

## Mapping of Mutations Contributing to the Temperature Sensitivity of the Sabin 1 Vaccine Strain of Poliovirus

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**The temperature-sensitive and attenuated phenotypes of the Sabin type 1 vaccine strain of poliovirus result from numerous point mutations which occurred in the virulent Mahoney virus parent. One of these mutations is located in a 3D polymerase (3D<sup>pol</sup>) codon (U-6203→C, Tyr-73→His) and is involved in attenuation in common mice (M. Tardy-Panit, B. Blondel, A. Martin, F. Tekaiia, F. Horaud, and F. Delpeyroux, *J. Virol.* 67:4630–4638, 1993). This mutation also appears to contribute to temperature sensitivity, in association with at least 1 other of the 10 mutations of the 3'-terminal part of the genome including the 3D<sup>pol</sup> coding and 3' noncoding regions. To map the other mutation(s), we constructed poliovirus mutants by mutagenesis and recombination of Mahoney and Sabin 1 cDNAs. Characterization of these poliovirus mutants showed that a second mutation in a 3D<sup>pol</sup> codon (C-7071→U, Thr-362→Ile) contributes to temperature sensitivity. A mutation in the 3' noncoding region of the genome (A-7441→G), alone or linked to another mutation (U-7410→C), also appeared to be involved in this phenotype. The temperature-sensitive effect associated with the 3'-terminal part of the Sabin 1 genome results from the cumulative and/or synergistic effects of at least three genetic determinants, i.e., the His-73 and Ile-362 codons of 3D<sup>pol</sup> and nucleotide G-7441. Sequence analysis of strains isolated from patients with vaccine-associated paralytic poliomyelitis showed that these genetic determinants are selected against in vivo, although the Ile-362 codon appeared to be more stable than either the His-73 codon or G-7441. These genetic determinants may contribute to the safety of Sabin 1 in vaccinees.**

Poliovirus (PV), an enterovirus of the *Picornaviridae* family, is the causative agent of poliomyelitis and is classified into three distinct serotypes. It replicates in the human digestive tract and may induce paralysis by infecting and destroying motor neurons (5, 10). Poliomyelitis has been effectively controlled by the use of inactivated or live attenuated vaccinees. Attenuated strains of all three serotypes have been selected by numerous passages of wild-type strains in monkey tissues in vivo and in vitro (43). These strains (Sabin 1, 2, and 3), which replicate in the human gut and induce a strong immunity, have a good safety record. However, in a small number of cases, vaccination is associated with paralytic poliomyelitis (34). Vaccine-associated paralytic poliomyelitis (VAPP) may result from the genetic variability (point mutations and recombination) of the Sabin strains. Indeed, neurovirulent vaccine-derived strains are found in the gut of healthy vaccinees and in the central nervous system of patients with VAPP (16, 31). VAPP is most frequently associated with Sabin 2 and Sabin 3 and rarely with Sabin 1 (3, 37). Analysis of the attenuation determinants of Sabin 1 could lead to a better understanding of the safety of this strain in vaccinees.

PV is composed of an icosahedral protein shell which contains the viral genome, 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 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 (2, 6, 38, 42). The short 3' noncoding (3' NC) region is involved in viral RNA replication (19, 44).

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 31 and 51). The genome of Sabin 3 differs from that of the virulent wild-type progenitor by 12 mutations, and it has been found that among these mutations, only three determine the attenuation phenotype (47, 50). In contrast, the genome of Sabin 1 differs from that of its parental virulent Mahoney virus by 55 point mutations (35). 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, 36). A point mutation in the 5' NC region of the PV-1 genome, at nucleotide position 480, is known to play an important role in the attenuation of Sabin 1 in monkeys and in mice (20, 30). Recently, by using transgenic mice carrying the human PV receptor gene, mutations at positions 189 and 21 and/or 935 of the PV-1 genome were found to play minor but significant roles in attenuation (18). Previous studies of PV-1 attenuation suggested that the 3'-terminal part of the Sabin 1 genome including the 3D<sup>pol</sup> coding region and the 3' NC region is also involved in the attenuation of PV-1 in monkeys (1, 8, 36). We searched for attenuation determinants in this part of the Sabin 1 genome, using a mouse-adapted PV-1/PV-2 chimeric virus. A mutation at nucleotide position 6203 (U→C) leading to an amino acid change at position 73 of 3D<sup>pol</sup> (Tyr-73→His) was identified as being implicated in the attenuation of Sabin 1 in common mice (46).

For PV vaccine strains, temperature sensitivity correlates with attenuation, and vaccine-derived mutant strains which have lost their temperature sensitivity have been shown to be neurovirulent (8, 16, 26, 36). Seven attenuating mutations have been well characterized in Sabin strains (20, 28, 29, 40, 46, 47, 50), and depending on the cell type used for the studies, five

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influence viral growth at elevated temperatures (27, 29, 32, 46). In Sabin 1, the His-73 codon of 3D<sup>pol</sup> resulting from a missense mutation in Mahoney appeared to contribute to temperature sensitivity, in association with at least 1 of the 10 mutations located in the 3'-terminal part of the genome (46). To map the other mutation(s) we constructed PV mutants by mutagenesis and recombination of Mahoney and Sabin 1 cDNAs. In the 3'-terminal part of the genome, at least three mutations contribute to the temperature-sensitive (*ts*) phenotype of Sabin 1. Sequence analysis of Sabin 1-derived strains and intertypic recombinants isolated from patients with VAPP showed that these three mutations are selected against in vivo.

## MATERIALS AND METHODS

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

Plasmid pKK17 contains the full-length Mahoney cDNA downstream from the simian virus 40 (SV40) late promoter (22). It also carries the origin of replication, enhancer sequences, and T-antigen gene from SV40. Plasmid pVS(1)IC-0(T) contains the entire genomic cDNA of the LS-c, 2ab Sabin strain of PV-1 (24). Plasmids pKSM and pVSM have already been described (46). Plasmid pKSM is derived from pKK17 and carries a PV Lansing type 2 cDNA fragment replacing a PV-1 fragment encoding part of one of the structural capsid proteins. In addition, the nucleotide at position 6203 (T-6203) of the 3D<sup>pol</sup> coding region has been changed to C-6203 as found in Sabin 1. Plasmid pVSM is identical to pVS(1)IC-0(T) except for nucleotide C-6203, which has been changed to the nucleotide (T-6203) found in the Mahoney cDNA.

**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 a Sequenase kit (United States Biochemical).

(i) **Plasmids pHSB, pHSB<sub>62</sub>, and pKS<sub>62</sub>.** The *Bgl*II-*Eco*RI fragment carrying the 3' end (nucleotides 5602 to 7441) and the poly(A) tract of PV cDNA was excised from each of the plasmids pVS(1)IC-0(T), pVSM, and pKSM and inserted in place of the corresponding fragment in pKK17, to give pHSB, pHSB<sub>62</sub>, and pKS<sub>62</sub>, respectively.

(ii) **Plasmid pHSBA.** The *Bgl*II-*Acc*I fragment (nucleotides 5602 to 6219) encoding the C-terminal end of the viral protease 3C<sup>pro</sup> and the N-terminal end of 3D<sup>pol</sup> was excised from pVS(1)IC-0(T) and inserted in place of the corresponding fragment in pKK17, giving plasmid pHSBA.

