Construction and Characterization of Poliovirus Subgenomic Replicons

GERARDO KAPLAN AND VINCENT R. RACANIELLO*

Department of Microbiology, Columbia University College of Physicians & Surgeons, New York, New York 10032

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Poliovirus RNAs containing in-frame deletions within the capsid-coding region were produced by *in vitro* transcription of altered poliovirus type 1 cDNA by using bacteriophage T7 RNA polymerase. Three RNAs were transcribed that contained deletions of 2,317 nucleotides (bases 747 to 3064), 1,781 nucleotides (bases 1,175 to 2,956), and 1,295 nucleotides (bases 1,175 to 2,470). All three subgenomic RNAs replicated after transfection into HeLa cells, demonstrating that sequences encoding the capsid polypeptides are not essential for viral RNA replication *in vivo*. Viral RNA containing the largest deletion (R1) replicated approximately three times better than full-length RNA produced *in vitro*. Northern blot (RNA blot) hybridization analysis of total cellular RNA from HeLa cells at different times after transfection with R1 demonstrated the presence of increasing amounts of the expected 5.1-kilobase subgenomic RNA. Analysis by immunoprecipitation of viral proteins induced after transfection of R1 RNA into HeLa cells revealed the presence of proteins 2A^{pro}, 2C, and 3D^{pol} and its precursors, suggesting that the polypeptide cleavages are similar to those occurring in virus-infected cells. Replication of P2/Lansing virion RNA was inhibited by cotransfection with the R1 replicon, as demonstrated by hybridization analysis with a serotype-specific oligonucleotide probe. A higher level of inhibition of RNA replication was observed when P2/Lansing RNA was cotransfected into HeLa cells with truncated R1 transcripts (R1-PvuII) that were missing 395 3' nucleotides and a poly(A) tail. These internally and terminally deleted RNAs inhibited the replication of subgenomic replicons R1, R2, and R3 and caused a reduction in plaque size when cotransfected with P1/Mahoney or P2/Lansing viral RNA, suggesting that individual cells had received both RNAs. No inhibition of plaque size was observed when replicon RNAs were used that were missing 1,384 or 1,839 3' nucleotides or contained plasmid-derived sequences downstream of the 3' poly(A). The *trans*-acting inhibitory effect of R1-PvuII on the replication of poliovirus P2/Lansing RNA did not involve entry of RNA into cells and appeared to reduce viral translation and RNA synthesis late in the infection cycle.

Poliovirus, the prototype of the *Picornaviridae* family, is a nonenveloped animal virus containing an RNA genome of 7,440 bases (13, 24). The viral RNA, which is covalently linked at its 5' end to a small protein known as VPg, contains an untranslated leader of 745 bases followed by a large open reading frame, a 71-base 3' untranslated region, and a poly(A) tract. The long open reading frame is translated into a polypeptide that is cleaved by the two viral proteases 3C^{pro} and 2A^{pro} to produce mature viral proteins.

Replication of the RNA genome of poliovirus has been studied for the last 30 years with the goal of identifying and purifying viral and cellular proteins involved in this process. Much less attention has been devoted to identifying sequences in the RNA genome that are required for its replication. Previous work on poliovirus defective interfering (DI) particles indicated that parts of the P1 region of the RNA, encoding the capsid proteins, could be deleted without affecting the ability of the DI RNAs to replicate (4-7, 15, 18, 21, 22). The ability to recover infectious poliovirus from cloned cDNA (25) enables a direct test of the importance of genomic sequences in viral replication. Recently, several poliovirus mutants were isolated which possess defects in RNA synthesis. The locations of the lesions responsible for the phenotypes of these mutants indicate that both the 5' and 3' noncoding regions are important for RNA replication (26, 29).

The demonstration that RNA transcripts synthesized *in vitro* are infectious when transfected into cultured cells suggests another approach to identifying sequences impor-

tant for replication that circumvents the need for isolating viable virus mutants with RNA⁻ phenotypes (12, 34). Since transfection of cultured cells with poliovirus is quite efficient, it should be possible to produce RNAs containing a desired mutation, transfect the altered RNA into cultured cells, and study replication and translation. Here, we used this approach to show that the entire capsid coding sequence of the poliovirus genome is not required for translation or RNA replication. Furthermore, cotransfection of these subgenomic RNAs with wild-type viral RNA resulted in inhibition of replication of the wild-type RNA. In the course of these experiments, it became apparent that the same subgenomic RNAs, produced by transcription of cDNA linearized at a specific internal site, are potent *trans* inhibitors of viral replication. Although the mechanism of this inhibition is not known, its study may elucidate molecular interactions that occur during replication *in vivo*.

