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# Complete nucleotide sequence of the attenuated poliovirus Sabin 1 strain genome

(vaccine strain of poliovirus/molecular cloning/genome sequence/mutation sites)

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**ABSTRACT** **The complete nucleotide sequence of the genome of the type 1 poliovirus vaccine strain (LSc,2ab) was determined by using molecular cloning and rapid sequence analysis techniques. The restriction fragments of double-stranded cDNA synthesized from the vaccine strain RNA were inserted into the adequate sites of cloning vector pBR322. Sequence analysis of the cloned DNAs revealed that the virion RNA molecule was 7,441 nucleotides long and polyadenylylated at the 3′ terminus. When the nucleotide sequence was compared with that of the genome of the virulent parental strain (Mahoney), 57 base substitutions were observed to be scattered all over the genome. Of these, 21 resulted in amino acid changes in a number of viral proteins. A cluster of amino acid changes is located in the viral coat proteins, especially in the $NH_2$-terminal half of the viral capsid protein VP1. These results may imply that the mutations in the VP1 coding region contribute to attenuation.**

The genome of poliovirus is a single-stranded RNA with positive polarity, in which all of the viral genetic information is stored (1). This genomic RNA is composed of ≈7,500 nucleotides, polyadenylylated at the 3′ terminus (2) and covalently attached to a genome-linked protein (VPg) at the 5′ terminus (3–6). Recently, the total genome sequence of the virulent Mahoney strain of type 1 poliovirus [PV1(M)] was determined (7, 8). Studies on the sequences of RNA and of amino acid in viral polypeptides have provided a precise viral protein map and proved that all of the known poliovirus polypeptides originated from a single precursor molecule NCVPOO [noncapsid viral protein (NCVP)] (7).

Attenuated polioviruses have been used effectively as oral live vaccines to prevent poliomyelitis. However, little is known, as yet, about the mechanisms responsible for attenuation. Poliovirus LSc,2ab strain [Sabin 1 strain, PV1(Sab)] is a live vaccine strain derived from the PV1(M) by spontaneous mutations during the attenuation process (9, 10).

To determine the molecular basis for the biological differences between virulent and attenuated poliovirus strains, the sequences of large and unique RNase T1- and A-resistant oligonucleotides of PV1(M) and PV1(Sab) have been compared. We have shown that mutations detected by oligonucleotide analysis were caused by single base substitutions and appeared to be scattered all over the genome (11).

For further comparative sequence studies, the restriction fragments obtained from double-stranded cDNA of the PV1(Sab) genome have been cloned (12). We report here the complete 7,441-nucleotide sequence of the PV1(Sab) genome, and the mutation sites were identified by comparison of our sequence with the known sequence of the PV1(M) genome (7, 8).

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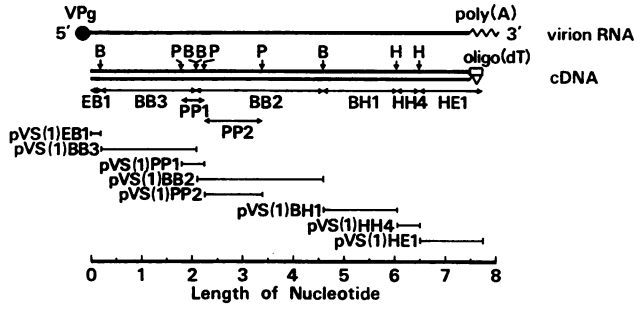


FIG. 1. Restriction cleavage sites on PV1(Sab) cDNA and the obtained cDNA clones. B, H, and P represent restriction cleavage sites for *Bam*HI, *Hin*dIII, and *Pst* I, respectively. The oligo(dT)·oligo(dA) at one end of EB1 fragment is not shown. Length of nucleotide is given in kilobases.

## MATERIALS AND METHODS

**Molecular Cloning of the PV1(Sab) Genome.** Double-stranded cDNA of PV1(Sab) genome was synthesized from the purified virion RNA as described (12). Restriction fragments of the double-stranded cDNA were obtained by digestion with *Hin*dIII, *Bam*HI, *Pst* I, or a mixture of *Hin*dIII and *Bam*HI (Takara Shuzo, Kyoto, Japan) and were inserted into the appropriate site of cloning vector pBR322 by virtue of their cohesive termini (12).