(iii) **Plasmids pHSP, pHSM, and pHSOM.** The *Pvu*II-*Eco*RI and *Mun*I-*Eco*RI fragments carrying the 3' end (nucleotides 7055 to 7441 and 7305 to 7441, respectively) and the poly(A) tract of PV cDNA were isolated from pVS(1)IC-0(T) and used to replace the corresponding fragments in pKS<sub>62</sub>, yielding pHSP and pHSM, respectively. To construct pHSOM, the *Mun*I-*Eco*RI fragment from pVS(1)IC-0(T) was used to replace the corresponding fragment in pKK17.

(iv) **Plasmid pHSBM<sub>70</sub>.** To introduce the base (T) found in the Mahoney cDNA at position 7071 into the 3D<sup>pol</sup> coding region of Sabin 1 cDNA, a DNA fragment was amplified from pVS(1)IC-0(T) by PCR as described by the manufacturer (Appligene). In this reaction, the upstream primer (Ma7071) corresponding to nucleotides 7044 to 7080 carried a T at nucleotide position 7071, as found in the Mahoney cDNA. The downstream primer corresponded to an SV40 DNA fragment (nucleotides 42 to 58). The *Pvu*II-*Eco*RI fragment (as used for pHSP) was prepared from the amplified DNA product and used to substitute the corresponding fragment in a cDNA subclone of pVS(1)IC-0(T). The integrity of the construct and the mutation were verified by DNA sequencing. The *Bgl*II-*Eco*RI fragment from pVS(1)IC-0(T) carrying the T-7071→C substitution was then isolated and inserted in place of the corresponding fragment in pKK17 to yield pHSBM<sub>70</sub>.

(v) **Plasmids pKS<sub>70</sub>, pK2S<sub>6270</sub>, and pKS<sub>62</sub> bis.** The base (C) found in the Sabin 1 genome at position 7071 was introduced into the 3D<sup>pol</sup> coding region of the Mahoney cDNA by using the same PCR strategy as used to construct pHSBM<sub>70</sub>. The upstream primer (Sa7071) corresponded to nucleotides 7044 to 7080 and carried a C at nucleotide position 7071 consistent with the Sabin 1 cDNA. The downstream primer (MaR1) corresponded to the last 6 nucleotides of the Mahoney cDNA, a poly(A) tail of 10 nucleotides, and an *Eco*RI site. The *Pvu*II-*Eco*RI fragment (as used for pHSP) was prepared from the amplified DNA product (the template was Mahoney cDNA) and used to substitute the corresponding fragment in pKK17 and pKS<sub>62</sub>, to yield pKS<sub>70</sub> and pK2S<sub>6270</sub>, respectively. A control plasmid, pKS<sub>62</sub> bis, was also constructed by using the same strategy but with primers Ma7071 and MaR1. pKS<sub>62</sub> bis was identical to pKS<sub>62</sub> except for the polyadenylation site of SV40, which was deleted downstream of the cDNA poly(A) tail. Most plasmids other than pKK17, pKS<sub>62</sub>, and pHSBA were similarly deleted. This deletion has previously been shown to have no effect on the infectivity of cDNA for primate cells (21) and was shown in this study to have no effect on the phenotype of the recombinant strain (see Results).

(vi) **Plasmids pK2S<sub>6241</sub>, pK3S<sub>41</sub>, pK3S<sub>10</sub>, and pK4S.** The base (G) found in

the Sabin 1 genome at position 7441 was introduced into the 3' NC region of the Mahoney cDNA by using the PCR strategy. The downstream primer (SaR1) used was identical to primer MaR1 but was designed for introducing a G at nucleotide position 7441. The two *Pvu*II-*Eco*RI fragments amplified from pKK17 DNA by using primers Ma7071-SaR1 and Sa7071-SaR1 were used to substitute the corresponding fragments in pKS<sub>62</sub>, to yield pK2S<sub>6241</sub> and pK3S<sub>41</sub>, respectively. Similarly, plasmids pK3S<sub>10</sub> and pK4S were constructed by using two *Pvu*II-*Eco*RI fragments amplified from pHSOM DNA with primers Sa7071-MaR1 and Sa7071-SaR1, respectively.

Viral RNA or plasmid DNA was sequenced to verify mutations and nucleotide fragments derived from PCR products.

**Transfection and virus stocks.** Plasmids containing full-length PV infectious cDNAs and regulatory sequences of SV40 were used to transfect simian Vero cells (1.5  $\mu$ g of DNA per 10<sup>6</sup> cells) by the calcium phosphate technique as previously described (9). Transfected cells were incubated at 34°C until the cytopathic effect was complete. Virus was harvested and amplified by one passage after transfection on Hep-2c cells infected at a multiplicity of infection of >1 PFU per cell. After about 18 h at 34°C, supernatants were submitted to three cycles of freezing-thawing and clarified; the resulting virus stocks were maintained at -30°C. Viruses vKS<sub>62</sub>, vHSB, etc., were thus recovered from transfection with plasmids pKS<sub>62</sub>, pHSB, etc., respectively. Viruses vS1 and vKK were recovered from pVS(1)IC-0(T) and pKK17, respectively.

The specific infectivity of some of the plasmids was measured. Cells were transfected with various dilutions of plasmid DNA and maintained in liquid medium for 18 h. Cells were then maintained under a 0.9% agar overlay for 2 days and stained. The number of viral plaques per amount of plasmid DNA was determined.

**Viral RNA extraction and sequencing.** Viral RNA was extracted as previously described (30). 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 (14). The presence of the Sabin-like nucleotide at position 7441 of the 3' NC region of the PV genome was confirmed by comparative sequencing analysis using the cDNA primer (dT)<sub>23</sub>dC as previously described (46).

**Phenotypic characterization.** The temperature sensitivity of viruses was evaluated by studying their reproductive capacity at various temperatures (standard Rct test) (33). Briefly, each virus stock, with a titer of  $8.7 \pm 0.5 \log_{10}$  50% tissue culture infective dose units (TCID<sub>50</sub>) per ml, was decimally diluted through 10<sup>-8</sup>. A 0.1-ml volume of each virus dilution was inoculated into 10 wells of each of two 96-well plastic plates. A 0.1-ml volume of medium containing  $4 \times 10^4$  Hep-2c cells was then added into each well. One plate was incubated at 34°C (optimal temperature) in an incubator, and the second was submerged in a water bath at 40°C (supraoptimal temperature). After 7 days of incubation, cells were stained and the titer (TCID<sub>50</sub> per milliliter) was calculated for each temperature. The Rct value is defined as the difference between the log<sub>10</sub> virus titers obtained at 34 and 40°C. Only viruses with Rct values above 2 (Rct > 2) are considered to be *ts* (33).

Plaque diameter was determined by using virus-infected Hep-2c or HeLa cells maintained under a 0.9% agar overlay and stained after 3 days of incubation at 34°C, or after 2 days at 39.5°C, in a 4% CO<sub>2</sub> incubator. For each virus, the diameter of all isolated plaques (at least 50 plaques) was measured. For each experiment, the means of the different plaque diameter samples were compared by one-way analysis of variance (45). In all cases, at least one sample differed significantly from the others ( $P < 0.001$ ). Statistical comparisons between plaque diameter samples were then made pairwise and tested for significance by Scheffé's test (45).

