JOURNAL OF VIROLOGY, Oct. 1990, p. 4922–4929
0022-538X/90/104922-08$02.00/0

Vol. 64, No. 10

Mapping of Mutations Associated with Neurovirulence in Monkeys Infected with Sabin 1 Poliovirus Revertants Selected at High Temperature

C. CHRISTODOULOU,[1]† * F. COLBERE-GARAPIN,[1] A. MACADAM,[2] L. F. TAFFS,[2] S. MARSDEN,[2] P. MINOR,[2] AND F. HORAUD[1]

Unité de Virologie Médicale, Institut Pasteur, 75724 Paris Cedex 15, France,[1] and National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, United Kingdom[2]

Poliovirus type 1 neurovirulence is difficult to analyze because of the 56 mutations which differentiate the neurovirulent Mahoney strain from the attenuated Sabin strain. We have isolated four neurovirulent mutants which differ from the temperature-sensitive parental Sabin 1 strain by only a few mutations, using selection for temperature resistance: mutant $S_1 37C_1$ was isolated at 37.5°C, $S_1 38C_5$ was isolated at 38.5°C, and $S_1 39C_6$ and $S_1 39C_{10}$ were isolated at 39.5°C. All four mutants had a positive reproductive capacity at supraoptimal temperature (Rct^+ phenotype). Mutant $S_1 37C_1$ induced paralysis in two of four cynomolgus monkeys, and the three other mutants induced paralysis in four of four monkeys. The lesion score increased from the $S_1 37C_1$ mutant to the $S_1 39$ mutants. To map the mutations associated with thermoresistance and neurovirulence, we sequenced all regions in which the Sabin 1 genome differs from the Mahoney genome. The $S_1 37C_1$ mutant had one mutation in the 5′ noncoding region and another in the 3′ noncoding region. Mutant $S_1 38C_5$ had these mutations plus another mutation in the 3D polymerase gene. The $S_1 39$ mutants had three additional mutations in the capsid protein region. The mutations were located at positions at which the Sabin 1 and Mahoney genomes differ, except for the mutation in the 5′ noncoding region. The noncoding-region mutations apparently confer a low degree of neurovirulence. The 3D polymerase mutation, which distinguishes $S_1 38C_5$ and $S_1 39$ mutants from $S_1 37C_1$, is probably responsible for the high neurovirulence of $S_1 38C_5$ and $S_1 39$ mutants. The capsid region mutations may contribute to the neurovirulence of the $S_1 39$ mutants, which was the highest among the mutants.

Poliovirus (PV) is a human enterovirus belonging to the *Picornaviridae* family. The ability of the three serotypes of PV to replicate in and destroy motor neurons characterizes PV neurovirulence (19), but the molecular basis of neurovirulence is still largely unknown (33). The disease, however, has been brought under control by two efficient viral vaccines: the inactivated and the oral attenuated vaccines (35, 37). To make the latter vaccine, attenuated strains of the three serotypes of PV were derived from the wild-type strains by multiple passages in monkey tissue culture (35). The viral genome is a single-stranded RNA of positive polarity which is 3′ polyadenylated. At the 5′ end of the genome, there is no cap structure, but a small protein, VPg, is covalently linked to the extremity (14). The nucleotide sequence has been determined for virulent and attenuated strains of all three PV serotypes (18, 34, 42, 43). The 5′ noncoding (NC) region is highly conserved among the three serotypes (31), and the alignment of RNA sequences of several picornaviruses suggested a model for the secondary structure containing three relatively independent domains involved in the cap-independent initiation of translation (16, 30). In the case of PV, particularly type 3, the secondary structure of the 5′ NC region could also be correlated with neurovirulence. It has been shown that a single point mutation at position 472 (C → U) affects the structure of the 5′ NC region, the level of in vitro translation, and the degree of neurovirulence (2, 3, 9, 45). A second mutation, which affects the capsid protein VP3, is responsible for the temperature-sensitive phenotype of the attenuated strain of PV type 3 (Sabin 3) and is also involved in determining whether the phenotype of PV type 3 is attenuated or neurovirulent (24).

The situation appears to be more complicated in the case of PV type 1. The virulent Mahoney and the attenuated Sabin 1 strains differ by 56 point mutations, which are scattered throughout the genome of 7,441 nucleotides and which correspond to 21 amino acid differences (14, 15, 43). Inter- and intratypic recombinants have been constructed by different groups in order to localize the sequences implicated in neurovirulence and attenuation (1, 3, 12, 15, 28). While it was proposed that the determinants of neurovirulence were spread over the entire viral genome (28, 32), the results suggested that position 480 in the highly ordered 5′ NC region could be a major determinant of PV type 1 neurovirulence (12).

We used a different strategy to study the molecular basis of PV type 1 neurovirulence, starting from the correlation between virulence and the capacity for viral multiplication at 40°C (20, 22). Spontaneous mutants can be easily selected because of the high mutation rate of single-stranded RNA genomes. We obtained several point mutations of the Sabin 1 strain after successive passages at supraoptimal temperatures. The mutants differed from the parental strain by several phenotypic markers, including plaque size, thermoresistance, and degree of neurovirulence in monkeys. The neurovirulence of the mutants increased gradually with the number of passages at elevated temperature, correlating with an accumulation of point mutations in their genomes. The results that we report here show that not only the 5′ half of the genome but also the 3′ NC region and the 3D

* Corresponding author.
† Present address: Unité de Biochimie Microbienne, Institut Pasteur, 75724 Paris Cedex 15, France.

polymerase gene may carry strong determinants of PV type 1 neurovirulence.

