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In Vitro Construction of Poliovirus Defective Interfering Particles

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To construct poliovirus defective interfering (DI) particles in vitro, we synthesized an RNA from a cloned poliovirus cDNA, pSM1(T7)1, which carried a deletion in the genome region corresponding to nucleotide positions 1663 to 2478 encoding viral capsid proteins, by using bacteriophage T7 RNA polymerase. The RNA was designed to retain the correct reading frame in nucleotide sequence downstream of the deletion. HeLa S3 monolayer cells were transfected with the deletion RNA and then superinfected with standard virus as a helper. The DI RNA was observed in the infected cells after three passages at high multiplicity of infection. The sequence analysis of RNA extracted from the purified DI particle clearly showed that this DI RNA had the same deletion in size and location as that in the RNA used for the transfection. Thus, we succeeded in construction of a poliovirus DI particle in vitro. To gain insight into the mechanism for DI generation, we constructed poliovirus cDNAs pSM1(T7)1a and pSM1(T7)1b that, in addition to the same deletion as that in pSM1(T7)1, had insertion sequences of 4 bases and 12 bases, respectively, at the corresponding nucleotide position, 2978. The RNA transcribed from pSM1(T7)1a was not a template for synthesis of poliovirus nonstructural proteins and therefore was inactive as an RNA replicon. On the other hand, the RNA from pSM1(T7)1b replicated properly in the transfected cells. Superinfection of the transfected cells with standard virus resulted in production of DI particles derived from pSM1(T7)1b and not from pSM1(T7)1a. These observations indicate that deletion RNAs that are inactive replicons have little or no possibility of being genomes of DI particles, suggesting the existence of a nonstructural protein(s) that has an inclination to function as a *cis*-acting protein(s). The method described here will provide a useful technique to investigate genetic information essential for poliovirus replication.

The poliovirus genome is an approximately 7.5-kilobase single-stranded RNA with positive polarity (13). The genome is polyadenylated at the 3′ terminus (28) and covalently attached to the virus-coded protein called VPg at the 5′ terminus (27). The virion RNA functions as mRNA after entering the cytoplasm of cells. The mRNA carries a single long open reading frame for the synthesis of virus-specific precursor polyprotein. The three domains P1, P2, and P3 exist in the precursor polyprotein. P1 is the precursor region for virus-specific capsid proteins, and P2 and P3 are regions for viral nonstructural proteins. A large single precursor polyprotein with a molecular weight of 247,000 is cotranslationally cleaved by proteases to form virus-specific proteins (12).

Cole et al. (5) reported the generation of defective interfering (DI) particles in a preparation of the Mahoney strain of type 1 poliovirus by serial passages at high multiplicity of infection. The purified DI particles can infect cells and can initiate the early cycle of replication but cannot synthesize the viral capsid proteins (2–4). Kajigaya et al. (9) observed the generation of DI particles from the Sabin strain of poliovirus type 1 and reported that the DI RNAs lack genome regions between nucleotide positions 1307 and 2630, a genome region encoding viral capsid proteins. The results were compatible with those obtained by the various studies on mapping of deletion regions in the DI genomes of the Mahoney strain of poliovirus type 1 (17, 20). Detailed structural studies of the cloned cDNAs of these DI genomes were performed by Kuge et al. (16). They observed that every DI genome retained the corrected reading frame for viral protein synthesis. These results strongly suggested that one or all of the viral nonstructural proteins were *cis* acting, at least at a certain stage in viral replication. Thus, DI particles are very useful tools to investigate mechanisms of viral replication. However, it has been impossible to study the biological characterization of individual DI particles because naturally occurring DI particles are mixtures of a huge number of different DI particles. Attempts to obtain a single species of poliovirus DI particle by plaque purification have so far been unsuccessful (S. Kajigaya and A. Nomoto, unpublished data).

Recently, van der Werf et al. (26) reported the synthesis of a large amount of infectious poliovirus RNA by using purified T7 RNA polymerase. The specific infectivity of the synthesized RNA is approximately 10^5 PFU/μg of RNA in HeLa cells. This infectivity is almost 100 times higher than that of the most efficient infectious cDNA clone of poliovirus (15). Availability of synthesized RNA of high specific infectivity may make it possible to construct mutant polioviruses with low viability. It is also possible to examine the ability of an RNA to act as a replicon by measuring the amount of RNA replicated in the transfected cells even if the RNA cannot produce infectious particles. In fact, Kaplan and Racaniello (10) produced poliovirus RNA containing in-frame deletions within the capsid-coding region to show that the entire capsid-coding sequence of the poliovirus genome is not required for translation or RNA replication. We took advantage of the RNA synthesized in vitro to construct artificial poliovirus DI particles to study poliovirus replication.

Here, we report successful recovery of DI particles from cells transfected with RNAs containing in-frame deletions in the viral capsid-coding region by superinfection with a helper virus. This method provides a new way to identify genome regions required for production of poliovirion parti-

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cles as well as to investigate biological characteristics of individual poliovirus DI particles.

