

## A Mutation in the RNA Polymerase of Poliovirus Type 1 Contributes to Attenuation in Mice

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The attenuated Sabin strain of poliovirus type 1 (PV-1) differs from the neurovirulent PV-1 Mahoney strain by 55 nucleotide mutations. Only one of these mutations (A-480→G), in the 5' noncoding (5' NC) region of the genome, is well characterized, and it confers a strong attenuating effect. We attempted to identify genetic attenuation determinants in the 3'-terminal part of the Sabin 1 genome including the 3D polymerase (3D<sup>pol</sup>) gene and the 3' NC region. Previous studies suggested that some of the 11 mutations in this region of the Sabin 1 genome, and in particular a mutation in the polymerase gene (U-6203→C, Tyr-73→His), are involved to some extent in the attenuation of PV-1. We analyzed the attenuating effect in the mouse model by using the mouse-adapted PV-1/PV-2 chimeric strain v510 (a Mahoney strain carrying nine amino acids of the VP1 capsid protein from the Lansing strain of PV-2). Mutagenesis of locus 6203 was performed on the original v510 (U-6203→C) and also on a hybrid v510/Sabin 1 (C-6203→U) carrying the downstream 1,840 nucleotides of the Sabin 1 genome including the 3D<sup>pol</sup> and 3' NC regions. Statistical analysis of disease incidence and time to disease onset in numerous mice inoculated with these strains strongly suggested that nucleotide C-6203 is involved in the attenuation of the Sabin 1 strain. Results also suggested that, among the mutations located in the 3D<sup>pol</sup> and 3' NC regions, nucleotide C-6203 may be the principal or the only one to be involved in attenuation in this mouse model. We also found that the effect of C-6203 was weaker than that of nucleotide G-480; the two nucleotides acted independently and may have a cumulative effect on attenuation. The U-6203→C substitution also appeared to contribute to the thermosensitivity of the Sabin 1 strain.

Poliovirus (PV), an enterovirus of the *Picornaviridae* family, is the causative agent of poliomyelitis. It replicates in the human digestive tract and may induce paralysis by infecting and destroying motor neurons (7, 15). Attenuated strains of all three serotypes have been selected by numerous passages of wild-type strains in monkey tissues *in vivo* and *in vitro* (49). These strains (Sabin 1, 2, and 3), which replicate in the human gut and induce strong immunity, have been efficiently used as oral vaccines. Studies of the genetic basis of attenuation of PV vaccine strains have been greatly facilitated now that the sequences of attenuated and virulent strain genomes are known (reviewed in references 38 and 44).

The PV genome is a polyadenylated single-stranded RNA of positive polarity (7,441 bases long for PV type 1 [PV-1]) (for a review, see reference 51). Two noncoding regions flank the single large open reading frame which codes for the viral structural (VP1 to VP4) and nonstructural polypeptides including the RNA-dependent RNA polymerase (3D<sup>pol</sup>). The 5' noncoding (5' NC) region, terminally linked to a small viral protein, VPg, is involved in viral replication and in cap-independent initiation of translation (3, 42). The short 3' NC region is thought to be involved in viral replication (50).

The genome of the Sabin 3 strain differs from that of the neurovirulent wild-type progenitor by 12 mutations, and it has been found that among these mutations only three determine the attenuation phenotype (55, 58). In contrast, the genome of the Sabin 1 strain differs from that of its parental virulent Mahoney virus (PV-1 Mahoney) by 55 point mutations (39). The characterization of recombinant viruses

(between attenuated and virulent strains of PV-1) has shown that determinants of attenuation are spread over the entire viral genome (1, 40). A point mutation in the 5' NC region of the Sabin 1 genome, at position 480 (A→G), is the only well-characterized mutation known to be involved in the attenuation of PV-1 (21, 32). A similar strong attenuating mutation is found in the 5' NC region of the Sabin 2 and Sabin 3 strain genomes at nucleotide positions 481 and 472, respectively (27, 31, 47, 58). In the Sabin 3 strain, this mutation has been shown to affect viral replication in human neuroblastoma cell lines (28) and the level of translation of the genome in an acellular system (54), probably as the result of the alteration of the secondary structure of the 5' NC region (43, 52). Previous studies of PV-1 attenuation suggested that the 3'-terminal part of the Sabin 1 genome including the 3D<sup>pol</sup> gene and the 3' NC region is also involved to some extent in the attenuation of PV-1 in monkeys (40). In particular, a mutation at position 6203 (U→C) leading to an amino acid change at position 73 of the 3D polymerase (Tyr-73→His) has been highlighted as a possible determinant of the thermosensitivity and/or attenuation of PV-1 (12, 57).

All three PV serotypes induce paralysis only in primates; thus genetic attenuation determinants of PV strains have been studied mainly in monkeys. However, some PV strains, in particular the Lansing strain of PV-2 (PV-2 Lansing), have been adapted to mice and can induce poliomyelitis in animals following intracerebral inoculation (44). Moreover, it has been shown that PV-1 Mahoney acquires the ability to cause paralysis in mice if the exposed B-C loop (amino acids 94 to 102) of its capsid protein VP1 is replaced by that of PV-2 Lansing (33, 35). Recent studies with mice confirming the attenuating role of the G-480 Sabin 1 nucle-

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otide have suggested that genomic determinants important for PV-1 attenuation could be analyzed with a mouse-adapted PV1/PV2 strain (32).

