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Construction of Viable Deletion and Insertion Mutants of the Sabin Strain of Type 1 Poliovirus: Function of the 5′ Noncoding Sequence in Viral Replication

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A number of deletion and insertion sequences were introduced into the 5′ noncoding sequence (742 nucleotides long) of the genome of the Sabin strain of type 1 poliovirus by using an infectious cDNA clone of the virus strain. The genomes of all three poliovirus serotypes contained highly homologous sequences (nucleotide positions 509 to 639) as well as highly variable sequences (positions 640 to 742) in the 5′ noncoding region. The viability of mutant viruses was tested by transfecting mutant cDNA clones into African green monkey kidney cells and then estimating the plaque sizes displayed on the cells. The results suggested that the highly variable sequence next to the VP4 coding region did not play an important role, at least in the in vitro culture system used, that the loci of highly conserved nucleotide sequences were not always expected to be the genome regions essential for viral replication, that the sequence between positions 564 and 599 carried genetic information to maintain the efficiency of certain steps in viral replication, and that the sequence between positions 551 to 563 might play an essential role in viral replication. Four-base deletion or insertion mutations were introduced into relatively variable sequences in the genome region upstream of position 509. The results suggest that variable sequences do not always indicate that the corresponding genome regions are less important. Apparent revertants (large-plaque variants) were easily generated from one of the viable mutants with the small-plaque phenotype. The determination of nucleotide sequences of the revertant genomes revealed the second mutation site. The results suggested that the different loci at around positions 200 and 500 might specifically interact with each other. This interaction may result in the formation of a functional structure that influences the efficiency of certain steps in the viral replication.

Poliovirus, a member of the *Picornaviridae*, contains a single-stranded RNA genome with positive polarity. The genomic RNA is composed of approximately 7,500 nucleotides to which a genome-linked protein (VPg) is covalently attached at the 5′ terminus (27) and poly(A) is attached at the 3′ terminus (28). This RNA is infectious in mammalian cells regardless of whether VPg is attached at the 5′ terminus (9). In the host cell cytoplasm, the viral RNA is translated into a single continuous polyprotein with a molecular weight of 247,000, and the polyprotein is subsequently cleaved by proteases to form viral structural and nonstructural proteins (4, 5, 25). Studies on the sequences of RNA and of amino acids in viral polypeptides have provided a precise viral protein map. The studies have also revealed a fairly long untranslated region (approximately 750 nucleotides long) at the 5′ terminus of the genome (5).

To date, the total nucleotide sequences of the genomes of both the virulent and attenuated strains of all three poliovirus serotypes have been elucidated (5, 8, 10, 13, 20, 22, 24). A comparative sequence study on the genomes of polioviruses of all three serotypes has been performed by Toyoda et al. (24). The study revealed that the nucleotide sequence in the 5′ noncoding region carried a highly variable sequence at nucleotide positions 640 to 742 and a highly conserved sequence at nucleotide positions 509 to 639 (see Fig. 1, 2, and 5). Although the sequence upstream of nucleotide position 509 showed high homology among viruses of different serotypes, several portions of this genome region were found to be relatively variable. The reasons for the sequence homology and variation are unknown. It is possible that homologous sequences in the untranslated region, which have been conserved through a long evolutionary process, play essential roles in viral replication such as in virus-specific RNA synthesis, virus-specific protein synthesis, and packaging, and that a long variable sequence discovered in the 5′ noncoding region next to the VP4 coding region (nucleotide positions 640 to 742) serves just as a spacer in the genome because of the conservation of the approximate length of the sequence in this locus. Thus, the biological significance of these genome structures in the 5′ noncoding region remains to be elucidated.

Racaniello and Baltimore (14) have shown that a complete, cloned cDNA copy of the genome of the virulent Mahoney strain of type 1 poliovirus is infectious in mammalian cells. A similar clone of high specific infectivity was also constructed by Semler et al. (17). Omata et al. (12) isolated an infectious cDNA clone of the genome of the attenuated Sabin strain of type 1 poliovirus [Sabin 1 strain; PV1(Sab)]. Furthermore, highly infectious cDNA clone of the Sabin 1 strain was reported by Kohara et al. (6), who used a plasmid vector constructed by Semler and Johnson (manuscript in preparation) containing the simian virus 40 origin of replication as well as the promoter and coding region for the simian virus 40 large T antigen. The availability of infectious cDNA clones of polioviruses provided a molecular genetic approach for investigating the relationship between structure and function of the viral genome by using a recombinant

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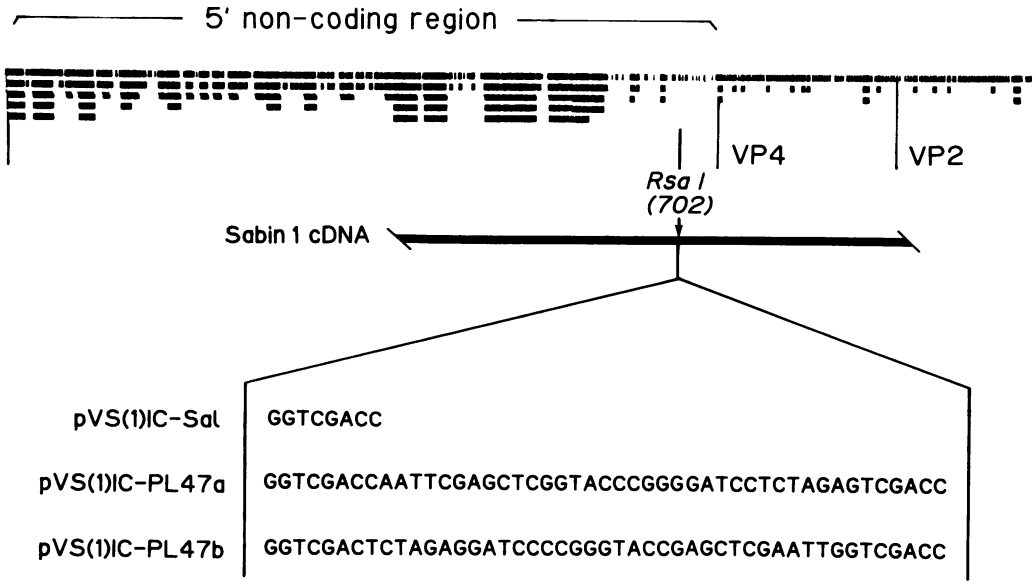

FIG. 1. Sequences of insertion mutant genomes. Sequences of the *Sal*I linker nucleotide and polylinker nucleotide were inserted into the *Rsa*I site at nucleotide position 702 as described in Materials and Methods. Nucleotide sequences inserted are shown with the corresponding plasmids. The insertion sequence of plasmid pVS(1)IC-47b is in the opposite orientation to that of plasmid pVS(1)IC-47a. Nucleotide sequence homology among genomes of various poliovirus serotypes is indicated as black bands at the top of the figure. The bands indicate homologous regions as follows: row 1 (top), more than 1 nucleotide; row 2, more than 3 nucleotides; row 3, 6 nucleotides; row 4, 12 nucleotides; row 5, 18 nucleotides (24).

DNA technology. Thus, it is now possible to modulate nucleotide sequences of poliovirus genomes in vitro. Indeed, intratypic recombinant polioviruses (7, 11) and intertypic recombinant polioviruses (8, 23) have been constructed. Furthermore, Semler et al. (18) reported the in vitro construction of a recombinant virus between poliovirus and coxsackievirus. Viable insertion mutants of the Mahoney strain of type 1 poliovirus in regard to the viral nonstructural protein coding region (2) and the 3' noncoding region (16) have also been constructed. These in vitro-modified viruses were successfully used for molecular genetic analyses of poliovirus replication.

