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Mapping of Attenuating Sequences of an Avirulent Poliovirus Type 2 Strain

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A mouse model for poliomyelitis was used to identify genomic sequences that attenuate neurovirulence of poliovirus strain P2/P712. This type 2 strain is avirulent in primates and mice yet grows as well as virulent strains in cell culture. The approach used was to exchange portions of the genome of the mouse-virulent P2/Lansing strain with the corresponding region from P2/P712 to identify sequences that could attenuate Lansing neurovirulence in mice. A full-length infectious cDNA of P2/P712 was assembled and used to construct recombinants between P2/P712 and P2/Lansing. The results of neurovirulence testing of 11 recombinants indicated that strong attenuating determinants are located in the 5′ noncoding region of P2/P712 and a region encoding capsid protein VP1 and $2A^{pro}$, 2B, and part of 2C. An attenuating determinant was further localized to between nucleotides 456 and 628 of P2/P712. A third sequence from P2/P712, nucleotides 752 to 2268, encoding VP4, VP2, and part of VP3, was weakly attenuating. The sequence from nucleotide 4454, approximately halfway through the 2C-coding region, to the end of the P2/P712 genome did not contain attenuating determinants. Nucleotide sequence analysis revealed that P2/P712 differs from the type 2 Sabin vaccine strain by only 22 nucleotides. Six differences lead to amino acid changes in the coding region, and four differences are in the 5′ noncoding region. These studies show that, like the type 1 and type 3 Sabin vaccine strains, the attenuated type 2 strain P712 contains multiple attenuating sequences, including strongly attenuating sequences in the 5′ noncoding region of the genome.

Poliomyelitis is an acute and sometimes fatal paralytic disease in humans caused by poliovirus, an enteric virus of the picornavirus family. The occurrence of epidemic poliomyelitis has been greatly reduced by extensive vaccination with inactivated virus preparations and live attenuated strains. The live oral poliovirus vaccine consists of one attenuated strain of each of the three serotypes of poliovirus. Although most cases of poliomyelitis now occur in undervaccinated populations, poliomyelitis may occasionally result after vaccination (1, 14). These cases appear to result from mutation of attenuated strains to neurovirulence (2). The genetic nature of the attenuation of poliovirus can be elucidated by a study of virulent and avirulent isolates.

Poliovirus, like other picornaviruses, is an unenveloped particle consisting of four capsid proteins in icosahedral symmetry around a single-stranded, message-sense RNA genome (reviewed in reference 6). The genome is 7.5 kilobases in length and is covalently linked to a viral protein. The RNA contains a 5′ untranslated region of about 750 nucleotides which contains information necessary for translation initiation and RNA replication (17, 19, 23, 30, 31). The virus infects a susceptible cell by interacting with specific receptor molecules on the cell surface, followed by endocytosis, release of the RNA genome into the cytoplasm, and translation. The genome is translated into a polyprotein that is cleaved by two viral proteinases into functional viral proteins.

The attenuated poliovirus vaccine strains developed by A. B. Sabin were isolated by repeated passage of wild virulent and avirulent strains in primates and primate cell culture (reviewed in reference 26). Those strains with the lowest neurovirulence were used as vaccine strains. These strains rarely cause paralytic disease yet replicate sufficiently to induce a protective immune response. The molecular basis for the attenuation of the Sabin vaccine strains has been studied for the past several years. The approach to this problem has been to construct viral recombinants, using the infectious cDNA of an attenuated vaccine strain and that of the virulent parent from which the vaccine was derived, and test the recombinants for neurovirulence in primates. Once genomic regions containing determinants of attenuation are identified, the nucleotide sequence in this area of the vaccine strain and the parent strain are compared. With this approach, two point mutations, one in the 5′ noncoding region and one in the capsid, that are responsible for the attenuation phenotype of the type 3 vaccine strain have been identified (35). Similar studies have revealed that attenuating mutations are scattered throughout the genome of the type 1 vaccine strain, with a particularly strong determinant in the 5′ noncoding region (5, 15).

