JOURNAL OF VIROLOGY, Aug. 1995, p. 4972–4978 Vol. 69, No. 8
0022-538X/95/$04.00+0

Determinants of Attenuation and Temperature Sensitivity in the Type 1 Poliovirus Sabin Vaccine

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Received 17 January 1995/Accepted 11 May 1995

To identify determinants of attenuation in the poliovirus type 1 Sabin vaccine strain, a series of recombinant viruses were constructed by using infectious cDNA clones of the virulent type 1 poliovirus P1/Mahoney and the attenuated type 1 vaccine strain P1/Sabin. Intracerebral inoculation of these viruses into transgenic mice which express the human receptor for poliovirus identified regions of the genome that conferred reduced neurovirulence. Exchange of smaller restriction fragments and site-directed mutagenesis were used to identify the nucleotide changes responsible for attenuation. P1/Sabin mutations at nucleotides 935 of VP4, 2438 of VP3, and 2795 and 2879 of VP1 were all shown to be determinants of attenuation. The recombinant viruses and site-directed mutants were also used to identify the nucleotide changes which are involved in the temperature sensitivity of P1/Sabin. Determinants of this phenotype in HeLa cells were mapped to changes at nucleotides 935 of VP4, 2438 of VP3, and 2741 of VP1. The $3D^{pol}$ gene of P1/Sabin, which contains three amino acid differences from its parent P1/Mahoney, also contributes to the temperature sensitivity of P1/Sabin; however, mutants containing individual amino acid changes grew as well as P1/Mahoney at elevated temperatures, suggesting that either some combination or all three changes are required for temperature sensitivity. In addition, the 3 -noncoding region of P1/Sabin augments the temperature-sensitive phenotype conferred by $3D^{pol}$. Although nucleotide 2741, $3D^{pol}$, and the 3 -noncoding region of P1/Sabin contribute to the temperature sensitivity of P1/Sabin, they do not contribute to attenuation in transgenic mice expressing the poliovirus receptor, demonstrating that determinants of attenuation and temperature sensitivity can be genetically separated.

Poliomyelitis, an acute disease of the central nervous system, can be controlled through the use of either inactivated virus preparations or live attenuated strains. Attenuated viruses of each of the three serotypes of poliovirus were isolated by Albert Sabin after repeated passage of virulent strains in nonhuman primate cells (44). The Sabin strains replicate sufficiently to induce a protective immune response yet rarely cause paralysis.

The poliovirion is a nonenveloped particle composed of four structural proteins (VP1, VP2, VP3, and VP4) arranged in icosahedral symmetry and a 7.5-kb single-stranded message sense RNA genome that is covalently linked to a viral protein, VPg, at its 5′ terminus. The viral RNA contains 5′- and 3′-noncoding regions (5′- and 3′NCR) important for viral translation and replication that flank a long open reading frame (see reference 50 and references therein). To infect susceptible cells, poliovirus interacts with a cell surface receptor that is a novel member of the immunoglobulin superfamily of proteins (28). Following release of the viral genome into the cell cytoplasm, it is translated into proteins that replicate and encapsidate the viral genome. Newly synthesized virus particles are believed to be released from cells by lysis; however, the observation that certain cell lines release poliovirus in the absence of lysis suggests an alternative mechanism (6, 20, 29).

Although the attenuated Sabin strains have an excellent safety record, vaccine-associated paralytic poliomyelitis (VAPP) may occur in recipients at a frequency of 1 case per 1.2 million first-vaccine doses (34). This disease is most often associated with types 2 and 3 and rarely with type 1 vaccine strains (2, 34). Analysis of viruses isolated from cases of VAPP demonstrated that they are revertants of the Sabin strains. One VAPP isolate of P2/Sabin, which is called P2/117, contains mutations to the parental sequence at nucleotide 481 of the 5′NCR and amino acid 143 of VP1, which are two positions that have been demonstrated to be important determinants of attenuation (24, 37, 42). VAPP isolates of P3/Sabin contain mutations to the parental sequence at nucleotide 472 of the 5′NCR and amino acid 91 of VP3 or at second sites that suppress the 91 mutation (4, 22). The mutations at base 472 and amino acid 91 of VP3 are important determinants of the attenuation in P3/Sabin (49).

The observation that vaccine-associated neurovirulent revertants of P1/Sabin are rarely detected has not been explained. Analysis of the complete nucleotide sequences of P1/Sabin and its virulent parent P1/Mahoney demonstrated that P1/Sabin differs at 55 nucleotide positions which code for 21 amino acid changes (35, 39). P1/Sabin, therefore, may possess a greater number of attenuation determinants than either types 2 or 3, which contain two and three determinants, respectively (24, 37, 42, 47, 49).

Studies of experimentally infected monkeys suggest that attenuation determinants of P1/Sabin are scattered throughout the genome (5, 16, 36). Analysis of attenuation has been facilitated by the development of transgenic mice that express the human receptor for poliovirus and develop a disease that is clinically and histopathologically similar to poliomyelitis (14, 17, 41, 43). Examination of the neurovirulence of P1/Mahoney and P1/Sabin and recombinants of these two viruses previously studied with monkeys indicates that Pvr transgenic mice can

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serve as an alternative to the monkey neurovirulence test (14, 41).