MATERIALS AND METHODS

Construction of plasmids. Infectious cDNA clones of the virulent Mahoney and attenuated Sabin strains of type 1 poliovirus are designated as pVM(1)pDS306(T) (11, 14, 22) and pVS(1)IC-0(T) (15), respectively; the vector plasmid, pSVA14, contains the replication origin, a promoter, and a coding sequence for the large T antigen of simian virus 40 (8). The ϕ10 promoter for T7 RNA polymerase was inserted just before the poliovirus cDNA sequence as previously described (8). Plasmids containing the promoter sequence derived from pVM(1)pDS306(T) and pVS(1)IC-0(T) were designated pVMT7(1)DS(T) and pVST7(1)0(T), respectively (8). In this work, plasmids pVMT7(1)DS(T) and pVST7(1)0 (T) were renamed pM1(T7)0 and pS1(T7)0, respectively.

A recombinant plasmid, pSM1(T7)0, was constructed by the replacement of the shorter *Ban*II fragment of pM1(T7)0 by the corresponding *Ban*II fragment of pVS(1)IC-0(T). To construct a deletion plasmid, pSM1(T7)1, we used plasmid pVS(1)DI-213, which contained a deletion sequence of the genome of a naturally occurring poliovirus DI particle. The strategies for the construction of pSM1(T7)0 and pSM1(T7)1 are shown in Fig. 1. An insertion sequence of 4 or 12 bases at the corresponding nucleotide position 2978 of plasmid pSM1(T7)1 was introduced as follows. The *Ava*I cleavage site at position 2978 was cleaved with *Ava*I, treated with *Escherichia coli* DNA polymerase I (Klenow fragment) to fill in the protruding ends, and rejoined with or without the insertion sequence of a *Sac*I linker (CGAGCTCG). The derivatives of plasmid pSM1(T7)1 carrying insertion sequences of 4 and 12 bases were designated pSM1(T7)1a and pSM1(T7)1b, respectively.

In vitro transcription. Template DNAs were linearized by designation with *Eco*RI or *Pvu*I. The digests were treated with 100 μg of proteinase K per ml at 37°C for 30 min and then with phenol-chloroform (1:1, vol/vol) once. DNAs were precipitated with ethanol and used for in vitro transcription as templates. Linearized DNAs (1 μg) were incubated in 25 μl of reaction solution containing 20 mM Tris hydrochloride (pH 8.0), 40 mM $MgCl_2$, 10 mM spermidine, 25 mM NaCl, 0.4 mM each ribonucleoside triphosphate, 30 mM dithiothreitol, 1 U of RNase inhibitor (Takara Shuzo Co.), and 10 to 15 U of T7 RNA polymerase at 37°C for 30 min. The RNA transcripts were examined by electrophoresis in a 0.8% agarose gel under denaturing conditions with formamide and Formalin (18) and used for the transfection of HeLa S3 monolayer cells.

T7 RNA polymerase was prepared from *E. coli* BL21 carrying plasmid pAR1219 (6, 8, 19, 24), kindly supplied by William Studier and John Dunn. In plasmid pAR1219, bacteriophage T7 gene 1 (RNA polymerase) is designed to be expressed under control of the *lacUV5* promoter. The enzyme, after induction by treatment of BL21(pAR1219) with isopropyl-β-D-thiogalactopyranoside, were purified from the cell lysate by fractionation with Polymin P and ammonium sulfate and then by chromatography on S-Sepharose (Pharmacia, Uppsala, Sweden) and CM-Toyopearl 650M and DEAE-Toyopearl 650M (Seikagaku Kogyo Co., Osaka, Japan) columns. A complete description of this procedure was personally communicated by J. J. Dunn.

Transfection. The closed circular form of plasmid DNA was transfected to HeLa S3 monolayer cells by the calcium phosphate precipitation method as described previously (7).