MATERIALS AND METHODS

Cells and viruses. HeLa S3 cells were grown in Spinner cultures and plated in 6-cm-diameter plastic tissue culture dishes (3×10^6 cells per dish) 24 h before use (16). Stocks of P1/Mahoney/41 and P2/Lansing/37 derived from infectious cDNAs (23, 25) were prepared in HeLa cell Spinner cultures and purified by equilibrium centrifugation in CsCl gradients. Virion RNA was extracted with phenol-chloroform-1% sodium dodecyl sulfate (SDS), precipitated with ethanol, and suspended in H₂O as described previously (25, 32).

Antisera. Anti-3D^{pol} serum was obtained from rabbits immunized with an *Escherichia coli* trpE-3D^{pol} fusion polypeptide that was isolated from bacteria harboring p3DX (Fig.

* Corresponding author.

FIG. 1. Construction of a *trpE*-replicase fusion expression plasmid. The poliovirus processing map is shown at the top. The amino acid composition of the predicted fusion product is shown at the bottom.

1). This plasmid was constructed by inserting a P1/Mahoney cDNA fragment, from base 5825 through the 3' end, into the expression vector pATH2 (31), followed by insertion of an 8-nucleotide *Xho*I linker at the *Sma*I site to place the poliovirus sequences and the *trpE* gene in the same reading frame. The resulting plasmid, p3DX, encodes a fusion protein consisting of 322 N-terminal amino acids of the *trpE* protein followed by 6 amino acids generated by polylinker sequences, 54 amino acids of 3C^{pro}, and the entire polymerase 3D^{pol}. To prepare antiserum against the fusion polypeptide, a 100-ml culture of *E. coli* containing this plasmid was starved for amino acids and induced with indoleacrylic acid. An insoluble protein fraction was prepared (30) and fractionated on a 7.5% SDS-polyacrylamide gel. The fusion polypeptide (1,000 µg) was excised from the gel, emulsified in Freund complete adjuvant, and inoculated into two female New Zealand White rabbits. After several boosts, the resulting antiserum immunoprecipitated the viral RNA polymerase 3D^{pol} and its precursors from infected cells (see Fig. 5, lane 6, and Fig. 12, lanes 12 and 13).

Rabbit anti-2C (3) and anti-2A^{pro} sera, gifts from D. Baltimore, were obtained from rabbits immunized with the corresponding proteins purified from infected cells.

Construction of plasmids containing P1 deletions. (i) **pT7R1.** P1/Mahoney cDNA in vector pSV20 (16; pSV20 [PVX2]) was cleaved with *Kpn*I (site at nucleotide 3068), and a *Sma*I linker was added to form pSV20[PV_{sma}39]. This plasmid was then cleaved with *Sma*I and digested with exonuclease Bal 31 (International Biotechnologies, Inc.), recircularized after the addition of *Sma*I linkers, and transformed into *E. coli*. Nucleotide sequence analysis was used to identify a clone containing a deletion from base 747 to base 4186, designated pSV20[PV_{sma}39dIB5]. To restore the Gln-Gly cleavage site at the C terminus of P1, pSV20 [PV_{sma}39dIB5] was cleaved with *Sma*I, ligated to *Eco*RI linkers, and cleaved with *Eco*RI (site at the 3' end of poliovirus cDNA) and the large fragment was gel purified. Next, pSV20[PV_{sma}39] was treated in the same way, and the small DNA fragment was purified. These two DNAs were ligated, producing pVR112, a full-length cDNA containing a deletion of nucleotides 747 to 3064. To restore the

reading frame across the deletion, this plasmid was opened at the *Eco*RI site at base 3064, blunted with Klenow fragment, and religated. Nucleotide sequence analysis confirmed that the final product, pVR115, contained an in-frame deletion of bases 747 to 3064. The *Kpn*I fragment of pVR115, from bases 68 to 3664, was then excised and used to replace the corresponding fragment in the T7-transcription plasmid pT7PV1-5 (34; gift of E. Wimmer).

(ii) **pT7R2.** pT7PV1-5 DNA was cleaved with *Sna*BI and *Nru*I at positions 2956 and 1174, respectively. The *Sna*BI ends were filled in with Klenow polymerase, and the DNA was recircularized and transformed into *E. coli*. Nucleotide sequence analysis was used to identify one plasmid that contained a 1,782-base-pair deletion in P1.

(iii) **pT7R3.** pT7PV1-5 was cleaved with *Nhe*I and *Nru*I at positions 2470 and 1174, respectively, the *Nhe*I site was filled in as described above, and the recircularized DNA was used to transform *E. coli*. Nucleotide sequence analysis was used to identify one plasmid that contained a 1,292-base-pair deletion in P1.

All plasmids were grown in *E. coli* DH1 (10) and purified by equilibrium centrifugation in CsCl in the presence of ethidium bromide (19).