With the exception of nucleotide 480 (16, 26), previous work has not identified specific nucleotide changes that are determinants of attenuation in P1/Sabin. In our study, variants of P1/Mahoney were generated by substituting gene segments or individual amino acids from P1/Sabin, and the neurovirulence of the viruses was determined for TgPvr mice. This analysis has identified specific amino acids within capsid proteins VP4, VP3, and VP1 that are determinants of attenuation. The viral variants were also used to map the mutations responsible for the temperature sensitivity (*ts*) of P1/Sabin (12). Determinants of the *ts* phenotype in HeLa cells were localized to VP4, VP3, VP1, and the $3D^{pol}$ gene and did not always covary with attenuation. These results suggest that temperature sensitivity may not always be an acceptable method for evaluating the presence of attenuating mutations. Furthermore, because TgPvr mice can detect attenuation determinants of P1/Sabin, neurovirulence assays in TgPvr mice may be useful for assuring the safety of the Sabin vaccines.

MATERIALS AND METHODS

Cells and viruses. HeLa S3 cells were grown in suspension cultures in Joklik minimal essential medium containing 5% horse serum. For growth in monolayers, HeLa cells were plated in Dulbecco minimal essential medium containing 10% horse serum. The infectious P1/Mahoney cDNA has been described previously (38). An infectious P1/Sabin cDNA was a gift from A. Macadam, National Institute for Biological Standards and Control, Hertfordshire, England. All viruses used in these experiments were derived by transfection of HeLa cells with the cloned poliovirus cDNAs. Viruses were twice plaque purified. All in vitro transfections and plaque purifications were performed at 32°C.

Virus growth and assay. Virus titers were determined by plaque assays on HeLa cell monolayers as described elsewhere (31). Virus stocks of high titer used for inoculation of TgPvr mice were prepared as described elsewhere, with the following modification: all preparations were incubated for 12 to 14 h at 32°C prior to collection of infected cells for centrifugation and isolation of virus (31).

Viruses were tested for temperature sensitivity by plaque assaying at 37 and 40°C. The efficiency of plating (EOP) was calculated by dividing the virus titer at 40°C by the virus titer at 37°C. Plaque sizes were calculated by measuring and averaging the diameter of 30 plaques on HeLa cells for each virus plated at 37°C.

One-step growth curve experiments were conducted by infecting monolayers of HeLa cells with viruses at a multiplicity of infection of 10 PFU per cell. At different times postinfection, the cells and supernatant were collected, frozen and thawed three times, and centrifuged to remove cell debris. Virus titers in the supernatants were determined by plaque assay on HeLa cell monolayers.

Nucleotide sequencing. Sequencing of recombinant and mutagenized cDNAs was performed with Sequenase according to the directions of the manufacturer (U.S. Biochemical Corp., Cleveland, Ohio). Isolation and chain termination sequencing of poliovirus genomic RNA were performed as described elsewhere, with oligonucleotide primers specific for P1/Mahoney (18) and with the following modifications. The cells were infected and incubated at 32°C until cytopathic effect was observed. The cells were then scraped from the plate and collected by low-speed centrifugation. The cells were resuspended in phosphate-buffered saline supplemented with 1% Nonidet P-40 and 0.5% deoxycholate. The cells were incubated on ice for 10 min to allow cell lysis and were then centrifuged to remove cellular debris. Proteinase K (50 μg/ml) was added to the supernatant, and the mixture was incubated at 50°C for 1 h. After two phenol extractions, the RNA was ethanol precipitated and sequenced directly. Virus acquired from transfection of cDNAs, high-titer stocks used for injections, and spinal isolates from paralyzed mice were all sequenced to ensure that the correct mutations were present.

Construction of recombinants. Plasmid DNAs were grown in *Escherichia coli* Dh5 and were purified by Qiagen according to the directions of the manufacturer (Qiagen, Inc., Chatsworth, Calif.). P1/Mahoney or P1/Sabin cDNA was cleaved with restriction endonucleases under conditions recommended by the manufacturer (New England BioLabs, Inc., Beverly, Mass.), and DNA fragments were separated by electrophoresis in low-melting-point agarose gels buffered with 40 mM Tris acetate–5 mM sodium acetate–2 mM EDTA (pH 7.8). For ligations, the agarose gel was melted at 50°C and the DNA was used directly without extraction from low-melting-point agarose. Ligations of DNA fragments were performed according to the instructions of the manufacturer of T4 DNA ligase (New England BioLabs, Inc.).

Six recombinant cDNAs were initially constructed by the substitution of restriction fragments of the cloned P1/Mahoney cDNA with P1/Sabin cDNA. The VP4 recombinant, FLMB3M, was constructed by exchanging a *Pfl*MI-to-*Nru*I fragment located from bases 496 to 1172. Along with the single amino acid change of VP4, this recombinant also contained a portion of the 5′NCR which included two base changes. The VP2 recombinant, FLMB4, was made by exchanging the *Nru*I-to-*Afl*II restriction fragment from bases 1172 to 1932. The VP3 recombinant, FLMB5M, was made by exchanging the *Afl*II-to-*Nhe*I restriction fragment from bases 1932 to 2470. The VP1 recombinant, FLMB7, was constructed using the *Nhe*I-*Sna*BI restriction fragment from bases 2470 to 2956. Although this recombinant did not include the entire VP1, it did include all of the amino acid differences of P1/Sabin. The nonstructural protein recombinants were constructed by exchanging the *Sna*BI-to-*Bgl*II fragment for proteins 2A through 3C (FLMB1M) and the *Bgl*II-to-*Eco*RI fragment for the 3D polymerase and 3′NCR (FLMB2M). The *Bgl*II site is located at base 5601, while the *Eco*RI site is located in the vector beyond the 3′NCR.