We introduced a number of deletion or insertion mutations into the 5' noncoding sequence of the Sabin 1 strain genome by using a highly infectious cDNA clone of the virus strain, and we tested the mutant viruses for their efficiency of replication. The genetic analysis, including identification of the second mutation sites in the genomes of variants derived from one of the in vitro mutants, provided important information about the function of the 5' noncoding sequence in poliovirus replication.

MATERIALS AND METHODS

DNA procedure. An infectious cDNA clone, pVS(1)IC-O(T), was constructed from a series of cDNA clones of PV1(Sab) (6, 12). Restriction endonucleases, T4 DNA polymerase, and *Sal*I linker nucleotide (GGTCGACC) were purchased from Takara Shuzo Co. (Kyoto, Japan), Toyobo Co. (Osaka, Japan), or Nippongene Co. (Toyama, Japan). Avian myeloblastosis virus reverse transcriptase and human placenta RNase inhibtor were from Seikagaku Kogyo Co. (Osaka, Japan). Calf intestine alkaline phosphatase was from Boehringer (Mannheim, Federal Republic of Germany). Labeled compounds were purchased from Amersham Corp., Arlington Heights, Ill. These enzymes and compounds were used according to the manufacturers' instructions. The modified calcium phosphate method of transfection was performed as described previously (6).

Subcloning of the 5'-proximal sequence of PV1(Sab) genome. To modulate the 5' noncoding sequence of the PV1(Sab) genome, the corresponding sequence of nucleotide positions 1 to 1813 was subcloned into plasmid pML2 as follows. Plasmid pVS(1)IC-O(T) was digested with *Pst*I, treated with T4 DNA polymerase to produce blunt ends at the cleavage sites, and digested with *Eco*RI. The cDNA fragment approximately 1,800 nucleotides long was isolated after the separation of DNA fragments by agarose gel electrophoresis. Plasmid pML2 was digested with *Sal*I, and the protruding ends were filled in by treatment with T4 DNA polymerase. The linearized DNA was digested with *Eco*RI, and the longer DNA fragment was isolated by electrophoresis on agarose gel. Both of the isolated DNA fragments were ligated together to form a plasmid called pEP(Sab), which had a PV1(Sab) genome sequence of nucleotide positions 1 to 1809.

Construction of cDNA mutant with long insertion sequence. A *Sal*I linker nucleotide 8 bases long was inserted into the *Rsa*I cleavage site at the corresponding nucleotide (position 702) as follows. Plasmid pEP(Sab) was digested with *Rsa*I and separated by 5% polyacrylamide gel electrophoresis. The cDNA fragments approximately 450 and 290 nucleotides long were eluted from the gel and ligated with the eight-base *Sal*I linker. The former fragments were digested with *Nco*I and *Sal*I, and the latter was digested with *Ban*II and *Sal*I. The larger DNA fragments (approximately 315 and 210 nucleotides long, respectively) of both digests were isolated by gel electrophoresis as described above. These isolated fragments were ligated with the longer DNA fragment of pEP(Sab) that had been digested with *Nco*I and *Ban*II. The modified pEP(Sab), designated as pEP(Sab)Sal, was digested with *Aat*II, and the shorter DNA fragment was ligated with the longer *Aat*II fragment of pVS(1)IC-O(T). Thus, the eight-base *Sal*I linker nucleotide was inserted into an *Rsa*I

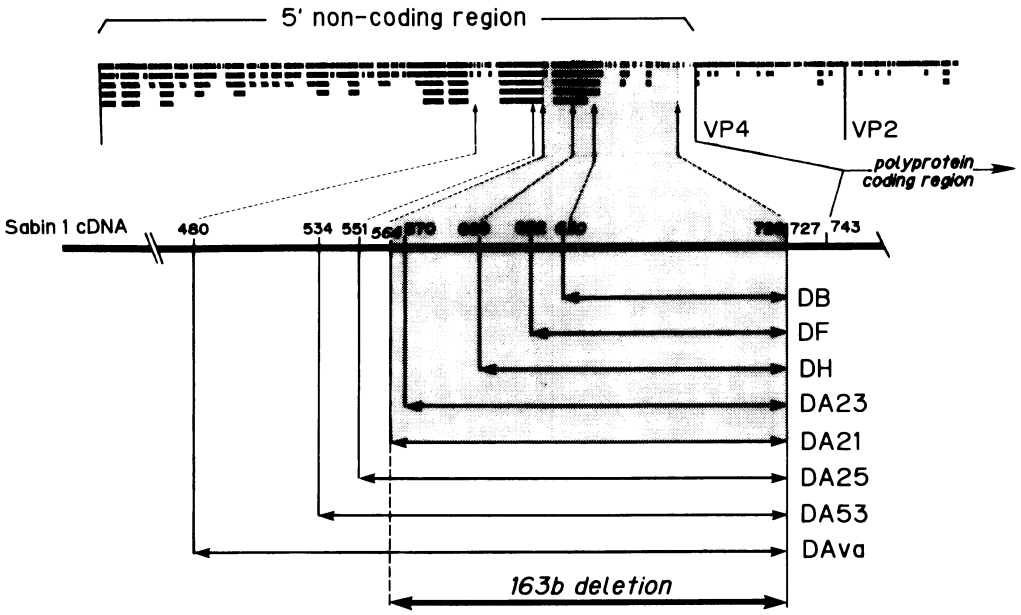

FIG. 2. Viability of deletion mutants. The nucleotide sequence homology among genomes of various poliovirus serotypes is shown as in Fig. 1. Deletion cDNA clones were constructed as described in Materials and Methods. The nomenclature of cDNA clones is shown on the right side of the figure. pVS(1)IC- is omitted from the nomenclature. Deleted sequences of the cDNA clones are indicated by lines with arrowheads on both ends. Viable deletion mutants lacking genome sequences are indicated as shadowed regions. Nucleotide positions are shown by numbers above the Sabin 1 cDNA line.

cleavage site at the corresponding nucleotide position 702 of the PV1(Sab) genome. This plasmid was designated as pVS(1)IC-Sal (Fig. 1).

A longer insertion sequence for the *Sal*I cleavage site of plasmid pVS(1)IC-Sal was prepared from the polylinker region of a cloning vector, pUC18. Plasmid pUC18 was digested with *Eco*RI, the protruding ends were filled in by treatment with T4 DNA polymerase, and the DNA was ligated with *Sal*I linker nucleotides. The modified pUC18 DNA was then digested with *Sal*I, and the 39-base *Sal*I fragment was purified by electrophoresis on a 5% polyacrylamide gel. The *Sal*I DNA fragment eluted from the gel was inserted into the *Sal*I cleavage site of plasmid pVS(1)IC-Sal. The plasmids thus obtained have a total 47-base insertion sequence at the corresponding nucleotide position 702 on the PV1(Sab) genome and were designated as pVS(1)IC-PL47a and pVS(1)IC-PL47b (Fig. 1).

Construction of cDNA mutant with long deleted sequences. Plasmid pEP(Sab)Sal that had been linearized by the digestion with *Sal*I was treated with exonuclease *Bal*31 and ligated with *Sal*I linker nucleotides. The DNAs, after treatment with *Sal*I, were circularized with T4 DNA ligase. One of the plasmid clones thus obtained carried the deleted sequence of nucleotide positions 697 to 726 and was designated as pEP(Sab)D56. On the other hand, plasmid pEP(Sab) was digested with *Bal* 1 at positions 629 and 1227, ligated with *Sal*I linker nucleotides, and then digested with *Pst*I. The DNA fragment approximately 1,400 nucleotides long was isolated by agarose gel electrophoresis. This DNA fragment was used to replace the corresponding *Pst*I-*Sal*I fragment of pEP(Sab)D56, and the resulting plasmid was designated as pEP(Sab)DB. The shorter *Aat*II fragment of pVS(1)IC-O(T) was replaced by the shorter Aat II fragment of pEP(Sab)DB; this plasmid, called pVS(1)IC-DB, lacked the genome region of the corresponding nucleotide positions 630 to 726 (Fig. 2).