In vitro RNA synthesis and transfection. In vitro synthesis of RNA from cDNA templates was performed using T7 RNA polymerase (Pharmacia, Inc.). Reaction mixtures (50 µl) contained 2 µg of plasmid DNA [linearized with *Eco*RI (Fig. 2) to leave two bases past the poliovirus poly(A)], 1 mM each nucleoside triphosphate, 1 U of RNasin (Promega Biotec) per µl, 0.5 µg of bovine serum albumin (RNase and DNase free; Boehringer Mannheim Biochemicals) (per µl), 5 mM dithiothreitol, 40 mM Tris hydrochloride (pH 8.0), 15 mM MgCl₂, and 30 U of T7 RNA polymerase. After incubation for 30 min at 37°C, the RNA was examined by electrophoresis in a 1% agarose gel (16) and used to transfect HeLa cells without further purification. Marker RNAs (see Fig. 11) were produced in the same manner except that [³²P]UTP was substituted for UTP. RNAs produced by transcription of pT7PV1-5, pT7R1, pT7R2, and pT7R3 were named PV1-5, R1, R2, and R3, respectively.

RNAs containing deletions at their 3' ends were produced by linearizing plasmids with *Pvu*II (site at nucleotide 7055) before transcription. The RNAs produced in this way were named R1-PvuII, R2-PvuII, and R3-PvuII. Other sites used to linearize pT7R1 before transcription were the *Hind*III site at nucleotide 6056 and the *Bgl*II site at nucleotide 5601.

Similar amounts of RNA (approximately 20 µg) were synthesized in each reaction, regardless of which DNA template was used and at which site the template was linearized. In all instances the predominant product of the transcription reaction was RNA of the expected length, as judged by agarose gel electrophoresis.

Unless otherwise indicated, one-fifth of a T7 transcription reaction was used to transfect each 6-cm plate of HeLa cells, using DEAE-dextran as a facilitator (33). In some experiments transfected cells were covered with an agar overlay and plaques were counted 48 h later as described previously (25).

Analysis of [³⁵S]methionine-labeled polypeptides. To examine polypeptides in HeLa cells transfected with RNAs, cell monolayers were washed with phosphate-buffered saline and incubated for 45 min at 37°C in 2 ml of methionine-free Dulbecco modified Eagle medium (GIBCO Laboratories) containing 50 µCi of [³⁵S]methionine (1,383 Ci/mmol; Amersham Corp.). After this time, the labeling medium was removed, and the cells were washed with phosphate-

buffered saline and scraped into the same buffer. Cytoplasmic extracts were prepared by suspending pelleted cells in 50 μ l of reticulocyte standard buffer–1% Nonidet P-40 and removing nuclei by centrifugation (26).

Proteins were immunoprecipitated by using 10 μ l of cytoplasmic extract, 5 μ l of immune rabbit serum, and 50 μ l of 10% (wt/vol) formaldehyde-denatured staphylococcus (Ig-Sorb; New England Enzyme Center) as described previously (2).

Proteins were fractionated in 15% SDS–polyacrylamide gels, which were stained with Coomassie blue, impregnated with Autofluor (National Diagnostics), dried, and exposed to X-Omat AR film (Eastman Kodak Co.) with intensifying screens for 3 to 10 days.

In pulse-chase experiments, polypeptides were labeled at 3 h posttransfection for 15 min, chased with 0.1 mM unlabeled methionine for increasing amounts of time, and analyzed by immunoprecipitation as described above.

Analysis of RNA by blot hybridization. For analysis of intracellular RNAs by the slot blot technique, HeLa cell cytoplasmic extracts, obtained as described above, were denatured with an equal volume of 22% formaldehyde–8 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C for 15 min. Samples were frozen at –70°C, thawed, and centrifuged for 30 min in a Microfuge (Brinkmann Instruments, Inc.). One-fifth of each denatured sample was filtered onto nitrocellulose paper by using a slot blot apparatus (Schleicher & Schuell, Inc.). The filters were baked, prehybridized, and hybridized as described previously (19). To quantitate the hybridization, the filter was cut and placed in 5 ml of OmniSolv (Fisher Scientific Co.) and the radioactivity was counted in a liquid scintillation counter.

For Northern blot (RNA blot) hybridization analysis, total-cell RNA was prepared by the guanidine thiocyanate–CsCl technique (19), fractionated in 1% agarose–formaldehyde gels, and transferred to nitrocellulose paper and hybridized as described previously (19).