In this work, we used this mouse model to study the role played by the mutation at position 6203 of the Sabin 1 genome in the attenuation of PV-1 (the U-6203→C substitution in the Sabin 1 genome compared with the PV-1 Mahoney genome will be referred to as the C-6203 mutation). We also studied the relationship of the C-6203 mutation to the other Sabin mutations in the 3D<sup>pol</sup> gene and in the 3' NC region as well as its relationship to the known attenuating mutation in the 5' NC region of the Sabin genome (the G-480 mutation). This was done by constructing mutant and recombinant strains by using the cDNAs of the Sabin 1 strain and of the mouse adapted PV-1/PV-2 strain. Characterization of the viral strains showed that the C-6203 mutation in the 3D polymerase gene is one of the mutations implicated in the thermosensitivity of the Sabin 1 strain. Results strongly suggested that the C-6203 mutation is involved in the attenuation of the Sabin 1 strain. Of the mutations located in the 3D<sup>pol</sup> and 3' NC regions, the C-6203 mutation may be the main or the only one to be involved in attenuation in this mouse model. It acts independently of the G-480 mutation, and the two mutations, i.e., G-480 and C-6203, may have a cumulative effect on attenuation.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *Escherichia coli* DH5 $\alpha$  (Bethesda Research Laboratories) was used for propagation of plasmids and transformation procedures.

Plasmid pAM510 contains the full-length PV-1 Mahoney cDNA in which the nucleotide sequence coding for amino acids 94 to 102 of the VP1 protein of PV-1 (nucleotides 2556 to 2786) is substituted by the corresponding nucleotide sequence from PV-2 Lansing (33). The cDNA is inserted downstream from the simian virus 40 late promoter, and this plasmid also carries the origin of replication, enhancer sequences, and T-antigen gene from simian virus 40. Plasmid pAM510Ab is a pAM510 derivative in which an A→G mutation has been introduced at position 480 in PV-1 Mahoney cDNA (32). Plasmid pVS(1)IC-0(T) contains the entire genomic cDNA of the LSc2ab Sabin strain of PV-1 (24) (this plasmid was a generous gift from A. Nomoto). Recent sequence analysis of the infectious Sabin 1 cDNA clone revealed an adenine residue at position 6734, not a guanine residue as reported originally (39).

**Construction of recombinant plasmids and directed mutagenesis.** DNAs were cleaved with restriction endonucleases under conditions recommended by the manufacturers. Ligations and transformations were performed by standard methods, and plasmid DNA sequences were determined by using the Sequenase kit (United States Biochemical).

**Plasmids pKSM and pAS.** The sequence between nucleotides 5548 and 6227 of PV-1 Mahoney cDNA was amplified by the polymerase chain reaction as described by the manufacturer (Genofit). In this reaction, the downstream primer corresponding to nucleotides 6195 to 6227 carried a T→C substitution at nucleotide position 6203, as found in the genome of the Sabin 1 strain. The ends of the 679-bp amplified fragment were converted to blunt ends by using the Klenow fragment of *E. coli* DNA polymerase I. The blunt-ended fragment was then inserted into the *Sma*I site of pBluescript II SK+ (Stratagene), and its nucleotide sequence was checked. The *Bgl*II-*Acc*I fragment (nucleotides 5602 to 6219) carrying the Sabin mutation C-6203 in the 3D<sup>pol</sup>

gene was isolated and used to replace the corresponding DNA fragments in pAM510 and pAM510Ab to yield plasmids pKSM and pAS, respectively.

**Plasmids pH, pHM, pHAM, and pHAM.** To introduce the base (T) found in the PV-1 Mahoney genome at position 6203 into the 3D<sup>pol</sup> gene of Sabin 1 cDNA, we took advantage of the presence of this mutation in the genome of the thermoresistant mutant (S<sub>1</sub>39C<sub>6</sub>) derived from the attenuated Sabin 1 strain (12). The sequence between nucleotides 6033 and 6556 of the S<sub>1</sub>39C<sub>6</sub> genome was amplified from purified S<sub>1</sub>39C<sub>6</sub> genomic RNA according to the method of J. Balanant et al. (4). The *Hind*III fragment (nucleotides 6057 to 6516) was prepared from the amplified fragment and used to substitute the corresponding fragment in a cDNA subclone of pVS(1)IC-0(T). The construct and mutation were verified by DNA sequencing. The *Bgl*II-*Acc*I fragment (nucleotides 5602 to 6219) carrying the C-6203→T substitution was then isolated and used to replace the corresponding fragment in pVS(1)IC-0(T) to yield pVSM.

The *Bgl*II-*Eco*RI fragment carrying the 3' end (nucleotides 5602 to 7441) and the poly(A) tract of the Sabin 1 cDNA was excised from pVS(1)IC-0(T) and inserted in place of the corresponding fragments in pAM510 and pAM510Ab. This yielded plasmids pH and pHAM, respectively. Similarly, the *Bgl*II-*Eco*RI fragment of pVSM, which carries the Sabin 1 sequences with the Mahoney-like nucleotide (T) at position 6203, was used to construct pHM and pHAM, respectively. These constructions resulted in the deletion of the polyadenylation site of simian virus 40, which has previously been shown to have no effect on the infectivity of cDNA for primate cells (22).

**Plasmid pSabin 1/PV2.** To construct the Sabin 1/PV-2 hybrid, in which the VP1 B-C loop of the Sabin 1 strain (amino acids 94 to 102) was replaced by that of PV-2 Lansing, we used the plasmid pJF C82 (17). Plasmid pJF C82 is identical to pVS(1)IC-0(T) except that a mutagenesis cartridge was generated by introducing the unique restriction sites *Sal*I and *Sac*I at positions 2753 and 2790 of the viral cDNA, respectively. Annealed synthetic oligonucleotides 5'-TCGATAATGATGCTCCAACAAAGCGTGCCAGTAA GCT and 5'-TACTGGCAGCCTTTGTTGGAGCATCATTA, corresponding to PV-2 Lansing sequences, were ligated to the *Sal*I-*Sac*I-digested pJF C82 to yield plasmid pSabin 1/PV2.