## RESULTS

**Construction and characterization of Mahoney/Sabin 1 recombinant viruses.** To localize genetic determinants of temperature sensitivity in the 3D<sup>pol</sup> region and the 3' NC region of the Sabin 1 genome, Mahoney/Sabin 1 recombinant and Mahoney mutant viruses were constructed by using plasmids carrying infectious cDNAs. The 3'-terminal part of the Sabin 1 genome (3D<sup>pol</sup> and 3' NC regions) differs from that of the parental Mahoney by 11 point mutations, three of which lead to amino acid changes in 3D<sup>pol</sup> (G-6143→A, Asp-53→Asn; U-6203→C, Tyr-73→His; C-7071→U, Thr-362→Ile) (Fig. 1). Six of the eight other mutations are silent mutations in the 3D<sup>pol</sup> region, and two are located in the 3' NC region (U-7410→C and A-7441→G). We focused our attention on the three residues of Sabin 1 corresponding to missense mutations in 3D<sup>pol</sup> codons, i.e., Asn-53 (A-6143), His-73 (C-6203), and Ile-362 (U-7071), and the two positions (C-7410 and G-7441)

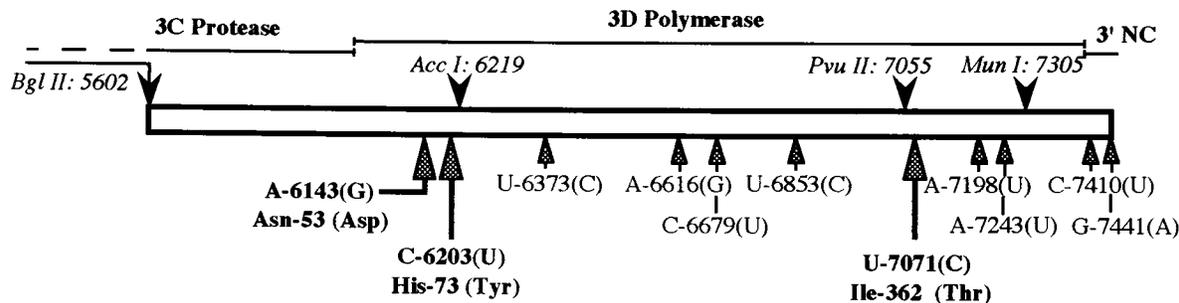


FIG. 1. Genome structure of the Sabin 1 cDNA region encoding the C-terminal part of 3C<sup>pro</sup>, 3D<sup>pol</sup>, and the 3' NC region. Mutations (with respect to Mahoney) are indicated, with the wild-type nucleotides and amino acids in parentheses. Large arrows indicate missense mutations; small arrows indicate silent mutations. The positions of the *Bgl*II, *Acc*I, *Pvu*II, and *Mun*I sites used for constructing recombinant cDNAs are shown.

corresponding to mutations in the 3' NC region of genome. The genome structures of the Mahoney/Sabin 1 recombinant viruses are schematized in Fig. 2 to 5.

Recombinant viruses were recovered from Vero cells transfected at 34°C with cDNA-containing plasmids and amplified on HEP-2c cells. In all cases, the cytopathic effect was seen 72 ± 20 h after transfection. In several cases, the specific infectivity of cDNAs was determined as the number of viral plaques per unit of plasmid DNA. There were no large differences between the specific infectivities of recombinant and mutant virus cDNAs and that of the Mahoney cDNA (from 0.5 × 10<sup>2</sup> to 1.5 × 10<sup>2</sup> PFU/nmol of plasmid DNA per 10<sup>6</sup> cells, for cDNAs of viruses vKK, vHSB, vHSP, vKS<sub>62</sub> bis, vHSBM<sub>70</sub>, vKS<sub>70</sub>, vK2S<sub>6270</sub>, vK3S<sub>41</sub>, vK3S<sub>10</sub>, and vK4S; described below). The viral titers (analyzed on HEP-2c cells at 34°C) of recombinant viruses and of the original cDNA-derived Mahoney were not significantly different when recovered from Vero cells (8.6 ± 0.4 log<sub>10</sub> TCID<sub>50</sub>/ml) or amplified on HEP-2c cells (8.7 ± 0.5 log<sub>10</sub> TCID<sub>50</sub>/ml). The sequences of the relevant regions (approximately from nucleotides 6050 to 6250, 7000 to 7125, and 7360 to 7410) of viral RNAs were confirmed by RNA sequencing. Nucleotide 7441 at the extreme end of the 3' NC region was checked as previously described (46) and was as expected. Nevertheless, to minimize the effects of possible suppressor mutations elsewhere in the genomes of recombinant strains, most results for each construct were checked by using two viral stocks derived from two different cDNA clones.

To test the effects of mutations on temperature sensitivity, the reproductive capacity of each recombinant virus was determined by titrating viral stocks at 34 and 40°C (Rct test) (Fig. 2). cDNA-derived Mahoney (vKK) was not *ts* (Rct < 2). In contrast, the Sabin 1 strain was highly *ts* (Rct > 5). The Mahoney/Sabin 1 recombinant virus vHSB carrying the 3'-terminal part of Sabin 1 genome (schematized in Fig. 1) was *ts* (Rct > 3). Reversion of the Sabin 1 His-73 codon of 3D<sup>pol</sup> to the wild-type Tyr-73 codon in Mahoney/Sabin 1 virus (vHSBM<sub>62</sub>) suppressed the *ts* phenotype (Rct < 2). However, conversion of the codon 73 of Mahoney to His (vKS<sub>62</sub>) did not result in a *ts* virus (Rct < 2). These results were in complete agreement with those previously described for mouse-adapted PV-1/PV-2 chimeric viruses (46) and confirmed that the His-73 codon of 3D<sup>pol</sup> is involved in the *ts* phenotype of Sabin 1, in association with at least 1 other of the 10 mutations which differentiate the 3'-terminal part of the Sabin 1 genome from that of Mahoney. The phenotype conferred by the His-73 codon in association with the Asn-53 codon of 3D<sup>pol</sup> (vHSBA) or with the two nucleotides (C-7410 and G-7441) of the 3' NC region of Sabin 1 (vHSM) was determined. The phenotypes of vHSBA and vHSM were similar to that of Mahoney vKK (Rct < 2) (Fig. 2).

However, the presence in vHSP of the His-73 codon and the five Sabin 1 nucleotides corresponding to the last 3'-terminal mutations led to a *ts* phenotype similar to that of the original Mahoney/Sabin 1 virus vHSB. The Sabin 1 residues carried by vHSP include those corresponding to the third missense mutation (Ile-362), to two silent mutations of 3D<sup>pol</sup>, and to the two nucleotides corresponding to the 3' NC mutations (C-7410 and G-7441). vHSM carrying the His-73 codon, C-7410, and G-7441 did not appear to be *ts* (Rct test). We therefore focused on the Ile-362 codon of 3D<sup>pol</sup> (U-7071).