Three different ³²P-labeled nucleic acid probes were used. One was a negative-strand RNA complementary to poliovirus virion RNA from the *Hind*III site at nucleotide 6516 through the 3' end; it was transcribed by using SP6 RNA polymerase as described previously (20) in reaction mixtures containing three unlabeled nucleoside triphosphates and 50 μ Ci of [³²P]UTP (800 Ci/mmol; New England Nuclear Corp.). The DNA template used was plasmid pSP65(3'17) (17), linearized at the *Hind*III site. The second probe was a synthetic oligonucleotide (5'-TAACAATGAGGTAATTCC-3') complementary to bases 711 to 728 of P2/Lansing RNA, labeled at the 5' end with [³²P]ATP and polynucleotide kinase (19). This oligonucleotide hybridizes to P2/Lansing but not P1/Mahoney RNA. The third probe was a double-stranded DNA labeled by random priming, using an oligolabeling kit (Pharmacia). The cDNA fragment used extended from a unique *Bgl*III site (nucleotide 5601) to the 3' end of P1/Mahoney.

RESULTS

Replication of poliovirus RNAs containing P1 deletions. As a first step in identifying genomic sequences required for replication of poliovirus RNA, we asked whether deletion of the P1 region of the genome, known to encode the viral capsid proteins, had an effect on replication. To address this question, in-frame deletions within P1 were introduced into infectious P1/Mahoney cDNA. Three deleted plasmids were constructed, pT7R1, pT7R2, and pT7R3, containing dele-

tions of 2,317, 1,781, and 1,295 base pairs, respectively (Fig. 2).

To determine whether RNAs containing these deletions could replicate in HeLa cells, the plasmids were linearized with *Eco*RI and transcribed in vitro by using T7 RNA polymerase. The resulting transcripts were transfected into HeLa cells, and at various times posttransfection cytoplasmic extracts were prepared and subjected to blot hybridization by the slot blot technique, using a negative-strand RNA probe representing the 3' end of P1/Mahoney. Transcripts from all three deleted plasmids (R1, R2, and R3) replicated in transfected cells, as shown by the increase in transcript levels beginning at 4 h posttransfection (Fig. 3). For comparison, transcripts from the unaltered pT7PV1-5 and virion RNA were transfected into HeLa cells (Fig. 3). Examination of the autoradiograph suggests that efficiency of replication is proportional to the size of the deletion. Quantitation of radioactivity on the filter by scintillation counting indicated that at 8 h posttransfection the ratio of R1, R2, R3, and PV1-5 RNAs was 4:1.9:1.7:1.2. It is not possible to compare replication of these RNAs with that of virion RNA since far less virion RNA was transfected onto cells (300 ng versus 4 to 5 μ g for T7-derived RNAs).

To examine the size of RNAs produced in transfected cells, total cellular RNA prepared at different times posttransfection was subjected to Northern hybridization analysis. In cells transfected with R1 RNA, a transcript of the expected size (5.1 kilobases [kb]) was observed at 4 h posttransfection and increased in abundance dramatically by 8 h posttransfection (Fig. 4). Similar results were observed for cells transfected with R2 and R3, except that the appropriate-size RNAs were detected (data not shown). The nature of the larger RNA species that appeared at 8 h posttransfection is not known, but it is clearly not a covalently linked dimer that has been proposed to be an intermediate in replication (1). Based on these results together with those shown in Fig. 3, it is clear that the 5.1-kb RNA R1 is able to replicate in cells without rearrangement of the RNA, such as further deletions or duplications. Thus, the subgenomic RNAs are replicons that contain all the information required for RNA replication.

Protein synthesis in cells transfected with subgenomic replicons. Polypeptide synthesis in cells transfected with the subgenomic replicon R1 was examined to determine the nature and levels of poliovirus proteins that were synthesized. When cells were labeled with [³⁵S]methionine 4 h after transfection and fractionated by SDS–polyacrylamide gel electrophoresis, it was not possible to detect virus-specific proteins over the background of untransfected-cell polypeptides (results not shown). Therefore, immunoprecipitations were performed using rabbit antiserum directed against a *trpE*-3D^{pol} fusion protein expressed in *E. coli* (see Materials and Methods). In extracts of virus-infected cells, this antiserum detected the polymerase 3D^{pol}, its precursors P3 and 3CD, and alternate cleavage products 3C' and 3D' (Fig. 5, lane 6). In cells transfected with replicon R1, R2, or R3 or with the full-length transcript PV1-5, the polymerase precursor 3CD was detected (Fig. 5, lanes 1 to 3 and 5). Overexposure of Fig. 5 revealed the presence of 3D^{pol}, 3C', and 3D' (results not shown).

The amount of 3CD observed in R1-transfected cells was greater than that found in R2- and R3-transfected cells, and the levels of this precursor in all three replicon-transfected cell groups exceeded that observed in PV1-5-transfected cells. These observations are consistent with the RNA levels shown in Fig. 3.