**Transfection and virus stocks.** Plasmids containing full-length PV infectious cDNAs and regulatory sequences of simian virus 40 were transfected into simian Vero cells (2.5  $\mu$ g of DNA per 10<sup>6</sup> cells) by the calcium phosphate technique as previously described (13). Transfected cells were incubated at 34°C until the cytopathic effect was complete (between 3 and 6 days). Virus was harvested and amplified by one or two passages after transfection, on HEP-2c cells infected at a multiplicity of infection of approximately 1 PFU per cell. After 18 h at 34°C, remaining cells and supernatants were submitted to three cycles of freezing-thawing and clarified; the resulting virus stocks were maintained at -30°C. When necessary, viruses were concentrated by centrifugation for 2 h at 30,000 rpm in a Kontron 50.38 rotor at 4°C and resuspended in cell culture medium (Dulbecco's modified minimum essential medium supplemented with 2% newborn calf serum and 50  $\mu$ g of gentamicin per ml).

Viruses v510, vKSM, etc., were thus recovered from transfection with plasmids pAM510, pKSM, etc., respectively. Viruses v510A and vS1 were recovered from pAM510Ab and pVS(1)IC-0(T), respectively.

**Viral RNA extraction, sequencing, and the restriction frag-**

**ment length polymorphism assay.** Viral RNA was extracted as previously described (32). Briefly, HEP-2c cells were infected with the appropriate virus stock and were lysed (5 h after infection) in the presence of 0.5% Nonidet P-40. Cytoplasmic RNAs were recovered by phenol-chloroform extraction in the presence of 1% sodium dodecyl sulfate and sequenced by using avian myeloblastosis virus reverse transcriptase (18). The presence of the Sabin-like nucleotide at position 7441 of the 3' NC region of the PV genome was confirmed by sequencing as previously described (10) but using a cDNA primer poly (dT)<sub>23</sub> · poly (dC); fragments synthesized from PV-1 Mahoney RNA are 1 base shorter than those derived from Sabin 1 RNA, a difference which can be detected on a sequencing gel. This is due to the presence of GAG(A)n and GAGG(A)n at the ends of the PV-1 Mahoney and Sabin 1 genomes, respectively.

In some cases, the nature of nucleotide 6203 was verified by restriction fragment length polymorphism analysis using the *RsaI* restriction enzyme (41).

**Thermosensitivity.** The thermosensitivities of viruses were evaluated by studying the reproductive capacity of each virus strain at various temperatures (standard Rct test). Rct is defined as the difference, after 7 days of incubation, between the log<sub>10</sub> virus titer (50% tissue culture infective dose per ml) at optimal temperature (34°C) and that at each of the supraoptimal (39.5 and 40°C) temperatures (37). Viral titers were determined by using HEP-2c cells. Viruses were considered thermosensitive when the Rct value (between 34 and 40°C) was >4 log<sub>10</sub>, not thermosensitive when the Rct value was <2 log<sub>10</sub>, and partially thermosensitive for intermediate cases.

**Mouse neurovirulence assay.** Groups of six 21-day-old female Swiss (OF1; IFFA CREDO) mice were inoculated intracerebrally with 0.03 ml of serial dilutions of virus stocks, and the animals were observed daily for 21 days for scoring paralysis or death. The amount of virus which caused paralysis or death in 50% of mice (PD<sub>50</sub>) was calculated by the method of Reed and Muench (45). Virus was isolated from the spinal cord of at least one paralyzed mouse for each viral stock used (32). After one passage on HEP-2c cells, viral RNA was extracted and checked for the presence of the introduced mutations.

**Statistical analysis.** The disease incidence (paralysis or death) and time to disease onset data were subjected to survival data analysis methods (29). Cumulative results of four experiments using three virus doses (10<sup>6</sup>, 10<sup>5</sup>, and 10<sup>4</sup> PFU) and six mice per dose for each virus strain were included (72 mice per virus). Statistical comparisons between virus-associated disease development were made pairwise according to the Kaplan-Meier method and tested for significance by using the log-rank, Tarone-Ware, and Wilcoxon tests (5). Statistical calculations were performed by using the 1L program of BMDP statistical software (5).

## RESULTS

**Construction of recombinant viruses.** We analyzed the possible attenuating effect of the mutation at position 6203 in the Sabin 1 genome (the C-6203 mutation) with a mouse model. We constructed a series of PV recombinants derived from plasmid pAM510, which carries the infectious cDNA of the mouse-adapted PV-1/PV-2 strain, v510 (33). The v510 genome consists of the wild-type PV-1 Mahoney genome in which the sequence coding for the amino acids 94 to 102 of the VP1 capsid protein was substituted by the corresponding nucleotide sequence from PV-2 Lansing. First, single-site

mutagenesis was performed on the original v510 cDNA to introduce the Sabin-like C-6203 mutation into the PV-1 Mahoney genome. This C-6203 mutation led to a Tyr-to-His amino acid change at position 73 of the polymerase of the resulting vKSM strain (Fig. 1).

Then, to analyze the possible relationship between the C-6203 and other mutations carried by the 3D<sup>pol</sup> gene and the 3' NC region of the Sabin 1 genome, we constructed a hybrid v510-Sabin 1 strain (vH) which carried the 3'-terminal 1,840 bases of the Sabin 1 genome. In this region, the genome of the attenuated Sabin 1 strain differs from that of the neurovirulent Mahoney virus by 11 point mutations including C-6203. Three mutations of the nine located in the 3D<sup>pol</sup> gene induce amino acid changes at codons 53, 73, and 362 (nucleotide positions 6143, 6203, and 7071, respectively). Two other mutations are located in the 3' NC region of the genome (nucleotide positions 7410 and 7441). To discriminate the specific effect of the C-6203 mutation from the possible effects of the other 10 mutations, the C-6203 nucleotide in the vH hybrid strain genome was replaced by the Mahoney T-6203 nucleotide to produce strain vHM (Fig. 1).