**Involvement of the Ile-362 codon of 3D<sup>pol</sup> (U-7071) in temperature sensitivity.** The involvement of the Ile-362 codon was investigated by using the Rct test (Fig. 3A). The *ts* phenotype conferred by the 3'-terminal part of the Sabin 1 genome in vHSB (Rct > 3) was lost when the Ile-362 codon was replaced

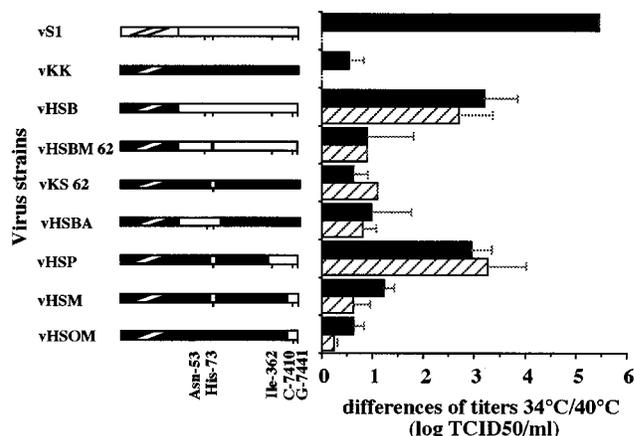


FIG. 2. Reproductive capacity of recombinant and parent viruses at various temperatures (Rct test). Simplified genome structures of the recombinant and mutant viruses are shown. Solid bars represent Mahoney wild-type nucleotide sequences. The oblique line indicates that the region corresponding to nucleotides 1 to 5601 is only partially represented. This region includes the 5' NC region, the region encoding the virus capsid proteins, and the region encoding part of the nonstructural proteins. Open bars represent Sabin 1. Vertical dashes indicate Sabin 1 amino acids (3D<sup>pol</sup> region) and the two Sabin 1 nucleotides in the 3' NC region (see Fig. 1). vHSB and vHSBM<sub>62</sub> carry the six Sabin 1 silent mutations in the 3D<sup>pol</sup> region. vHSP carries two of these mutations (positions 7198 and 7243). Titers of virus stocks were determined in HEP-2c cells at 34 and 40°C (Rct test). Rct values are expressed as the differences between the logarithms of virus titers at 34 and 40°C (TCID<sub>50</sub> per milliliter). Viruses with Rct values above 2 are considered to be *ts*. The corresponding standard deviations are reported to indicate variations between at least two independent experiments with the same viral stock. Solid and hatched bars show the values obtained for the same virus strain but with two different viral stocks derived from two independent cDNA clones.

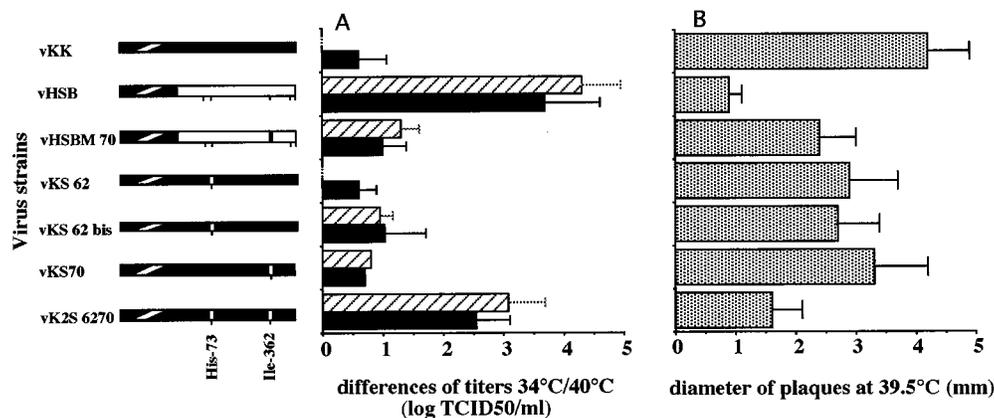


FIG. 3. Effect of the substitution Thr-362→Ile (3D<sup>pol</sup> region) on temperature sensitivity. Simplified genome structures of the viruses are as described for Fig. 2. vHSB and vHSBM<sub>70</sub> carry the six Sabin 1 silent mutations in 3D<sup>pol</sup>. vKS<sub>62</sub> bis is identical to vKS<sub>62</sub> except that the polyadenylation site of SV40 was deleted from the corresponding plasmid (see Materials and Methods). (A) Reproductive capacity of recombinant and parent viruses (Rct test). Rct values (TCID<sub>50</sub> per milliliter ± standard deviation) were obtained from at least two experiments (except in the case of vKS<sub>70</sub>). For each virus, two different virus stocks were used (solid and hatched bars). (B) Plaque size was measured for each virus strain on infected HeLa cells maintained for 48 h at 39.5°C under agar. The mean diameter (± standard deviation) of at least 50 plaques was calculated.

by the wild-type Thr-362 codon (vHSBM<sub>70</sub>) (Rct < 2). Like the His-73 codon, the Sabin 1 Ile-362 codon alone in the Mahoney context (vKS<sub>70</sub>) did not confer a *ts* phenotype (Rct < 2). However, the association of His-73 and Ile-362 (in vK2S<sub>6270</sub>) resulted in temperature sensitivity (Rct > 2). However, the His-73–Ile-362 double mutant appeared less *ts* than Mahoney/Sabin 1 vHSB (Fig. 3A). This finding suggested that other mutations also contribute to temperature sensitivity.

The plaque sizes of the Mahoney virus vKK and of the Mahoney/Sabin 1 virus vHSB were similar on HEp-2c cells maintained at 34°C (data not shown). However, the plaques formed at 39.5°C by vHSB were smaller than those formed by vKK. The number of plaques of vHSB at 39.5°C was about 0.5 log<sub>10</sub> PFU lower than at 34°C, consistent with a poor decrease of titer between 34 and 39.5°C (1.4 log<sub>10</sub> TCID<sub>50</sub>/ml) as scored by the Rct test (data not shown). HeLa cells were also used to analyze the plaque size phenotype (at 34 and 39.5°C), and the results were similar to those obtained with HEp-2c cells. Because the HeLa cell monolayers resisted infection better than HEp-2c cells monolayers when maintained at high temperature under agar, HeLa cells were used to determine plaque diameters of recombinant viruses at 39.5°C (HeLa cells are closely related to HEp-2c cells; see reference 17). Statistical comparisons between plaque diameter samples were made pairwise and tested for significance (see Materials and Methods). There was no significant difference between the plaque diameters of vKS<sub>62</sub> and vKS<sub>62</sub> bis (Fig. 3B). The plaque size of the Mahoney/Sabin 1 virus vHSBM<sub>70</sub> carrying the wild-type Thr-362 codon was intermediate between that of the Mahoney/Sabin 1 virus vHSB ( $P < 0.01$ ) and that of the Mahoney virus vKK ( $P < 0.01$ ). The Sabin 1 His-73 codon, or the Ile-362 codon, alone in a wild-type context (vKS<sub>62</sub> or vKS<sub>62</sub> bis, or vKS<sub>70</sub>) resulted in plaque diameters smaller than those of vKK ( $P < 0.01$ ). Thus, the His-73 and Ile-362 codons are independent determinants of the *ts* plaque size phenotype. The plaque size of the virus carrying both codons (vK2S<sub>6270</sub>) was smaller than that of each virus carrying only one ( $P < 0.01$ ) but nevertheless larger than that of the Mahoney/Sabin 1 virus vHSB ( $P < 0.01$ ) (Fig. 3B and 4). There was thus a good correlation between loss of titer at 40°C (Rct test) and plaque size reduction at 39.5°C. However, the plaque size at 39.5°C was a more discriminative marker than the Rct test for differentiating vi-

rus with different degrees of temperature sensitivity. The findings also suggested that at least one other mutation, in addition to His-73 and Ile-362 in 3D<sup>pol</sup>, affects the replication of the Mahoney/Sabin 1 virus vHSB at high temperature.