FIG. 2. Location of deletions in poliovirus cDNAs. The poliovirus processing map is shown at the top. Cleavage sites for mature polypeptides and restriction enzymes correspond to the nucleotide positions in P1/Mahoney RNA (13, 24). Restriction sites used in the constructions, as well as those used to linearize the plasmids for in vitro RNA synthesis were *Eco*RI (E), *Pvu*II (P), *Hind*III (H), *Bgl*II (B), *Nhe*I (N), *Sna*BI (S), and *Nru*I (N). The solid arrowheads on the left represent the T7 promoter.

The kinetics of processing of the P2 region was examined by pulse-chase studies. In these experiments, cells were labeled with [35 S]methionine at 4 h posttransfection and chased with cold methionine for different amounts of time and cell extracts were prepared and immunoprecipitated with either anti-2A^{pro} or anti-2C serum.

Polypeptide 2A^{pro} was identified in virus-infected cells (Fig. 6, lanes 8 to 14) and in R1-transfected cells (lanes 1 to 7) by immunoprecipitation with anti-2A^{pro} serum. It is clear that 2A^{pro} from R1-transfected cells is the same size as its

counterpart in virus-infected cells, and it appears to turn over rapidly in both instances. Apparently the absence of most of the capsid polypeptides did not prevent cleavage of the Tyr-Gly bond between P1 and P2. Anti-2A^{pro} serum also immunoprecipitated other viral proteins from infected and transfected cells, most likely because of the presence of contaminating antigens in the 2A^{pro} preparation used to generate the antiserum (Fig. 6). For example, it was possible to identify capsid polypeptides VP1, VP2, and VP3 in virus-infected extracts (for example, lane 12); as expected, these polypeptides were not present in cells transfected with R1 (lanes 1 to 7). When the same cytoplasmic extracts were immunoprecipitated with anti-2C serum, polypeptide 2C was

FIG. 3. Slot blot analysis of poliovirus RNA in transfected HeLa cells. Cytoplasmic extracts of transfected HeLa cells were denatured with formaldehyde-SSC, slot blotted on nitrocellulose paper, and hybridized with a negative-strand RNA probe representing the 3' end of P1/Mahoney RNA. Extracts were prepared at 2, 4, 6, and 8 h posttransfection from mock-transfected cells or cells transfected with replicon R1, R2, or R3, full-length in vitro transcript PV1-5, or 300 ng of virion RNA (vRNA1).

FIG. 4. Northern blot analysis of RNA from R1-transfected HeLa cells. At 30 min and 1, 2, 4, 6, and 8 h after transfection of HeLa cells with R1, total cellular RNA was prepared, fractionated in a 1% agarose-formaldehyde gel, and blotted onto nitrocellulose. An SP6-transcribed 32 P-labeled negative-strand RNA was used as the probe. Lane M, RNA from mock-transfected cells.

FIG. 5. 3D^{pol}-related polypeptides induced after transfection with subgenomic replicons R1, R2, and R3. Polyacrylamide gel electrophoresis of [³⁵S]methionine-labeled immunoprecipitates from cytoplasmic extracts of transfected HeLa cells was performed. Anti-*trpE*-3D^{pol} antibody was used. Extracts were prepared from cells transfected with R1 (lane 1), R2 (lane 2), or R3 (lane 3), mock-transfected cells (lane 4), PV1-5 RNA-transfected cells (lane 5), and P1/Mahoney-infected cells (lane 6). Lane 7, [³⁵S]methionine-labeled, total cytoplasmic extract of P1/Mahoney-infected HeLa cells.

identified in virus-infected and R1-transfected cells (Fig. 7). As reported previously (3), this antiserum also immunoprecipitates other viral proteins. In contrast to the rapid turnover of 2A^{pro}, 2C and its precursor 2BC were very stable in both virus-infected and R1-transfected cells.

A polypeptide unique to R1-transfected cells was observed in anti-2A^{pro} immunoprecipitates at time zero (Fig. 6,

FIG. 6. 2A^{pro}-related polypeptides in cells transfected with R1. HeLa cells were transfected with R1 or infected with P1/Mahoney (V1), labeled 3 h later with [³⁵S]methionine for 15 min, and chased with cold methionine for 0, 15, 30, 45, 60, and 75 min. Cytoplasmic extracts were prepared, immunoprecipitated with anti-2A^{pro} antibody, and analyzed by SDS-polyacrylamide gel electrophoresis. Lanes 1 to 7, extracts from cells transfected with R1 and chased for the times (minutes) indicated; lanes 8 to 14, virus-infected cells chased for the times (minutes) indicated; lane 15, mock-transfected cells; lane 16, cytoplasmic extract from [³⁵S]methionine-labeled, virus-infected cells. The arrow indicates a polypeptide unique to R1-transfected cells, observed at time zero

FIG. 7. 2C-related polypeptides in cells transfected with R1. The procedures and lanes are the same as those described in the legend to Fig. 6, except that anti-2C antibody was used for immunoprecipitation.

lane 1, arrow). This polypeptide is unstable, since it was not observed after a 15-min chase (lane 2). A polypeptide of similar molecular weight was immunoprecipitated from the same extracts by using anti-VP1 serum (data not shown). These results, coupled with the molecular weight of the polypeptide, suggest that it consists of 105 amino acids of VP1 present in the R1 genome linked to 2AB by the Tyr-Gly cleavage site. Cleavage of this bond by 2A^{pro} liberates 2AB and the VP1 fragment, which was not detected (data not shown).