Furthermore, we looked for possible joint effects of the strong attenuating mutation G-480 of the 5' NC region and the mutations carried by the 3'-terminal part of the Sabin 1 genome. Strains similar to those described above were constructed by using a plasmid which carries the cDNA corresponding to the original mouse-adapted strain v510 with the additional mutation G-480 (strain v510A) (32). These constructions resulted in strains vAS, vHA, and vHAM, all carrying the G-480 nucleotide in the 5' NC region but having different 3' genomic ends (Fig. 1).

Recombinant viruses were recovered from Vero cells transfected at 34°C with the above plasmids and amplified on HEP-2c cells. Viral RNAs obtained from viral stocks were checked in the region of introduced mutations by RNA sequencing. The nature of nucleotides at positions 480 and 6203 as well as the Sabin 1-specific nucleotide markers at positions 6143, 7410, and 7441 was verified as appropriate. No change in the regions of the genome that were checked (approximately from nucleotide 450 to 550, 6050 to 6250, and 7360 to 7410) could be detected in viral stocks. Sabin-like base G-7441 was checked in genomic RNAs of the recovered virus pools (see Materials and Methods); banding patterns on sequencing gels were similar to those for genomic RNA of the Sabin 1 strain of the laboratory.

The viral titers of all recombinant strains on HEP-2c cells at 34°C were not significantly different from that of the original v510 strain. All virus strains including the original v510 and v510A strains showed a small-plaque phenotype on HEP-2c cells (data not shown).

**Thermosensitivities of viruses.** To test the effects of introduced mutations on the thermosensitivities of the strains, the reproductive capacity of each recombinant strain at 34°C and 39.5 or 40°C (Rct marker) was determined (see Materials and Methods for details) (Fig. 2). The Sabin 1 strain (vS1) was thermosensitive and exhibited a loss of more than 5 log<sub>10</sub> 50% tissue culture infective dose units per ml between 34 and 40°C, whereas the original v510 strain was not thermosensitive as previously reported (24, 33).

The introduction of the Sabin-like nucleotide at position 480 of the 5' NC region (v510A) had a very slight effect on thermosensitivity, with a loss of around 1 log<sub>10</sub> 50% tissue culture infective dose unit per ml between 34 and 40°C. These results confirmed that the G-480 nucleotide is a very weak determinant of thermosensitivity (21, 32). However, hybrid strains (vH and vHA) carrying the 3'-terminal part of