Once a recombinant had been shown to contain attenuating mutations or a growth defect at 40°C, recombinants with smaller fragment exchanges or site-directed mutants were constructed. For the VP3 recombinants, the *Avr*II site located at base 2042 was used to generate full-length cDNAs which contained the P1/Sabin *Afl*II-to-*Avr*II restriction fragment in a P1/Mahoney background and the *Avr*II-to-*Nhe*I restriction fragment of P1/Sabin in the P1/Mahoney background. These recombinants, FLMB5M-1944S and FLMB5M-2438S, separated the two amino acid changes located in VP3 of P1/Sabin. These regions also contained other differences between P1/Sabin and P1/Mahoney; however, only those at bases 1944 and 2438 coded for amino acid changes (35). When exchange of smaller fragments was not possible because of convenient restriction sites, site-directed mutagenesis was performed with either M13mp18 (25) or pBluescript SK^+ (Stratagene, La Jolla, Calif.). A *Sal*I restriction enzyme fragment was subcloned into pBluescript SK^+ for site-directed mutagenesis of the three mutations contained in FLMB3M. Two of these changes were in the 5′NCR. The *Sal*I subclone was created by using the *Sal*I restriction enzyme site in the P1/Mahoney vector (pBR322) at base 651. This restriction site is 350 bases 5′ of the 5′NCR of P1/Mahoney. The 3′ *Sal*I restriction site used was at base 2751 of P1/Mahoney. This restriction site was engineered into P1/Mahoney (30). Site-directed mutagenesis was performed by using P1/Mahoney-specific oligonucleotide primers from bases 639 to 657 and 667 to 684 containing the P1/Sabin mutations at bases 649 and 674, respectively (a C-to-A change at 649 and a C-to-U change at base 674). The VP4 site-directed mutant was constructed by using a P1/Mahoney-specific oligonucleotide primer which spanned bases 922 to 943 and contained the P1/Sabin mutation (G to U at base 935). After site-directed mutagenesis, the fragment was recloned into full-length P1/Mahoney cDNA by using the 5′ *Sal*I site and the *Nhe*I site located at base 2470 of P1/Mahoney so that the engineered 3′ *Sal*I site was not contained within the site-directed full-length P1/Mahoney cDNA. The full-length cDNAs were called FL649S, FL674S, and FL935S to identify the altered bases in P1/Mahoney.

Seven differences in VP1 were site directed by cloning the *Pst*I fragment from bases 2243 to 3417 of P1/Mahoney into M13mp18. The P1/Mahoney-specific oligonucleotide primers used for these constructs were as follows. The base change from an A in P1/Mahoney to a G in P1/Sabin was site directed with an oligonucleotide primer which spanned bases 2574 to 2593, with the change of A to G at base 2585; the full-length P1/Mahoney cDNA with this mutation was called FL2585S. The mutation at base 2741 was generated with an oligonucleotide primer which spanned bases 2732 to 2749, with the A-to-G change at base 2741; the full-length P1/Mahoney cDNA with this mutation was called FL2741S. The mutation at base 2749 was generated with an oligonucleotide primer which spanned bases 2742 to 2761, with the G-to-A change at base 2749; the full-length P1/Mahoney cDNA with this mutation was called FL2749S. The mutation at base 2762 was generated by using an oligonucleotide primer which spanned bases 2755 to 2773, with the C-to-U change at base 2762; the full-length P1/Mahoney cDNA with this mutation was called FL2762S. The mutation at base 2775 was generated with an oligonucleotide primer which spanned bases 2769 to 2788, with the change of C to A at base 2775; the full-length P1/Mahoney cDNA with this mutation was called FL2775S. The change at base 2795 was generated with an oligonucleotide primer which spanned bases 2788 to 2806, with the G-to-A change at base 2795; the full-length P1/Mahoney cDNA with this mutation was called FL2795S. The change at base 2879 was generated with an oligonucleotide primer which spanned bases 2873 to 2889, with the C-to-U change at base 2879; the full-length P1/Mahoney cDNA with this mutation was called FL2879S. After confirmation of the P1/Sabin mutation in each site-directed subclone, the *Nhe*-to-*Sna*BI fragment from bases 2470 to 2956 was recloned into full-length P1/Mahoney.