Similarly, the *Sal*I linker nucleotide was introduced into the *Fok*I cleavage site at the corresponding nucleotide position 617 or the *Hph*I site at position 599. PV1(Sab) sequences upstream of these newly introduced *Sal*I sites were ligated with PV1(Sab) sequence downstream of the *Sal*I site of pEP(Sab)D56 as described above. Plasmids obtained were designated as pEP(Sab)DF and pEP(Sab)DH, respectively. The shorter *Aat*II fragment of pVS(1)IC-O(T) was then replaced by the corresponding DNA fragment with deletion of pEP(Sab)DF or pEP(Sab)DH as described above. These deletion cDNA mutants were called pVS(1)IC-DF and pVS(1)IC-DH, respectively (Fig. 2). The deleted sequences of pVS(1)IC-DF and pVS(1)IC-DH are the corresponding nucleotide positions 622 to 726 and 600 to 726, respectively (Fig. 2).

After digestion with *Sal*I, plasmid pEP(Sab)DH was treated with *Bal* 31. The *Bal* 31-treated pEP(Sab)DH DNAs were ligated with *Sal*I linker nucleotides and circularized by using the cohesive cleavage site of *Sal*I. PV1(Sab) sequences upstream of the *Sal*I cleavage sites of the deletion plasmids constructed from pEP(Sab)DH were ligated with PV1(Sab) sequence downstream of the *Sal*I cleavage site of pEP(Sab)D56. The deletion plasmids thus obtained were designated as pEP(Sab)DA23, pEP(Sab)DA21, pEP(Sab) DA25, and pEP(Sab)DA53. Similarly, plasmid pEP(Sab) DAva was constructed by using the *Ava*I cleavage site at nucleotide position 476. Replacements of *Aat*II fragments between these deleted subclones and pVS(1)IC-O(T) were carried out as described above to construct pVS(1)IC-O(T) were carried out as described above to construct pVS(1)IC-DA23, pVS(1)IC-DA21, pVS(1)IC-DA25, pVS(1)IC-DA53, and pVS(1)IC-DAva, whose deleted sequences were the corresponding nucleotide positions 570 to 726, 564 to 726, 551 to 726, 534 to 726, and 480 to 726, respectively (Fig. 2).

Construction of cDNA with four-base deletion or insertion sequences. Plasmid pEP(Sab) was linearized by using the

unique cleavage site of *Kpn*I, *Bam*HI, *Nco*I, or *Ava*I, treated with T4 DNA polymerase to modify the terminal structure of DNAs, and circularized with T4 DNA ligase. The plasmid with a modified sequence at the corresponding restriction cleavage site was digested with *Aat*II. The shorter *Aat*II fragment was ligated with the longer *Aat*II fragment of plasmid pVS(1)IC-O(T). Thus, we constructed plasmids pVS(1)IC-ΔK, pVS(1)IC-ΔB, pVS(1)IC-ΔN, and pVS(1)IC-ΔA, which carried a four-base deletion at the *Kpn*I site at position 70, a four-base insertion at the *Bam*HI site at position 220, a four-base insertion at the *Nco*I site at position 388, and a four-base insertion at the *Ava*I site at position 475, respectively (see Fig. 5).

Cells and virus stocks. AGMK cells (African green monkey kidney cells) were maintained in Dulbecco modified Eagle medium supplemented with 5% newborn calf serum. AGMK cells were transfected with 10 μg of closed circular forms of plasmid pVS(1)IC-O(T) or mutant cDNA clones per 60-mm plastic dish, and viruses were recovered from the cells by the modified calcium phosphate method described above. The recovered viruses were used as virus stocks. Usually, titers of the virus stocks were 1×10^8 to 3×10^8 PFU/ml.

Large-plaque variants were prepared by isolating a single large plaque and expanding it on AGMK cells by conventional methods.

Virus infection and growth. To measure virus titers, AGMK monolayer cells in 60-mm plastic dishes were washed with Eagle minimum essential medium twice, covered with 0.5 ml of virus solution diluted from high-titer virus stocks, and kept at room temperature for 1 h. After incubation at 36.5°C for 30 min, the cells were washed with minimum essential medium twice and covered with minimum essential medium containing 1% agarose and 5% newborn calf serum. After 3 to 5 days of incubation at 35.5°C, plaques were visualized by staining cells with crystal violet.

To determine the extent of virus production in AGMK cells, approximately 5×10^6 AGMK cells in a 60-mm plastic dish were infected with viruses at 5 to 10 PFU per cell. After the infection, the cells were washed with ice-cold minimum essential medium five times to remove free viruses, covered with the same medium containing 5% newborn calf serum, and incubated at 35.5°C. The infected cells were frozen at appropriate times, and the virus solution was prepared by freeze-thawing three times followed by centrifugation to remove cell debris. The titers of viruses produced in the infected cells were measured as described above.

Nucleotide sequence analysis. Approximately 4×10^7 AGMK cells were infected with viruses at 10 to 20 PFU per cell and incubated at 33.5°C for 7 to 12 h, when almost all of the cells showed cytopathic effects (CPE). Cytoplasmic RNAs containing poliovirus RNA were prepared from the infected cells by the method of Berk et al. (1). Briefly, the infected cells were harvested with a rubber policeman, washed with ice-cold phosphate-buffered saline three times, and suspended in 2 ml of buffer containing 0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris hydrochloride (pH 7.4), and 0.5% Nonidet P-40. After incubation at 0°C for 5 min followed by centrifugation to remove the nuclear fraction, the supernatant was mixed with an equal volume of urea solution (0.35 M NaCl, 10 mM Tris hydrochloride [pH 7.4], 10 mM EDTA, 7 M urea, 1% sodium dodecyl sulfate). The mixture was extracted with phenol-chloroform three times, and RNAs were precipitated in ethanol. With this procedure, approximately 100 μg of cytoplasmic RNA containing poliovirus RNA was obtained.

A modified dideoxy method was employed for sequencing RNAs. Synthetic DNA primer (2 pmol) was mixed with cytoplasmic RNAs (5 to 10 μg) in 10.5 μl of buffer containing 75 mM Tris hydrochloride (pH 8.3), 12 mM $MgCl_2$, and 25 mM dithiothreitol and incubated at 60°C for 5 min. For DNA synthesis, the primer-RNA solution was added with 2 μl of [α-^{32}P]dCTP (400 Ci/mmol), 0.5 μl of human placenta RNase inhibitor (72 U/μl), and 1.0 μl of avian myeloblastosis virus reverse transcriptase (20 U/μl). A 3.5-μl portion of this mixture was mixed with 2 μl of each dideoxynucleoside mixture containing 0.1 mM each of dGTP, dATP, and dTTP and the appropriate concentrations of dideoxynucleoside triphosphates (0.014 mM ddGTP, 0.01 mM ddATP, 0.006 mM ddTTP, and 0.0005 mM ddCTP for G, A, T, and C reactions, respectively). The reaction for cDNA synthesis was carried out at 42°C for 15 min, and then 1 μl of chase mixture containing 1 mM each of four deoxynucleoside triphosphates was added. The reaction was continued at 42°C for 15 min and stopped by the addition of 6 μl of stop solution containing 95% formamide, 20 mM Tris borate (pH 8.3), 0.03% bromophenol blue, and 0.03% xylene cyanol. The final mixture was heated to 95°C for 3 min, and samples of 1 to 2 μl were applied to sequencing gels (15).