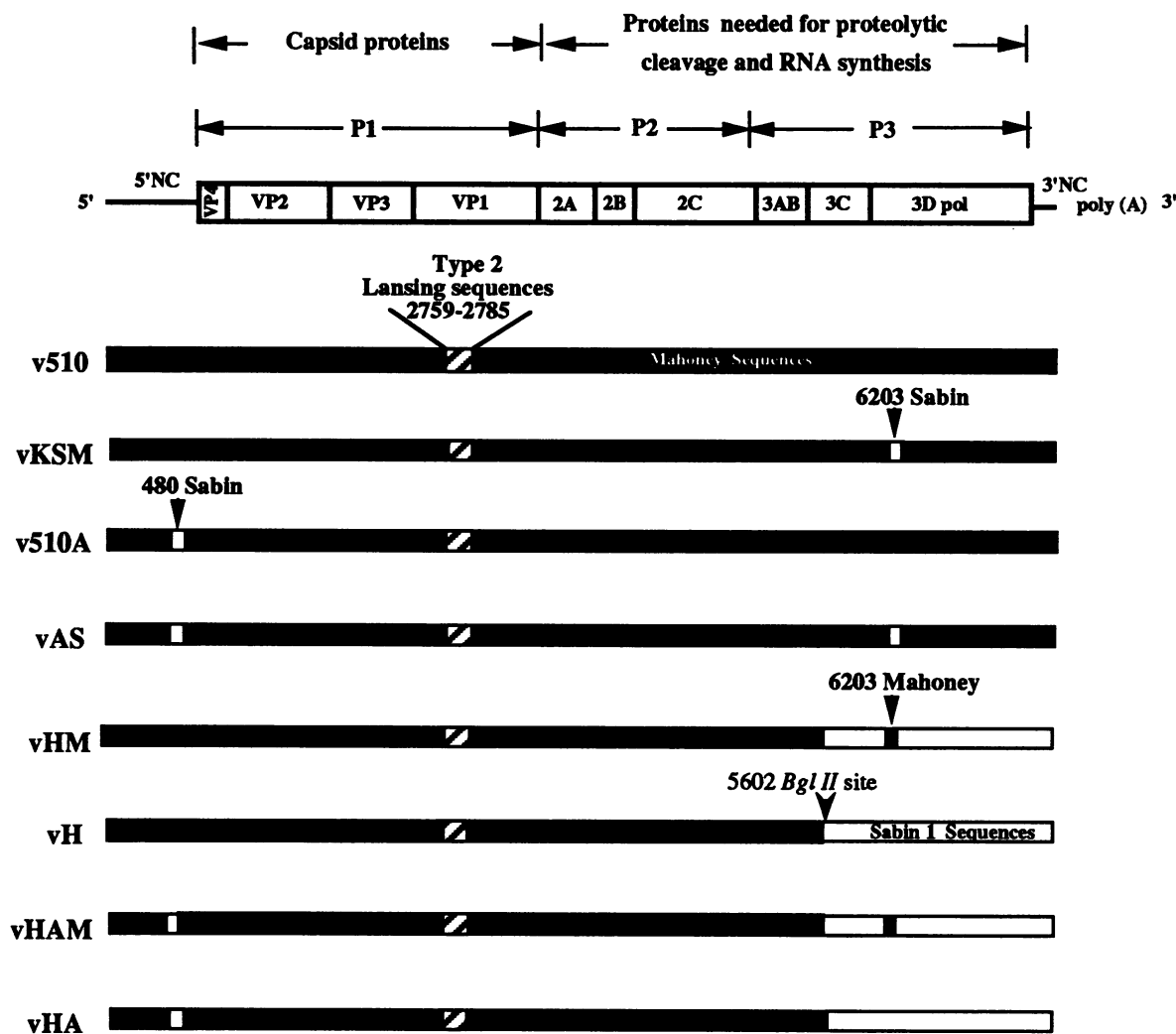


FIG. 1. Genome structures of recombinant viruses. The structure of the poliovirus RNA genome with the 5' NC and 3' NC regions and the polyprotein-coding region (open bars) is shown. Proteolytic cleavage of the polyprotein gives four capsid proteins VP1 to VP4 (region P1) and the nonstructural proteins (regions P2 and P3) including the polymerase (3D<sup>pol</sup>). The origins of genome sequences of the recombinant viruses are indicated as follows: closed bars, PV-1 Mahoney; open bars, strain Sabin 1; hatched bars, strain Lansing type 2. The genotype and nucleotide position of the introduced mutation and the position of the *Bgl*II site used to construct the v510/Sabin cDNA are indicated. Each pair of recombinant strains differ only by the presence of a Mahoney or Sabin 1-like mutation at nucleotide position 6203 of the genome. The effect of the nature of base 6203 was analyzed in the background of the original PV-1/PV-2 strain (v510 and vKSM) and of the v510/Sabin 1 hybrid strain (vHM and vH) as well as by using similar strains carrying, in addition, the Sabin mutation at position 480 of the 5' NC region (v510A, vAS, vHA, and vHAM).

the Sabin 1 genome were shown to be partially thermosensitive compared with the original v510, v510A, and Sabin 1 strains. This partial thermosensitivity was abrogated when the Sabin mutation at position 6203 of the hybrid genome was replaced with the wild-type nucleotide (vHM and vHAM). Thus, the mutation C-6203 (at codon 73 of the Sabin 1 polymerase) is involved in the thermosensitivity of the vaccinal strain. However, the introduction of the C-6203 mutation alone in the 3D<sup>pol</sup> gene of v510 and v510A (wild-type context) (vKSM and vAS) did not result in thermosensitivity. This suggests that the C-6203 mutation is phenotypically linked with other mutations carried by the 3' part of the Sabin 1 genome extending from the 3D<sup>pol</sup> gene to the 3' NC region.

**Neurovirulence of viruses in mice.** To test the neurovirulence of virus strains, mice were intracerebrally inoculated

with various dilutions of viral stocks. This experiment was repeated for each virus by making use of two different viral stocks obtained by independent cell transfection experiments. The PD<sub>50</sub> was determined for each strain (Fig. 3). The PD<sub>50</sub> of strains (v510A, vAS, vHAM, and vHA) carrying a Sabin-like nucleotide at position 480 were roughly 2 log<sub>10</sub> PFU higher than that of the parental v510 strain. This confirmed the strong attenuating effect of this mutation as previously shown with mice by using v510A (32) and with monkeys by using PV-1 Mahoney/Sabin 1 recombinant strains (21).

Virus strains which differed only at nucleotide 6203 of the 3D<sup>pol</sup> gene were compared pairwise. The PD<sub>50</sub> of viral strains which carry a Sabin-like residue at position 6203 of the 3D<sup>pol</sup> gene were systematically about 0.7 log<sub>10</sub> PFU higher than those of the corresponding viruses with the

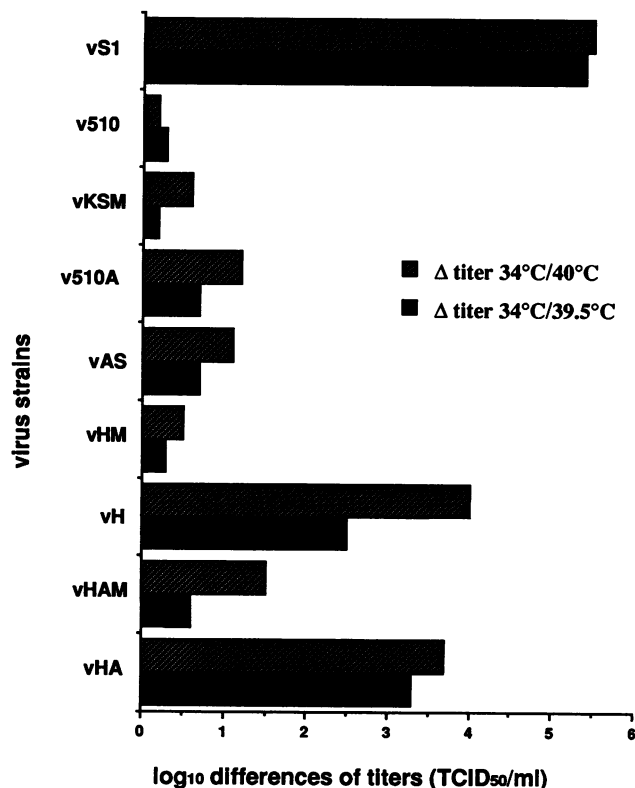


FIG. 2. Reproductive capacity of recombinant and parent viruses at different temperatures (Rct marker). Titers of virus stocks were measured after viruses were grown for 7 days in HEP-2c cells at 34, 39.5, and 40°C. Results of Rct markers are expressed as the differences between logarithmic virus titers at 34 and 39.5°C (closed bars) or between those at 34 and 40°C (hatched bars). Recombinant virus strains are diagrammed in Fig. 1 except for vS1, which corresponds to the cDNA-derived Sabin 1 strain. TCID<sub>50</sub>, 50% tissue culture infective dose.

