient to support RNA replication when all of the viral replication proteins were provided in *trans* by a helper RNA. In contrast to RNA 1, RNA 3 should allow the cellular ribosomes to terminate synthesis of the fragment polyprotein at the nor-

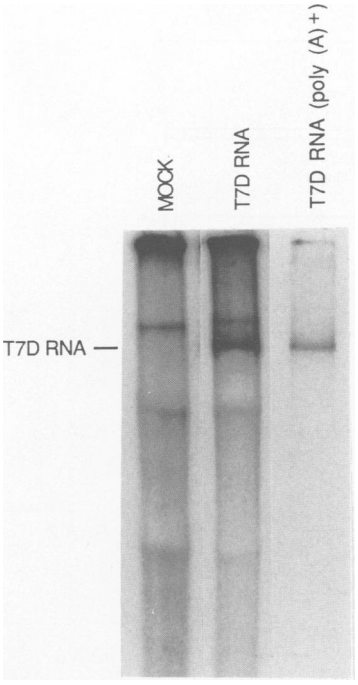

FIG. 2. Formaldehyde-agarose gel electrophoresis of ^{32}P-labeled RNA synthesized in T7D-polio RNA-transfected cells. Whole-cell RNAs were isolated from HeLa cells which were transfected with T7D-polio RNA and from a mock-transfected control. RNA synthesized in the transfected cells was labeled by adding $^{32}P_i$ to the cell culture following treatment with actinomycin D as described in Materials and Methods. Labeled polyadenylated RNA from the T7D-polio RNA-transfected cells was further purified by chromatography on oligo(dT)-cellulose. The poly(A)$^+$ RNA and the whole-cell RNAs isolated from the transfected and mock-transfected cells were analyzed by gel electrophoresis and located by autoradiography.

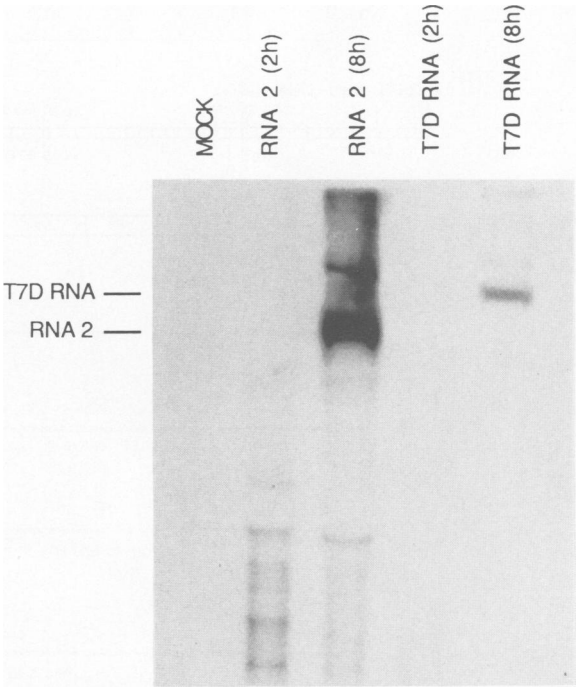

FIG. 3. Northern blot analysis of replication of T7D-polio RNA and RNA 2 in transfected cells. HeLa cells were transfected with T7D-polio RNA or RNA 2 as described in Materials and Methods. Whole-cell RNA was isolated at 2 and 8 h posttransfection, fractionated by electrophoresis on a formaldehyde-agarose gel, blotted onto nitrocellulose, and probed with a ^{32}P-labeled poliovirus minus-strand RNA transcript.

mal termination codon. Because of the tight linkage between protein synthesis and RNA replication in poliovirus-infected cells, we hypothesized that the release of ribosomes from a viral RNA molecule at the normal termination codon might be required for replication of a subgenomic RNA in vivo. However, there was no increase in the amount of RNA 3 detected between 2 and 8 h posttransfection in the presence of T7D-polio RNA (Fig. 5). The RNA detected in the blot was input RNA which was reproducibly found to be more stable than T7D-polio RNA, RNA 2, RNA 4, and RNA 5 (compare with the 2-h time points in Fig. 3 and 7 and the 4-h time point in Fig. 6). For reasons that have not been determined, input RNA 3 remained at a steady-state level from time zero to 8 h posttransfection, both in the presence and in the absence of helper RNA (Fig. 5). The fact that RNA 3 was not complemented again suggested that a *cis*-acting function is required for poliovirus RNA replication in vivo.