The changes at bases 6143 and 6203 of $3D^{pol}$ were made by cloning the *Hin*dIII restriction fragment which spanned bases 6056 to 6516 into M13mp18 and then by site-directed mutagenesis with P1/Mahoney-specific oligonucleotide primers. The change at base 6143 was generated with an oligonucleotide primer from bases 6133 to 6155, with the G-to-A change at base 6143; the full-length P1/Mahoney cDNA with this mutation was called FL6143S. The change at base 6203 was generated with an oligonucleotide primer which spanned bases 6196 to 6215, with the U-to-C change at base 6203; the full-length P1/Mahoney cDNA with this mutation was called FL6203S. The recombinant which contained only the mutation at base 7071 of P1/Sabin was constructed by replacing the *Pvu*II-to-*Mun*I fragment of P1/Mahoney with the corresponding restriction fragment from P1/Sabin; the full-length P1/Mahoney cDNA with this mutation was called FL7071S. This exchanged fragment extends from bases 7052 to 7305. The recombinant which contains only the $3D^{pol}$ of P1/Sabin, FL3D^{pol}S, was constructed by ex-

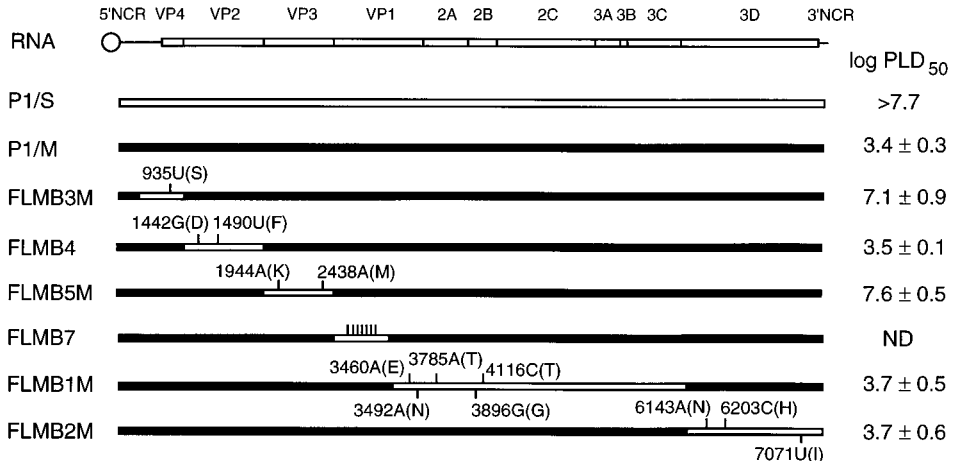

FIG. 1. Genomic structures and neurovirulence of P1/Mahoney (P1/M)-P1/Sabin (P1/S) recombinant viruses. At the top is a genetic map of poliovirus genomic RNA. The genomic RNA of each recombinant is shown schematically, with P1/Mahoney sequences in black boxes and P1/Sabin sequences in open boxes. Numbers, bases, and amino acids shown indicate the changes in P1/Sabin compared with those in P1/Mahoney. The seven mutations in VP1 are not listed. Virus names are listed on the left, and the PLD_{50}s are listed on the right, with standard deviations. ND, not determined.

changing the *Bgl*II-to-*Mun*I restriction fragment of P1/Mahoney, from bases 5601 to 7305, with the corresponding fragment from P1/Sabin. The reported mutation at position 6734 in P1/Sabin (35), which leads to an amino acid change from Lys to Glu, is not present in the viral RNA of P1/Sabin vaccine lots (21) and was therefore not analyzed. Position 7396 in P1/Sabin cDNA was changed from T to G to reflect the base found in P1/Sabin viral RNA (21).

Transfection of cDNAs. Transfection of cDNAs of P1/Mahoney and P1/Sabin, the recombinant cDNAs, and the mutagenized cDNAs was as described elsewhere, with modifications (19, 31). After transfection of HeLa cells, incubations were performed at 32°C in 5% CO_2. Cells were observed for cytopathic effect for 5 days, supernatants were collected, and virus was plaque purified twice. For every virus reported, at least two independently derived cDNAs were analyzed.

Neurovirulence assay. Groups of eight 21-day-old TgPvr mice (four males and four females) were inoculated intracerebrally with 50 μl of virus. Tenfold dilutions of virus were made in phosphate-buffered saline plus 0.2% horse serum such that each group of mice received approximately 10^2 to 10^9 PFU. Mice were observed for 21 days for paralysis and/or death. Spinal cords were recovered from one representative mouse for each virus tested, and virus was reisolated from the spinal cord and resequenced to ensure that the mutations being tested were still present. The amount of virus that caused paralysis or death in 50% of the mice (PLD_{50}) was calculated by the method described by Reed and Muench (40). At least three PLD_{50} determinations were performed for each virus.

RESULTS

Neurovirulence of recombinant and site-directed viruses. To identify attenuating mutations in P1/Sabin, P1/Mahoney-P1/Sabin recombinants and site-directed mutants were constructed by manipulation of full-length cDNAs of P1/Sabin and P1/Mahoney (Fig. 1; Table 1). Viruses were derived by transfection of HeLa cell monolayers with in vitro-transcribed RNA from each of the full-length P1/Mahoney cDNAs. At least two independently derived cDNAs were used for each virus, and viral RNA was sequenced to confirm the presence of the introduced mutation(s).

To determine the neurovirulence of each virus, eight 21-day-old TgPvr mice were inoculated intracerebrally with 50 μl of 10-fold serial dilutions of the virus, and the PLD_{50} of each virus was calculated (Fig. 1; Table 1). In this analysis, intracerebral injection of a very dilute inoculum of P1/Mahoney caused paralysis or death in TgPvr mice, while paralysis was rarely observed with P1/Sabin even at the highest titer inoculated. Virus was isolated from the spinal cords of paralyzed mice, and the presence of the introduced mutations was confirmed by nucleotide sequence analysis. By this analysis, all viruses isolated from the spinal cords of paralyzed mice resembled the inoculated virus (data not shown).