To obtain the clone pVS(1)HE1 (see Fig. 1) that contains the sequence corresponding to the 3′ end of the viral genome, the synthesized double-stranded DNA was ligated to *Eco*RI linker nucleotides (Collaborative Research, Waltham, MA) after treatment with *Escherichia coli* DNA polymerase I (Bethesda Research Laboratories) to exonucleolytically remove any 3′ protruding ends and to "fill in" any 5′ protruding ends (13). The double-stranded DNA ligated with *Eco*RI linker nucleotide(s) was digested with a mixture of *Hin*dIII and *Eco*RI, purified by Sephadex G-150 column chromatography (13), and inserted into pBR322 that had been treated with the same endonucleases. One clone, pVS(1)HE1, was selected from ampicillin-resistant and tetracycline-sensitive transformants (ampicillin and tetracycline at 30 and 15 μg/ml, respectively) (12).

To isolate the pVS(1)EB1 (see Fig. 1) that contains the sequence corresponding to the 5′ end of the viral genome, the primer extension method (8) was employed by using a *Hin*fI fragment (positions 221–267) of cloned BB3 fragment (see Fig. 1) as a primer for the reverse transcription. The sequence of the extension products was determined by the method of Maxam and Gilbert (14). For cloning of the EB1 fragment, the extended

Abbreviations: PV1(Sab), poliovirus type 1 Sabin strain; PV1(M), poliovirus type 1 Mahoney strain; VPg, genome-linked viral protein; NCVP, noncapsid viral protein.