Deletion in the P1-2A coding sequence. Because our large in-frame construct did not replicate in the presence of helper RNA, we decided to proceed by expanding the size of the in-frame deletion in RNA 2 into the 2A coding sequence. We constructed an in-frame deletion in the P1-2A coding regions that removed nucleotides 910 to 3519 and specifically removed nucleotides 3386 to 3519 in 2A (RNA 4). This RNA was self-replicating but at a reduced level relative to RNA 2 (Fig. 6). A time course experiment was used to compare the replication of RNA 4 alone versus that of RNA 4 plus RNA 2 (Fig. 6). A low level of RNA 4 replication was detected by 8 h when RNA 4 was transfected alone. When RNA 4 was cotransfected with RNA 2, however, the level of replication for RNA 4 dramatically increased; this was seen as early as the 6-h time point. In the cotransfection experiment, the amounts of RNA 4 and RNA 2 detected at the 6-, 8-, and 10-h time points were essentially the same (Fig. 6). The results indicated that it was possible to complement the 2A deletion mutation in *trans* by cotransfection with RNA 2. Complementation was also observed by cotransfection with T7D-polio RNA (data not shown).

Deletion in the P1-2A-2B coding sequence. We continued our analysis of nested deletions by expanding the deletion to include sequences in the 2B coding region. The RNA 5 construct deleted bases 1175 to 3925, which included 3833 to 3925 in 2B. RNA 5 was neither self-replicating nor capable of replication after cotransfection with either RNA 2 or T7D-polio RNA (Fig. 7). This result suggested either that 2B is a *cis*-acting protein required for poliovirus RNA replication or that a *cis*-active RNA sequence required for replication was removed by this deletion.

Translation of poliovirion RNA and transcript RNAs in vitro. In vitro translation reactions were used to determine the effects of the various deletions on the synthesis of poliovirus proteins. It was important to demonstrate that the in-frame deletions did not interfere with the synthesis and processing of proteins downstream from the deletions. Translation of poliovirion RNA in vitro resulted in the synthesis of all of the poliovirus proteins found in vivo (Fig. 8A and B). T7D-polio RNA, the full-length infectious tran-

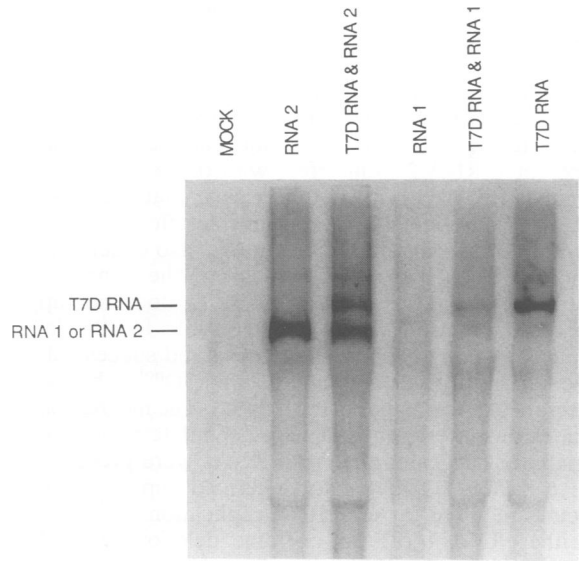

FIG. 4. Northern blot analysis of replication of RNA 1 and RNA 2 in the absence or presence of T7D-polio RNA. HeLa cells were transfected with RNA 1 (P1 out-of-frame deletion), RNA 2 (P1 in-frame deletion), or T7D-polio RNA as indicated. Whole-cell RNA was isolated at 8 h posttransfection and was analyzed as described in the legend to Fig. 3.