Substitution of sequences encoding VP4 and VP3 from P1/Sabin into P1/Mahoney resulted in viruses with attenuated neurovirulence (Fig. 1). By a similar analysis, VP2, 2A→3C, and $3D^{pol}$-3′NCR do not contain determinants of attenuation (Fig. 1). Exchange of VP1 resulted in a virus with an unstable small plaque phenotype that could not be reliably studied. The determinants of attenuation in VP1 were therefore analyzed by site-directed mutagenesis (see below).

Compared with P1/Mahoney, the attenuated recombinant FLMB3M contains two base changes in the 5′NCR and one amino acid change in VP4. To determine which of these changes is responsible for attenuation, these mutations were introduced individually into P1/Mahoney. Neurovirulence assays demonstrated that the changes in the 5′NCR at bases 649 and 674 did not contribute to attenuation, while the amino acid change at position 935 of VP4 from Ala to Ser was dramatically attenuating (Table 1).

The attenuated VP3 recombinant FLMB5M (Fig. 1) contains the following two amino acid differences from P1/Mahoney: Thr to Lys at position 1944 and Leu to Met at position 2438. Two viruses were constructed that contain the individual

TABLE 1. Log PLD_{50}s of P1/Mahoney, P1/Sabin, and mutant viruses used in this study

Virus	Genome region	Position	Nucleotide[a]		Amino acid[a]		Log PLD_{50}
			P1/M	P1/S	P1/M	P1/S	
P1/Mahoney							3.7 ± 0.1
P1/Sabin							>7.7
FL649S	5′NCR	649	C	U			3.7 ± 0.5
FL674S	5′NCR	674	C	U			3.4 ± 0.3
FL935S	VP4	935	G	U	A	S	6.8 ± 0.3
FLMB5M-1944S	VP3	1944	C	A	T	K	3.8 ± 0.2
FLMB5M-2438S	VP3	2438	U	A	L	M	5.3 ± 0.2
FL2585S	VP1	2585	A	G	T	A	3.8 ± 0.1
FL2741S	VP1	2741	A	G	T	A	3.9 ± 0.6
FL2749S	VP1	2749	G	A	M	I	4.2 ± 0.3
FL2762S	VP1	2762	C	U	P	S	3.5 ± 0.2
FL2775S	VP1	2775	C	A	T	K	3.4 ± 0.1
FL2795S	VP1	2795	G	A	A	T	5.2 ± 0.2
FL2879S	VP1	2879	C	U	L	F	4.9 ± 0.1

[a] P1/M, P1/Mahoney; P1/S, P1/Sabin.

TABLE 2. Plaque sizes at 37°C and EOPs at 40°/37° of P1/Mahoney, P1/Sabin, and mutant viruses

Virus[a]	Plaque size	EOP
P1/Mahoney	0.35	0.9
P1/Sabin	0.18	$<10^{-8}$
FLMB3M	0.23	0.001
FLMB4	0.40	1.1
FLMB5M	0.15	$<10^{-9}$
FLMB1M	0.35	1
FLMB2M	0.31	0.2
FL649S	0.35	1
FL674S	0.31	0.6
FL935S	0.20	0.002
FLMB5M-1944S	0.30	1
FLMB5M-2438S	0.30	0.2
FL2585S	0.37	0.9
FL2741S	0.25	0.5
FL2749S	0.32	0.9
FL2762S	0.38	1
FL2775S	0.29	0.8
FL2795S	0.29	1.2
FL2879S	0.14	0.8
FL3D^{pol}S	0.30	0.5
FL6143S	0.35	0.9
FL6203S	0.35	1
FL7071S	0.32	1.3

[a] Viruses in rows 3 to 7 are recombinants described in the legend to Fig. 1. Viruses in rows 8 to 18 are mutants described in Table 1. FL3D^{pol}S is a recombinant virus in which 3D^{pol} of P1/Mahoney has been substituted with 3D^{pol} of P1/Sabin. In virus FL6143S, the G of P1/Mahoney at position 6143 has been changed to an A, the base in P1/Sabin. In virus FL6203S, the U of P1/Mahoney at position 6203 has been to changed to a C, the base in P1/Sabin. In FL7071S the *Pvu*II-to-*Mun*I restriction fragment of P1/Mahoney, from bases 7052 to 7305, has been replaced by the corresponding restriction fragment of P1/Sabin. There is only one amino acid change in this fragment caused by the mutation at nucleotide position 7071.

mutations (FLMB5M-1944S and FLMB5M-2438S [Table 1]); however, only the latter virus was attenuated, although not to the same extent as FLMB5M.

To determine whether VP1 of P1/Sabin contains attenuating mutations, the seven differences with P1/Mahoney were individually assessed. This analysis demonstrated that the mutation from Ala to Thr at base 2795 produces an attenuated virus, while the change at base 2879 (Leu to Phe) produces a moderately attenuated virus. Changes at base 2585, 2741, 2749, 2762, or 2775 did not produce attenuated viruses.