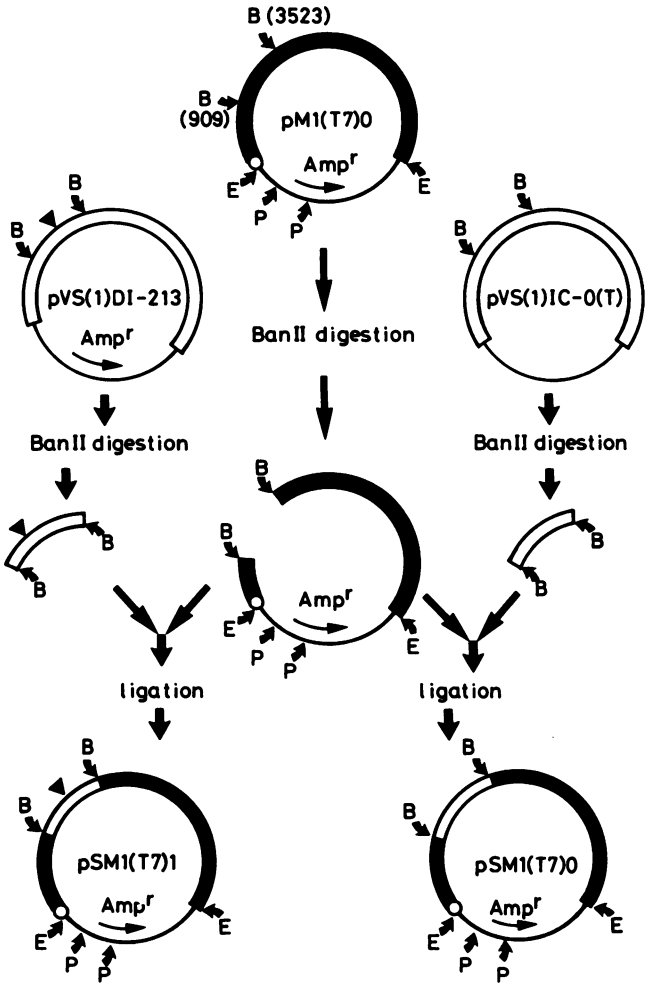

FIG. 1. Strategies for constructing recombinant cDNA clones between the Mahoney and Sabin 1 strains. A recombinant cDNA clone, pSM1(T7)0, was constructed by assembling segments of cDNAs from the Mahoney (■) and Sabin 1 (□) strains. Sequences derived from plasmid vectors including pBR322, pUC19, and pSVA14 are indicated by lines. Deletion sites on plasmid pVS(1)DI-213 are indicated by solid triangles. B, E, and P represent cleavage sites of the restriction enzymes *Ban*II, *Eco*RI, and *Pvu*I, respectively. ○, Positions of the ϕ10 promoter for T7 RNA polymerase.

The DEAE-dextran method of RNA transfection was performed by the method described by van der Werf et al. (26).

Cells and viruses. Suspension-cultured HeLa S3 cells (3×10^6 cells) were plated in a 60-mm plastic tissue culture dish to measure the specific infectivities of poliovirus cDNAs and RNAs 1 day before the transfection. The cells transfected with cDNAs and RNAs were cultured at 35.5°C for 4 and 3 days, respectively, under 1% agarose overlays containing Dulbecco modified Eagle medium supplemented with 5% newborn calf serum. Plaques were visualized after the incubation by staining cells with crystal violet, and the specific infectivities were calculated on the basis of the numbers of plaques (11). To recover DI particles, we superinfected the transfected cells with the Mahoney strain of poliovirus type 1 2 h after the transfection at a multiplicity of infection of 3. Serial passages to amplify DI particles were carried out at multiplicities of infection of 10 to 100. Stock viruses including the Mahoney strain, the Sabin 1 strain, and DI45, a

TABLE 1. Infectivity of poliovirus genomes[a]

Template DNA	Infectivity (PFU/μg)
pM1(T7)0-*Eco*RI[b]	1×10^5 to 3×10^5
pS1(T7)0-*Eco*RI	1×10^5 to 3×10^5
pSM1(T7)0-*Eco*RI	1×10^5 to 3×10^5
pSM1(T7)1-*Eco*RI	0

[a] The input genome was synthesized RNA.
[b] Plasmid pM1 (T7) 0 was linearized by digestion with *Eco*RI and then used as a template in in vitro transcription.

mixture of the Sabin virus and its DI particles, were prepared in suspension-cultured HeLa S3 cells as previously described (9, 16).

Viruses were purified from cytoplasmic extracts of the infected HeLa S3 cells. The cells were disrupted in an RSB plus Mg^{2+} buffer containing 10 mM Tris hydrochloride (pH 7.4), 10 mM NaCl, and 1.5 mM $MgCl_2$ by 10 gentle strokes in a Dounce homogenizer and then centrifuged at $1,000 \times g$ for 10 min to remove cell debris. The supernatant was adjusted to final concentrations of 0.3 NaCl, 10 mM EDTA, and 2% sarcosyl and then centrifuged at 35,000 rpm for 3 h at 20°C in a Spinco type 45Ti rotor. The pellet was dissolved in 1 ml of 10 mM sodium phosphate buffer (pH 7.0), and the virus solution was subjected to chromatography on a DEAE-Sepharose CL6B column. Viruses in void fractions were further purified by equilibrium centrifugation in a CsCl density gradient (1.33 g/cm^3 at 25°C) at 33,000 rpm for 15 h at 4°C in a Spinco type 50 Ti rotor as described previously (16).

Analysis of viral RNAs. RNAs were prepared from virus-infected HeLa S3 cells 7 to 8 h postinfection as previously described (1, 16). The cells were washed with phosphate-buffered saline and lysed in a Nonidet P-40 solution containing 0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris hydrochloride (pH 7.4), and 0.5% Nonidet P-40. After low-speed centrifugation to remove the nuclear fraction, the supernatant was mixed with an equal volume of a urea solution containing 0.35 M NaCl, 10 mM Tris hydrochloride (pH 7.4), 10 mM EDTA, 1% sodium dodecyl sulfate, and 7 M urea and treated with phenol-chloroform (1:1, vol/vol). RNAs were precipitated from the aqueous phase with ethanol, dissolved in a binding buffer containing 0.5 M NaCl, 10 mM Tris hydrochloride (pH 7.5), 0.5% sodium dodecyl sulfate, and 1 mM EDTA, and applied onto an oligo(dT)-cellulose column. Poly(A)$^+$ RNAs were eluted by a buffer containing 10 mM Tris hydrochloride (pH 7.5), 0.5% sodium dodecyl sulfate, and 1 mM EDTA. RNAs thus obtained were analyzed by 0.8% agarose gel electrophoresis under denaturing conditions as described above followed by staining with ethidium bromide. RNAs used for dot-blot hybridizations were prepared from the transfected HeLa S3 cells 8 h postinfection as previously described (8). Hybridization experiments were performed with nylon membrane filters as previously described (8).