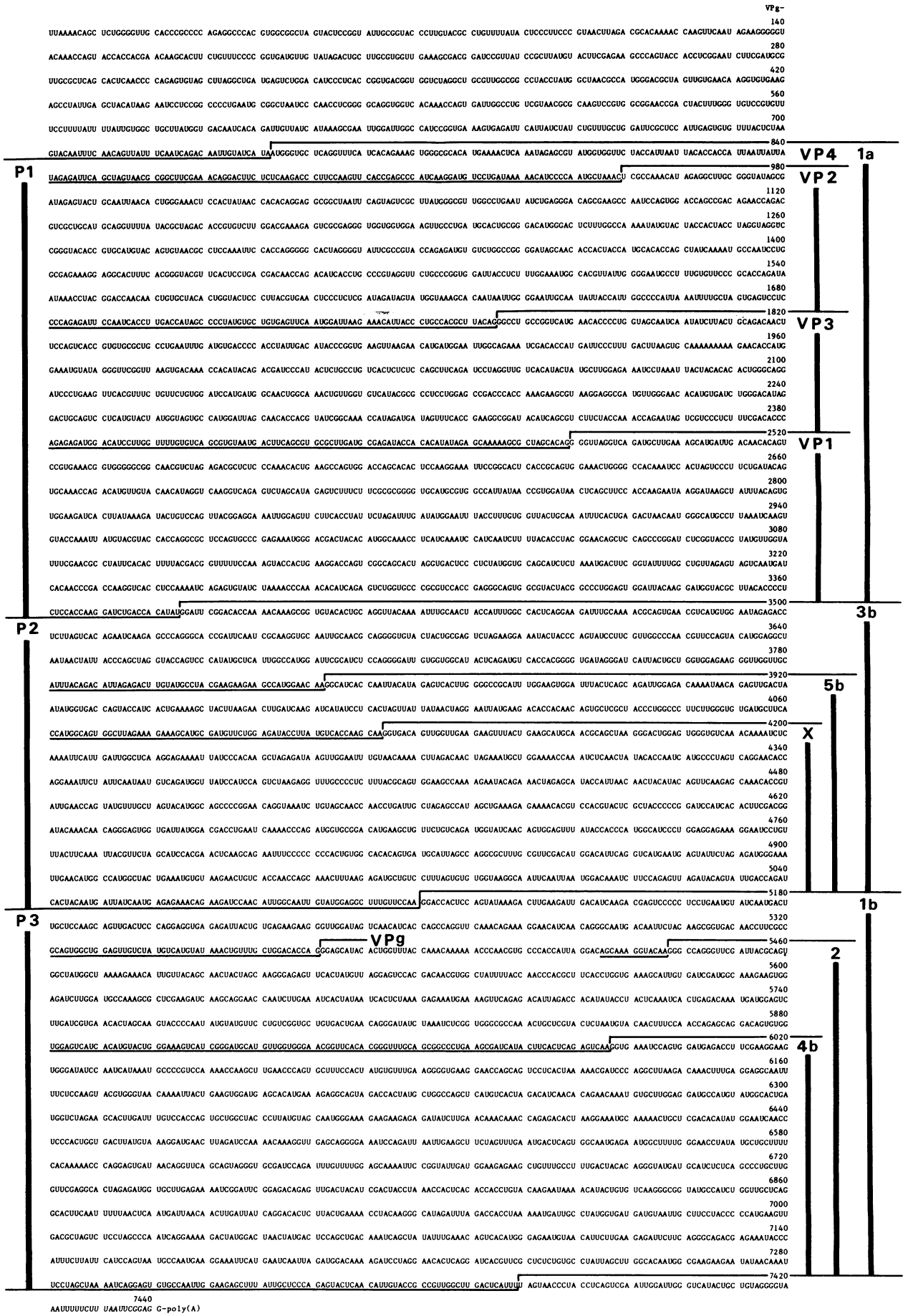


FIG. 2. (*Legend appears at the bottom of the next page.*)

DNA fragment was purified by polyacrylamide gel electrophoresis and tailed with poly(dT). The fragment was used as a template for the synthesis of the second strand with reverse transcriptase in the presence of oligo$(dA)_{10}$ (P-L Biochemicals). The product obtained, after treatment with the DNA polymerase I, was ligated to *Eco*RI linker nucleotides at both termini, treated with a mixture of *Eco*RI and *Bam*HI, and inserted into pBR322 as described above.

**Sequence Analysis of the PV1(Sab) Genome.** Nucleotide sequences of the cloned PV1(Sab) cDNA were determined by the method of Maxam and Gilbert (14). In addition, sequences of restriction fragments of the synthesized single-stranded cDNA were determined to ascertain whether the primary structure of the PV1(Sab) genome was correctly reflected in that of the cloned DNA. This was carried out by digestion of the single-stranded cDNA with *Hae* III, *Hin*fI, or *Hha* I, which was followed by use of rapid sequence analysis techniques (unpublished results). Primer extension products that included sequences of restriction sites were analyzed to confirm the restriction map shown in Fig. 1.

## RESULTS

**Sequence Analysis of Cloned DNA.** The HE1 fragment in the plasmid pVS(1)HE1 was shown to contain poly(dT) at one end (data not shown) to which *Eco*RI linker nucleotide was ligated. This result indicated that this insert corresponded to the 3′ end portion of the viral genome (Fig. 1). van der Werf *et al.* (15) have succeeded in the direct cloning of the 5′ end of poliovirus RNA using RNA·DNA hybrids. Because we were unable to obtain directly 5′-terminal clones, we used a restriction fragment (positions 221–267) from clone pVS(1)BB3 that was 5′-end-labeled and employed as a primer to synthesize a primer extension fragment. The nucleotide sequence of this extended material was determined chemically (14). Comparison of the sequence with that of the previously established 5′-terminal sequences (15–17) indicated that the extension proceeded to the very 5′ end of the viral genome. Sequence analysis of the EB1 fragment in a plasmid pVS(1)EB1 revealed that this insert contained the sequence corresponding to that of the first 220 bases of the viral genome.

We were unable to obtain clones that contained overlapping sequences that included the restriction cleavage sites of the genome region consisting of the fragments BB2, BH1, HH4, and HE1. Therefore, the single-stranded cDNAs having the nucleotide sequences of these sites were synthesized by the primer extension method. Sequence analysis of these cDNAs proved that sequences of any two neighboring cloned DNAs were directly adjacent to each other in this part of the viral genome.

**Selection of Correct Clones and Primary Structure of PV1(Sab) Genome.** To know whether the primary structure of the cloned DNAs correctly reflected those of the viral genome, sequences of single-stranded cDNA synthesized directly from genome RNA were determined after digestion with *Hae* III, *Hin*fI, or *Hha* I, as described. By this alternative method, sequences totaling more than 4,000 nucleotides were determined (data not shown). Among the sequences, three disagreements were discovered compared with the sequences of cloned DNAs, and all of them were found to exist in the BB2 fragment. Therefore, the BB2 DNA fragment was recloned and the recloned BB2 DNA was analyzed again. The result indicated that this new clone reflected the viral genome correctly.