The dideoxy method (15) was also employed to confirm nucleotide sequences of all modified DNAs by using synthetic DNA primers.

RESULTS

Variable sequence at nucleotide positions 640 to 742. A highly variable sequence in the untranslated genome region next to the VP4 coding region has been considered to be a possible spacer of the genome, since the approximate length is conserved among genomes of various poliovirus serotypes (24).

As a first step to investigate the function of this genome region in viral replication, we constructed mutant cDNAs with insertion sequences at the corresponding nucleotide position 702 as described in Materials and Methods, that is, pVS(1)IC-Sal, pVS(1)IC-PL47a, and pVS(1)IC-PL47b (Fig. 1). AGMK cells were transfected with closed circular forms of these plasmids. All of these plasmids were infectious in AGMK cells, and infectious virus particles were produced in the transfected cells. The recovered viruses were designated as PV1(Sab)IC-Sal, PV1(Sab)IC-PL47a, and PV1(Sab)IC-PL47b, respectively (Fig. 1). RNA sequences of the modified genome region were determined as described in Materials and Methods. The RNA in the genome region had the nucleotide sequence which correctly reflected those of the modified cDNAs (data not shown; Fig. 1).

We tested biological characteristics of the recovered viruses with regard to plaque size, the rate by which CPE progressed, and final yields of infectious virus particles from AGMK cells. These phenotypes of the insertion mutants were indistinguishable from those of the parent virus PV1(Sab)IC-O, which was produced in AGMK cells transfected with plasmid pVS(1)IC-O(T) (6, 7). Similar observations were obtained in experiments with in vitro mutants, with a 72-base insertion sequence at nucleotide position 702 of PV1(Sab) genome (S. Kuge, N. Kawamura, and A. Nomoto, manuscript in preparation). These results indicated that the insertion of mutations up to 72 bases long into the highly variable sequence in the 5′ noncoding region of poliovirus genome had no effect on the viability of this virus, suggesting that the conservation of the approximate length of the sequence of this genome region is not important for viral replication, at least in the in vitro culture system used. To

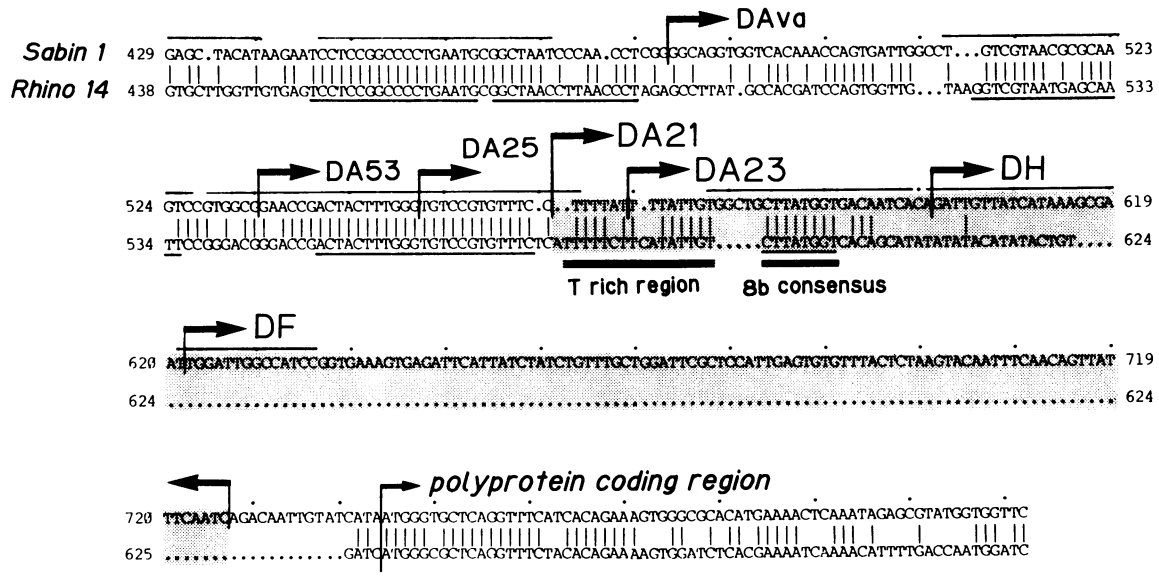

FIG. 3. Nucleotide sequence homology between genomes of PV1(Sab) and human rhinovirus type 14. The nucleotide sequences of PV1(Sab) (10) and human rhinovirus type 14 (3, 21) are aligned to show maximum homology. Lines above the PV1(Sab) sequence or below the human rhinovirus type 14 sequence indicate sequences where more than 7 bases are common among poliovirus serotype genomes or between human rhinovirus type 14 and type 2 (19), respectively. Viable deletion mutants lacking genome sequences are indicated as shadowed regions. PV1(Sab)IC- is omitted as in Fig. 2. Nucleotide positions are indicated by numbers on the right and left sides of the figure.

confirm this assumption, we constructed a mutant cDNA with deletion in this variable genome region, that is, pVS(1)IC-DB (Fig. 2).

AGMK cells were transfected with pVS(1)IC-DB. pVS(1)IC-DB was infectious, and the virus PV1(Sab)IC-DB recovered from the transfected cells also had biological properties in regard to phenotypes described above indistinguishable from those of the parent virus PV1(Sab)IC-O (data not shown). The RNA sequence of the modified genome region of PV1(Sab)IC-DB was determined and found to have the nucleotide sequence expected from cDNA (data not shown).

Thus, neither insertion mutations nor deletion mutations introduced into the highly variable sequence of the 5′ noncoding region appeared to have any effect on the viral replication in the in vitro culture system used. These results suggested that the genome region did not serve even as a spacer of the genome under the experimental conditions used. It may not be surprising that this genome region has no biological function in replication of picornviruses in vitro, since the genomes of rhinovirus belonging to other genera of the *Picornaviridae* lack the 5′ noncoding sequence of the corresponding nucleotide positions 616 to 739 of the PV1(Sab) genome (Fig. 3) (3, 19, 21).

Highly conserved sequence at nucleotide positions 509 to 639. We constructed a number of cDNA mutants with deletions of various sizes as described in Materials and Methods (Fig. 2). AGMK cells were transfected with these deletion cDNA clones to determine whether viable viruses could be generated in the transfected cells. The cells transfected with plasmids pVS(1)IC-DF, pVS(1)IC-DH, pVS(1)IC-23, and pVS(1)IC-DA21 produced infectious virus particles (Fig. 2). The recovered viruses were designated as PV1(Sab)IC-DF, PV1(Sab)IC-DH, PV1(Sab)IC-DA23, and PV1(Sab)IC-DA21, respectively. The results suggested that the highly conserved sequence between nucleotide positions 580 and 639 did not play an essential role in viral replication under the experimental conditions used. Thus, we concluded that high conservation of a nucleotide sequence in the poliovirus genome does not always indicate an essential biological function encoded by the genome locus. On the other hand, AGMK cells transfected with plasmids pVS(1)IC-DA25, pVS(1)IC-DA53, and pVS(1)IC-DAva did not show CPE under liquid medium overlays or plaques under agar overlays. Thus, infectivity was not detectable for these plasmids (Fig. 2). Since approximately 100 plaques were displayed on monolayers of AGMK cells transfected with 10 μg of plasmid pVS(1)IC-O(T) (6), the specific infectivities of these plasmids must be lower than 1% of that of pVS(1)IC-O(T). To exclude the possibility that very low or no infectivity of these plasmids was due to the shorter length of the genome, a polylinker sequence 64 bases long prepared from plasmid vector pUC18 was inserted into the *Sal*I cleavage site of plasmid pVS(1)IC-DA25. This modified plasmid clone did not show any infectivity (data not shown). These results suggested that the highly conserved sequence between nucleotide positions 509 and 563 includes sequences essential for viral replication.