Temperature sensitivity of mutant viruses. Replication of P1/Sabin is restricted at elevated temperatures (12). To identify the mutations responsible for the *ts* phenotype, plaque assays of parental and mutant viruses were conducted on HeLa cells at 37 and 40°C, and EOPs were calculated (Table 2).

Mutations in VP4, VP3, VP1, 3D^{pol}, and 3′NCR contribute to the *ts* phenotype of P1/Sabin (EOP, $<10^{-8}$ [Table 2]). Site-directed mutagenesis demonstrated that the change at base 935 (EOP, 0.002) is mainly responsible for the *ts* phenotype of the VP4 recombinant, FLMB3M (EOP, 0.001). Of the seven differences in VP1 between P1/Mahoney and P1/Sabin, only the mutation at base 2741 confers a slight *ts* phenotype (EOP, 0.5). While substitution of VP3 in P1/Mahoney with the sequence from P1/Sabin confers a severe *ts* phenotype (FLMB5M; EOP, $<10^{-9}$) when introduced individually, the mutation at base 2438 produced a mild *ts* phenotype (EOP, 0.2) and the mutation at base 1944 had no effect (EOP, 1). 3D^{pol} contains determinants of the *ts* phenotype (FL3D^{pol}S; EOP, 0.5); however, none of the individual mutations within 3D^{pol} caused a growth defect at 40°C. The *ts* phenotype of 3D^{pol} was enhanced by inclusion of the 3′NCR from P1/Sabin (EOP, 0.2).

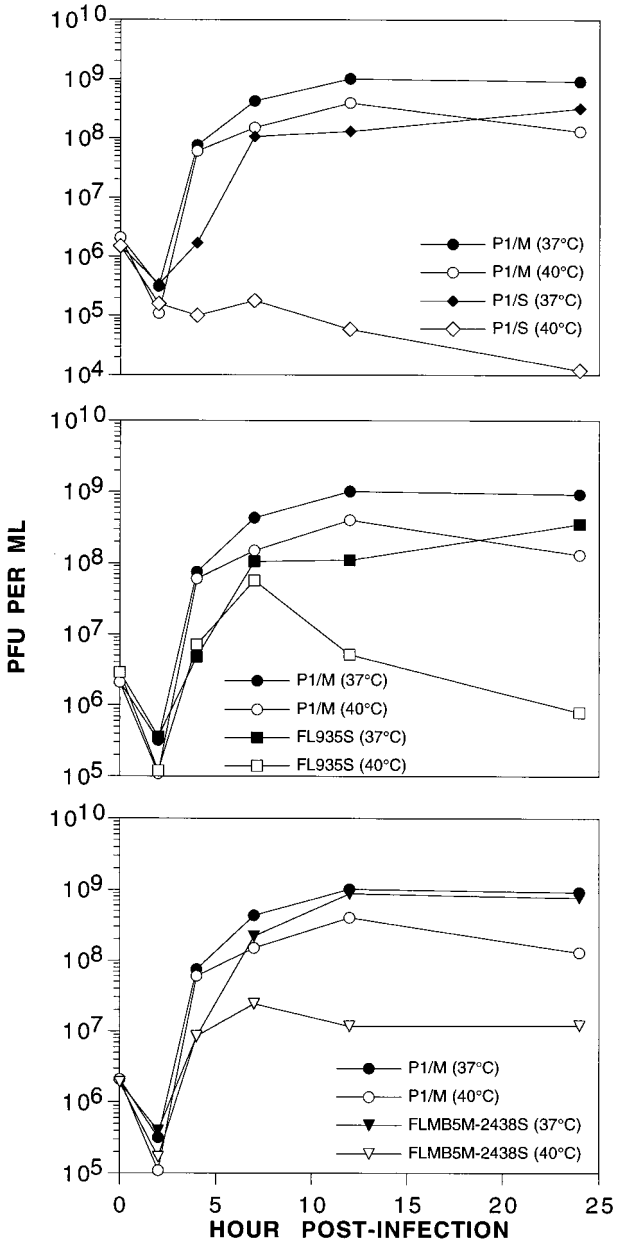

FIG. 2. One-step growth curves of P1/Mahoney (P1/M), P1/Sabin (P1/S), FL935S, and FLMB5M-2438S at 37 and 40°C in HeLa cells. Total virus production at different times postinfection was determined by a plaque assay on HeLa cells.

To determine whether the *ts* phenotype, as determined by a plaque assay, is indicative of growth defects, one-step growth curve experiments were carried out with selected viruses (Fig. 2). Growth of P1/Sabin is completely blocked at 40°C, while that of P1/Mahoney is only slightly inhibited. For the first 7 h postinfection, replication of FL935S at 40°C is slightly lower than that of P1/Mahoney; however, later the titer of FL935S drops dramatically. FLMB5M-2438 shows only a slight rise in virus titer at 40°C during the first 7 h postinfection, and then no further increase occurs.

Some P1/Sabin mutations impart a small-plaque phenotype

to P1/Mahoney at 37°C (Table 2). The plaque size of P1/Sabin at 37°C is half that of P1/Mahoney, and both mutations within VP3 (FLMB5M) and one mutation in VP1 (base 2879) appear to be responsible for this phenotype. As observed for the attenuation and *ts* phenotypes mediated by VP3 of P1/Sabin, the individual base changes within VP3 do not confer the small-plaque phenotype at 37°C.