MATERIALS AND METHODS

Cells, viruses, and viral RNA. HEp-2c cells were grown in Eagle basal medium supplemented with 5% calf serum. Stocks of the Sabin 1 (original strain plus three passages) and the mutant strains of PV were prepared in HEp-2c cells. The virus stocks were clarified at 10,000 × *g* for 30 min, and virions were sedimented by centrifugation at 100,000 × *g* for 2 h and purified on a CsCl density gradient in a VTi65 rotor at 242,000 × *g* for 18 h. The PV RNA was extracted twice with phenol-chloroform and once with chloroform and was then ethanol precipitated.

Reproductive capacity at different temperatures (Rct marker). The thermoresistance of viruses was evaluated by an Rct test. Rct is defined as the difference, after 7 days of incubation, between the virus titer at 34°C and that at 40°C, in HEp-2c cells (26).

Test of neurovirulence in Monkeys. Seronegative monkeys [*Macaca fascicularis* (Cynomolgus)] were inoculated intraspinally with 0.1 ml of a virus suspension (10^6 50% infectious doses) by the standard technique of Beswick and Coid (4).

Labeling of viral proteins. (i) Capsid proteins. HEp-2c cells in 75-cm^2 flasks were washed twice and inoculated with virus. After 30 min, Eagle medium with 2% newborn calf serum was added and the cells were incubated for 90 min. This medium was replaced by medium without methionine for 30 min, followed by the addition of 150 μCi of [^{35}S]methionine (Amersham). When all cells showed cytopathic effects, the virus was recovered and purified as described above.

(ii) Intracellular viral proteins. Confluent cultures of HEp-2c cells in 75-cm^2 flasks were infected in 1 ml at a multiplicity of 5 to 10 PFU per cell. After 30 min of adsorption at 37°C, 10 ml of Eagle medium with 2% newborn calf serum was added. After 2 h, this medium was replaced by medium without methionine. Serum (2%) and 450 μCi of [^{35}S]methionine were added 30 min later, and the preparation was left for 3 h. The cells were then washed twice with cold phosphate-buffered saline and lysed in 2.5 ml ice-cold TNE buffer (0.01 M Tris hydrochloride, pH 7.4, 0.1 M NaCl, 0.001 M EDTA, 0.5% [vol/vol] Nonidet P-40, 0.1 mM isopropyl fluorophosphate, 0.2 mM phenylmethylsulfonyl fluoride). Cell debris was pelleted by centrifugation for 5 min at 10,000 × *g*, and cytoplasmic extracts were stored at −20°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were denatured for 5 min in 0.04 M Tris hydrochloride (pH 6.8)–2% (wt/vol) SDS–5% (vol/vol) β-mercaptoethanol–15% (vol/vol) glycerol–0.01% bromophenol blue. Electrophoresis was performed on a 12.5% polyacrylamide-SDS gel for 10 h at 8 mA with a standard Tris-glycine buffer system (17). After migration, the gel was fixed for 30 min in a 50% methanol–7% acetic acid solution, dried, and autoradiographed with Fuji-X films. [^{35}S]methionine-labeled capsid proteins from wild-type virus were used as markers.

Two-dimensional isoelectric focusing. Protein samples were incubated in 8 M urea at 25°C for 1 h in the presence of 100 μg of pancreatic RNase per ml and 4% β-mercaptoethanol. Gels for isoelectric focusing contained 6.5% acrylamide, 15% urea, and 4% ampholytes (pH 3.5 to 10). For the first dimension, isoelectric focusing was performed in gel rods of 1.5 mm in diameter and 13 cm in length. The rods were overlaid with 7 M urea containing 2% ampholytes (46). The electrode solutions consisted of 0.2 M sodium hydroxide at the cathode and 0.1 M phosphoric acid at the anode. The samples were applied to the top of the gel. The potential was 200 V for 1 h and 400 V overnight with an additional hour at 800 V. In the second dimension, the gel rods were set up on top of stacking gels of 12.5% polyacrylamide-SDS and overlaid with 1% agarose in gel buffer (10% glycerol, 2% SDS, Tris hydrochloride [2.5 mM, pH 6.8]). Samples were denatured by heating for 2 min at 100°C in a gel buffer containing 1% SDS, 20% glycerol, 1% β-mercaptoethanol, and 0.1% bromophenol blue. Electrophoresis was carried out at 8 mA overnight.

RNA sequencing. The dideoxy-chain termination method (38, 48) was employed, with modifications, for sequencing RNA. Synthetic 16- to 18-nucleotide DNA primers specific for precise regions of the PV genome, with approximately 200-nucleotide intervals, were used (Table 1). A 1-μg sample of PV RNA was mixed with 40 ng of primer and incubated at 65°C for 10 min. A 2-μl sample of hybridization buffer (0.5 M Tris hydrochloride, pH 8.3, 60 mM $MgCl_2$, 0.4M KCl, 1 mM dithiothreitol, 25 U of RNase block [Genofit]) was added, and the hybridization mixture was incubated at 0°C for 10 min. The mixture was divided into four samples for the A, C, G, and T reactions, and 2 μl of reverse transcriptase solution (5 μl of hybridization buffer, 5 μl of H_2O, 0.5 μl of avian myeloblastosis virus reverse transcriptase [17 U/μl], 2 μl of [α-^{32}P]dTTP [800 mCi/ml, Amersham]) was added. One microliter of deoxynucleoside-dideoxynucleotide solution was added to each reaction, as follows: 100 μM (each) dCTP and dGTP, 50 μM (each) dTTP and dATP, and 10 μM ddATP were added to the A reaction; 100 μM (each) dATP and dGTP, 50 μM (each) dCTP and dTTP, and 10 μM ddCTP were added to the C reaction; 100 μM (each) dATP and dCTP, 50 μM (each) dGTP and dTTP, and 10 μM ddGTP were added to the G reaction; 100 μM (each) dATP, dCTP, and dGTP, 50 μM dTTP, and 10 μM ddTTP were added to the T reaction. The reactions were carried out at 37°C for 15 min and then at 42°C for 45 min. Then 1 μl of chase mixture containing the four deoxynucleoside triphosphates (1 mM each) was added. After 15 min, the reaction was stopped by the addition of 3 μl of a solution containing 0.1% xylene cyanol, 0.1% bromophenol blue, 1 mM EDTA, and 80% formamide in running 1× TBE buffer (0.089 M Tris hydrochloride, pH 8.3, 0.089 M boric acid, 0.002 M EDTA). The final mixture was denatured at 100°C for 2 min, and the samples were applied to sequencing gels containing 7 M urea, 7% acrylamide, and 0.35% bisacrylamide in 1× TBE. Electrophoresis was carried out at 1,400 to 1,800 V in 1× TBE. Electrophoresis was stopped when the xylene cyanol reached 19 cm. The gel was then fixed for 20 min in 10% acetic acid–10% methanol, dried in a Zabona drying-gel apparatus for 1 h, and autoradiographed with Fuji-X film for 24 or 48 h.