The sequence of the 3' NC region is highly conserved in most known PV strains and forms a pseudoknot structure involved in viral RNA replication (19, 48). We therefore investigated the possible role of the two nucleotide residues (C-7410 and G-7441) which differentiate the 3' NC region of Sabin 1 genome from that of Mahoney.

#### Involvement of the 3' NC region in temperature sensitivity.

The involvement of the two Sabin 1 nucleotides C-7410 and G-7441 was first analyzed in the context of wild Mahoney strains carrying the Sabin 1 residues His-73 and Ile-362 of 3D<sup>pol</sup> (Fig. 5A). The Rct value of virus carrying C-7410 (vK3S<sub>10</sub>) was similar to that of its reference strain vK2S<sub>6270</sub> and was thus intermediate between those of viruses vHSB (or vHSP) and vKK. Rct values of virus carrying G-7441 (vK3S<sub>41</sub>) appeared to be intermediate between those of vK2S<sub>6270</sub> (or vK3S<sub>41</sub>) and vHSB (or vHSP). However, virus carrying C-7410 plus G-7441 (vK4S) displayed Rct values close to those of the Mahoney/Sabin 1 viruses vHSB and vHSP.

There was no significant difference between the plaque diameter phenotype (at 39.5°C) of any two viral stocks of the same virus (Fig. 5A). Therefore, plaque diameters from the same virus were grouped for further comparisons. There were no differences between vK2S<sub>6270</sub> and vK3S<sub>10</sub> or between vHSB and vHSP. Plaque diameters of vK2S<sub>6270</sub> (or vK3S<sub>10</sub>) differed significantly ( $P < 0.01$ ) from those of vKK and vHSB (or vHSP and vK3S<sub>41</sub>). This result thus confirmed that vK2S<sub>6270</sub> has a plaque size phenotype (at 39.5°C) intermediate between those of vKK and vHSB. There was no difference between plaque diameters of vK3S<sub>41</sub>, vHSB, and vHSP. However, plaques of vK4S appeared to be larger than those of vHSB, vHSP, and vK3S<sub>41</sub> and smaller than those of vK3S<sub>10</sub> and vK2S<sub>6270</sub> ( $P < 0.01$ ). Parts of the genomes of two independent stocks of vK3S<sub>41</sub> were sequenced, and each carried one unexpected silent mutation in the 3D<sup>pol</sup> region: A→C at position 7295 and A→G at position 7306. These mutations were present in plasmid DNA and were probably introduced during the PCR step used for constructing cDNAs. They are thus unlikely to contribute to the phenotype of the vK3S<sub>41</sub> virus stocks analyzed.

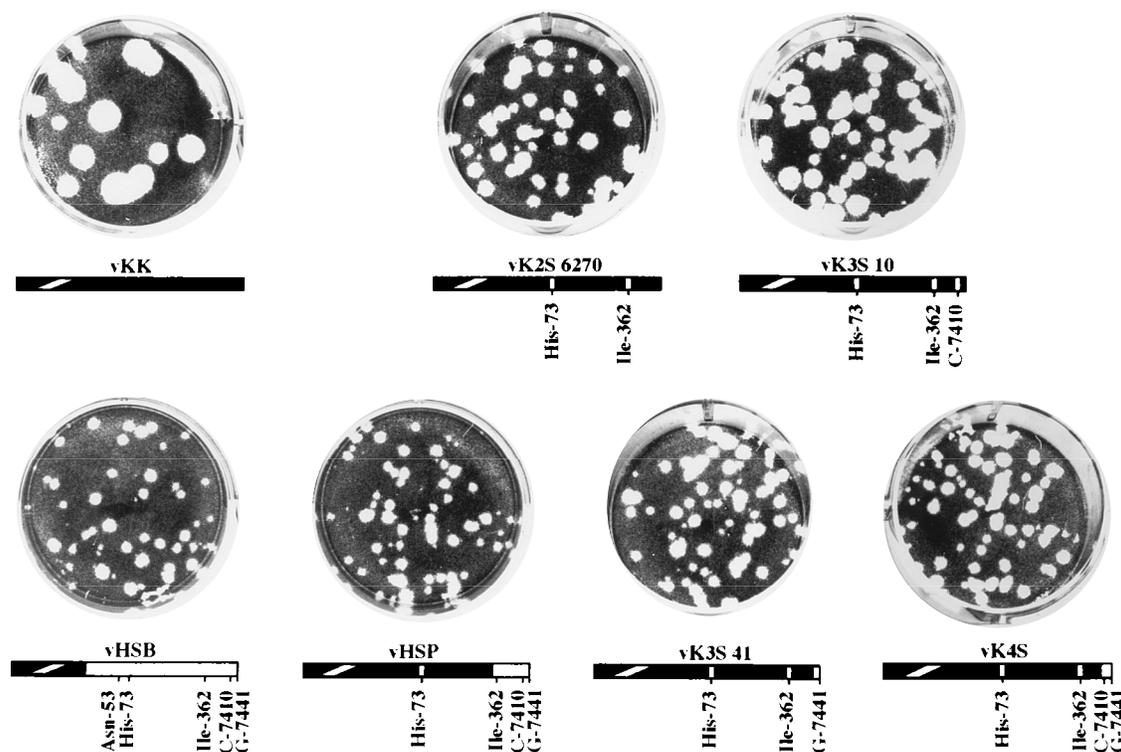


FIG. 4. Plaque morphologies of recombinant, mutant, and parent viruses on HeLa cells at 39.5°C. Shown are photographs of plates used for the experiment presented in Fig. 5A.

The involvement of the two Sabin 1 nucleotides C-7410 and G-7441 was also analyzed in the context of wild Mahoney strains carrying the Sabin 1 residues His-73 of 3D<sup>pol</sup> (Fig. 5B). None of those viruses were *ts* according to the Rct test (Rct < 2; Fig. 2). However, the plaque sizes at 39.5°C of the wild Mahoney carrying the Sabin 1 His-73 codon (vKS<sub>62</sub> bis) were reduced by the presence of both C-7410 and G-7441 (vHSM) ( $P < 0.01$  in both experiments; Fig. 5B) and, to a lesser extent, by the presence of G-7441 alone (vK2S<sub>6241</sub>) ( $P < 0.01$  and not significant, depending on the experiment and/or viral stock). vHSBM<sub>70</sub> (Fig. 3) carries the same Sabin 1 nucleotides as vHSM, and it also carries the Asn-53 codon plus the six Sabin 1 nucleotides corresponding to silent mutations in 3D<sup>pol</sup>. vHSBM<sub>70</sub> produced plaques smaller than those of vKS<sub>62</sub> or vKS<sub>62</sub> bis ( $P < 0.01$  and  $0.01 < P < 0.05$ , respectively; Fig. 3). However, C-7410 and G-7441 had no significant effect in the wild-type Mahoney context (compare vKK with vHSOM in Fig. 5B; no significant difference in both experiments).