Plaques formed by virus infections with PV1(Sab)IC-DF, PV1(Sab)IC-DH, PV1(Sab)IC-DA23, PV1(Sab)IC-DA21, and the parent virus PV1(Sab)IC-O on monolayers of AGMK cells are shown in Fig. 4. Viruses PV1(Sab)IC-DA23 and PV1(Sab)IC-DA21 formed plaques that were apparently smaller than those of the parent virus PV1(Sab)IC-O, whereas plaques produced by PV1(Sab)IC-DF and PV1(Sab)IC-DH were similar in size to those of virus PV1(Sab)IC-O. The large-plaque phenotype of the latter two viruses indicates that the highly homologous sequence of positions 600 to 639 may not play an important role in viral replication in the cell culture system. It should be noted again that the genome of human rhinovirus lacks the corresponding genome region of positions 616 to 739 of PV1(Sab) genome (Fig. 3). The small-plaque phenotype of the former two deletion mutants could be attributed to lowered efficiencies in certain viral replication steps. Indeed, the generation times of these viruses appeared to be longer than that

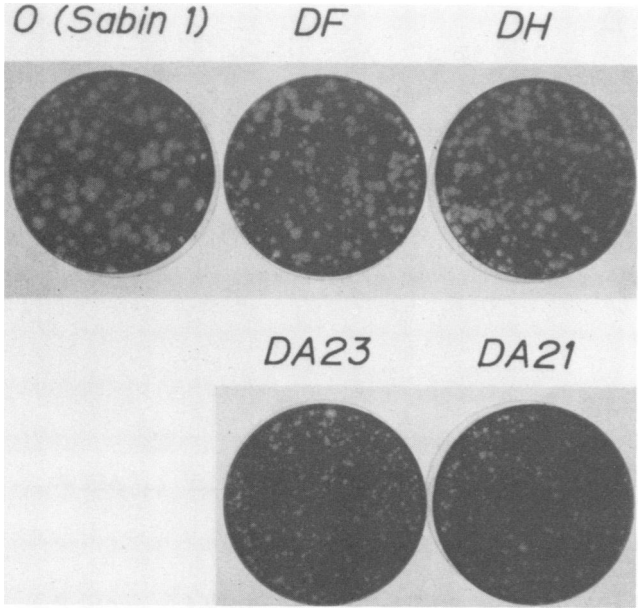

FIG. 4. Plaques of deletion mutants displayed on AGMK cells. After the virus infection, AGMK cells were cultured at 35.5°C for 3 days under agarose overlays and stained as described in Materials and Methods. PV1(Sab)IC- is omitted as in Fig. 2.

of the parent virus (N. Iizuka, S. Kuge, and A. Nomoto, unpublished result). It is therefore of interest to identify the specific sequences contributing to the efficiency of the viral replication.

The nucleotide sequences of positions 429 to 810 of the PV1(Sab) genome (10) and of positions 438 to 696 of the human rhinovirus 14 genome (3, 21) are shown in Fig. 3. In the nucleotide sequence of positions 564 to 599 in the genome of PV1(Sab)IC-DH but missing in the genome of PV1(Sab)IC-DA21, two interesting nucleotide sequences are observed. One is the nucleotide sequence of positions 564 to 577, which is rich in uridine residues and is indicated as the T-rich region in Fig. 3. Many uridine residues are also observed in this region of the genomes of other poliovirus strains (5, 8, 13, 20, 22, 24), rhinoviruses (3, 19, 21), and coxsackieviruses (26; N. Iizuka, S. Kuge, and A. Nomoto, Virology, in press; A. M. Lindberg, P. O. K. Stålhandske, and U. Pettersson, Virology, in press). The interesting nucleotide sequence is at positions 583 to 590, indicated as the 8b consensus in Fig. 3. This sequence is perfectly conserved in this region of the genome of picornaviruses (see above). The biological significances of these nucleotide sequences are unknown. It is possible, however, that at least one of two sequences serves as a signal to enhance the efficiency in certain viral replication steps such as the initiation of viral RNA synthesis or viral protein synthesis and functions associated with assembly.

A fairly long conserved sequence is observed upstream of the T-rich region (Fig. 3). Since no viable virus was recovered from cells transfected with pVS(1)IC-DA25, this conserved sequence may have an essential role in viral replication. The biological function of this genome region also remains obscure.

Relatively variable sequences upstream of nucleotide position 509. The nucleotide sequence upstream of nucleotide position 509 includes several genome regions of relatively variable sequences. To test the biological significances of these genome regions with regard to viral replication, four-base deletion or insertion sequences were introduced into these regions by making use of the restriction cleavage sites of *Kpn*I (nucleotide position 70), *Bam*HI (position 220), *Nco*I (position 388), and *Ava*I (position 475).

Transfection experiments of these modified cDNA clones into AGMK cells were performed as described above. Infectious virus particles were recovered from the cells transfected with pVS(1)IC-ΔB or pVS(1)IC-ΔN, but not from the cells transfected with pVS(1)IC-ΔK or pVS(1)IC-ΔA. These observations indicate that the genome regions of relatively variable sequences do not always suggest that the loci are less important for viral replication. The viruses recovered from cells transfected with pVS(1)IC-ΔB and pVS(1)IC-ΔN were designated as PV1(Sab)IC-ΔB and PV1(Sab)IC-ΔN, respectively (Fig. 5).

The plaques produced by viruses PV1(Sab)IC-ΔB and PV1(Sab)IC-ΔN on AGMK monolayer cells are shown in Fig. 6; both insertion mutants showed smaller plaques than those of the parent PV1(Sab)IC-O, especially in the case of PV1(Sab)IC-ΔB. These observations suggest that the insertion of four bases at the *Bam*HI or the *Nco*I site in the 5′ noncoding sequence of the PV1(Sab) genome results in viral replication with lower efficiency by an unknown mechanism.

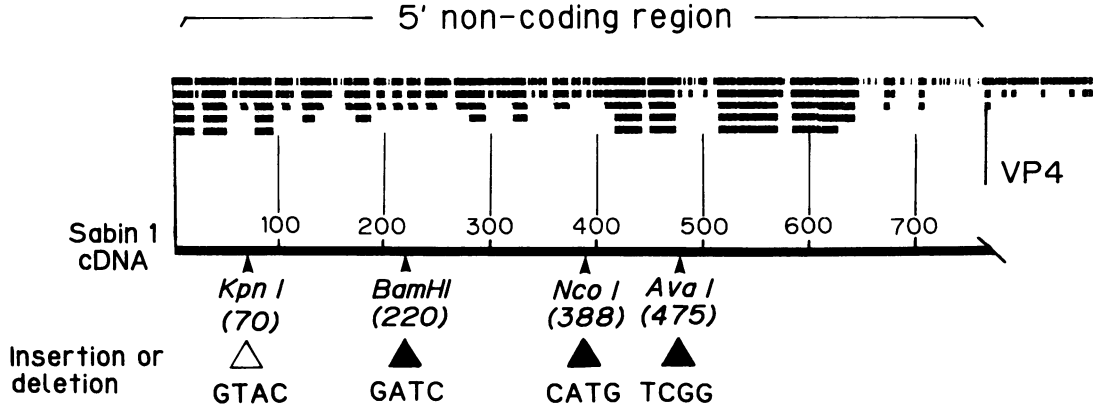

FIG. 5. Nucleotide sequences of the genomes of site-directed mutants. The nucleotide sequence homology among genomes of various poliovirus serotype is shown as in Fig. 1. The numbers in parentheses represent nucleotide positions of the cleavage sites of restriction endonucleases. Open and closed triangles indicate positions where four bases are deleted and inserted, respectively. The nucleotide sequences deleted or inserted are shown under the triangles.