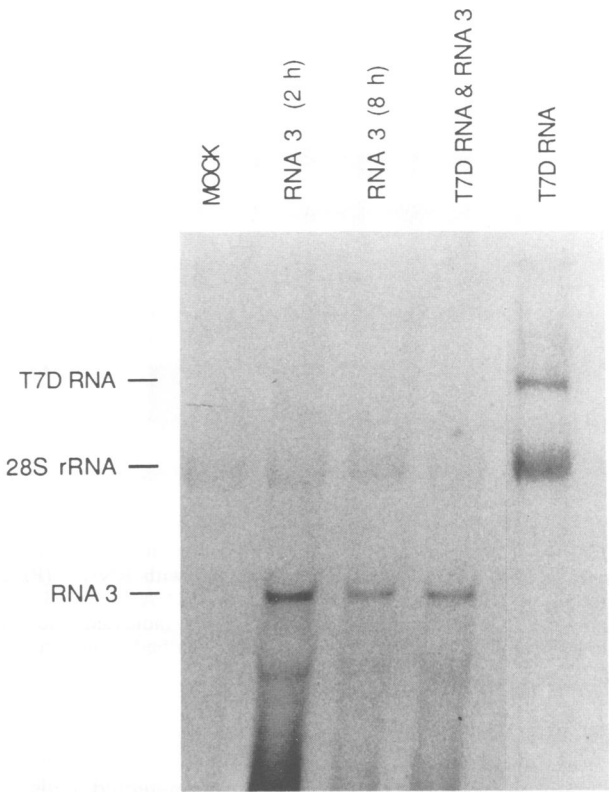

FIG. 5. Inability of RNA 3 to replicate alone or in cells cotransfected with T7D-polio RNA. Cells were transfected with RNA 3 (P1-P2-P3 in-frame deletion) or cotransfected with RNA 3 and T7D-polio RNA as indicated. Whole-cell RNA was isolated at 2 or 8 h and characterized by Northern blot analysis as described in the legend to Fig. 3. A lane containing RNA isolated from T7D-polio RNA-transfected cells was included as a marker for full-length poliovirus RNA.

script, generated the same products as virion RNA but in reduced amounts (Fig. 8A and B).

RNA 2 contained an in-frame deletion in the P1 coding region of the poliovirus genome (Fig. 1). As expected, translation of RNA 2 yielded all of the poliovirus proteins except P1, VP0, VP1, and VP3 (Fig. 8A). Protein 2A was synthesized in the reaction containing RNA 2 and comigrated with the 2A protein translated from virion RNA and T7D-polio RNA (Fig. 8B). This indicated that cleavage occurred at the P1-P2 junction, a 2A protease cleavage site downstream of the deletion (Fig. 1). In addition, 3C′ and 3D′ were present, indicating the synthesis of functional 2A protease (Fig. 1). Thus, the in-frame capsid deletion of RNA 2 resulted in the absence of the P1 proteins but had normal translation and processing of the poliovirus proteins downstream of the deletion.

RNA 4 contained an in-frame deletion in the P1-2A coding region of the poliovirus genome (Fig. 1). Translation of RNA 4 yielded all of the proteins predicted for this construct (Fig. 8A and B). As expected, 2A was not synthesized but a new protein with a molecular weight slightly higher than that of 2A was present (Fig. 8B). This protein is likely a fusion of the NH_3-terminal portion of VP4, which was upstream of the deletion, with the COOH-terminal portion of 2A. This fusion protein would be 160 amino acids long and 11 amino acid residues longer than 2A. The complete absence of 3C′ and 3D′ indicates that the deletion of the NH_3-terminal portion of the 2A protein abolished all 2A protease activity. All of the other viral proteins downstream of the capsid and 2A deletion were translated and processed normally.

The translation of RNA 5, which contained an in-frame deletion in the P1-2A-2B coding region, resulted in the synthesis of all of the viral proteins predicted for this construct (Fig. 8A and B). P1, VP0, VP1, VP3, 2A, and 2B were not synthesized. 2B has only one methionine residue and was not visualized in this experiment; however, the absence of 2BC was consistent with the deletion in the 2B coding region. 2C was synthesized as usual. Thus, processing by 3C protease occurred at the 2BC junction site. The P2 protein was smaller than that from wild-type constructs, owing to the absence of 2A and part of 2B. As expected, translation of RNA 5 did not yield 2A (Fig. 8B) and 2A protease activity was absent. Thus, the large in-frame deletion of RNA 5 was confirmed and it did not interfere with translation and processing of the proteins downstream from the deletion.