These results suggested that the Sabin 1 nucleotide G-7441, alone or in combination with C-7410, plays a small but significant role in the temperature sensitivity of Sabin 1. C-7410 alone did not seem to be responsible for temperature sensitivity. Thus, the *ts* phenotype of viruses carrying the 3'-terminal part of the Sabin 1 genome results from contributions of at least 3 of the 11 residues which differentiate Sabin 1 from Mahoney: His-73 (C-6203), Ile-362 (U-7071) of 3D<sup>pol</sup>, and, at least, nucleotide G-7441 in the 3' NC region.

**Stability of Sabin 1-specific nucleotides in vivo.** Sequence analysis of viruses isolated from patients with VAPP indicates that genetic determinants of temperature sensitivity and/or attenuation of vaccine PV strains are selected against in vivo (12, 13, 15, 26, 28, 37). To determine the possible selection pressure in vivo against some of the Sabin 1 nucleotides which

differentiate the 3'-terminal part of Sabin 1 genome from that of Mahoney, we analyzed the sequences of 22 strains isolated from patients with VAPP. These strains have already been described as Sabin 1-derived strains (6 strains) or as intertypic Sabin 2/Sabin 1 or Sabin 3/Sabin 1 recombinants (16 strains) (15, 16, 37). The intertypic recombinants carry at least part of the 3D<sup>pol</sup> coding region and the entire 3' NC region of the Sabin 1 genome (15, 16). Nucleotide position 6203 (codon 73 of 3D<sup>pol</sup> [3D<sup>pol</sup>-73]) and, in some cases, nucleotide position 7441 (3' NC region) have already been analyzed in these strains (15, 16, 37). In this study, nucleotide positions 6143, 6203, and 7071, corresponding to amino acid codons 53, 73, and 362 of 3D<sup>pol</sup>, and nucleotide positions 7410 and 7441 in the 3' NC region were checked in all VAPP strains by RNA sequencing (Table 1).

In all cases, Sabin 1 nucleotides were found at positions 6143 (3D<sup>pol</sup>-53) and 7410 (3' NC). Wild-type nucleotides were found at positions 6203 (3D<sup>pol</sup>-73) and 7441 (3' NC) in 15 and 14 of the 22 cases, respectively. In two cases, the wild-type nucleotide was found at position 7071 (3D<sup>pol</sup>-362). No other changes were discovered at the three nucleotide positions of each codon corresponding to amino acids 53, 73, and 362 of 3D<sup>pol</sup>.

Multiple intertypic recombination events have been found in natural vaccine-derived strains (7, 16). To check that the wild-type nucleotides found at these three positions (6203, 7071, and 7441) result from reversion to the wild type and not from multiple intertypic recombination events with the Sabin 2 or Sabin 3 genome, the surrounding positions which display nucleotide variations differentiating PV-1 (Mahoney and Sabin 1) strains from Sabin 2 and Sabin 3 were investigated. In all but one case, nucleotide 6203 (3D<sup>pol</sup>-73) was surrounded by the PV-1 sequence. In VAPP strain 317, a Sabin 2-like sequence

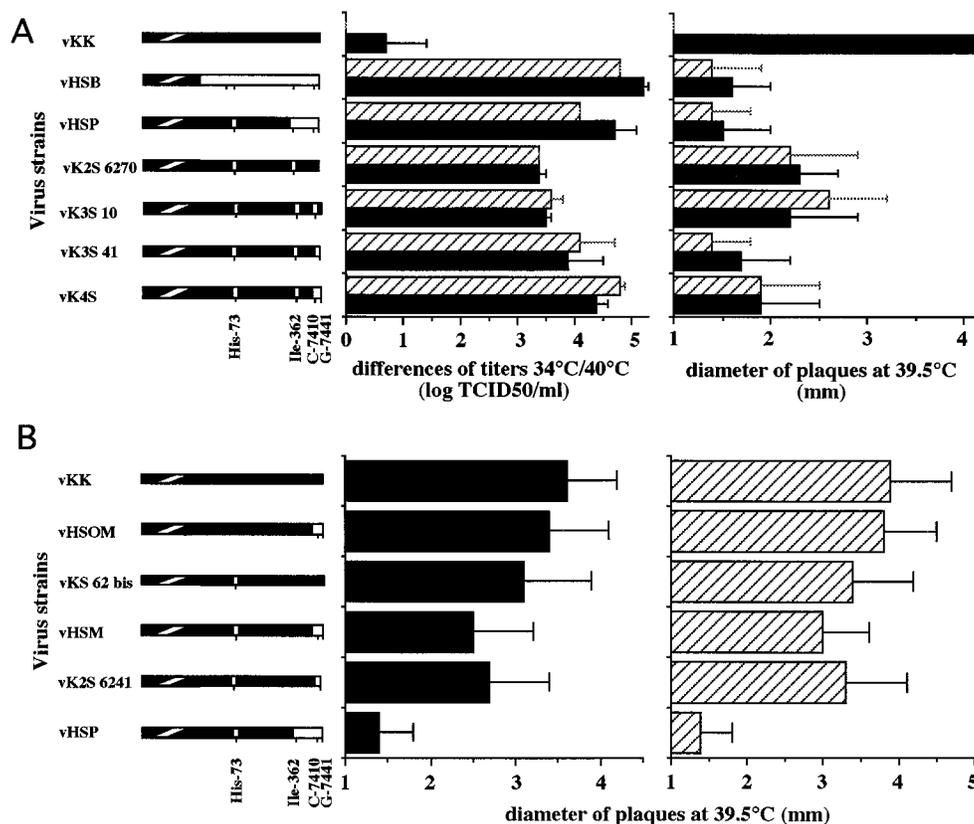


FIG. 5. Involvement of the 3' NC region in temperature sensitivity. Simplified genome structures of the viruses are as described for Fig. 2. Viruses vHSB and vHSP carry Sabin 1 silent mutations (see the legend to Fig. 2). (A) The effect of Sabin 1 nucleotides at positions 7410 and 7441, linked to His-73 and Ile-362, was analyzed in Mahoney strain. Rct values ( $\pm$  standard deviation) are presented as described for Fig. 2. The mean diameter of plaques ( $\pm$  standard deviation) on HeLa cells at 39.5°C was calculated by using two different virus stocks in parallel for each virus strain (solid and hatched bars). (B) The effect of Sabin 1 nucleotides at positions 7410 and 7441 was analyzed in the Mahoney background with and without the His-73 residue of 3D<sup>pol</sup>. Plaque sizes on HeLa cells at 39.5°C were determined. Results of two independent experiments using two different virus stocks (except in the case of vKK Mahoney) are presented.

was found around this position. Nevertheless, in this strain and in strain 330, both of which displayed the wild-type nucleotide at position 7071 (3D<sup>pol</sup>-362), position 7071 was surrounded by PV-1 nucleotides. The presence of Sabin 1 nucleotides at positions 7198 and 7243 (silent mutations in 3D<sup>pol</sup> region) was checked in 16 and 20 strains, respectively, and was as expected. The presence of the Sabin 1 nucleotide at position 7410 (3' NC) was verified in most cases (Table 1). It is therefore more likely that the genomic regions analyzed in VAPP strains were derived from the Sabin 1 genome. The wild-type nucleotides found in place of Sabin 1 nucleotides thus result from true reversions.