RESULTS

Selection of mutants derived from Sabin type 1 PV. The attenuated strain of PV type 1 (Sabin LSc 2ab strain) multiplies well at 34 to 35°C (26, 47). However, above this temperature, viral multiplication becomes less and less efficient. To isolate temperature-resistant derivatives of this virus, we subjected the LSc 2ab strain to successive passages and plaque purification at 37.5, 38.5, and 39.5°C (Fig. 1). The LSc 2ab virus was passaged twice at 37.5°C and then

TABLE 1. Genome regions sequenced in parental and mutant strains

Sequenced nucleotides[a]	Genome region[b]	Differences between Mahoney and Sabin 1 strains					
		Nucleotide			Amino acid		
		Position	Mahoney	Sabin 1	Position	Mahoney	Sabin 1
1–742	5′ NC	21	U	C			
		26	A	G			
		189	C	U			
		480	A	G			
		619	C	U			
		674	C	U			
780–958	VP4	934	G	U	65	A	S
1031–1298	VP2	1208	A	C	87	R	R
		1228	G	C	93	G	G
1402–1507	VP2	1442	A	G	154	N	D
		1465	C	U	161	R	R
		1490	C	U	170	L	F
		1507	G	A	176	L	L
1570–1700	VP2						
1736–2014	VP2	1747	C	U	225	I	I
	VP3	1885	A	U	40	V	V
		1941	C	A	59	A	A
		1943	C	A	60	T	K
2100–2252	VP3						
2264–2612	VP3	2353	U	C	196	T	T
		2438	U	A	255	L	M
	VP1	2545	A	G	22	T	T
		2585	A	G	36	T	A
2635–3040	VP1	2741	A	G	88	T	A
		2749	G	A	90	M	I
		2762	C	U	95	P	S
		2775	C	A	99	T	K
		2795	G	A	106	A	T
		2879	C	U	134	L	F
3080–3274	VP1	3163	U	C	228	L	L
3317–3670	2A	3445	C	U	20	H	H
		3460	U	A	25	D	E
		3492	G	A	36	S	N
3715–4206	2A	3765	C	A	127	G	G
		3784	U	A	134	S	T
	2B	3905	A	G	22	S	G
		3907	C	A	22	S	G
		3918	C	U	29	T	T
		4002	C	U	57	N	N
		4115	U	C	95	I	T
4423–5187	2C	4446	U	C	107	T	T
		4788	A	G	222	T	T
		5106	U	C	308	F	F
	3A	5136	A	G	9	K	K
5377–5622	VPg	5419	C	A	17	R	R
	3C	5439	A	G	1	G	G
6097–6486	3D	6141	G	A	53	D	N
		6202	U	C	73	Y	H
		6372	C	T	129	D	D
6547–7245		6615	G	A	210	V	V
		6678	U	C	231	A	A
		6730	A	G	250	K	E
		6851	C	U	289	G	G
		7070	C	U	362	T	I
		7197	U	A	404	T	T
		7242	U	A	419	L	L
7383–7413	3′ NC	7410	U	C			
7431–7441		7441	A	G			

[a] All regions were sequenced by the dideoxynucleotide method for the four mutants and the Sabin 1 strains.
[b] Protein region corresponding to sequenced nucleotides.

cloned by plaque isolation. The clones thus obtained were designated $S_1$37C ($1 \rightarrow n$). The clone $S_1$37C_1 was used as an inoculum, passaged twice at 38.5°C, and then cloned. The resulting clones were designated $S_1$38C ($1 \rightarrow n$). Mutants $S_1$39C ($1 \rightarrow n$) were obtained in the same way by using as the parental virus a $S_1$38C clone passaged at 39.5°C (Fig. 1) (8). Mutants $S_1$37C_1, $S_1$38C_5, $S_1$39C_6, and $S_1$39C_{10} were studied in detail at passage 5 or 6 after cloning.

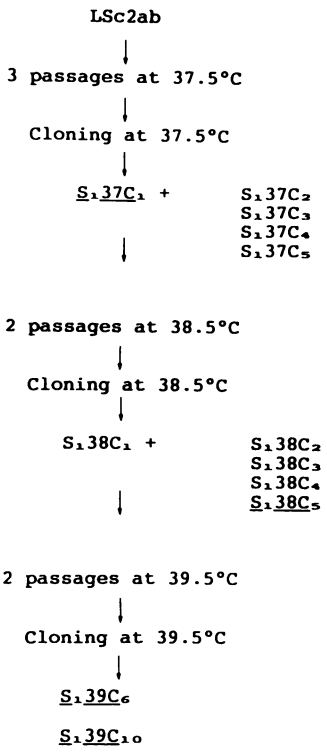

FIG. 1. Schema for isolation of PV mutant clones derived from the thermosensitive and attenuated Sabin 1 (LSc 2ab) strain. Mutants were selected by successive passages and plaque purification at the indicated temperatures. The four viral clones studied in detail are underlined. Virus stocks at five or six passages after cloning were used for all studies.