A similar approach has not been used to identify the determinants of attenuation in the type 2 Sabin strain, since the parent of this vaccine strain is not neurovirulent (25). However, it is known that a 5′ noncoding mutation known to attenuate the type 3 Sabin strain for humans also attenuates neurovirulence of the mouse-virulent P2/Lansing strain in mice (7). This observation suggests a different approach to identifying determinants of attenuation in viruses for which a neurovirulent parent is not available. Here this approach is used to map attenuation sequences in the genome of P712, a type 2 strain that is avirulent in mice and monkeys. An infectious cDNA of P2/P712 was generated and used to construct viral recombinants with the P2/Lansing strain of poliovirus. By testing the neurovirulence of these recombinants in mice, it was possible to identify regions of the P2/P712 genome that confer the attenuation phenotype. Nucleotide sequence analysis of P2/P712 reveals that this virus is closely related to the Sabin type 2 vaccine strain.

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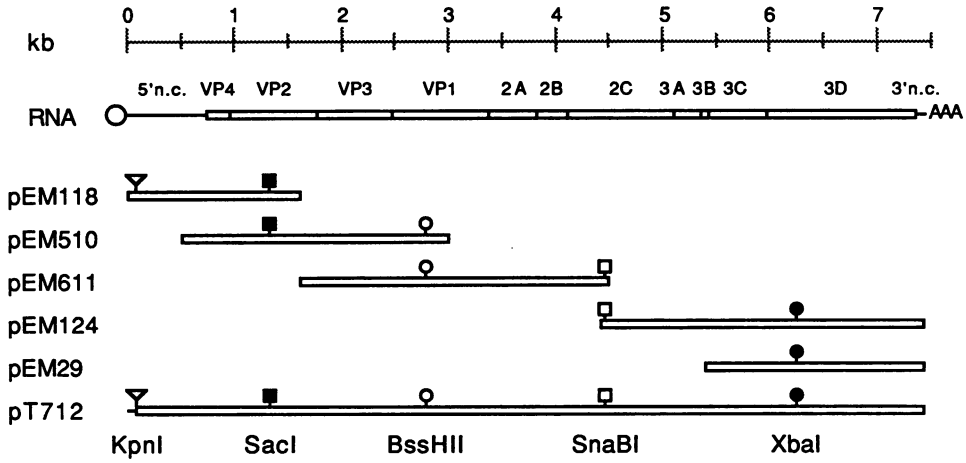

FIG. 1. Construction of full-length infectious cDNA of the P2/P712 genome. Positions of the poliovirus strain P2/P712-specific inserts from cDNA clones pEM118, pEM510, pEM611, pEM124, and pEM29 are shown in relation to a genetic map of viral genomic RNA and to the full-length infectious cDNA insert in pT712. The first 68 nucleotides (*Kpn*I site) of pT712 are derived from a type 1 cDNA (32). Restriction enzyme cleavage sites used to construct the insert in pT712 are shown. kb, Kilobases; n.c., noncoding.

MATERIALS AND METHODS

Cells and virus. 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 P2/P712 virus used for cDNA synthesis was obtained from the American Type Culture Collection, Rockville, Md.; no passage history was available. An uncloned stock of this virus prepared in HeLa S3 cells in our laboratory did not cause disease in mice (data not shown) and resembled the P2/Sabin strain in the World Health Organization monkey neurovirulence test (P. Minor, personal communication). All other viruses used in these experiments were derived by transfection of HeLa cells with cloned poliovirus cDNAs.

Virus growth and assay. Virus titers were determined by plaque assay on HeLa cell monolayers. Tenfold dilutions of viruses prepared in phosphate-buffered saline plus 0.2% horse serum were used to inoculate 6-cm-diameter dishes of cells and allowed to adsorb at 37°C. The cells were then covered with 5 ml of 0.9% Bacto-Agar (Difco Laboratories, Detroit, Mich.) in Dulbecco minimal essential medium plus 5% horse serum. After incubation at 37°C in 5% CO_2 for 2 to 3 days, plates were stained with crystal violet as described previously (21).