Viral RNAs were also prepared from purified viruses. The viruses purified as described above were dialyzed against 1 liter of STE solution containing 0.1 M NaCl, 10 mM Tris hydrochloride (pH 7.4), and 1 mM EDTA to remove CsCl and treated with phenol-chloroform (1:1, vol/vol) in the presence of 0.5% sodium dodecyl sulfate. Viral RNAs were precipitated with ethanol and analyzed by gel electrophoresis under denaturing conditions as described above. In some cases, nucleotide sequences of the viral RNAs were determined by the method of Sanger et al. (25) with synthetic oligonucleotide primers complementary to nucleotide sequences from positions 2542 to 2571 and from 1767 to 1791 of the type 1 poliovirus genome (21). Avian myeloblastosis virus reverse transcriptase (Seikagaku Kogyo Co., Tokyo, Japan) was used for the chain elongation reaction.

RESULTS

Infectivity of poliovirus genomes. A plasmid containing the entire cDNA sequence of poliovirus type 1 (Mahoney strain) under control of the ϕ10 promoter for T7 RNA polymerase was constructed by van der Werf et al. (26). The RNA transcribed from the linearized plasmid contains the entire poliovirus genome with two extra guanine residues and a vector-derived sequence at the 5′ and 3′ ends of the poliovirus RNA sequence, respectively, and shows a specific infectivity for HeLa S3 cells of approximately 10^5 PFU/μg of RNA. Recently, we also constructed similar plasmids named pM1(T7)0 and pS1(T7)0 that had cDNAs derived from the genomes of the Mahoney and Sabin 1 strains of poliovirus type 1, respectively, as previously described (8). The plasmids pM1(T7)0 and pS1(T7)0 were linearized by digestion with *Eco*RI to prepare templates for in vitro transcription by T7 RNA polymerase as described in Materials and Methods.

To test the infectivity of these transcripts, we transfected HeLa cells with the RNA by the DEAE-dextran method as described in Materials and Methods. The RNAs transcribed

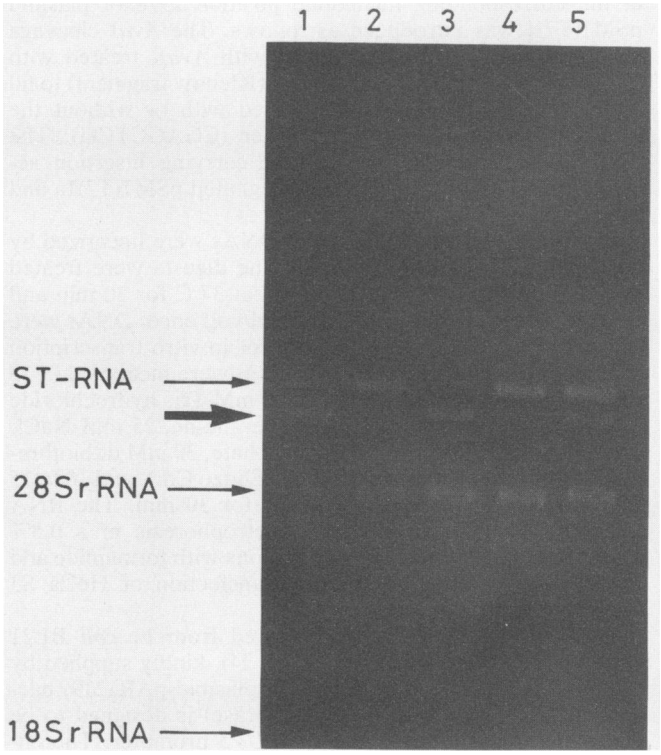

FIG. 2. Analysis of RNAs in virus-infected cells. HeLa S3 cells (2×10^5 cells) were transfected with 1 μg (lanes 2 and 4) or 5 μg (lanes 3 and 5) of RNA synthesized from plasmid pSM1(T7)1 linearized by *Eco*RI (lanes 2 and 3) or *Pvu*I (lanes 4 and 5) and then superinfected with the standard virus as described in Materials and Methods. Poly(A)$^+$ RNAs were prepared from the infected cells at passage 5 and separated by 0.8% agarose gel electrophoresis as described in Materials and Methods. RNAs extracted from DI45 are shown in lane 1 as markers. Positions of the ST RNA, 28S rRNA, and 18S rRNA are indicated, and the position of DI RNA is shown by a thick arrow. After electrophoresis, RNAs were visualized by staining with ethidium bromide.