DISCUSSION

Mutations that attenuate the neurovirulence of Sabin's vaccine strains in humans have been identified, by necessity, with animal models. It is well known that the neurovirulence of polioviruses can be influenced not only by the animal host used but also by the route of inoculation (45). Significant variation in susceptibility to poliovirus in Pvr transgenic mice has been observed. For example, the PLD_{50}s of P1/Mahoney in our TgPvr mice are 3.4 $\log_{10}$ PFU (Fig. 1) and 2.0 $\log_{10}$ PFU in other lines of Pvr transgenic mice (14). In addition, intraspecies variation in susceptibility to poliovirus in nontransgenic mice has been reported elsewhere (15). Nevertheless, studies with monkeys and mice have identified the same determinants of attenuation in the Sabin vaccine strains, although the absolute level of attenuation imparted by a particular mutation may vary in different hosts (Fig. 1 and Table 1) (14, 24, 42).

With the exception of a determinant at position 480 of the 5′-nontranslated region (16), previous work has implicated only broad regions of the viral genome that are involved in attenuation of P1/Sabin neurovirulence in monkeys and mice (14, 36). In the work reported here, we have identified specific mutations that attenuate the neurovirulence of P1/Sabin by analyzing the intracerebral neurovirulence of P1/Sabin-P1/Mahoney recombinants and single-base mutants of P1/Mahoney in a transgenic mouse model. These viral variants were also used to identify mutations responsible for the restricted growth of P1/Sabin at elevated temperatures in HeLa cells.

By selection of P1/Sabin revertants that replicate at elevated temperatures, some information on the determinants of P1/Sabin temperature sensitivity was previously obtained (5). Mutations in the 5′NCR, base 2438 of VP3, bases 2741 and 2795 of VP1, base 6203 of $3D^{pol}$, and base 7441 of the 3′NCR were associated with phenotypic reversion of the temperature sensitivity of P1/Sabin, although the contribution of individual mutations was not assessed. Our results show that VP3 and base 935 of VP4 are clearly involved in the growth defect at 40°C, while changes in $3D^{pol}$ and base 2741 of VP1 each impart a slight growth defect at the elevated temperature. The involvement of the 3′NCR in the *ts* phenotype of P1/Sabin was confirmed. When the two mutations in VP3 are separated, only the change at 2438 results in a *ts* phenotype. This phenotype is not as drastic as that observed when VP3 is exchanged. We conclude that both mutations in VP3 are required for full expression of the *ts* phenotype.

Mutations that attenuate P1/Sabin neurovirulence in TgPvr mice were localized to base 935 of VP4 (amino acid 4065), base 2438 of VP3 (amino acid 3225), and base 2795 of VP1 (amino acid 1106), while a weaker mutation was identified at VP1 base 2879 (amino acid 1134). Although the *ts* phenotype was conferred by changes at base 2741 and $3D^{pol}$ plus the 3′NCR, none of these mutations were determinants of attenuation in our TgPvr mouse model.

The attenuation and *ts* determinant at base 935 was not previously identified, although the results of earlier studies of viral recombinants suggested that it might play a role in reduced neurovirulence in monkeys and Pvr transgenic mice (14, 36). This change affects amino acid 65 of VP4, a protein that is lost when poliovirus contacts its cell receptor and is converted to an altered particle. The mutation might affect the efficiency of alteration, thereby reducing viral replication at elevated temperatures or in the central nervous system. Amino acid 4065 is in the vicinity of internal VP1 mutations known to influence receptor utilization and might affect interactions with Pvr (32). Alternatively, because amino acid 4065 is near a seven-stranded beta sheet formed by components of VP1, -2, and -3 that are believed to stabilize the association of pentamers (11), this mutation might affect the assembly of new virus particles. In agreement with this hypothesis, preliminary studies of FL935S in temperature shift experiments with HeLa cells suggest that the defect observed at elevated temperatures occurs at later stages of the viral life cycle (3).

Analysis of a viral recombinant in which VP2, VP3, and VP1 of P1/Mahoney are substituted with the sequences from P1/Sabin previously suggested the presence of weak attenuation determinants in this region (14). Curiously, we find that a VP3 recombinant, FLMB5M, is strongly attenuated. The reason for this difference is not clear; however, it is possible that removing VP3 from the context of the P1/Sabin capsid sequence results in a general replication defect which may not be present in the P1/Sabin genome.

The attenuation and temperature sensitivity of FLMB5M, which contains two amino acid changes, are greater than that of either single mutant (FLMB5M-1944S or FLMB5M-2438S). The latter observations are consistent with the finding that only one mutation, at base 2438, is lost in mutants selected for growth at higher temperatures (5). The attenuation and *ts* phenotypes of the double VP3 mutant suggest that amino acids 3060 and 3225 both participate in the same viral function. Amino acid 3225, which alone imparts some attenuation and temperature sensitivity, is at the interface between protomers, a region of the virion that is important in regulating receptor interactions, alteration, and assembly (8, 23). Amino acid 3060 is on the virion surface at the threefold axis of symmetry, distant from amino acid 3225. Recently, the same mutation at 3060 has been shown to allow P1/Mahoney to replicate in nontransgenic mice (9), suggesting that 3060 may be involved in receptor interactions. Alternatively, silent mutations present in the VP3 recombinant FLMB5M that are not common to either of the single mutants might cause alterations of viral RNA structure that enhance the attenuation and temperature sensitivity of the recombinant.