Thus, every portion of the viral genome was cloned into a plasmid pBR322, and the complete nucleotide sequence of the PV1(Sab) genome was determined by using the cloned DNAs. The total PV1(Sab) genome sequence of 7,441 nucleotides is shown in Fig. 2. This nucleotide sequence of PV1(Sab) is very similar to that of PV1(M) (7, 8), an observation that was expected from oligonucleotide analyses (6, 11). A possible initiator codon for the polyprotein was found at position 743, which is followed by an open reading frame that terminated at a UAG in position 7,370 (Fig. 2). The amino acid sequence (data not shown) of the viral polyprotein NCVPOO predicted from the identified open reading frame was also very similar to that of PV1(M) (7, 8). Furthermore, amino acid pairs that serve as possible signals for proteolytic processing of the PV1(M) polyprotein (7) were perfectly conserved in the amino acid sequence of the PV1(Sab) polyprotein. Thus, the genetic map established for PV1(M) (7) is applicable to PV1(Sab), as indicated in Figs. 2 and 3.

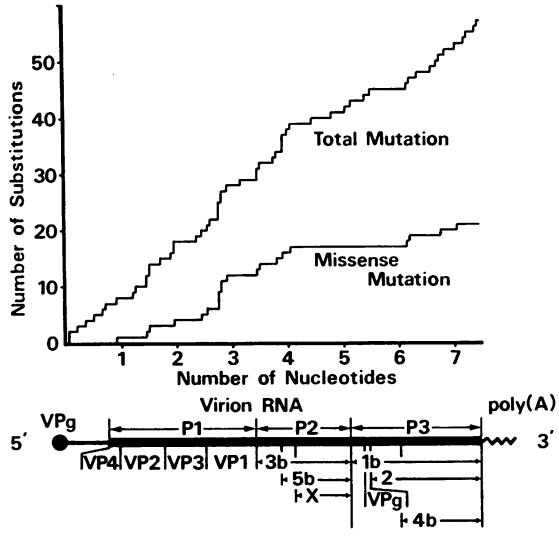


FIG. 3. Location of mutations of the PV1(Sab) genome. The number of total mutations or missense mutations accumulated from the 5′ terminus of the PV1(Sab) genome was plotted for comparison with the sequence of the PV1(M) genome (8). Gene organization follows that of the PV1(M) genome (7). Number of nucleotides is given in kilobases.

## DISCUSSION

**Cloning and Sequence of the PV1(Sab) Genome.** We have determined the complete PV1(Sab) genome sequence using molecular cloning technique. The sequence data suggest that the ninth AUG triplet from the 5′ terminus of the RNA serves as an initiator codon for the synthesis of the large polyprotein NCVPOO. This was suggested previously in the translation of PV1(M) RNA (7, 8) and has now been proven by sequence analyses of the viral capsid proteins and of mRNA (18). According to the "scanning model" (19), eukaryotic ribosomes bind to the 5′ terminus of mRNA and migrate along the mRNA sequence until they encounter the first AUG triplet which, solely by virtue of its position, is the initiator codon. Recently, a more elaborate version of the scanning model (20) was proposed in which the nucleotides flanking the AUG initiator codon are proposed to play an important role in the recognition by eukaryotic ribosomes, the most favorable sequence for initiation being $\begin{matrix}A\\G\end{matrix}$-N-N-A-U-G-G. An AUG at position 390 has such a flanking sequence but it is followed closely by in-phase terminator codons.

FIG. 2 (*on preceding page*). Nucleotide sequence of PV1(Sab) genome. The RNA sequence is deduced from the DNA sequence of cloned cDNAs. Genome organization indicated here follows that of the PV1(M) genome reported by Kitamura *et al.* (7).

In addition, a computer search of the genome identified a very stable secondary structure surrounding the upstream AUG at position 390. The free energy of this secondary structure was calculated as −29.2 kcal/mol (Fig. 4) (21). Therefore, it may be possible that ribosomes "jump" over the stable secondary structure without recognition of the AUG at position 390. Alternatively, initiation of protein synthesis on poliovirus RNA may occur by a quite different mechanism from that on other eukaryotic mRNAs, because poliovirus RNA, as well as other picornavirus RNAs, lacks the $m^7Gppp$ cap at the 5′ terminus (22, 23). Ribosomes thus might bind to the RNA independently of the cap at the site close to the AUG at position 743 so as to use this AUG as the initiation codon.

The results of oligonucleotide analysis of the PV1(Sab) genome had suggested that the inoculation stock used in the experiment seemed to be pure enough for structural studies of the genome (6, 11). However, an intrinsic problem in using the molecular cloning technique for viral genomes is the threat of selecting a cloned DNA whose sequence reflects that of a mutated virus genome that was a minor component in the inoculation stock. Moreover, the molecular cloning procedure itself could lead to minor base changes. Therefore, we employed an alternative sequence analysis method (unpublished results) by which the sequence of the synthesized single-stranded cDNA was directly determined without recourse to molecular cloning. The results were compared with that obtained from the cloned DNA. By this strategy we discovered a few wrong nucleotides in the cloned BB2 fragment.