Phenotype of the mutants. The LSc 2ab Sabin strain differs from its parent, the Mahoney strain of PV type 1, by several phenotypic characteristics, including plaque size, thermoresistance (Rct marker), and neurovirulence in monkeys. To see whether the temperature-resistant mutants behaved like their parental Sabin 1 strain or like the temperature-resistant Mahoney strain, we studied their phenotypic properties. Temperature-resistant mutant clones, like the parental Sabin 1 strain, produced plaques that were 2.5 times smaller than those of the virulent Mahoney wild-type strain (data not shown). The reproductive capacity of PV strains at different temperatures (Rct marker) has been correlated with neurovirulence (11, 12, 28, 47). When the difference in virus logarithmic titer at permissive (34°C) and supraoptimal (40°C) temperatures is lower than 2, the Rct marker is considered positive (Rct^+) (47). Neurovirulent viruses are generally Rct^+ (11, 12, 36). The Rct marker was positive for all four mutants and, as expected, negative for the LSc 2ab parental strain (Table 2). Because neurovirulence depends on several parameters, such as the viral strain, the host species, and the route of inoculation (33), we chose a standard test (4) to quantitate paralysis and the pathological lesions in the monkey central nervous system.

The parental Sabin strain was used as a negative control and the virulent Mahoney strain was used as a positive control in tests for neurovirulence in monkeys. All four mutants were neurovirulent (Table 2). However, mutant $S_1 37C_1$ induced paralysis in only two of four monkeys, while the three other mutants induced paralysis in four of four monkeys. The lesion scores were higher for the mutants isolated at 39.5°C than for those isolated at 38.5 and 37.5°C (Table 2). The degree of neurovirulence thus increased with the temperature used for mutant isolation and perhaps also with the number of passages.

In an attempt to approximately localize the mutations which result in amino acid changes on the genomes of the four mutants, we analyzed the migration profiles of viral polypeptides obtained by SDS-PAGE or by isoelectric focusing. We chose this approach because the polypeptides of the Mahoney strain and those of the Sabin strain migrate differently (46). In addition, it has been shown that even a few amino acid changes can dramatically alter the migration of a PV polypeptide on SDS-PAGE and isoelectric focusing (7, 46). The capsid proteins of the mutants comigrated in SDS-PAGE with those of the Sabin parental strain, with the exception of polypeptide VP1 of mutants $S_1 39C_6$ and $S_1 39C_{10}$ (data not shown). The VP1s of both of these mutants migrated at an intermediate position, between those of the Mahoney and Sabin VP1 proteins.

These results indicate that mutations had occurred in the VP1-coding region for the two clones selected at 39.5°C and that such mutations changed the amino acid sequence of VP1. For $S_1 37C_1$ and $S_1 38C_5$, mutations could not be correlated with changes in any of the structural or nonstructural viral polypeptides, since all mutant polypeptides comigrated with those of Sabin 1 both in SDS-PAGE and in two-dimensional electrophoresis (data not shown).

Genomic sequence of the mutants. A comparison of the nucleotide sequence of the LSc 2ab Sabin 1 genome with that of the Mahoney reveals 56 nucleotide substitutions (14,

TABLE 2. Phenotypic markers and point mutations on the genome of thermoresistant PV 1 mutants in comparison with reference strains

Strain	Rct[a]	Neuro-virulence[b]	Lesion score[c]	Nucleotide[d] at region (position):						
				5′ NC		VP3 (2438)	VP1		$3D^{pol}$ (6203)	3′ NC (7441)
				480	525		2741	2795		
Sabin 1	6.4	0	0.56	G	U	A	G	A	C	G
$S_1 37C_1$	1.3	2	1.52	G	**C**	A	G	A	C	**A**
$S_1 38C_5$	0.1	4	2.14	G	**C**	A	G	A	**U**	**A**
$S_1 39C_6$	0.2	4	2.52	G	**C**	**C**	**U**	**G**	**U**	**A**
$S_1 39C_{10}$	0.2	4	2.51	G	**C**	**C**	**U**	**G**	**U**	**A**
Mahoney	0.2	4	2.50	**A**	U	**U**	**A**	**G**	**U**	**A**

[a] Reproductive capacity at supraoptimal temperature (see Material and Methods).
[b] Number of monkeys ($n = 4$) in which paralysis was induced, as evaluated by the standard test described by Beswick and Coid (4).
[c] Evaluated in monkeys by the standard test described by Beswick and Coid (4).
[d] Boldface type indicates a point mutation at the position indicated, compared with the LSc 2ab Sabin sequence.