DISCUSSION

In this study, we investigated poliovirus RNA replication in cultured cells transfected with a specific viral transcript or in cells cotransfected with both mutant and helper RNAs. Mutant RNAs containing specific deletions were evaluated for the ability to replicate alone and in the presence of helper RNA by using Northern blot analysis. This approach had the advantage of providing a means of directly investigating the roles of specific genomic sequences in viral RNA replication without the necessity of packaging the product RNA in virions or first isolating conditional-lethal mutants. By cotransfecting RNA-negative mutants with helper RNA, both *cis*- and *trans*-active genetic elements required for RNA replication were identified.

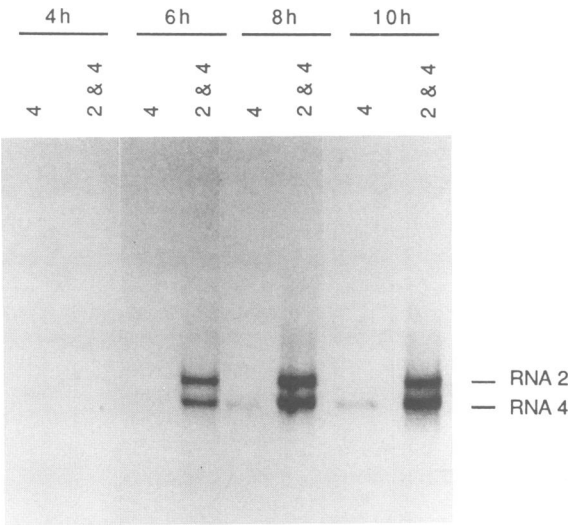

FIG. 6. Time course of RNA 4 replication in the absence or presence of RNA 2. Cells were transfected with RNA 4 (P1-2A deletion) or cotransfected with RNA 4 and RNA 2 as indicated. Whole-cell RNA was isolated at the times indicated and was characterized by Northern blot analysis as described in the legend to Fig. 3.

Both full-length T7D-polio RNA and RNA 2 (P1 in-frame deletion) were efficient replicons in transfected cells. In agreement with previous work, the subgenomic-size replicon, RNA 2, replicated more efficiently than full-length transcripts (14). In addition, since transfection with RNA 2 did not result in virion formation or cell lysis it continued to replicate for several hours beyond the standard 8-h transfection time used in this study. This would make RNA 2 a valuable parental clone for increasing the sensitivity of

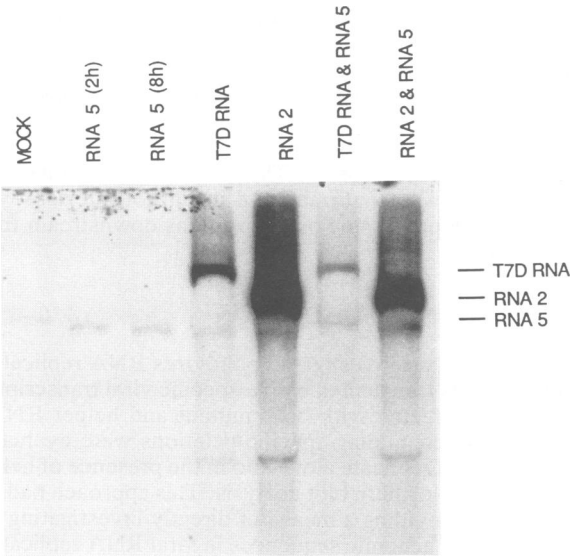

FIG. 7. Absence of RNA 5 replication in transfected cells in either the absence or the presence of T7D-polio RNA or RNA 2. Cells were transfected with RNA 5 or cotransfected with RNA 5 and T7D-polio RNA or RNA 2 as indicated. Whole-cell RNA was isolated at 2 or 8 h and analyzed as described in the legend to Fig. 3.

mutations in other regions of the viral genome which are being assessed for their effects on RNA replication. In cotransfection experiments, RNA 2 and T7D-polio RNA were found to interfere to some degree with each other's replication. From the results of previous studies with defective interfering RNAs (8, 9) and subgenomic replicons (14), we expected RNA 2 to interfere with the replication of the full-length RNA. However, it was clear that the full-length RNA also had a small but reproducible effect on the replication of the subgenomic replicon. We also observed interference of the helper RNA by each of the nonreplicating constructs (i.e., RNAs 1, 3, and 5). It does not appear, however, that interference is responsible for the lack of complementation, since we have observed successful complementation of other mutants (2C and $3D^{pol}$) when similar interference of the helper occurred (9a). The mechanism that mediates this interference has not been determined, but the interference suggested that both RNAs were present in the same cells and competed for a limited supply of cellular cofactors required for viral RNA replication.