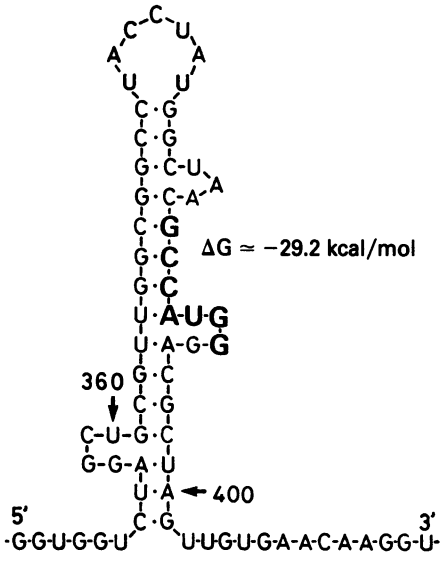


FIG. 4. Secondary structure of the nucleotide sequence including AUG at position 390. The calculated free energy (ΔG) for the stem and loop structure (21) is indicated to the right of the figure. A seven-nucleotide sequence, which emerges as the favored sequence for eukaryotic initiation sites of protein synthesis (20), is drawn in boldface letters.

**Comparison of Genome Sequences Between PV1(M) and PV1(Sab).** To date, two slightly different genome sequences of PV1(M) have been published. One was reported by Kitamura

Table 1. Comparison of genome sequences between PV1(M) and PV1(Sab)

| Nucleotide position | Substitutions: Base M | Base Sab | Amino acid M | Amino acid Sab | Nucleotide position | Substitutions: Base M | Base Sab | Amino acid M | Amino acid Sab |
|---|---|---|---|---|---|---|---|---|---|
| 21 | U | C | | | 3,445 | C | U (3) | | |
| 26 | A | G | | | 3,460 | U | A (3) | Asp | Glu |
| 189 | C | U | | | 3,492 | G | A (2) | Ser | Asn |
| 355 | C | U | | | 3,766* | C | A (3) | | |
| 480 | A | G | | | 3,785 | U | A (1) | Ser | Thr |
| 649 | C | U | | | 3,896 | A | G (1) | Ser | Gly |
| 674 | C | U | | | 3,898 | C | A (3) | | |
| 935 | G | U (1) | Ala | Ser | 3,919 | C | U (3) | | |
| 1,208 | A | C (1) | | | 4,003 | C | U (3) | | |
| 1,228 | G | C (3) | | | 4,116 | U | C (2) | Ile | Thr |
| 1,442 | A | G (1) | Asn | Asp | 4,444 | U | C (3) | | |
| 1,465 | C | U (3) | | | 4,789 | A | G (3) | | |
| 1,490 | C | U (1) | Leu | Phe | 5,107 | U | C (3) | | |
| 1,507 | G | A (3) | | | 5,137 | A | G (3) | | |
| 1,747 | C | U (3) | | | 5,420 | C | A (1) | | |
| 1,885 | A | U (3) | | | 5,440 | A | G (3) | | |
| 1,942 | C | A (3) | | | 6,143 | G | A (1) | Asp | Asn |
| 1,944 | C | A (2) | Thr | Lys | 6,203 | U | C (1) | Tyr | His |
| 2,353 | U | C (3) | | | 6,373 | C | U (3) | | |
| 2,438 | U | A (1) | Leu | Met | 6,616 | G | A (3) | | |
| 2,545 | A | G (3) | | | 6,679 | U | C (3) | | |
| 2,585 | A | G (1) | Thr | Ala | 6,734 | A | G (1) | Lys | Glu |
| 2,741 | A | G (1) | Thr | Ala | 6,853 | C | U (3) | | |
| 2,749 | G | A (3) | Met | Ile | 7,071 | C | U (2) | Thr | Ile |
| 2,762 | C | U (1) | Pro | Ser | 7,198 | U | A (3) | | |
| 2,775 | C | A (2) | Thr | Lys | 7,243 | U | A (3) | | |
| 2,795 | G | A (1) | Ala | Thr | 7,410* | U | C | | |
| 2,879 | C | U (1) | Leu | Phe | 7,441* | A | G | | |
| 3,163 | U | C (3) | | | | | | | |

M and Sab represent PV1(M) and PV1(Sab), respectively. The PV1(M) genome sequence by Racaniello and Baltimore (8) is used here. Position of the nucleotide is shown by the nucleotide number from the 5′ terminus of the genome. Nucleotide numbers of the PV1(M) genome are corrected, considering 30 additional nucleotides that should be inserted at position 2,104 (8). Numbers in parentheses represent positions of in-phase codon. Predicted amino acid changes resulting from base substitutions are also shown. Three nucleotides of PV1(M) with asterisks are different from those in the sequence reported by Kitamura *et al.* (7).