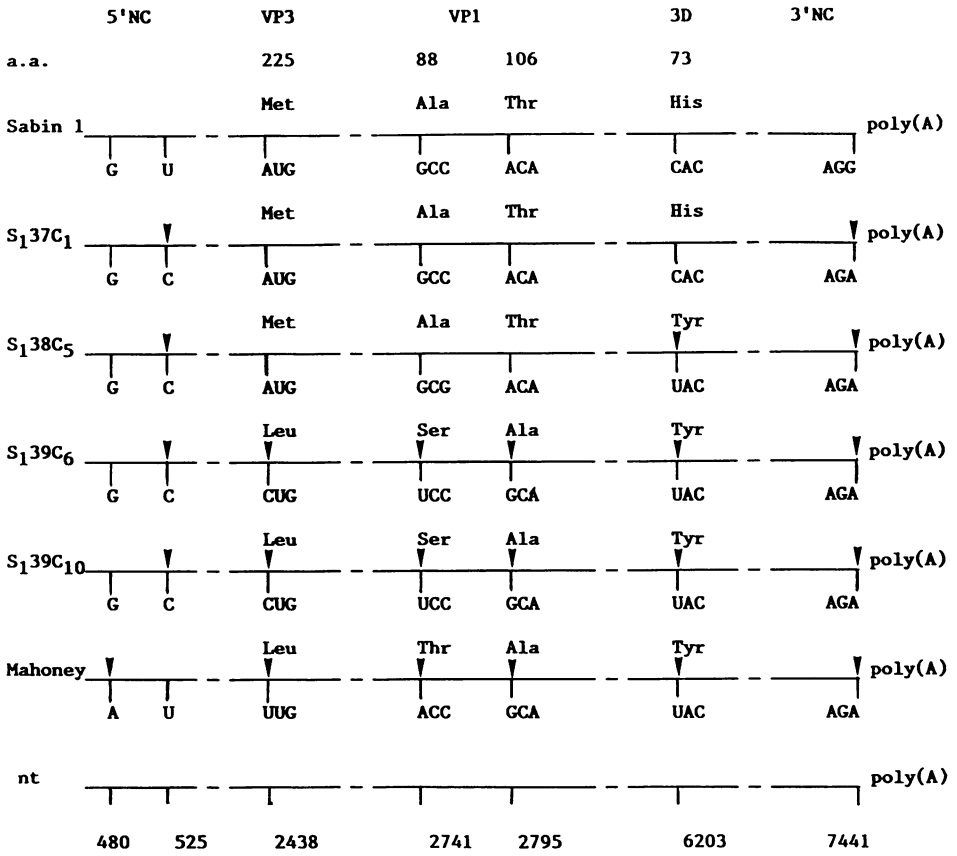

FIG. 2. Mutation positions on the genome of the four temperature-resistant mutants derived from the parental Sabin 1 strain. Mutations are indicated with arrowheads. With the exception of that at position 525, all mutations found were at positions at which the virulent Mahoney and the attenuated Sabin 1 strain differ from each other. The genomic regions in which mutations were found and the corresponding amino acids (a.a.) are shown. Nucleotide (nt) positions corresponding to mutations are indicated at the bottom of the figure.

15, 43). Twenty-one of these change an amino acid. The genomic RNAs of the four temperature-resistant mutants were sequenced directly, by using the dideoxynucleotide method and primers upstream of all positions at which the Mahoney and Sabin genomes differ. Primers were chosen that corresponded to regions of the genome separated by 200 nucleotides. Approximately 80% of the genome of each mutant was sequenced and compared with the sequence of the parental LSc 2ab Sabin 1 genome. Sequenced regions corresponding to 5,880 of the 7,441 nucleotides of the genome are presented in Table 1. With only one exception, all of the mutations found in the genome were located at positions at which substitutions differentiate the Mahoney and the Sabin 1 RNAs.

When compared with the Sabin 1 strain, all four mutants carried one mutation in the 5′ NC region, at position 525 (U → C). At this position, the Mahoney and the Sabin 1 genomes are identical (U). Another mutation carried by all four mutants was found in the 3′ NC region, at position 7441 (G → A); this is the last nucleotide before the poly(A) sequence of the PV type 1 genome. This substitution is a true reversion toward the Mahoney genotype. In all regions of the genome of mutant $S_1 37C_1$ that have been sequenced (approximately 78%; Fig. 2), only these two mutations were found; they are probably responsible for the Rct^+ and partially neurovirulent phenotype of this mutant.

Mutant $S_1 38C_5$ had the same mutations as $S_1 37C_1$ in the 5′ and 3′ NC regions and one additional point mutation in the 3D polymerase gene, at nucleotide 6203 (C → U) (Fig. 2). This mutation induced the alteration His → Tyr of amino acid 73 of the enzyme.

The two mutants $S_1 39C_6$ and $S_1 39C_{10}$ were found to be identical to each other. They carried the mutations in the NC regions and 3D polymerase gene described above, as well as three additional mutations in the capsid protein region. Two of the mutations found in $S_1 39C_6$ and $S_1 39C_{10}$ are in the VP1 gene (Fig. 2). The first one, at nucleotide 2741 (G → U), induced an Ala → Ser change at amino acid 88 of VP1. The second one, at nucleotide 2795, induced a Thr → Ala change at amino acid 106. In the latter case, the mutation was a true reversion toward the Mahoney genotype (Fig. 2). The third capsid mutation found in the $S_1 39$ mutants is in the VP3 gene at nucleotide 2438 (A → C) and induced the alteration Met → Leu (Fig. 2). This mutation restored the Mahoney amino acid sequence, although the Leu codons used are different in the mutants and the wild-type strain.

DISCUSSION

We have isolated a series of PV revertants derived from the Sabin 1 strain by passages at supraoptimal temperature (20, 21). Four of these revertants are neurovirulent. The degree of neurovirulence increased with the selection temperature and with the accumulation of point mutations on the genomes; thus, the mutants selected at the highest temperature (39.5°C) had the greatest number of mutations and the

highest degree of neurovirulence. Five of six mutations identified were located at positions at which the Mahoney and Sabin 1 genomes differ. These results strongly suggest that despite a high mutation frequency, the evolution of the PV genome under the specific selective pressure of high temperature can be limited to specific positions.