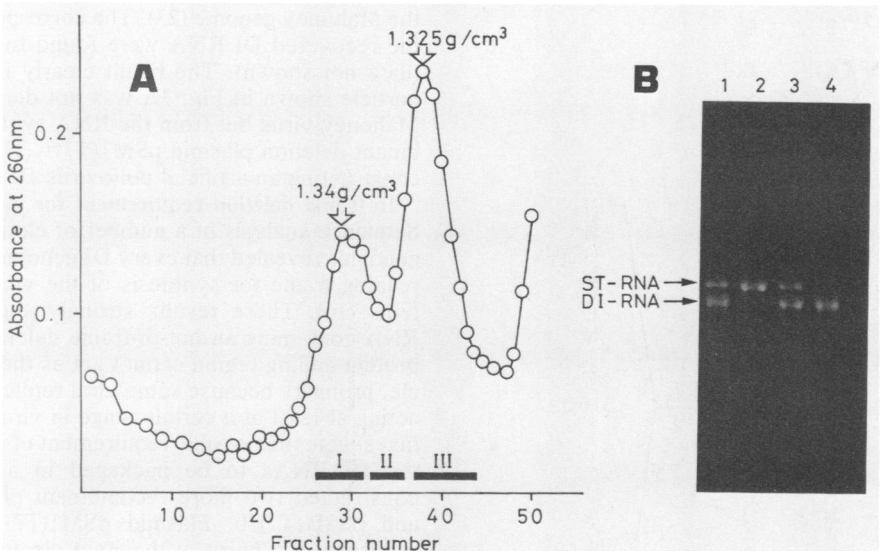

FIG. 3. Identification of an artificial DI particle. (A) Profile of CsCl equilibrium density gradient of viruses of passage 6. Virus particles were purified and analyzed as described in Materials and Methods. The gradient was fractionated from bottom to top. The A_{260} of each fraction was measured with a spectrophotometer. Densities of peaks are indicated by open arrows. Fractions indicated by bold bars were pooled and named fraction I (fractions 25 to 31), fraction II (fractions 32 to 36), and fraction III (fractions 37 to 44). (B) RNAs extracted from DI45 (lane 1), fraction I (lane 2), fraction II (lane 3), and fraction III (lane 4) were subjected to 0.8% agarose gel electrophoresis as described in Materials and Methods.

from pM1(T7)0 and pS1(T7)0 with *Eco*RI ends had specific infectivities of 1×10^5 to 3×10^5 PFU/μg of RNA (Table 1), whereas plasmid DNA itself had a specific infectivity of approximately 1×10^3 PFU/μg of DNA (data not shown) (see reference 15). These results were compatible with the previous observations reported by van der Werf et al. (26). The RNAs produced from pM1(T7)0 and pS1(T7)0 with *Pvu*I ends also showed specific infectivity similar to that of RNAs from the plasmids with *Eco*RI ends (data not shown).

Recombinant cDNA containing a deletion within the P1 region. Kuge et al. (16) have isolated a number of cloned cDNAs for the DI genomes spontaneously generated by serial passages of the Sabin strain of type 1 poliovirus. Of these, pVS(1)DI-213 contains a deletion of 816 bases from the corresponding nucleotide positions 1663 to 2478, which encode parts of capsid proteins VP2 and VP3 (16). Digestion of this plasmid with *Ban*II generated a DNA fragment of about 1.8 kilobases containing the deletion site. The shorter *Ban*II DNA fragment of plasmid pM1(T7)0 was replaced by this fragment. The resultant recombinant plasmid was named pSM1(T7)1. A recombinant plasmid pSM1(T7)0 which had no deletion sequence was also constructed by replacing the *Ban*II fragment of pM1(T7)0 by the corresponding fragment of pVS(1)IC-0(T). Strategies for the construction of these recombinant plasmids are shown in Fig. 1, and structures of these cDNAs are shown in Fig. 5. RNA synthesized from pSM1(T7)0 showed a high infectivity similar to that of RNA derived from parental pM1(T7)0 or pS1(T7)0 (Table 1). However, no infectivity was detected by the RNA transcribed from pSM1(T7)1, because this genome lacked the information for parts of viral capsid proteins VP2 and VP3 (Table 1).