These results strongly suggest that only three of the five Sabin 1 nucleotides considered are subject to significant selection pressure *in vivo*. These three nucleotides correspond to those which have been shown to contribute to temperature sensitivity. It should be noted that the Sabin 1 residue U-7071 (Ile-362) appeared to be more stable than C-6203 (His-73) and G-7441 (3' NC). In 14 of 22 cases, two of these three nucleotides were found to have reverted to the wild type in the same strain. These nucleotides appeared more unstable in intertypic recombinants (16 of 16 cases) than in nonrecombinant Sabin 1-derived strains (1 of 6 cases).

## DISCUSSION

Previous studies have shown that the temperature sensitivity of the poliovirus vaccine Sabin 1 strain results from numerous

genetic determinants (36). Among them, only the G-480 in the 5' NC region, and His-73 codon of 3D<sup>pol</sup> have been characterized and contribute to the *ts* phenotype (20, 27, 46). In this work, we have shown that the *ts* effect associated with the 3'-terminal part of the Sabin 1 genome (3D<sup>pol</sup> and 3' NC regions), results from the cumulative and/or synergistic effects of at least three determinants, including, in addition to the His-73 codon, the Ile-362 codon of 3D<sup>pol</sup> and nucleotide G-7441 in the 3' NC region. Sequence analysis of strains isolated from patients with VAPP indicated that these three determinants are selected against *in vivo*.

It has been previously shown that determinants of temperature sensitivity map in the 3'-terminal part of the Sabin 1 genome and in each of two other genomic fragments including the 5' NC region and the capsid protein-encoding region (36). In particular, the *ts* effect associated with nucleotides 1 to 1122 is not due only to nucleotide G-480 of the 5' NC region (20). The genomic fragment encompassing nucleotides 1122 to 3664 encoding a large part of the capsid proteins also carries at least one determinant of temperature sensitivity (36). These data and our present study showed that the temperature sensitivity of Sabin 1 results from many determinants. In contrast, Sabin 3 was shown to carry as few as two determinants of temperature sensitivity, depending on the cell type used for the studies (27, 32).

Of the three Sabin 1 residues, Asn-53, His-73, and Ile-362, resulting from missense mutations in 3D<sup>pol</sup> codons, only His-73

TABLE 1. Analysis of nucleotide positions 6143, 6203, 7071, 7410, and 7441 in PV strains isolated from patients with VAPP

Strain	Serotype <sup>a</sup>	Nucleotide <sup>b</sup>				
		6143 (3D <sup>pol</sup> -53)	6203 (3D <sup>pol</sup> -73)	7071 (3D <sup>pol</sup> -362)	7410 (3' NC)	7441 (3' NC)
AF	S1	SAB	WT	SAB	SAB	WT
NIR	S1	SAB	SAB	SAB	SAB	SAB
BA	S1	SAB	SAB	SAB	SAB	SAB
IG	S1	SAB	SAB	SAB	SAB	SAB
PG	S1	SAB	SAB	SAB	SAB	SAB
2-II <sub>s</sub>	S2/S1	SAB	WT	SAB	ND	WT
2-II <sub>c</sub>	S2/S1	SAB	WT	SAB	ND	WT
2-III <sub>s</sub> ''	S2/S3/S1	SAB	WT	SAB	SAB	SAB
2-IV <sub>s</sub> ''	S2/S1	SAB	WT	SAB	SAB	SAB
2-IV <sub>s</sub> '''	S3/S1	SAB	WT	SAB	SAB	WT
2-IV <sub>c</sub>	S2/S1	SAB	WT	SAB	SAB	WT
3-IV <sub>c</sub>	S1	SAB	SAB	SAB	SAB	SAB
232	S2/S1	SAB	WT	SAB	SAB	WT
233	S2/S1	SAB	WT	SAB	SAB	WT
234	S2/S1	SAB	WT	SAB	SAB	WT
235	S2/S1	SAB	WT	SAB	SAB	WT
239	S2/S1	SAB	WT	SAB	SAB	WT
245	S2/S1	SAB	WT	SAB	SAB	WT
316	S3/S1	SAB	WT	SAB	SAB	WT
317	S3/S2/S1	NA <sup>c</sup>	NA	WT	SAB	SAB
321	S3/S1	SAB	WT	SAB	SAB	WT
330	S3/S1	SAB	SAB	WT	SAB	WT

<sup>a</sup> S1, Sabin 1; S2/S1, Sabin 2/Sabin 1 intertypic recombinant strain, etc.

<sup>b</sup> SAB, Sabin 1 nucleotide; WT, wild-type nucleotide; ND, not determined.

<sup>c</sup> NA, not applicable because the strain 317 genome displays a Sabin 2-like sequence in this region.

and Ile-362 appear to affect viral functions at high temperature. Recently, clustered charged-to-alanine mutagenesis of amino acids 51 plus 53, 53 plus 55 plus 56, and 71 plus 72 in Mahoney gave viruses with *ts* small-plaque, *ts* plaque reduction, and nonviable phenotypes, respectively (11). This finding emphasizes the importance of the peptide fragment encompassing amino acids 51 to 72 in the activities of viral polymerase 3D<sup>pol</sup> and/or of viral protease 3CD<sup>pro</sup> (51). His-73 could affect some of these activities at high temperature. Previous *in vitro* studies suggested that the presence Asn-53 and/or His-73 may reduce the initiation of viral RNA synthesis above 38°C, through a *ts* block of uridylylation of the viral protein VPg (49). Nevertheless, in our study, Asn-53 did not contribute to temperature sensitivity. The Mahoney mutant carrying Asn-53 and His-73 did not differ significantly from the mutant carrying only His-73 (Fig. 2), and the mutant carrying Asn-53 plus His-73 plus Ile-362 did not differ from the mutant carrying only His-73 plus Ile-362 (not shown). The *ts* effect observed by Diamond and Kirkegaard (11) after mutagenesis of residue Asp-53 to Ala could be due to major changes in the physicochemical properties of the 3D<sup>pol</sup> peptide chain and/or to the simultaneous mutagenesis of neighboring residues. The substitution Asp-53→Asn in Sabin 1 may cause less drastic changes in the properties of the peptide chain. Ile-362 may affect the polymerase activity of 3D<sup>pol</sup>, as this residue is located close to domain D (amino acid positions 351 to 361), a domain common to many viral RNA polymerases (39). Insertion mutagenesis of Mahoney between residues 354 and 355 (in domain D) gave a noncomplementable small-plaque mutant with delayed RNA synthesis (4). Plaque size analysis of recombinant and mutant viruses indicates that His-73 and Ile-362 are involved independently in temperature sensitivity. However, results of Rct tests suggest that when both residues are ex-

pressed, they may have a synergistic effect on temperature sensitivity and may then affect some other viral functions. We are currently studying the viral functions which are affected at high temperature by His-73 and Ile-362. Although it is unlikely, our study does not exclude the possibility that mutations leading to Ile-362, as well as to His-73, act on RNA secondary structure rather than on the 3D<sup>pol</sup> or 3CD<sup>pro</sup> enzyme activity.