*et al.* (7), who used the modified dideoxynucleotide method for sequence analysis of the cDNA directly synthesized from the genome. The other sequence was reported by Racaniello and Baltimore (8), who applied the Maxam and Gilbert method on the cloned cDNA; this method is the same method we have used. Because it is possible that different methods for sequence determination give rise to disagreements in the determined sequences, the sequence by the latter group was employed in this study and was compared with that of the PV1(Sab) genome. As a result, we identified 57 base substitutions dispersed all over the genome (Table 1; Fig. 3). These base substitutions can be marked with great confidence because 54 of them were also found when the sequence proposed by Kitamura *et al.* (7) was used for the comparison (Table 1).

Many base substitutions in the P2-X and P3-1b coding regions of the genome occurred in the third letter position of the in-phase codons (Table 1). This has resulted in a low frequency of amino acid changes in these coding regions. As shown in Table 1 and Fig. 3, most missense mutations are located in polypeptides P1-1a and in the $NH_2$-terminal half of P2-3b. No missense mutations occur in P2-X and in the $NH_2$-terminal half of P3-1b. A similar observation has been made by comparative sequence studies on PV1(Sab) and poliovirus type 2 Sabin strain genomes (unpublished results). This result may indicate that conservation of amino acid sequences of P2-X and portions of P3-1b is more important for the poliovirus replication than that of the viral coat proteins (P1-1a).

**Mutation Sites Contribute to Attenuation.** A major marker for attenuated poliovirus vaccine is the sensitivity of viral multiplication to temperature (24). In the case of PV1(Sab), the assembly of viral capsid proteins into capsomeres appears to be a temperature-sensitive step (24). In addition to a difference in optimal temperature of growth, PV1(M) and PV1(Sab) are distinguishable from each other by some of their antigens (25) and by virion aggregation (26). Thus, many known differences *in vitro* between the two strains can be explained by the surface changes of the virion particle resulting from amino acid substitutions in the coat proteins. In agreement, Kew *et al.* (27) have recently suggested that 10–13 amino acid replacements in the coat proteins occurred during the attenuation process leading to PV1(Sab). Because we have found that 12 of 21 amino acid replacements occurred in the coat proteins, we suggest that the surface changes of the virion may play an important role in the multiplicity of quantitative changes which are reflected in the characteristics of attenuated strains.

Our comparative data shown here indicate that base substitutions that resulted in amino acid changes were predominantly located in the 5′-terminal half of the genome. Of particular interest is a cluster of them in the 5′-terminal half of the viral capsid protein VP1 coding region (Table 1; Fig. 3). These results, together with abundant evidence that VP1 contributes importantly to the surface properties of the virion (28), suggest that base substitutions occurring in the coding region for the $NH_2$-terminal half of VP1 may contribute to the quantitative changes in certain biological properties that are associated with attenuation of virulence in different hosts and by different routes (29).

Evidence recently presented by Racaniello and Baltimore (30) suggests that a complete, cloned cDNA of the PV1(M) genome is infectious in mammalian cells. A reasonable approach to identification of the mutation(s) responsible for attenuation seems to be the *in vivo* quantitative neurovirulence tests (9, 29, 31) of recombinant viruses that could be recovered by transfection of the infectious cloned recombinant cDNAs made from cloned cDNAs of PV1(M) and PV1(Sab). Therefore, as a first step in identification of such mutation(s), we are currently attempting to construct an infectious cloned cDNA of PV1(Sab) in which the PP2 or BB2 fragment is replaced by the corresponding fragment of the PV1(M) genome. It is also of interest to test the influence of base substitutions in noncoding regions on the virulence of the virus, especially the base change observed at position 26 which affects the free energy of the secondary structure reported previously (16). Thus, recombinant DNA technology that includes site-directed mutagenesis becomes an extremely powerful tool for studying the relationship between the structure of the poliovirus genome and its biological significance.

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