Interestingly, large-plaque variants of PV1(Sab)IC-ΔB rapidly arose during passaging of PV1(Sab)IC-ΔB (Fig. 6 and 7). It is possible that these variants regain replication efficiency to some extent by some additional mutations.

Large-plaque variants derived from PV1(Sab)IC-ΔB. Four different large-plaque variants were isolated by plaque purification from the virus preparation obtained by one passage of PV1(Sab)IC-ΔB on AGMK cells at multiplicity of infection of 10^{-3} and designated as PV1(Sab)IC-ΔBL1, PV1(Sab)IC-ΔBL2, PV1(Sab)IC-ΔBL3, and PV1(Sab)IC-ΔBL4. One small-plaque virus that seemed to be the parent virus PV1(Sab)IC-ΔB was also isolated and designated as PV1(Sab)IC-ΔBS. These purified viruses were expanded once on AGMK cells by infection at a multiplicity of 10^{-3} and tested for their plaque sizes on the same cells (Fig. 7); a small number of large-plaque variants are already observed in the preparation of virus PV1(Sab)IC-ΔBS. The plaque sizes of isolated large-plaque variants are larger than those of PV1(Sab)IC-ΔB, although the sizes are slightly smaller than those of PV1(Sab)IC-O. Virus PV1(Sab)IC-ΔBL2 displayed relatively small plaques among those large-plaque variants when incubation was continued (Fig. 7B).

The small-plaque phenotype may be attributed to lowered efficiency of certain viral replication steps, resulting in reduction of the rate of viral production in the infected cells. To test this possibility, we measured titers of viruses produced in the infected AGMK cells in a single cycle of infection at 35.5°C (Fig. 8). In cells infected with virus PV1(Sab)IC-O, virion assembly starts at about 3 h postinfection and lasts until 6 to 8 h postinfection. The cells infected with PV1(Sab)IC-ΔB produced 100-fold fewer plaque-forming particles at 4 h postinfection and more than 10-fold fewer at 6 h postinfection as compared with virus

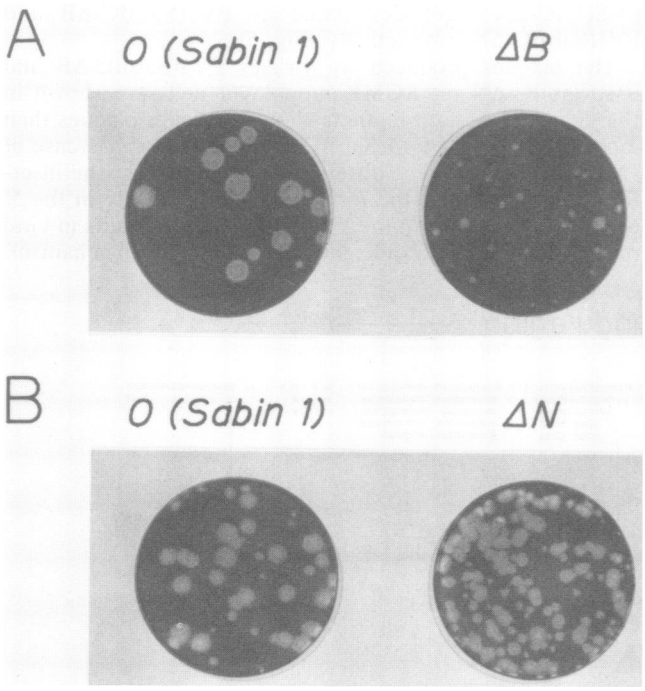

FIG. 6. Plaques of site-directed mutants displayed on AGMK cells. Plaques of PV1(Sab)IC-ΔB (A) and PV1(Sab)IC-ΔN (B) on AGMK cells are shown. The plaque formation procedure was similar to the conditions described in the legend to Fig. 4. PV1(Sab)IC- is omitted as in Fig. 2.

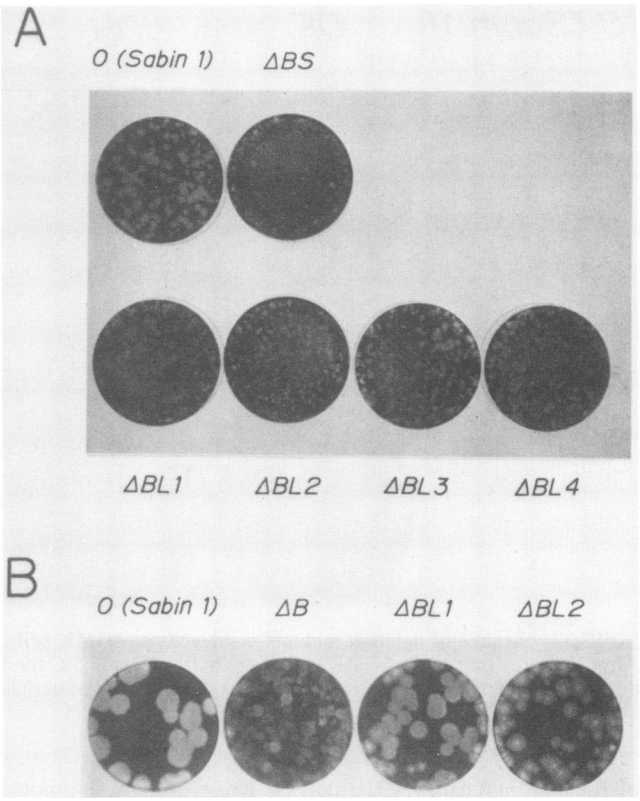

FIG. 7. Plaques of large-plaque variants derived from PV1(Sab)IC-ΔB. One small-plaque virus (ΔBS) and four large-plaque viruses (ΔBL1, ΔBL2, ΔBL3, and ΔBL4) were isolated from a preparation of virus PV1(Sab)IC-ΔB as described in Materials and Methods. Plaques displayed on AGMK cells after incubation for 3 days (A) and 5 days (B) are shown. PV1(Sab)IC- is omitted as in Fig. 2.

production in cells infected with PV1(Sab)IC-O (Fig. 8). The CPE induced by viruses was also examined (Fig. 8). At 8 h postinfection, almost all cells infected with PV1(Sab)IC-O were rounded and detached. At the same time, however, only 10 to 30% of cells infected with PV1(Sab)IC-ΔB showed apparent CPE. The cells infected with two large-plaque variants, PV1(Sab)IC-ΔBL1 and PV1(Sab)IC-ΔBL2, produced an intermediate number of plaque-forming virus particles and showed an intermediate extent of CPE until 8 h postinfection (Fig. 8). The virus infection cycle of PV1(Sab)IC-ΔBL2 appeared to progress slower than that of PV1(Sab)IC-ΔBL1 (Fig. 8). The number of infectious particles produced in these infected cells reached essentially the same level at 24 h postinfection (Fig. 8). Thus, the small-plaque phenotype of mutant virus PV1(Sab)IC-ΔB can be explained by the prolongation of each cycle of infection, probably because of a reduction of efficiency in certain steps of the viral replication. The large-plaque variants therefore seemed to regain to some extent the efficiency by the second-site mutations that might suppress the small-plaque phenotype of PV1(Sab)IC-ΔB.