The analysis of the role of the 3' NC region in the temperature sensitivity of Sabin 1 did not provide results as unambiguous as those obtained for the His-73 and Ile-362 codons of 3D<sup>pol</sup>. The *ts* effect associated with the presence of the 3' NC region of Sabin 1 in recombinants was observed only when the His-73 codon was also present. This could be due to the sensitivity of the tests used, which may not detect the small *ts* effect associated with the 3' NC region alone. Alternatively, the 3' NC Sabin 1 residues may have to be linked to the His-73 codon for expression of temperature sensitivity. Nevertheless, our results strongly suggest that G-7441 alone or in association with C-7410 is involved in the Sabin 1 *ts* phenotype (C-7410 was shown here not to contribute on its own). A *ts* RNA synthesis mutant has already been isolated from Mahoney cDNAs mutagenized in the 3' NC region (44). The temperature sensitivity of such a mutant may be due to disruption of a pseudoknot structure at the 3' terminus of the PV RNA genome (19). Similarly, in Sabin 1, G-7441 may disrupt a hairpin loop structure possibly required for initiation of RNA synthesis (19).

The six Sabin 1 nucleotides corresponding to silent mutations in 3D<sup>pol</sup> appear to contribute only weakly or not at all to the *ts* phenotype of Sabin 1. Virus vHSP differs from the original Mahoney/Sabin 1 virus vHSB by the absence of the Asn-53 missense mutation and the four Sabin 1 silent mutations at nucleotide positions 6373, 6616, 6679, and 6853. The *ts* phenotype of vHSP is similar to that of vHSB (similar Rct values and plaque sizes at 39.5°C). This finding strongly suggests that these four Sabin 1 mutations (and the Asn-53 codon) are not involved in the Sabin 1 *ts* phenotype. Virus vK4S differs from vHSP by the absence of the two other silent mutations (nucleotide positions 7198 and 7243). The vK4S phenotype differs somewhat from that of vHSP (similar Rct values but different plaque sizes at 39.5°C) (Fig. 4 and 5A). Thus, these two silent mutations are somewhat involved in the phenotype of vHSP, unless the differences are due to slight heterogeneity of viral stocks. However, the two corresponding Sabin 1 nucleotides were always found at positions 7198 and 7243 in several VAPP strains (16 and 20 strains, respectively). This observation suggests that the two silent mutations are not subject to strong selection pressure *in vivo* and, thus, that they contribute weakly or not at all to the Sabin 1 phenotype. The polyprotein coding region of the Sabin 1-derived VAPP strain AF (Table 1) carries 52 silent mutations compared with Sabin 1, including 11 mutations which are located in the 3D<sup>pol</sup> region (not shown). However, none of these 11 silent mutations are at the same positions as the Sabin 1 silent mutations (versus Mahoney).

It is difficult to assess the effects during viral replication in standard conditions and in infected hosts of viral mutations expressed at high temperature (39.5 or 40°C). Sabin 1 G-7441 and the His-73 codon of 3D<sup>pol</sup> have been shown to revert to the wild type in neurovirulent Sabin 1 mutants selected at 37.5 and 38.5°C, respectively (8). After many passages of Sabin 1 in cultured cells at 34 and 37°C, a significant proportion of viruses carry wild-type A-7441 and the Tyr-73 codon, respectively (41). After intracerebral inoculation of normal mice with mouse-adapted PV-1/PV-2 recombinant viruses carrying the 3'-terminal part of the Sabin 1 genome, wild-type A-7441, but not the

Tyr-73 codon, was found in some viral populations isolated from spinal cords of paralyzed animals (46). In all of the cases described above, G-7441 appeared to be more unstable than the His-73 codon. Nevertheless, in the Sabin 1-derived genomes of strains isolated from patients with VAPP, we found the wild-type Tyr-73 codon as often as A-7441 (15 and 14 of 22 cases, respectively) (this work). In these VAPP strains, the Ile-362 codon of 3D<sup>pol</sup> appeared to have reverted less frequently to the wild type (two cases) than the His-73 codon and G-7441. However, the fact that all of these three Sabin 1 residues are selected against *in vivo* indicates that viruses with the corresponding wild-type amino acids and/or nucleotides have some selective advantage in the human host.

By using mouse adapted PV-1/PV-2 recombinant viruses, the His-73 codon was shown to contribute to the attenuation of PV-1 in animals, but there was no evidence for a role for the Ile-362 codon or G-7441 (46). Nevertheless, although attenuation determinants of PV vaccine strains are not necessarily determinants of temperature sensitivity (20, 28, 40, 46, 47, 50), all identified determinants involved in temperature sensitivity are also involved in the attenuation of these strains (27, 32, 46). The Ile-362 codon of 3D<sup>pol</sup> and G-7441 of the 3' NC region are determinants of temperature sensitivity and selected against *in vivo*, suggesting that they might contribute to the attenuation of Sabin 1. Nevertheless, the His-73 codon appears to be a more significant determinant of attenuation, at least in the mouse model (46). The cumulative effect of the important attenuation determinant G-480 in the 5' NC region of Sabin 1 genome (20, 30) and of multiple weaker determinants like those described in this study may contribute to the safety of Sabin 1 in vaccinees.

Nevertheless, it is paradoxical that a 3'-terminal fragment of the Sabin 1 genome is frequently found in VAPP intertypic recombinant strain genomes (30 of 62 intertypic VAPP recombinants analyzed) (15, 16, 23, 25, 26). We would have expected that a genomic fragment having a negative effect on replication *in vivo* would be counterselected in intertypic recombinants and thus that corresponding fragments of Sabin 2 or Sabin 3 which are not believed to interfere with viral replication would be favored (28, 40, 50). Nevertheless, in this study, the 3'-terminal part of the Sabin 1 genome was always found in a mutated form in the VAPP intertypic recombinant strains. Interestingly, the determinants of temperature sensitivity that we studied appeared more unstable in these intertypic recombinants (16 of 16 cases) than in Sabin 1-derived nonrecombinant VAPP strains (1 of 6 cases), and this observation needs further investigation.

In conclusion, we looked for genetic determinants of the temperature sensitivity of the Sabin 1 vaccine strain; an in-depth analysis involving characterization of numerous recombinant and mutant strains indicates that at least three determinants, including two 3D<sup>pol</sup> codons, all in the 3'-terminal part of the Sabin 1 genome, contribute to the *ts* phenotype of Sabin 1. These three determinants may affect multiplication of Sabin 1 in vaccinees, since they were found to have reverted to the wild type in Sabin 1-derived or intertypic recombinant strains isolated from patients with VAPP.

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