Generation of DI particle. To generate the DI particle derived from an RNA synthesized in vitro, we transfected semiconfluent HeLa S3 monolayer cells with 1 to 5 μg of RNA which had been transcribed from pSM1(T7)1 linearized by digestion with either *Eco*RI or *Pvu*I. After the transfection, the Mahoney Strain of poliovirus type 1 was superinfected as described in Materials and Methods. Viruses produced in the cells were passaged to amplify the DI particles that were expected to exist. To determine the presence of the DI RNA in the infected cells, poly(A)$^+$ cellular RNA of the infected cells (4×10^7 cells) at passage 5 was isolated and subjected to gel electrophoresis under denaturing conditions as described in Materials and Methods. RNAs (Fig. 2, thick arrow) that migrated to the position of DI RNA were clearly detected in lanes 2 to 5 of Fig. 2. Northern (RNA) blot hybridization experiments confirmed that these RNAs contained poliovirus genome sequence (data not shown). The RNAs were visualized at passage 3 by staining with ethidium bromide, although the proportion of DI RNA to the standard virus RNA (ST RNA) was much less than that at passage 5 (data not shown). These observations indicated that the shorter RNA was able to interfere with the replication of ST RNA and therefore that the virus preparation after two passages already contained poliovirus DI particle(s). Considering the efficiency for poliovirus DI generation (5, 9, 17), it is unlikely that the DI particle existing in the passage virus preparation derived from the helper virus used for the superinfection. Indeed, DI RNA was not detected in cells infected with the Mahoney virus that had been serially passaged five times by gel electrophoresis under conditions similar to those described in the legend to Fig. 2 (data not shown).

To confirm that a DI particle(s) existed in the passaged virus preparation, we analyzed the virus preparation of passage 6 by equilibrium centrifugation in a CsCl density gradient as described in Materials and Methods (Fig. 3A). Two peaks at densities of 1.34 and 1.325 g/cm^3 were seen on the gradient and appeared to contain the ST and DI particles (see references 9 and 16). The fractions of different densities were pooled separately into three classes (fractions 25 to 31 [fraction I], 32 to 36 [fraction II], and 37 to 44 [fraction III]). RNAs were extracted from virus particles of each fraction and separated by agarose gel electrophoresis under denaturing conditions as described in Materials and Methods. The

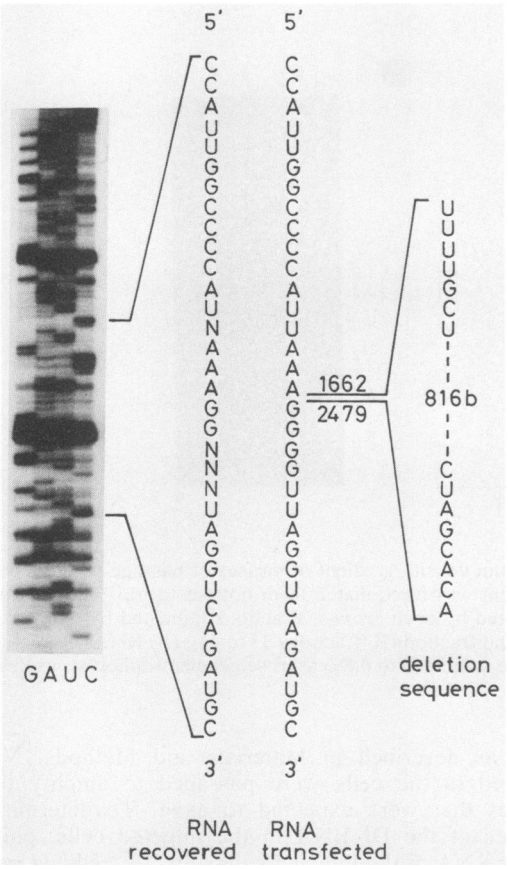

FIG. 4. Nucleotide sequence of genomic RNA. Nucleotide sequence ladders shown on the left side of the figure were obtained by the method described in Materials and Methods. Lanes C, U, A, and G were renamed G, A, U, and C, respectively, in the figure to obtain the nucleotide sequence of the positive-strand polarity. RNA sequences of the rearranged site deduced from DNA sequences of pSM1(T7)0 and pSM1(T7)1 are shown next to the nucleotide sequence of RNA from the recovered DI particle (fraction III in Fig. 3A). Nucleotides that were not identified by the sequence ladders are indicated by N. Numbers represent nucleotide positions of the deletion site.

RNA from fraction I (lane 2) and fraction III (lane 4) migrated to positions of ST RNA and DI RNA, respectively. The data clearly show that a DI particle(s) exists in the passaged isolates.

Sequence analysis of DI RNA. Although the rapid appearance of a DI particle strongly indicated that the DI RNA was derived from the transfected RNA and not from DI particles newly generated from the standard Mahoney virus used as a helper, nucleotide sequence analysis was performed on the DI RNA recovered from the DI particle as described in Materials and Methods. The nucleotide sequence determined in the vicinity of the deletion site is shown in Fig. 4. The deletion observed in the DI RNA recovered from the DI particle was exactly the same as that in the synthesized RNA used for the transfection in regard to its location and size. Furthermore, we examined nucleotide sequences differing between the genomes of the Mahoney and Sabin 1 strains to exclude the possibility that the DI particle obtained was generated from the helper Mahoney virus. Nucleotides at positions 2545 and 2585 have been determined to be guanine residues in the Sabin 1 genome (21) and adenine residues in the Mahoney genome (23). The corresponding nucleotides in the recovered DI RNA were found to be guanine residues (data not shown). The result clearly indicated that the DI particle shown in Fig. 3A was not derived from the helper Mahoney virus but from the RNA synthesized from recombinant deletion plasmid pSM1(T7)1. Thus, we succeeded in constructing an artificial poliovirus DI particle.