None of our mutants was mutated at nucleotide 480, which is the equivalent of the type 3 mutation at position 472 (43). The moderately neurovirulent $S_1 37C_1$ mutant differed from the parental attenuated Sabin 1 strain by only two mutations in the 5′ and 3′ NC regions. Because we did not have an intermediate mutant with a single substitution, we could not evaluate the contribution of each of these mutations to the mild neurovirulence of the $S_1 37C_1$ mutant. At least one of the two mutations is probably implicated in the loss of the attenuated phenotype, since all four mutants have these two mutations and are temperature resistant and partially or fully neurovirulent.

The mutation in the 5′ NC region was found at a position at which the Mahoney and the Sabin 1 genomes are identical. In the secondary structure predicted by Skinner et al. (41), residue 480 pairs with residue 525, and the mutation observed in $S_1 37C_1$, $S_1 38C_5$, $S_1 39C_6$, and $S_1 39C_{10}$ at residue 525 restores the base pair disrupted in the Sabin type 1 strain. By changing the secondary structure of the 5′ NC region, this mutation may facilitate the cap-independent initiation of translation (30, 31) or viral replication or both, thus rendering $S_1 37C_1$ moderately virulent. The mutation in the 3′ NC region at position 7441 may also contribute to the PV type 1 neurovirulence. Recently, evidence was presented that the length of the poly(A) tail is important for virus replication and that molecules with short poly(A) sequences are unstable and unfavorable templates for the initiation of minus-strand RNA synthesis (39). The sequence of the 3′ NC region is highly conserved among picornaviruses, and its secondary structure may be important for the binding of RNA-dependent RNA polymerase to the 3′ end of the genome, translocation to the beginning of the poly(A) tract, and the initiation of minus-strand RNA synthesis by a complex including a host factor (10, 27, 39, 40). The mutation at position 7441 could restore the wild-type secondary structure or the affinity of the host cell factor for RNA or both and thus favor the initiation of replication. A comparable situation occurs in the case of PV type 3, in which the last nucleotide before the poly(A) tail is an A in the virulent Leon strain and a G in the attenuated Sabin 3 strain (6). It has been suggested that the last nucleotide before the poly(A) tail contributes to the neurovirulent phenotype (5, 6). This hypothesis is strengthened by the finding of similar 3′-end mutations in vaccine-associated cases of poliomyelitis (5).

Mutant $S_1 38C_5$ had three mutations: the 5′ and 3′ NC-region mutations at positions 525 and 7441, respectively, and a mutation at position 6203 within the gene which encoded the RNA-dependent RNA polymerase ($3D^{pol}$). Mutant $S_1 38C_5$ was much more virulent than $S_1 37C_1$ as measured by the proportion of paralyzed monkeys and by lesion scores. These results strongly suggest that the mutation in the 3D polymerase gene contributes to the neurovirulent phenotype. Omata et al. (28) have constructed recombinant viruses between the Mahoney and Sabin 1 strains. The results of the neurovirulence tests in monkeys indicated that determinants at multiple loci along a large portion of the viral genome are involved in the expression of neurovirulence of type 1 PV. Two mutations, including the one at position 6203, in the N-terminal half of the 3D polymerase gene, appeared to strongly influence the initiation of viral RNA replication above 38°C (44). If neurovirulence depends on the rate of virus multiplication before the immune response intervenes, it would not be surprising to find that viral functions involved in replication are implicated in neurovirulence.

The two mutants $S_1 39C_6$ and $S_1 39C_{10}$ were found to be identical to each other by sequence analysis. These mutants carry the 5′ NC-region mutation, the two 3′ mutations, and three additional mutations in the capsid region: one in VP3 (position 2438) and two in VP1 (positions 2741 and 2795). The VP1 mutation at position 2795 has already been found in isolates from children vaccinated with the Sabin 1 strain of PV (13). Such a finding is not surprising, since the intestine temperature already represents a selective pressure for the Sabin strain.

The three capsid mutations are together responsible for increased neurovirulence in monkeys, since the mutants isolated at 39.5°C had a lesion score higher than that of $S_1 38C_5$, which does not carry the capsid mutations. The two mutations in VP1, which were responsible for an altered VP1 migration in SDS-PAGE, flank the antigenic site 1, which has been implicated in neurovirulence of PV type 2 in mice (23, 25). The mutation positions in VP3 are different for these type 1 mutants and the Sabin type 3 strain in which the attenuating mutation is also responsible for the virus temperature sensitivity (positions 2438 and 2034, respectively). Not all of the mutations may result in thermoresistance, yet they were all selected for by passage at high temperature. Since, excluding temperature, the isolation of mutants was performed under the same culture conditions as those used to grow the parent strain, it is possible that some mutations represent only the natural heterogeneity of the viral RNA genome population (29).

In conclusion, the results presented here strongly suggest that the 5′ and 3′ NC-region mutations are able to induce a partial reversion towards neurovirulence. The mutation in the 3D polymerase gene together with the 5′ and 3′ NC-region mutations restored a high degree of neurovirulence. These results suggest that neurovirulence may arise from the cooperation of several essential functions affecting the speed of virus multiplication in neurons. Thus, reversion towards neurovirulence may result from changes in the secondary structure of the 5′ NC region and enhancement of the efficiency of translation initiation, as in PV type 3 (31), or from the restoration of an optimal RNA synthesis, as suggested here. The data presented up until now, however, do not exclude the possibility that reversion towards neurovirulence could arise in other ways. The identification of point mutations directly implicated in the PV type 1 reversion towards neurovirulence may provide the basis for understanding this phenomenon at the molecular level.

ACKNOWLEDGMENTS

We thank O. Fichot for help during our first steps in RNA sequencing. We are grateful to R. Crainic and T. Couderc for helpful discussions. We thank K. Kean for critical reading of the manuscript.

This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (CRE no. 863003 and 881006).