Our results also identify determinants of attenuation at amino acids 1106 and 1134, which is consistent with earlier results suggesting the presence of determinants within VP2, VP3, and VP1 (14). A weak *ts* phenotype is also conferred by amino acid 1088 of P1/Sabin. A previously isolated neurovirulent variant of P1/Sabin selected at elevated temperatures contained mutations at amino acids 1088 and 1106, indicating that these sites might be involved in attenuation in monkeys (5). Our results suggest that the reversion at amino acid 1106 and not at amino acid 1088 is responsible for the increased neurovirulence of this mutant. Amino acids 1088 and 1106 are at or near the VP1 B-C loop, a region that is highly exposed at the fivefold axis of symmetry of the particle (13). Although the VP1 B-C loop is not required for replication in cultured human cells (10), mutations in this region have been shown to influence host range, suggesting an involvement in receptor interactions (7, 27, 33). These mutations may contribute to attenuation and temperature sensitivity by influencing the interaction of poliovirus with its cell receptor. The attenuation determinant at amino acid 1134 also confers a small-plaque phenotype at 37°C. This amino acid lies within a hydrophobic pocket below the canyon, a surface depression which sur-

rounds the fivefold axis of symmetry of the capsid. Amino acids within the hydrophobic pocket and the canyon regulate early events in poliovirus infection, including binding and production of altered particles (8, 11). The mutation at amino acid 1134 may result in reduced neurovirulence and the small-plaque phenotype at 37°C by affecting these steps in infection.

As shown previously (5, 36, 46) and as confirmed in this study, determinants of temperature sensitivity are located in $3D^{pol}$ of P1/Sabin, which differs by three amino acids from its parent P1/Mahoney. Our results indicate that the *ts* phenotype is not expressed in P1/Mahoney variants containing individual $3D^{pol}$ mutations. This observation agrees with a previous report that the change at 6203 is not sufficient for temperature sensitivity (46). Some P1/Sabin variants selected for growth at elevated temperatures contain back-mutations at 6203 (5). Taken together, these results indicate that 6203 plus one or two additional mutations in $3D^{pol}$ of P1/Sabin are required for expression of the *ts* phenotype, and back-mutation of only the 6203 mutation is sufficient for phenotypic reversion. It has been shown that the P1/Sabin $3D^{pol}$ causes a *ts* defect in the initiation of positive-strand viral RNA replication at elevated temperatures (48).

Our results indicate that there are no significant determinants of attenuation in the $3D^{pol}$ and 3′NCR of P1/Sabin. In a previous study, a recombinant of P1/Mahoney containing the $3D^{pol}$ and 3′NCR of P1/Sabin failed to cause paralysis in all four monkeys inoculated, although the spread values and lesion scores were similar to those of P1/Mahoney, which paralyzed three of four monkeys inoculated (36). These authors concluded that $3D^{pol}$ of P1/Sabin contains a determinant of attenuation. A study of revertants of P1/Sabin selected at high temperatures suggested that base 6203 may be involved in attenuation in the monkey model (5). Studies with Pvr transgenic mice (14) and a nontransgenic mouse model (46) indicate that $3D^{pol}$ of P1/Sabin is only slightly attenuating. These contrasting results are probably due to the use of different animal models, i.e., monkeys and different strains of Pvr transgenic and normal mice, which vary in their sensitivities to certain attenuation determinants, and may also reflect differences in quantification of neurovirulence.

The results of these studies with TgPvr mice, together with previous work with normal mice, Pvr transgenic mice, and monkeys, permit a comparison of attenuation determinants of all three Sabin poliovirus vaccine strains. Mutations at positions 480 (5′NCR [16]), 935 (VP4), 2438 (VP3), 2795 (VP1), and 2879 (VP1) attenuate P1/Sabin. The attenuation phenotype of P2/Sabin has been ascribed to mutations at 481 (5′NCR) and 2908 (VP1). In P3/Sabin, mutations at 472 (5′NCR), 2034 (VP3), and 2493 (VP1) are determinants of attenuation. If attenuating mutations identified in mice and monkeys also influence neurovirulence in humans, then there may be a correlation between the frequency of vaccine-associated poliomyelitis, which is most often associated with P2/Sabin and P3/Sabin, and the number of attenuation determinants in each serotype.

It will be interesting to determine how the mutations identified in this study confer the attenuation phenotype. Initial attempts to identify cell lines in which these mutations impart differences in viral replication at physiological temperatures have been unsuccessful. Although P1/Sabin but not P1/Mahoney has a replication defect in the human neuroblastoma cell line SK-N-MC (1, 3), all of the attenuated recombinants and site-directed mutants described in this study had no replication defect. Other systems are currently being sought to elucidate the molecular mechanisms of attenuation.

ACKNOWLEDGMENTS

We thank A. Macadam for P1/Sabin cDNA and sequence information; Yuang-Jing He for breeding TgPvr mice; and Elizabeth Colston, Roy Bohenzky, Ornella Flore, Saul Silverstein, and Hamish Young for discussions and contributions to this work.

This work was supported by Public Health Service grant AI20017 from the National Institute of Allergy and Infectious Disease.

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