Neither RNA 1 (P1 out-of-frame deletion) nor RNA 3 (P1-P2-P3 in-frame deletion) replicated alone or when cotransfected with T7D-polio RNA. Both of these results suggested that there was a *cis*-active requirement for poliovirus RNA replication. The results with RNA 1 were of particular importance, since the out-of-frame deletion in P1 blocked the synthesis of the P2- and P3-encoded replication proteins without directly altering the RNA sequence in this region of the genome. It was clear that the RNA sequence deleted in RNA 1 was not directly required to support RNA replication, since two other subgenomic RNAs with large P1 deletions replicated efficiently both alone (RNA 2) and in the presence of helper RNA (RNA 4). It is possible that the large deletion in RNA 1 had a long-range effect on the overall structure of this RNA and that this in turn blocked its replication. However, this did not appear to be the case since RNA 2 contained a deletion of a similar size in the P1 region (Fig. 1) and yet was a more efficient replicon than full-length RNA. In addition, RNA 4, which contained an even larger deletion, replicated efficiently in the presence of helper RNA. Thus, we concluded that one or more of the viral RNA replication proteins was *cis* active during the poliovirus RNA replication cycle.

Our results indicated that RNA 4 (P1-2A in-frame deletion) was a self-replicating subgenomic RNA. Although RNA 2 replicated more efficiently than full-length viral RNA, RNA 4 was found to be a less efficient replicon. In the presence of helper RNA, however, there was a large increase in the replication of RNA 4. Thus, our results indicated that it was possible to complement a 2A deletion mutation in *trans* by cotransfecting cells with either T7D RNA or RNA 2. This was an important result, since it demonstrated that it was possible to use the cotransfection procedure to complement a specific mutation in the viral genome. The enhanced replication of RNA 4 that was observed in the presence of helper RNA cannot be explained by recombination, since a recombinant which no longer contained the deletion would migrate at a different position in the gel used in the Northern blot analysis. Our in vitro analysis of the proteins translated from RNA 4 confirmed that the deletion in this construct had inactivated the 2A protease activity, since 3C′ and 3D′ were not present. This, in turn, suggested that the 2A-mediated cleavage of the polyprotein to form 3C′ and 3D′ is not an absolute requirement for viral RNA replication. This was in agreement with a previous study which showed that mutagenesis of the 2A