In-frame deletion requirement for genome of DI particle. Sequence analysis of a number of cloned cDNAs to the DI genomes revealed that every DI genome retained the correct reading frame for synthesis of the viral nonstructural proteins (16). These results strongly suggest that poliovirus RNA containing an out-of-frame deletion within the capsid protein-coding region cannot act as the genome of DI particle, probably because some viral replication protein(s) is *cis* acting at least at a certain stage in viral replication (16). To investigate the possible requirement of in-frame deletions for the DI RNAs to be packaged in a virion particle, we constructed two more recombinant plasmids, pSM1(T7)1a and pSM1(T7)1b. Plasmid pSM1(T7)1a has an insertion sequence of 4 bases at the *Ava*I cleavage site of the corresponding nucleotide position 2978 in the nucleotide sequence of pSM1(T7)1, and therefore the RNA transcript from pSM1(T7)1a has an incorrect reading frame for viral protein synthesis in the nucleotide sequence downstream of position 2978. Plasmid pSM1(T7)1b has an insertion sequence of 12 bases at the same site as the 4-base insertion in pSM1(T7)1. Thus, the RNA from pSM1(T7)1b has an extra nucleotide sequence encoding four amino acids but the correct reading frame is retained. Structures of the cDNAs are shown in Fig. 5.

RNAs transcribed from *Eco*RI-linearized plasmids pSM1(T7)0, pSM1(T7)1, pSM1(T7)1a, and pSM1(T7)1b were transfected into HeLa S3 cells, and their abilities as RNA replicons were examined by comparing the amounts of RNAs replicated in the transfected cells as described in Materials and Methods (8, 10). Synthesized RNAs replicated well in the transfected cells except for RNA from pSM1(T7)1a (Fig. 5). The results are very reasonable because all viral replication proteins can be produced in the cells transfected with RNAs from pSM1(T7)0, pSM1(T7)1, and pSM1(T7)1b but not in the cells transfected with RNA from pSM1(T7)1a.

To determine whether all the replication proteins are able to act in *trans*, we superinfected the cells transfected with these RNAs with the standard poliovirus. After four serial passages at high multiplicities of infection, cytoplasmic RNAs were examined by 0.8% agarose gel electrophoresis (Fig. 6). The DI RNA derived from plasmid pSM1(T7)1a was not detected on the gel, although the ST RNA was produced well (Fig. 6, lane 3). Other RNAs containing in-frame deletions were easily detected on the gel (Fig. 6, lanes 2 and 4). Sequence analysis of the DI RNA from purified virions derived from plasmid pSM1(T7)1b confirmed that 12 extra bases were correctly inserted at the right position (data not shown), indicating that the recovered DI RNA was derived from pSM1(T7)1b. These observations support our previous notion that at least one replication protein of poliovirus has little or no ability to function in *trans* at a certain stage in viral replication.

DISCUSSION

We succeeded in constructing artificial poliovirus DI particles. Furthermore, it was proved that it was very unlikely or altogether impossible that RNAs containing out-of-frame