Second-site suppressor mutations. To identify the second mutation sites, the nucleotide sequences of the 5′ noncoding sequences of the genomes of the large-plaque variants were determined as described in Materials and Methods (Fig. 9). Every genome of large-plaque variant was found to retain a four-base insertion sequence at the *Bam*HI cleavage site of

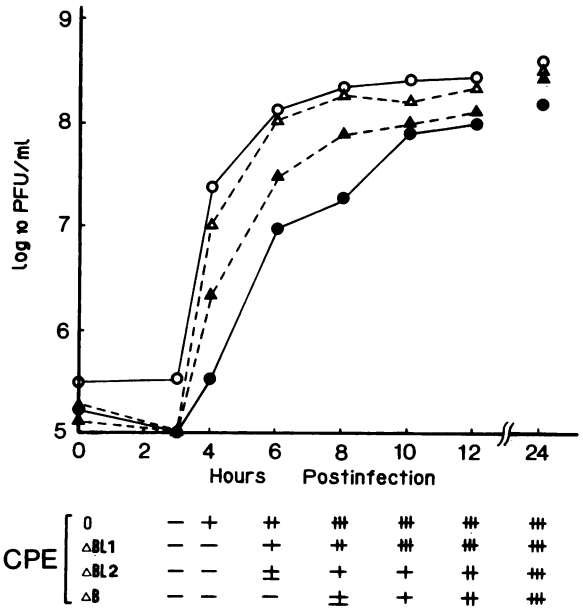

FIG. 8. Virus production and appearance of CPE in infected AGMK cells. AGMK cells were infected with PV1(Sab)IC-O(T) (○), PV1(Sab)IC-ΔB (●), PV1(Sab)IC-ΔBL1 (△), or PV1(Sab)IC-ΔBL2 (▲) as described in Materials and Methods. The titers of viruses produced in the infected cells were measured at indicated times after the infection. CPE was scored as follows: −, cells with no detectable difference from uninfected cells; ±, less than 10% of cells rounded; +, 10 to 30% of cells rounded or detached; ++, 30 to 70% of cells rounded or detached; +++, most cells rounded, detached, or lysed. PV1(Sab)IC- is omitted as in Fig. 2.

nucleotide position 220. In the sequence of three genomes of large-plaque variants PV1(Sab)IC-ΔBL1, PV1(Sab)IC-ΔBL3, and PV1(Sab)IC-ΔBL4, two point mutations were observed at nucleotide positions 186 and 525, at which U residues in PV1(Sab)IC-ΔB genome were changed to C residues (Fig. 9). In the genome of variant PV1(Sab)IC-ΔBL2, the U at position 186 and the G at position 480 were changed to C and A, respectively (Fig. 9). No other alteration of nucleotide sequence was detected in the 5′ noncoding sequence. The results indicated that two nucleotide substitutions in each genome that occurred at nucleotide positions 186 and around 500 gave rise to the recovery of the normal generation time of PV1(Sab)IC-O to some extent, which was prolonged by the introduction of the four-base insertion sequence at nucleotide position 220. These observations suggest that the specific interaction between these two genome loci at positions around 200 and around 500 influences the formation of some functional structures that maintain the efficiency of certain steps in viral replication.

DISCUSSION

We modulated the 5′ noncoding sequences of the PV1(Sab) genome, which included highly homologous sequences as well as highly variable sequences among genomes of poliovirus serotypes, to investigate the biological significance of the genome regions in viral replication. A number of mutant cDNA clones constructed in vitro were tested for their infectivity in AGMK cells. The mutant viruses recovered from the transfected cells were further characterized in terms of their plaque size, which was a marker of viral proliferation rate. From these experiments, it could be concluded that the genome regions of highly homologous sequences do not always have essential roles, although such regions appear to harbor very important signals in certain viral replication steps.

The mutant viruses that lacked the genome region at nucleotide positions 600 to 726 appeared to be fully viable as compared with PV1(Sab). The genome region contains the highly variable sequence (positions 640 to 726) and the highly homologous sequence (positions 600 to 639). Interestingly, the corresponding genome region [positions 616 to 739 of the PV1(Sab) genome] is missing in the genomes of human rhinoviruses (3, 19, 21), which are members of the *Picornaviridae* (Fig. 3). The genome region may therefore

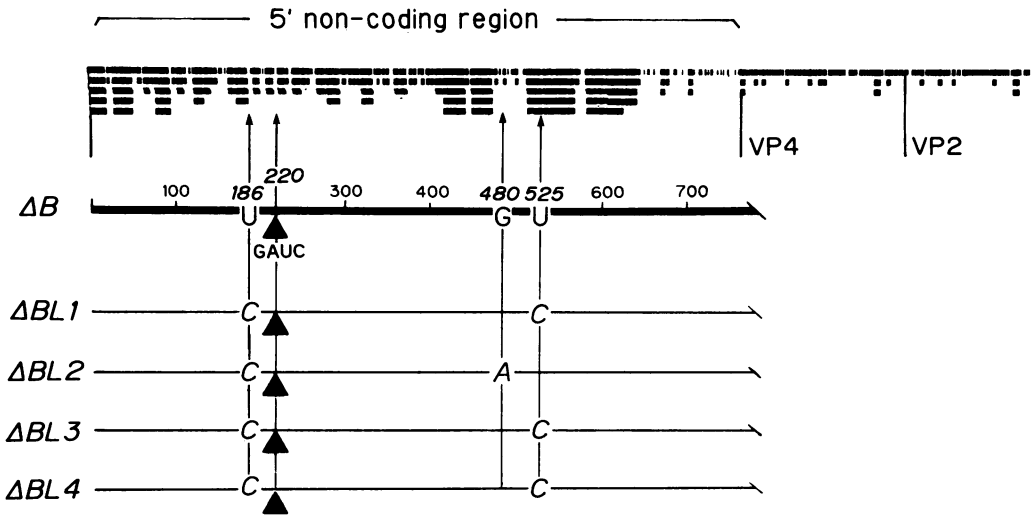

FIG. 9. Second-site suppressor mutations detected in the 5′ noncoding sequence of large-plaque variant genomes. Nucleotide sequence homology among genomes of various poliovirus serotypes is shown as in Fig. 1. The results of sequence analyses of the genomes of virus PV1(Sab)IC-ΔB and its large-plaque variants are shown. The four bases (GAUC) inserted at nucleotide position 220 remained intact in all variant genomes as indicated by closed triangles. Nucleotide numbers of PV1(Sab) genome (10) are shown above PV1(Sab)IC-ΔB genome. PV1(Sab)IC- is omitted as in Fig. 2.

not play an essential role in the replication of picornaviruses in cell culture systems. The lack of this genome region may cause the distortion of virion particles; this may result in instability of the particles in acidic conditions since rhinoviruses are not stable in acidic conditions as compared with polioviruses. We therefore tested the stability of viruses PV1(Sab)IC-DH and PV1(Sab)IC-DA21 at pH 2.0. The stability in acid was similar to that of PV1(Sab) particles (data not shown). Thus, the biological function of this genome region is unclear. It is possible, however, that the region may have an important function in poliovirus replication in nature but not in the cell culture system used in this study.

Viruses PV1(Sab)IC-DA21 and PV1(Sab)IC-DA23 showed small-plaque phenotypes, whereas virus PV1(Sab)IC-DH showed plaques almost similar in size to those of PV1(Sab). The small-plaque phenotypes of the former two viruses may be due to the lowered rate of viral proliferation as described above. Therefore, two specific nucleotide sequences (T-rich region and 8b consensus in Fig. 3) might act as important signals to maintain the viral proliferation rate. The functions of these two sequences, however, remain to be elucidated. It might be possible to construct new polio vaccine strains by introducing this kind of deletion into the viral genome, because viral attenuation may be attributed to lowered efficiency of certain steps essential for viral multiplication (11). The attenuation phenotype must be very stable upon repeated passages if the efficiency of viral replication is reduced by the introduction of deletion. Indeed, the small-plaque phenotype of virus PV1(Sab)IC-DA21 was found to be very stable during passaging. Monkey neurovirulence tests of the virus are currently under investigation.