LITERATURE CITED

1. **Agol, V. I., S. G. Drozdov, V. P. Grachev, M. S. Kolesnikova, V. G. Kozlov, N. M. Ralph, L, I. Romanova, E. A. Tolskaya, A. V. Tyufanov, and E. G. Viktorova.** 1985. Recombinants between attenuated and virulent strains of poliovirus type 1: derivation and characterization of recombinants with centrally located crossover points. Virology **143**:467–477.
2. **Almond, J. W.** 1987. The attenuation of poliovirus neuroviru-

lence. Annu. Rev. Microbiol. **41**:153–180.
3. **Almond, J. W., G. D. Westrop, D. M. A. Evans, G. Dunn, P. D. Minor, D. Magrath, and G. C. Schild.** 1987. Studies on the attenuation of the Sabin 3 oral polio vaccine. J. Virol. Methods **17**:183–189.
4. **Beswick, T. S. L., and C. R. Coid.** 1961. The influence of technique on the sensitivity of monkeys to intraspinally inoculated poliovirus. J. Hyg. **59**:395–399.
5. **Cammack, N., A. Phillips, G. Dunn, V. Patel, and P. D. Minor.** 1988. Intertypic genomic rearrangements of poliovirus strains in vaccinees. Virology **167**:507–514.
6. **Cann, A. J., G. Stanway, P. J. Hughes, P. D. Minor, D. M. A. Evans, G. C. Schild, and J. W. Almond.** 1984. Reversion to neurovirulence of the live-attenuated Sabin type 3 oral poliovirus vaccine. Nucleic Acids Res. **20**:7787–7792.
7. **Colbère-Garapin, F., C. Christodoulou, R. Crainic, A. C. Garapin, and A. Candrea.** 1988. Addition of a foreign oligopeptide to the major capsid protein of poliovirus. Proc. Natl. Acad. Sci. USA **85**:8668–8672.
8. **Crainic, R., B. Blondel, A. Candréa, G. Dufraisse, and F. Horaud.** 1985. Antigenic modifications of attenuated Sabin type 1 poliovirus by in vitro passages at supraoptimal temperatures. Dev. Biol. Standard. **60**:343–347.
9. **Evans, D. M., G. Dunn, P. D. Minor, G. C. Schild, A. J. Cann, G. Stanway, J. W. Almond, K. Currey, and J. V. Maizel, Jr.** 1985. Increased neurovirulence associated with a single nucleotide change in a non coding region of the Sabin type 3 poliovaccine genome. Nature (London) **314**:548–550.
10. **Hey, T. D., O. C. Richards, and E. Ehrenfeld.** 1986. Synthesis of plus- and minus-strand RNA from poliovirion RNA template in vitro. J. Virol. **58**:790–796.
11. **Horaud, F., R. Crainic, and B. Blondel.** 1985. Polio vaccine: from empiricism and efficacy to scientific knowledge. Ann. Inst. Pasteur Virol. **136E**:527–545.
12. **Kawamura, N., M. Kohara, S. Abe, T. Komatsu, K. Tago, M. Arita, and A. Nomoto.** 1989. Determinants in the 5′ noncoding region of poliovirus Sabin 1 RNA that influence the attenuation phenotype. J. Virol. **63**:1302–1309.
13. **Kew, O. M., and B. K. Nottay.** 1984. Evolution of the oral polio vaccine strains in humans occurs by both mutation and intramolecular recombination, p. 357–367. *In* R. Chanock and R. Lerner (ed.), Modern approaches to vaccines. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Kitamura, N., B. L. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J. Dorner, E. A. Emini, R. Hanecak, J. J. Lee, S. van der Werf, C. W. Anderson, and E. Wimmer.** 1981. Primary structure, gene organization and polypeptide expression of poliovirus RNA. Nature (London) **291**:547–553.
15. **Kohara, M., T. Omata, A. Kameda, B. L. Semler, H. Itoh, E. Wimmer, and A. Nomoto.** 1985. In vitro phenotypic markers of a poliovirus recombinant constructed from infectious cDNA clones of the neurovirulent Mahoney strain and the attenuated Sabin 1 strain. J. Virol. **53**:786–792.
16. **Kuge, S., N. Kawamura, and A. Nomoto.** 1989. Genetic variation occurring on the genome of an in vitro insertion mutant of poliovirus type 1. J. Virol. **63**:1069–1075.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **La Monica, N., C. Meram, and V. Racaniello.** 1986. Mapping of sequences required for mouse neurovirulence of poliovirus type 2 Lansing. J. Virol. **57**:515–525.
19. **La Monica, N., and V. R. Racaniello.** 1989. Differences in replication of attenuated and neurovirulent polioviruses in human neuroblastoma cell line SH-SY5Y. J. Virol. **63**:2357–2360.
20. **Lwoff, A.** 1959. Factors influencing the evolution of viral diseases at the cellular level and in the organism. Bacteriol. Rev. **23**:109–124.
21. **Lwoff, A.** 1969. Death and transfiguration of a problem. Bacteriol. Rev. **33**:390–403.
22. **Lwoff, A., and M. Lwoff.** 1958. L'inhibition du développement du virus poliomyélitique à 39° et le problème du rôle de l'hyperthermie dans l'évolution des infections virales. C. R. Acad. Sci. **246**:190–192.
23. **Martin, A., C. Wychowski, T. Couderc, R. Crainic, J. Hogle, and M. Girard.** 1988. Engineering a poliovirus type 2 antigenic site on a type 1 capsid results in a chimaeric virus which is neurovirulent for mice. EMBO J. **7**:2839–2847.
24. **Minor, P. D., G. Dunn, D. M. A. Evans, D. I. Magrath, A. John, J. Howlett, A. Phillips, G. Westrop, K. Wareham, J. W. Almond, and J. M. Hogle.** 1989. The temperature sensitivity of the Sabin type 3 vaccine strain of poliovirus: molecular and structural effects of a mutation in the capsid protein VP3. J. Gen. Virol. **70**:1117–1123.
25. **Murray, M. G., J. Bradley, X. F. Yang, E. Wimmer, E. G. Moss, and V. R. Racaniello.** 1988. Poliovirus host range is determined by a short amino acid sequence in neutralisation antigenic site 1. Science **241**:213–214.
26. **Nakano, J. H., M. H. Hatch, W. Thieme, and B. Nottay.** 1978. Parameters for differentiating vaccine-derived and wild poliovirus strains. Prog. Med. Virol. **24**:178–206.
27. **Oberste, M. S., and J. B. Flanegan.** 1988. Measurement of poliovirus RNA polymerase binding to poliovirion and nonviral RNAs using a filter-binding assay. Nucleic Acids Res. **16**: 10339–10352.
28. **Omata, T., M. Kohara, S. Kuge, T. Komatsu, S. Abe, B. L. Semler, A. Kameda, H. Itoh, M. Arita, E. Wimmer, and A. Nomoto.** 1986. Genetic analysis of the attenuation phenotype of poliovirus type 1. J. Virol. **58**:348–358.
29. **Parvin, J. D., A. Moscona, W. T. Pan, J. M. Leider, and P. Palese.** 1986. Measurement of the mutation rates of animal viruses: influenza A virus and poliovirus type 1. J. Virol. **59**:377–383.
30. **Pelletier, J., and N. Sonenberg.** 1989. Internal binding of eucaryotic ribosomes on poliovirus RNA: translation in HeLa cell extracts. J. Virol. **63**:441–444.
31. **Pilipenko, E. V., V. M. Blinov, L. I. Romanova, A. N. Sinyakov, S. V. Maslova, and V. I. Agol.** 1989. Conserved structural domains in the 5′-untranslated region of picornaviral genomes: an analysis of the segment controlling translation and neurovirulence. Virology **168**:201–209.
32. **Racaniello, V. R.** 1987. Viral sequences required for neurovirulence of poliovirus. BioEssays **5**:217–246.
33. **Racaniello, V. R.** 1988. Poliovirus neurovirulence. Adv. Virus Res. **34**:217–246.
34. **Racaniello, V. R., and D. Baltimore.** 1981. Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proc. Natl. Acad. Sci. USA **78**:4887–4891.
35. **Sabin, A. B., and L. R. Boulger.** 1973. History of Sabin attenuated poliovirus oral live vaccine strains. J. Biol. Stand. **1**:115–118.
36. **Sabin, A. B., and A. Lwoff.** 1958. Relation between reproductive capacity of polioviruses at different temperatures in tissue culture and neurovirulence. Science **129**:1287–1288.
37. **Salk, J. E.** 1955. Consideration in the preparation and use of poliomyelitis virus vaccine. J. Am. Med. Assoc. **1548**:1239–1248.
38. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
39. **Sarnow, P.** 1989. Role of 3′-end sequences in infectivity of poliovirus transcripts made in vitro. J. Virol. **63**:467–470.
40. **Sarnow, P., H. D. Bernstein, and D. Baltimore.** 1986. A poliovirus temperature-sensitive RNA synthesis mutant located in a noncoding region of the genome. Proc. Natl. Acad. Sci. USA **83**:571–575.
41. **Skinner, M. A., V. R. Racaniello, G. Dunn, J. Cooper, P. D. Minor, and J. W. Almond.** 1989. A new model for the secondary structure of the 5′ noncoding RNA of poliovirus is supported by biochemical and genetical data which also show that RNA secondary structure is important in neurovirulence. J. Mol. Biol. **207**:379–392.
42. **Stanway, G., P. J. Hughes, R. C. Mountford, P. Reeve, P. D. Minor, G. C. Schild, and J. W. Almond.** 1984. Comparison of the complete nucleotide sequences of the genomes of the