These experiments indicate that proteolytic cleavages in the polyprotein encoded by R1 appear to be normal. Whether the rates of cleavage were affected (e.g., by the different conformation of precursors caused by the P1 deletion) was not determined. This question could be addressed by comparing R1-transfected and PV1-5-transfected cells; however, the levels of polypeptides in PV1-5-transfected cells were extremely low (Fig. 5, lane 5).

Subgenomic replicons interfere with replication of full-length poliovirus RNA. Naturally occurring poliovirus DI particles, generated after deletions of no more than 20% of the capsid sequences (18), interfere with the replication of standard particles by competing for the cellular machinery and for viral capsids (5, 6). To determine whether R1 could interfere with the replication of wild-type RNA, R1 RNA was cotransfected into HeLa cells with P2/Lansing RNA extracted from virions. Samples of the medium were taken at different times posttransfection, and the amount of infectious virus present was determined by plaque assay. As a control, R1 RNA produced by linearizing the DNA template with *Pvu*II (which cleaves in the carboxy-terminal region of 3D^{pol} [nucleotide 7055]) was cotransfected with P2/Lansing virion RNA. Cotransfection with R1 had only a slight effect on virus yield and the development of cytopathic effect (CPE) (Fig. 8). Unexpectedly, cotransfection with R1-*Pvu*II reduced virus yields by 2 log₁₀ PFU and, furthermore, significantly delayed the development of CPE.

The interference phenomenon was further investigated in a plaque size reduction assay. In this experiment, a fixed amount of type 1 virion RNA (100 pg) was cotransfected with various amounts of subgenomic replicons (10, 5, 2.5,

CPE:

T 2	±	+	++	++++
T 2 + R1	-	±	++	++++
T 2 + R1-PvuII	-	-	-	++

FIG. 8. Effect of cotransfection on growth of poliovirus in HeLa cells. HeLa cells were transfected with 500 ng of poliovirus type 2 virion RNA (T2), cotransfected with 500 ng of poliovirus type 2 virion RNA and one-fifth of a pT7R1 transcription reaction mixture (T2 + R1), or cotransfected with 500 ng of poliovirus type 2 virion RNA and one-fifth of a pT7R1-PvuII transcription reaction mixture (T2 + R1-PvuII). Samples of cell medium were taken at 4, 8, 20, and 30 h posttransfection, and virus titers were determined by plaque assay. CPE was estimated for each time point: —, no change; + + + +, total CPE.

and 1 μ l of a T7 RNA synthesis reaction mixture as described in Materials and Methods) and the cells were incubated under an agar overlay. Plaque size was reduced when 2.5 μ l or more of R1-PvuII RNA was cotransfected (Fig. 9A; compare with the virion RNA control [vRNA1] in Fig. 9B). In contrast, plaque size was reduced only slightly when 10 μ l of R1 RNA was cotransfected (Fig. 9A). RNA produced by linearization of pT7R1 with *Hind*III (which cleaves in the amino-terminal region of 3D^{pol}) or *Bgl*II (which cleaves in 3C^{pro}) did not reduce plaque size in cotransfections (Fig. 9A). Cotransfection with replicons R2 and R3 did not cause plaque size reduction (Fig. 9B); however, R2-PvuII and R3-PvuII displayed the inhibitory phenotype (Fig. 9B). Subgenomic replicons terminating at the *Pvu*II site also reduced the plaque size when cotransfected with P2/Lansing virion RNA (results not shown).

Intracellular RNAs were examined in cotransfected cells to determine the molecular basis for the inhibition of poliovirus replication by R1-PvuII. For this analysis we used a P2/Lansing-specific oligonucleotide (see Materials and Methods) which did not hybridize with poliovirus type 1 RNA. Cytoplasmic extracts were prepared at different times posttransfection, applied to nitrocellulose in a slot blot format, and hybridized to a ³²P-labeled oligonucleotide probe. No hybridization was observed in cytoplasmic extracts from cells transfected with R1 or R1-PvuII alone, whereas replication of P2/Lansing virion RNA (vRNA2) was easily detected (Fig. 10). Replication of type 2 RNA was inhibited 2-fold by cotransfection with R1 and 10-fold by cotransfection with R1-PvuII. In a similar assay, R1-PvuII was shown to interfere with the replication of R1, R2, R3, PV1-5, and P1/Mahoney viral RNA (data not shown). These results indicate that the subgenomic replicons in some way interfere with viral RNA synthesis.