wild-type nucleotide. Although this difference was found for almost all comparative measurements, it was small compared with the variation from one experiment to another. Therefore, the Kaplan-Meier method was used for survival data analysis to compare disease incidence (paralysis or death) and time to disease onset in groups of mice inoculated with v510, vKSM, vH, and vHM. This method was not applicable to other strains because of the differing dilutions of the inocula. The significance of comparisons was tested by the log-rank test (see Materials and Methods for details) (Fig. 4). Viruses carrying a wild-type nucleotide at position 6203 of the genome induced a higher incidence of disease in mice than strains with a Sabin-like residue ( $P < 0.003$  for v510 or vHM versus vKSM or vH). Wilcoxon and Tarone-Ware tests gave similar results (not shown). This demonstrated the clear, although slight, attenuating effect of the Sabin C-6203 residue in mice.

Moreover, no significant differences between disease incidences in groups of mice inoculated with virus strains carrying the same 6203 residue but differing at other nucleotide positions were observed ( $P > 0.14$  for v510 versus vHM or vKSM versus vH) (Fig. 4). Therefore, the presence of the other 10 mutations carried by the 3D<sup>pol</sup> gene and the 3' NC region of the Sabin 1 genome did not seem to play any role in attenuation. Statistical analysis of disease incidence

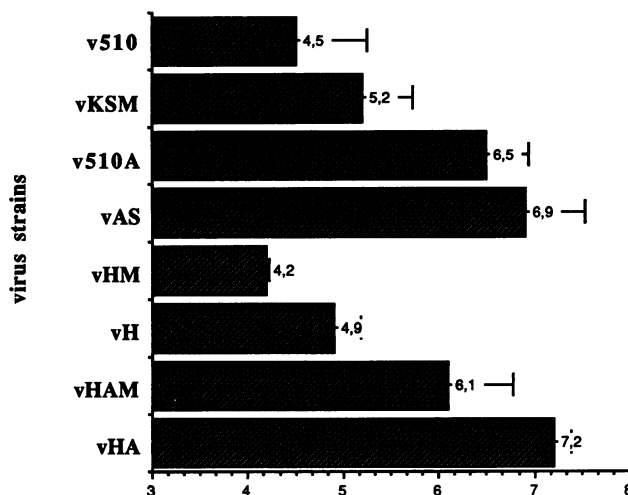


FIG. 3. PD<sub>50</sub>. The PD<sub>50</sub> was determined for each virus strain in Swiss mice. Results are expressed as the arithmetic means of log<sub>10</sub> PFU values from at least two experiments using two different viral stocks. The corresponding standard deviations are reported to indicate variations between experiments.

confirmed the results observed by comparing PD<sub>50</sub> values (Fig. 3). Unless suppressive mutations had appeared elsewhere in the genomes of recombinant strains, the C-6203 Sabin mutation may be the single attenuating factor of the 3'-terminal fifth of the Sabin 1 genome in this mouse model.

Analysis of PD<sub>50</sub> values also showed that both the C-6203 and the G-480 Sabin mutations acted independently to attenuate PV-1 (Fig. 3). Moreover, the PD<sub>50</sub> of viruses (vAS and vHA) carrying these two mutations suggested that, when both are expressed, the C-6203 and G-480 mutations have a cumulative effect on attenuation rather than a synergistic effect.

In order to check the presence of the introduced mutations in recombinant viruses after replication in mice, viruses were recovered from the spinal cord of at least one paralyzed mouse for each viral stock (mostly 4 days after inoculation) and their genomes were subjected to sequencing (in a few cases, position 6203 was checked by restriction fragment length polymorphism analysis). In all cases, bases 480 and 6203 were found to be as expected. However, the genome of virus recovered from one of nine mice inoculated with viruses carrying the Sabin-like base G-480 carried a mutation at position 525 (see Discussion). In some cases, Sabin-like bases at positions 6143, 7071 (3D<sup>pol</sup>-coding mutations), and 7410 (3' NC region) were also verified in genomic RNAs of viruses isolated from six, four, and eight paralyzed mice, respectively. No change occurred at these positions. The Sabin-like base G-7441 was checked in RNAs of viruses recovered from 12 paralyzed mice (three for each virus strain). In most cases, no change compared with the inocula was detected. However, for two mice inoculated with vH and for one mouse inoculated with vHA and vHAM, a mixture of G and A at position 7441 (between 30 and 50% A-7441) was found. The appearance of this subpopulation did not seem to depend either on the viral stocks, on the amounts of viruses inoculated, or on the nature of nucleotide 6203. Viruses with wild-type-like A-7441, which might have appeared from a subpopulation in viral stocks, might have a slight advantage in replication in mice. However, viruses with A-7441 were isolated from only a few of the paralyzed