neurovirulent poliovirus P3/Leon/37 and its attenuated Sabin vaccine derivative P3/Leon $12a_1b$. Proc. Natl. Acad. Sci. USA **81**:1539–1543.

43. **Toyoda, H., M. Kohara, Y. Kataoka, T. Suganuma, T. Omata, N. Imura, and A. Nomoto.** 1984. Complete nucleotide sequences of all three poliovirus serotype genomes. J. Mol. Biol. **174**: 561–585.
44. **Toyoda, H., C.-F. Yang, N. Takeda, A. Nomoto, and E. Wimmer.** 1987. Analysis of type 1 poliovirus by using an in vitro molecular genetic approach. J. Virol. **61**:2816–2822.
45. **Westrop, G. D., K. A. Wareham, D. M. A. Evans, G. Dunn, P. D. Minor, D. I. Magrath, F. Taffs, S. Marsden, M. A. Skinner, G. C. Schild, and J. W. Almond.** 1989. Genetic basis of attenuation of the Sabin type 3 oral poliovirus vaccine. J. Virol. **63**:1338–1344.
46. **Wiegers, K. J., and R. Dernick.** 1981. Poliovirus-specific polypeptide infected HeLa cells analysed by isoelectric focusing and 2D-analysis. J. Gen. Virol. **52**:61–69.
47. **World Health Organization, Expert Committee on Biological Standardization.** 1983. Requirements for poliomyelitis vaccine (oral). World Health Organization, Geneva.
48. **Zimmern, D., and P. Kaesberg.** 1978. 3′ Terminal nucleotide sequence of encephalomyocarditis virus RNA determined by reverse transcriptase and chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **75**:4257–4261.