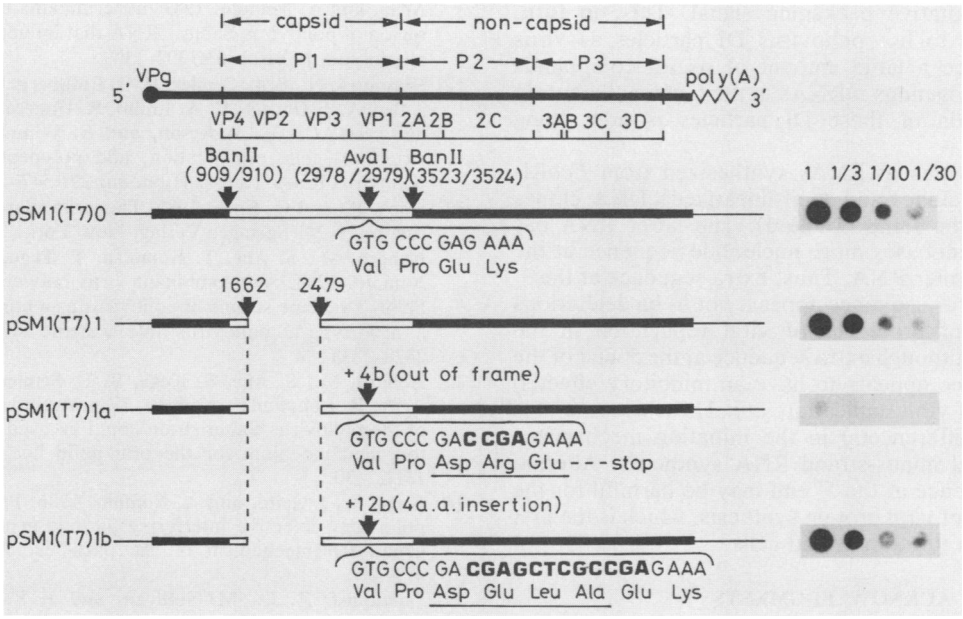

FIG. 5. Genome structures of deletion mutants and their activity as RNA replicons. The genome organization of poliovirus is shown at the top of the figure. VPg is a genome-linked protein attached at the 5′ terminus. Structures of RNA transcripts derived from plasmids indicated on the left of the figure are shown. Closed and open bars represent segments from the Mahoney and Sabin 1 genomes, respectively. Numbers indicated over the RNAs or in parentheses are the corresponding nucleotide positions from the 5′ end of the genome of the Sabin 1 strain (21). Nucleotide sequences at the corresponding *AvaI* site of position 2978 and the deduced amino acid sequences are shown under the RNAs. Results of dot-blot hybridization experiments are shown on the right side of the figure. Hybridization was performed as described in Materials and Methods. Dilutions of the RNA preparations extracted from the transfected cells are indicated by magnification of dilution. a.a., Amino acid.

deletions within the capsid-coding region were the genomes of poliovirus DI particles. A small number of particles carrying RNAs containing out-of-frame deletions may occur in cells transfected with such RNAs by the superinfection of helper viruses. These particles, however, appear to be selected out during serial passages. These results are compatible with our previous assumption that at least one nonstructural protein of poliovirus has a tendency to act in *cis* at a certain stage(s) in viral replication (16). Alternatively, the proper substrate for binding of replicase is a ribosome-associated viral RNA. In this case, the initiation of RNA replication must require concurrent translation of the RNA. In any event, it is now possible to investigate biological characteristics of individual DI particles.

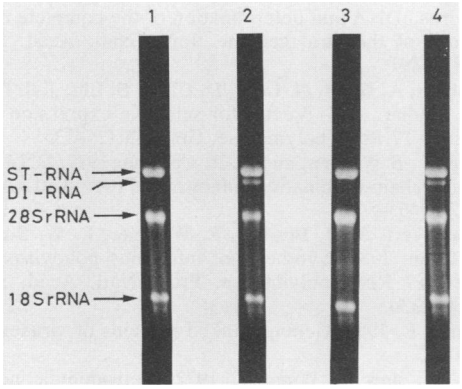

FIG. 6. Requirements of in-frame deletions for DI genomes. HeLa S3 cells (2×10^5 cells) were transfected with 2 μg each of RNAs derived from plasmid pSM1(T7)0 (lane 1), pSM1(T7)1 (lane 2), pSM1(T7)1a (lane 3), and pSM1(T7)1b (lane 4) and then superinfected with the standard virus as described in the legend to Fig. 2. After four serial passages, poly(A)$^+$ RNAs were prepared, separated, and detected as described in the legend to Fig. 2. Positions of ST RNA, DI RNA, 28S rRNA, and 18S rRNA are indicated on the left side of the figure.

Our previous studies on poliovirus DI particle genomes (16) showed that deletions occurred between nucleotide positions 1226 and 2705 with a size distribution of 4.2 to 13.2% and that every DI genome examined carried an intact capsid protein VP4-coding region. Kaplan and Racaniello (10), however, demonstrated that nucleotide sequences encoding almost whole capsid polypeptides were not essential for viral RNA replication in the transfected cells. These results may indicate that varieties in the size and location of deletions are limited if poliovirus RNA is to act as the genomes of the DI particles. Indeed, our preliminary result indicated that synthesized RNAs containing large deletions within the capsid-coding region were not able to be packaged under the conditions described here. Underlying mechanisms for this observation are unclear at present. However, it is possible that a minimum length of RNA is required to be packaged into the poliovirus capsid. Alternatively, a specific packaging signal might exist within nucleotide sequences encoding the viral capsid proteins. We are currently investigating these problems using the experimental system described here. Thus, this method for the construction of artificial poliovirus DI particles is a powerful tool to study mechanisms of poliovirus replication.

It is possible to construct artificial DI particles whose genomes carry foreign mRNAs for nucleotide sequences encoding the viral capsid proteins if the RNAs retain the correct reading frame for synthesis of the viral nonstructural

proteins and a putative packaging signal. This, in turn, makes it possible to use poliovirus DI particles as virus vectors to produce a large amount of useful compounds directed by the exogenous mRNAs in the human alimentary canal. Construction of these DI particles is now being investigated.

Specific infectivities of RNAs synthesized from *Eco*RI-linearized cDNA clones and *Pvu*I-linearized cDNA clones were essentially the same (Table 1). The latter RNA has approximately 4 kilobases more nucleotide sequence at the 3′ end than the former RNA. Thus, extra sequence at the 3′ end of the poliovirus sequence appears not to be deleterious for the subsequent processes of viral replication in the transfected cells, although extra sequence at the 5′ end of the poliovirus sequence appears to have an inhibitory effect(s) on the initiation of viral replication (26). These observations may indicate a difference(s) in the initiation mechanisms between plus- and minus-strand RNA syntheses. Alternatively, extra sequence at the 5′ end may be harmful for the efficient initiation of viral protein synthesis, which is the first event occurring in the transfected cells.

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