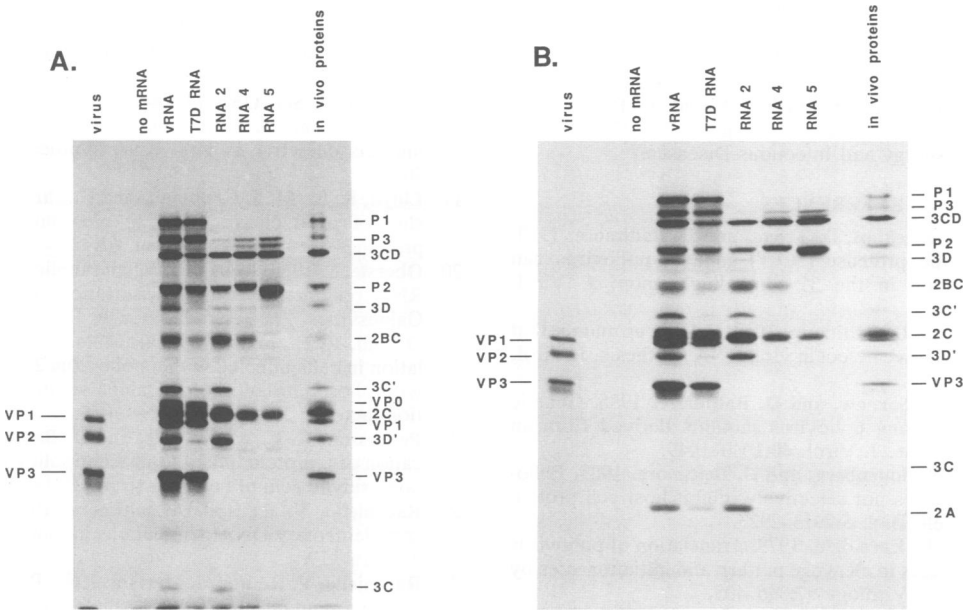

FIG. 8. Translation of RNA transcripts and poliovirion RNA in vitro. In vitro translation reactions were performed as described in Materials and Methods. Translation products from reactions containing no added RNA or containing poliovirion RNA, T7D-polio RNA, RNA 2 (P1 deletion), RNA 4 (P1-2A deletion), or RNA 5 (P1-2A-2B deletion) were analyzed on both a 10% polyacrylamide gel (A) and a 10 to 20% gradient polyacrylamide gel (B) as indicated. The capsid proteins from purified [^{35}S]Met-labeled poliovirions were used as capsid protein markers. A cytoplasmic extract was prepared from poliovirus-infected HeLa cells labeled with [^{35}S]Met from 2.5 to 4.5 h postinfection and used as markers for the poliovirus proteins synthesized in vivo. The mobilities of 3D, 3CD, 3C, 3C', and 3D' were identified by immunoprecipitation with anti-3C and anti-3D antibodies.

cleavage site in the P3 region blocked the formation of 3C' and 3D' but did not affect the viability of the mutant virus (16). The lack of 2A activity in cells transfected with RNA 4 would not result in the normal poliovirus-induced inhibition of host protein synthesis (4, 15, 19, 21, 34). This would result in a lower level of viral protein synthesis and may explain the reduced level of RNA replication observed with RNA 4. Cotransfection with helper RNA should result in the synthesis of active 2A protease and the subsequent inhibition of host protein synthesis. This, in turn, should result in an increase in the translation and replication of RNA 4. This is essentially the same mechanism previously described by Trono et al. (29) to explain the indirect complementation of mutations in the 5' noncoding region by coinfection with virus containing a wild-type 2A coding region.

Our experiments with RNA 5 (P1-2A-2B in-frame deletion) indicated that this transcript did not replicate in transfected cells. In contrast to RNA 4, there was no indication that RNA 5 replicated when cotransfected with either RNA 2 or T7D-polio RNA. Our analysis of the proteins translated from RNA 5 in vitro confirmed that the P1, 2A, and 2B proteins were missing and that the balance of the remaining viral proteins were synthesized and processed as expected. Taken together with our ability to complement the 2A deletion in RNA 4, these results support the hypothesis that 2B is a *cis*-active viral protein that is required for viral RNA replication to proceed. This possibility is in agreement with our inability to complement an out-of-frame deletion in the capsid-coding region (RNA 1), which indicated that one or more of the viral RNA replication proteins was *cis* active (see discussion above). It is possible that the deletion in the 2B coding sequence removed a *cis*-active RNA sequence, but this appears to be less likely than a *cis*-active function for protein 2B since small insertion mutations in 2B (6 or 12 bases) were also reported to be noncomplementable in coinfection experiments (3, 13).

The mounting evidence that 2B (or 2BC) and perhaps specific functions of other virus-encoded replication proteins (e.g., 3D) are *cis* acting supports a model for RNA replication which proposes that the replication of a molecule of plus-strand RNA requires a complex of viral proteins, one or more of which are translated directly from the same RNA molecule (3). This model predicts that minus-strand synthesis is directly linked to viral protein synthesis. Thus, for a molecule of plus-strand RNA to serve as a template for minus-strand synthesis, it must first be translated to synthesize the *cis*-active replication proteins. The direct entry of a newly synthesized poliovirus plus-strand RNA into the replication complex would be blocked according to this model. In addition, other polyadenylated RNAs that may be present in the cell (e.g., cellular mRNA) could not serve as templates for minus-strand synthesis by the viral replication complex. This mechanism would have the obvious advantage of providing absolute specificity for replication of viral RNA and would prevent replication of mutant plus strands that contain defective *cis*-active genetic elements required for RNA replication. Considering the high error frequency of poliovirus RNA polymerase (32, 33), this could be a significant factor in ensuring that a large number of viable progeny are produced during each round of replication. Additional studies are needed to identify and characterize the *cis*- and *trans*-active functions of the other poliovirus proteins required for poliovirus RNA replication in vivo.

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