To gain insight into the inhibition phenomenon, we studied in detail RNA and proteins in cells cotransfected with R1-PvuII and P2/Lansing virion RNA. At various times after

transfection, using duplicate cell monolayers, total cellular RNA was prepared for Northern hybridization, and cells were labeled with [³⁵S]methionine for analysis of proteins. A 7.5-kb poliovirus RNA was detected 4 and 8 h after transfection with virion RNA (Fig. 11, lanes 2 and 3). When virion RNA was cotransfected with R1-PvuII, the amount of 7.5-kb RNA was slightly reduced at 4 h posttransfection and more reduced at 8 h (lanes 5 and 6). In cells transfected with R1-PvuII only, a faint smear beginning at 5.1 kb and extending toward the bottom of the gel was detected (lanes 7 to 12; lanes 10 to 12 are a long exposure of lanes 7 to 9) which probably represents input RNA.

Immunoprecipitation analysis of [³⁵S]methionine-labeled extracts with anti-*trpE*-3D^{pol} serum revealed the presence of the precursor 3CD at 4 and 8 h after transfection with type 2 virion RNA (Fig. 12, lanes 4 and 5). When virion RNA was cotransfected with R1-PvuII, the amount of 3CD observed was similar at 4 h but clearly reduced at 8 h posttransfection (lanes 7 and 8). An additional replicase-related polypeptide slightly smaller than 3CD was observed in cotransfected cells (lane 8, arrowhead). This polypeptide might be the 3CD polypeptide encoded by the R1-PvuII genome, which is expected to lack 105 C-terminal amino acids. In support of this conclusion is the observation of a similar polypeptide 8 h after transfection with R1-PvuII only (lane 11 arrowhead).

A curious observation was the presence of a 45,000-dalton 3D^{pol}-related polypeptide in all cells 2 h after transfection (Fig. 12, lanes 3, 6, and 9). A polypeptide of this size has been observed during *in vitro* translation of poliovirus RNA in reticulocyte lysates and has been identified as a product of internal initiation (8). It is not known whether the polypeptide observed here originates from internal initiation. It should be noted, however, that synthesis of the 45,000-dalton polypeptide is not associated with the inhibitory phenotype, because the polypeptide was also present in HeLa extracts transfected only with poliovirus virion RNA (lane 3).

DISCUSSION

Early studies indicated that the genome of poliovirus DI particles may contain a maximum deletion of 15 to 20% of the viral genome (7, 18). Analysis of DI-particle RNA structure showed that the deletions were located in the area of the genome encoding the capsid polypeptides, approximately 1,300 to 3,100 bases from the 5' end of the viral RNA (21). Sequence analysis of DI-particle RNA enabled precise location of the deletions, which occurred between nucleotides 1226 and 2705, with a size distribution of 4.2 to 13.2% (15). Here we show that a subgenomic replicon containing a deletion of nearly the entire P1 region (nucleotide 747 to nucleotide 3065), comprising 31.2% of the genome, can replicate in HeLa cells. Why then are the extent and location of P1 deletions restricted in naturally occurring DI-particle RNA? One possibility is that for the DI-particle RNAs to be encapsidated into virions, a specific packaging signal in the P1 region must be conserved. Alternately, there may be a minimum amount of viral RNA that can be packaged into the poliovirus capsid. To address these possibilities, it will be necessary to determine whether the replicons reported here can be packaged into virions.

When R1 RNA and virion RNA were mixed and transfected into HeLa cells, replication of the virion RNA was inhibited. More surprising was the finding that an R1 transcript truncated at a *Pvu*II site within 3D^{pol} was able to interfere significantly with wild-type viral replication. R1-

FIG. 9. Plaque size reduction assay. HeLa cells were cotransfected with poliovirus type 1 virion RNA (100 pg) and different amounts of a T7 RNA transcription reaction mixture (10, 5, 2.5, or 1 μ l). (A) R1, R1-PvuII, R1-HindIII, and R1-BglII were produced by transcription of pT7R1 linearized with *Eco*RI, *Pvu*II, *Hind*III, or *Bgl*II, respectively. (B) R2 and R2-PvuII were produced by transcription of pT7R2 linearized with *Eco*RI or *Pvu*II, respectively; R3 and R3-PvuII were produced by transcription of pT7R3 linearized with *Eco*RI or *Pvu*II, respectively. Mock-transfected cells or cells transfected with 100 pg of poliovirus type 1 virion RNA (vRNA1) are also shown.