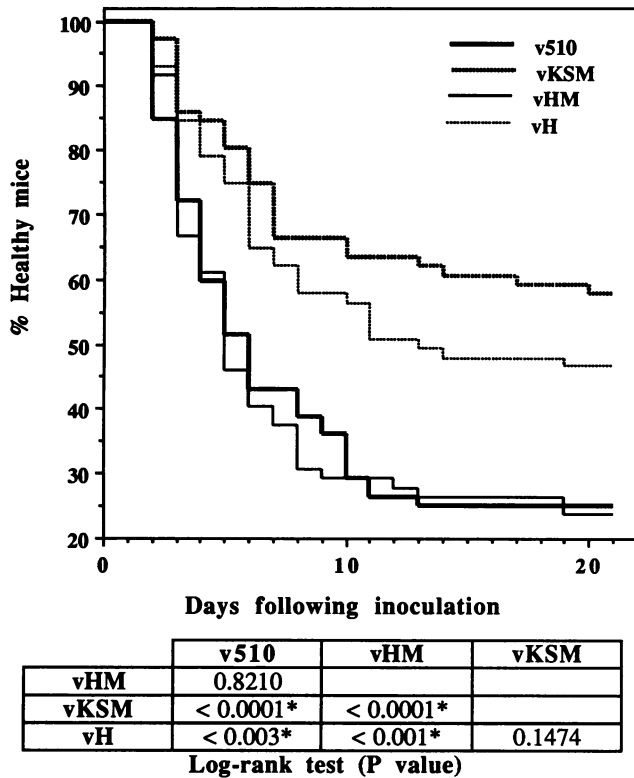


FIG. 4. Cumulative percentages of healthy mice following virus infection and log-rank *P* values from statistical comparisons. Mice were intracerebrally inoculated with the indicated viruses and observed daily for paralysis or death for 21 days. The percentages of healthy mice were calculated in four experiments using three virus doses (72 mice per virus strain). Comparisons were made according to the Kaplan-Meier method, and differences between virus-associated disease development were tested pairwise for significance by the log-rank test. \*, statistically significant ( $P < 0.05$ ).

mice, and full reversion to the wild type was never observed. Therefore, it seems unlikely that base A-7441 plays a role in the neurovirulence of the recombinant strains studied.

To determine the level of attenuation of strains carrying the two identified attenuating mutations (G-480 and C-6203), we tried to compare their neurovirulence with that of the Sabin 1 strain. To circumvent host restriction genetic determinants which could block infection of mice with the Sabin 1 strain, we constructed a new hybrid Sabin 1/PV-2 strain by substituting VP1 amino acids 94 to 102 of the Sabin 1 strain with those of PV-2 Lansing (the same type 2 residues which confer a mouse-adapted phenotype to the wild-type strain of PV-1). No paralyzes in mice inoculated with the highest dose of this hybrid strain ( $PD_{50} > 8.3 \log_{10}$  PFU) or in those inoculated with the parental Sabin 1 strain were observed. Thus the Sabin 1/PV-2 strain might be more attenuated in mice than strains (vAS and vH) carrying both the C-6203 and the G-480 Sabin mutations. However, more experiments are needed to ascertain that type 2 residues inserted in the Sabin 1 capsid allow the Sabin 1 strain to bypass mouse-specific host restriction.

#### DISCUSSION

To determine the role played by the 3' part of the Sabin 1 genome (the 3D polymerase gene and 3' NC region) in

attenuation, we circumvented the need for monkeys by using a strain of PV-1 rendered neurovirulent for mice by substituting nine amino acids of its capsid protein VP1 with those of the mouse-adapted PV-2 Lansing (32, 33). We focused our attention on the Sabin 3D<sup>pol</sup> C-6203 mutation, which has previously been implicated as a possible determinant of the thermosensitivity and/or attenuation of PV-1 (12, 57). We also studied the relationship between the C-6203 mutation and the strongly attenuating G-480 determinant in the 5' NC region of the Sabin 1 genome. Comparisons of the neurovirulence of recombinant and mutagenized strains in numerous mice allowed us to perform survival data analysis and thus to show that the C-6203 mutation is involved in attenuation. C-6203 may be the only, or at least the main, mutation of the 11 mutations carried by the 3D<sup>pol</sup> gene and the 3' NC region to be involved in attenuation in this mouse model.

The attenuating effect in mice of the C-6203 mutation appeared to be weaker than that of the G-480 mutation. The difference between the  $PD_{50}$  of viruses carrying the C-6203 mutation and that of the original PV-1/PV-2 strain was about  $0.7 \log_{10}$  PFU (compared with a difference of  $2 \log_{10}$  PFU for viruses with the G-480 mutation). Although small, this difference in neurovirulence was statistically significant as determined by comparing disease incidences plus times to disease onset (Kaplan-Meier method). A similar methodology has already been used to compare susceptibility and resistance to PV-induced paralysis of inbred mouse strains (20). Only relatively strong attenuating mutations have previously been analyzed by comparing  $PD_{50}$  values for mice (32, 34, 47). Our results show that survival data analysis could be helpful for analyzing weak determinants of PV attenuation. This statistical method could be adapted to compare the neurovirulence of PV mutant strains, or vaccine lots, in transgenic mice carrying the human PV receptor (these animals are susceptible to all PV serotypes) (25, 46).

The results previously obtained with mice in analyses of attenuation determinants carried by the 5' NC regions of all three serotypes (27, 32, 47) correlate well with those for monkeys (21, 31, 58). A recombinant PV-1 Mahoney carrying the 3'-terminal part of the Sabin 1 strain (the 3D<sup>pol</sup> and 3' NC regions) was shown to be weakly attenuated in monkeys (40). Our results with a similar construction (strain vH) but a different animal model confirmed that the 3' part of the Sabin 1 genome, and in particular the C-6203 mutation, is involved in the attenuation of PV-1 in mice. Despite the fact that with this kind of approach we cannot rule out that suppressive mutations could occur elsewhere in the genome of the recombinant strains used, several precautions were taken to minimize the roles of such events (see Results). Our data thus suggested that the other 10 mutations carried by the 3D<sup>pol</sup> and 3' NC regions of the Sabin 1 genome did not seem to play significant roles in attenuation. These mutations include, in particular, the G-7441 mutation (at the end of the 3' NC region), which has been shown to revert to the wild type in recombinant type 3-type 1 strains isolated from vaccinees (10) and, in conjunction with other mutations, in neurovirulent Sabin 1 mutants (12). Partial reversion of G-7441 was also found for viruses derived from some of our paralyzed mice, but this did not seem to be significantly linked with neurovirulence. The attenuated strain of PV-3 also carries a mutation just upstream from the poly(A) tract (A→G at position 7432). This mutation was found not to be significantly involved in attenuation of PV-3 in monkeys (58).