The second mutation sites were identified by analyzing nucleotide sequences of the 5′ noncoding region of the genomes of large-plaque variants derived from virus PV1(Sab)IC-ΔB, although additional mutations might reside in other portions of the genome. Every variant genome harbors two point mutations at positions around 200 and around 500. The efficiency in some replication steps lowered by the four-base insertion at position 220 appeared to be restored to some extent by these second-site mutations. These results suggest that the mutation sites in different genome loci may interact to function in some replication steps. The formation of certain functional structures such as initiation signals of virus-specific replication events may be influenced by the specific interactions between different genome loci. This phenomenon might be observed in the case of every single-stranded RNA genome. Additional molecular genetic experiments of the type reported here may elucidate the specific interactions in the 5′ noncoding sequence that have important functions during poliovirus replication.

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LITERATURE CITED

1. **Berk, A. J., F. Lee, T. Harrison, J. Williams, and P. Sharp.** 1979. Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell **17**:935–944.
2. **Bernstein, H. D., N. Sonenberg, and D. Baltimore.** 1985. Poliovirus mutant that does not selectively inhibit host cell protein synthesis. Mol. Cell. Biol. **5**:2913–2923.
3. **Callahan, P. L., S. Mizutani, and R. J. Colonno.** 1985. Molecular cloning and complete sequence determination of RNA genome of human rhinovirus type 14. Proc. Natl. Acad. Sci. USA **82**:732–736.
4. **Hanecak, R., B. L. Semler, C. W. Anderson, and E. Wimmer.** 1982. Proteolytic processing of poliovirus polypeptides: antibodies to polypeptide P3-7c inhibit cleavage at glutamine-glycine pairs. Proc. Natl. Acad. Sci. USA **79**:3973–3977.
5. **Kitamura, N., B. L. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J. Dorner, E. A. Emini, R. Hanecak, J. J. Lee, S. van der Werf, C. W. Anderson, and E. Wimmer.** 1981. Primary structure, gene organization and polypeptide expression of poliovirus RNA. Nature (London) **291**:547–553.
6. **Kohara, M., S. Abe, S. Kuge, B. L. Semler, T. Komatsu, M. Arita, H. Itoh, and A. Nomoto.** 1986. An infectious cDNA clone of the poliovirus Sabin strain could be used as a stable repository and inoculum for the oral polio live vaccine. Virology **151**:21–30.
7. **Kohara, M., T. Omata, A. Kameda, B. L. Semler, H. Itoh, E. Wimmer, and A. Nomoto.** 1985. In vitro phenotypic markers of a poliovirus recombinant constructed from infectious cDNA clones of the neurovirulent Mahoney strain and the attenuated Sabin 1 strain. J. Virol. **53**:786–792.
8. **LaMonica, N., C. Meriam, and V. R. Racaniello.** 1986. Mapping and sequences required for mouse neurovirulence of poliovirus type 2 Lansing. J. Virol. **57**:515–525.
9. **Nomoto, A., N. Kitamura, F. Golini, and E. Wimmer.** 1977. The 5′-terminal structures of poliovirion RNA and poliovirus mRNA differ only in the genome-linked protein VPg. Proc. Natl. Acad. Sci. USA **74**:5345–5349.
10. **Nomoto, A., T. Omata, H. Toyoda, S. Kuge, H. Horie, Y. Kataoka, Y. Genba, Y. Nakano, and N. Imura.** 1982. Complete nucleotide sequence of the attenuated poliovirus Sabin 1 strain genome. Proc. Natl. Acad. Sci. USA **79**:5793–5797.
11. **Omata, T., M. Kohara, S. Kuge, T. Komatsu, S. Abe, B. L. Semler, A. Kameda, H. Itoh, M. Arita, E. Wimmer, and A. Nomoto.** 1986. Genetic analysis of the attenuation phenotype of poliovirus type 1. J. Virol. **58**:348–358.
12. **Omata, T., M. Kohara, Y. Sakai, A. Kameda, N. Imura, and A. Nomoto.** 1984. Cloned infectious complementary DNA of the poliovirus Sabin 1 genome: biochemical and biological properties of the recovered virus. Gene **32**:1–10.
13. **Racaniello, V. R., and D. Baltimore.** 1981. Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proc. Natl. Acad. Sci. USA **78**:4887–4891.
14. **Racaniello, V. R., and D. Baltimore.** 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. Science **214**:916–919.
15. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
16. **Sarnow, P., H. D. Bernstein, and D. Baltimore.** 1986. A poliovirus temperature-sensitive RNA synthesis mutant located in a noncoding region of the genome. Proc. Natl. Acad. Sci. USA **83**:571–575.
17. **Semler, B. L., A. J. Dorner, and E. Wimmer.** 1984. Production of infectious poliovirus from cloned cDNA is dramatically increased by SV40 transcription and replication signals. Nucleic Acids. Res. **12**:5123–5141.
18. **Semler, B. L., V. H. Johnson, and S. Tracy.** 1986. A chimeric plasmid from cDNA clones of poliovirus and coxsackievirus produces a recombinant virus that is temperature-sensitive. Proc. Natl. Acad. Sci. USA **83**:1777–1781.
19. **Skern, T., W. Sommergruber, D. Blaas, P. Gruendler, F. Fraundorfer, C. Pieler, I. Fogy, and E. Kuechler.** 1985. Human rhinovirus 2: complete nucleotide sequence and proteolytic processing signals in the capsid protein region. Nucleic Acids Res. **13**:2111–2126.
20. **Stanway, G., A. J. Cann, R. Hauptmann, P. Hughes, L. D. Clarke, R. C. Mountford, P. D. Minor, G. C. Schild, and J. W.**

Almond. 1983. The nucleotide sequence of poliovirus type 3 Leon $12a_1b$: comparison with poliovirus type 1. Nucleic Acids Res. **11**:5629–5643.

21. **Stanway, G., P. J. Hughes, R. C. Mountford, P. D. Minor, and J. W. Almond.** 1984. The complete nucleotide sequence of a common cold virus: human rhinovirus 14. Nucleic Acids Res. **12**:7859–7875.
22. **Stanway, G., P. J. Hughes, R. C. Mountford, P. Reeve, P. D. Minor, G. C. Schild, and J. W. Almond.** 1984. Comparison of the complete nucleotide sequences of the genomes of the neurovirulent poliovirus P3/Leon 37 and its attenuated Sabin vaccine derivative P3/Leon $12a_1b$. Proc. Natl. Acad. Sci. USA **81**:1539–1543.
23. **Stanway, G., P. J. Hughes, G. D. Westrop, D. M. A. Evans, G. Dunn, P. D. Minor, G. C. Schild, and J. W. Almond.** 1986. Construction of poliovirus intertypic recombinants by use of cDNA. J. Virol. **57**:1187–1190.
24. **Toyoda, H., M. Kohara, Y. Kataoka, T. Suganuma, T. Omata, N. Imura, and A. Nomoto.** 1984. Complete nucleotide sequences of all three poliovirus serotype genomes: implication for genetic relationship, gene function and antigenic determinants. J. Mol. Biol. **174**:561–585.
25. **Toyoda, H., M. J. H. Nicklin, M. G. Murray, C. W. Anderson, J. J. Dunn, F. W. Studier, and E. Wimmer.** 1986. A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell **45**:761–770.
26. **Tracy, S., H.-L. Liu, and N. M. Chapman.** 1985. Coxsackievirus B3: primary structure of the 5′ non-coding and capsid protein-coding regions of the genome. Virus Res. **3**:263–270.
27. **Wimmer, E.** 1982. Genome-linked proteins of viruses. Cell **28**:199–201.
28. **Yogo, Y., and E. Wimmer.** 1972. Polyadenylic acid at the 3′-terminus of poliovirus RNA. Proc. Natl. Acad. Sci. USA **69**:1877–1882.