FIG. 10. Inhibition of poliovirus type 2 RNA replication. HeLa cells were transfected with poliovirus type 2 virion RNA (vRNA2), R1, or R1-PvuII, mock transfected (M), or cotransfected with type 2 poliovirus virion RNA and R1 (vRNA2 + R1) or R1-PvuII (vRNA2 + R1-PvuII). Cytoplasmic extracts were prepared at 2, 4, 6, and 8 h posttransfection, denatured with formaldehyde-SSC, slot blotted onto nitrocellulose paper, and hybridized with a type 2-specific oligonucleotide.

PvuII RNA inhibited the replication of type 1 and type 2 poliovirus, as well as that of the three subgenomic replicons R1, R2, and R3. The ability of R1-PvuII to inhibit viral RNA and protein synthesis during a single round of replication indicates that transfected cells had taken up both RNAs. Inhibition of viral replication did not occur at the level of

FIG. 11. Northern blot analysis of RNAs in cotransfected HeLa cells. HeLa cells were transfected with different RNAs, and at 2, 4, and 8 h posttransfection total-cell RNA was prepared and subjected to Northern blot analysis, using a DNA probe representing the 3' end of poliovirus virion RNA. Lanes 10 to 12 are a long exposure of lanes 7 to 9. Lane 13, Mock-transfected cell RNA. Lanes 14 and 15, ^{32}P -labeled transcripts of R1-PvuII and PV1-5. vRNA2, P2/Lansing virion RNA.

FIG. 12. Analysis of polypeptides in cotransfected HeLa cells. HeLa cells were transfected with different RNAs, and at 2, 4, and 8 h posttransfection cells were labeled with [^{35}S]methionine and cytoplasmic extracts were prepared and immunoprecipitated with anti-*trpE*-3D^{pol} serum. Immunoprecipitates were fractionated by electrophoresis on SDS-polyacrylamide gels. Lane 1, Uninfected cell lysate without immunoprecipitation; lane 2, immunoprecipitate of uninfected cells; lanes 3 to 11, extracts from transfected cells; lanes 12 and 13, immunoprecipitates of type 1- and type 2-virus-infected extracts, respectively. The arrowheads are explained in the text.

entry of RNA into the cell. This conclusion is supported by the observation that there was no reduction in the size and number of plaques on cells cotransfected with virion RNA and up to 10 μl of R1-HindIII or R1-BglIII (Fig. 9). In cotransfection experiments, viral RNA synthesis was inhibited beginning at 6 h posttransfection, and protein synthesis was reduced 2 h later. These results suggest that the inhibition occurs at late stages in the viral replication cycle.

What is the mechanism of inhibition of poliovirus replication by R1-PvuII? One possibility is that the deleted replicase and/or its precursors are potent inhibitors because they interact strongly with viral RNA or host factors and prevent interaction with the wild-type polymerase. This possibility seems unlikely because even if truncated 3CD (Fig. 12, lanes 8 and 11) is produced, it is made in small amounts at 8 h postinfection, by which time there is significant inhibition of viral RNA and protein synthesis. A second possibility is that the PvuII end of the interfering RNAs represents a strong replicase-binding site that sequesters the active replicase, other viral proteins, or host factors. Such a mechanism might explain our observation that inhibition was caused specifically by R1 RNAs ending at the PvuII site in 3D^{pol} and not by RNAs terminating at other sites in the 3' end of the genome. However, there is no evidence that R1-PvuII is able to replicate in transfected cells, and therefore the inhibition would have to be mediated by input R1-PvuII RNAs. Finally, it is possible that the R1-PvuII RNA, by virtue of its structure, induces a cellular antiviral activity. It is not likely that interferon plays a role in the inhibition for the following

reasons. (i) Wild-type poliovirus is not only a poor inducer of interferon (11) but is relatively resistant to this antiviral agent (unpublished results). (ii) The *trans*-acting inhibition was observed in cotransfected cells during the first round of replication (Fig. 10 to 12). It is possible, however, that interferon acts in subsequent infection cycles in cotransfected cells, contributing to the delay in the appearance of CPE and to the reduction of viral titers and plaque size (Fig. 8 and 9). (iii) The *trans*-acting inhibition is sequence dependent, because it was induced by R1-PvuII but not by R1-HindIII and R1-BglII, which differ at their 3' ends. R1-PvuII may activate a specific nuclease in transfected cells that degrades viral RNAs. Posttranscriptional regulation of gene expression by control of mRNA stability has been demonstrated for certain lymphokines, cytokines, and proto-oncogenes (9, 14, 27) and seems to be a general regulatory mechanism. A labile nuclease, different from the well-characterized RNase L activated in interferon-treated cells, has been suggested to mediate the specific mRNA shutoff (28). Further investigation of the mechanism of R1-PvuII inhibition may distinguish between these possibilities and elucidate processes that are masked in wild-type-virus-infected cells.

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