Although the estimation of  $PD_{50}$  values suggested that the G-480 and C-6203 mutations have a cumulative effect on

Sabin 1 attenuation, virus strains (vAS and vHA) carrying both mutations were not fully attenuated in mice. Therefore, it is likely that the level of replication of the Sabin 1 strain in the nervous system is also affected by other genetic determinants which have been roughly localized on other parts of the genome (21, 40). The presence of numerous attenuating determinants might prevent full reversion of the PV-1 vaccine strain to neurovirulence during replication in the host. This reasoning has been used to explain why the Sabin 1 strain is less implicated in vaccine-associated polio cases than the Sabin 2 and, in particular, Sabin 3 strains (53). However, reversion to the wild type of the G-480 and/or C-6203 Sabin residues has already been found for some Sabin 1-derived strains. True reversion to the wild type of the G-480 Sabin residue or related suppressive mutations at position 525 have been described for some PV strains isolated from cultured cells, from healthy vaccinees, and from patients with vaccine-associated polio cases (12, 26, 36, 41) (a mutation at position 525 [U→C] is believed to restore RNA base pairing disrupted by the Sabin mutation at nucleotide position 480 [A→G] [43, 52]). A similar suppressive mutation at position 525 was also found in one of nine viruses isolated from the spinal cords of paralyzed mice inoculated with viruses carrying the G-480 Sabin mutation. No reversion of the C-6203 3D<sup>pol</sup> mutation was detected among the few virus strains isolated from the spinal cords of paralyzed mice inoculated with our mutant strains. Nevertheless, reversion at this locus to the wild type (C-6203→T, His-73→Tyr) has been found for one of five Sabin 1 strains and for 11 of 14 Sabin 2/Sabin 1 or Sabin 3/Sabin 1 recombinant strains isolated in vaccine-associated polio cases (16, 30, 41). The reversion of the G-480 and/or C-6203 nucleotides to the wild type might contribute to the acquisition of some degree of neurovirulence by Sabin 1 strains in vaccinees (14).

Previous work has suggested that the reversion at the 3D<sup>pol</sup> locus in vaccinees could be linked to temperature selection pressure (12). This mutation appeared to be a critical factor in the moderate thermosensitivity expressed by the 3'-terminal part of the Sabin 1 genome (the 3D<sup>pol</sup> gene and 3' NC region); reversion at this locus to the wild type suppressed this thermosensitivity. However, the introduction of this reversion alone into the original Sabin 1 strain did not induce any apparent change in its thermosensitivity (not shown). Thus, the C-6203 mutation is only one of numerous mutations involved in the thermosensitivity of the Sabin 1 strain. This is in agreement with the results of previous studies showing that determinants of the Sabin 1 virus thermosensitivity map in the 3'-terminal part of the genome and also in each of two genomic fragments including the 5' NC region and the capsid protein-encoding region (38, 40). Moreover, the C-6203 mutation introduced alone into the wild-type background strain v510 did not cause a thermosensitive phenotype. This suggests that thermosensitivity results from interaction with at least one other of the 10 mutations carried by the 3D<sup>pol</sup> and the 3' NC region of the PV genome. We are currently mapping the loci which are phenotypically linked to the C-6203 mutation. Amino acid changes at positions 53 and 362 of the polymerase and the G-7441 mutation appeared to be good candidates for this investigation. However, there are numerous other silent mutations in the 3D<sup>pol</sup> gene and 3' NC region which may be involved in RNA secondary structure and/or in RNA replication. Thermosensitive RNA synthesis mutants have been isolated from PV cDNAs whose 3' NC regions had been mutagenized (50). Although it is unlikely, we also cannot

exclude that mutations leading to amino acid changes (including the C-6203 mutation) may act on RNA secondary structure rather than on the 3D<sup>pol</sup> enzyme activity.

The thermosensitive phenotype linked to the C-6203 mutation did not strictly correlate with attenuation in mice; vKSM carries this single mutation and is partially attenuated but not thermosensitive. A similar result has already been described for the G-480 Sabin mutation (21, 32). It is unclear which of the viral functions are affected by the C-6203 mutation and what their roles in attenuation and/or in thermosensitivity may be. Poliovirus RNA polymerase mutants which have an RNA synthesis defect have been described, and some of them were thermosensitive (2, 6, 9, 11, 23). None of the identified mutations map close to position 6203. The C-6203 mutation also does not appear to be close to mutations affecting the *in vitro* polymerase activity (8, 48) or close to the critical YGDD motif common to many viral polymerases (19). *In vitro* studies nevertheless suggested that at least one of two Sabin mutations affecting the 3D<sup>pol</sup> gene at nucleotide positions 6143 (G→A, Asp-53→Asn) and 6203 may influence the initiation of viral RNA synthesis above 38°C through a thermosensitive block of uridylation of the viral protein VPg (57). These observations and our results suggest that the 3D<sup>pol</sup> C-6203 mutation might affect viral RNA replication and therefore PV multiplication in murine neurons.

In conclusion, we have looked for PV-1 attenuation determinants using a mouse model. By evaluating neurovirulence in numerous mice with tests that take into account disease incidence and time to disease onset, we were able to show that a mutation in the RNA polymerase of PV-1 (the C-6203 mutation) contributes weakly but significantly to the attenuation of the Sabin 1 strain in this animal model.

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