Virus stocks of high titer, used for inoculation of mice, were prepared either in suspension cultures or in monolayers of HeLa cells. Cell monolayers or pellets were washed with phosphate-buffered saline and incubated with virus at a multiplicity of infection of 10 PFU per cell. After adsorption, cells were incubated in medium at 37°C for 5 to 7 h. Infected cells were collected by centrifugation, washed with phosphate-buffered saline, subjected to three cycles of freeze-thawing, clarified, and stored at −70°C. All other virus stocks were prepared in cell monolayers, using progeny of a single plaque as described elsewhere (9).

Viruses were tested for temperature sensitivity by plaque assay at both 33 and 39.5°C as described previously (8). The efficiency of plating was calculated by dividing the virus titer at 33°C by the titer at 39.5°C.

cDNA synthesis. Poliovirus type 2 strain P2/P712 was prepared in suspension cultures of HeLa cells as described above and purified by CsCl centrifugation; viral RNA was isolated as described elsewhere (22). Viral RNA was used as a template for reverse transcriptase by a modification of a previously described procedure (28). A 500-μl reaction mixture containing 50 μg of RNA, 50 mM Tris chloride (pH 8.3), 10 mM $MgCl_2$, 50 mM KCl, 0.5 mM each deoxynucleoside triphosphate, 0.4 mM dithiothreitol, 4 mM sodium pyrophosphate, 30 μg of oligo$(dT)_{12-18}$ per ml, 50 μCi of [α-^{32}P]dATP (800 Ci/mmol), 1 U of RNasin (Promega Biotec) per μl, and 2 U of avian reverse transcriptase (Pharmacia Fine Chemicals, Piscataway, N.J.) per μl was incubated at 42°C for 60 min. Synthesis was monitored by alkaline agarose gel electrophoresis followed by autoradiography. The double-stranded cDNA was completed by treating the RNA-cDNA hybrid in a 100-μl reaction mixture containing 20 mM Tris chloride (pH 7.4), 7 mM $MgCl_2$, 100 mM KCl, 50 μg of bovine serum albumin per ml, 100 μM each deoxynucleoside triphosphate, 150 μM β-NAD, 200 ng of RNA-cDNA, 5 μg of *Escherichia coli* DNA ligase per ml, 250 U of *E. coli* DNA polymerase I per ml, and 5 U of RNase H per ml. The mixture was incubated at 12°C for 60 min and then at 22°C for 90 min. The double-stranded cDNA was tailed in a 100-μl reaction mixture containing cDNA, 140 mM potassium-cacodylic acid (pH 6.9), 1 mM CoCl, 0.2 mM dithiothreitol, 50 μg of bovine serum albumin per ml, 150 μM dCTP, and 800 U of terminal transferase per ml and was incubated at 38°C for 30 min. The dG-oligo (dG)-tailed cDNA was then cloned in pUC9 (33).

Five cDNA clones representing all but the 5′ four nucleotides of the P2/P712 genome were selected. The relative position of each of the clones was determined by restriction analysis and nucleotide sequencing (Fig. 1). The 5′-most cDNA, pEM118, began at the fifth nucleotide in the poliovirus sequence, and the 3′-most cDNA, pEM29, ended with a 12-nucleotide poly(dA) tail. A full-length cDNA was constructed by joining cDNA clones, using common restriction enzyme cleavage sites (*Sac*I, *Bss*HII, *Sna*BI, and *Xba*I; Fig. 1). Nucleotides 1 to 68 (*Kpn*I site) were supplied from a P1/Mahoney cDNA, and the resulting full-length cDNA of P2/P712 was placed next to a bacteriophage T7 promoter in pJB127 (32; gift of E. Wimmer); the plasmid containing a

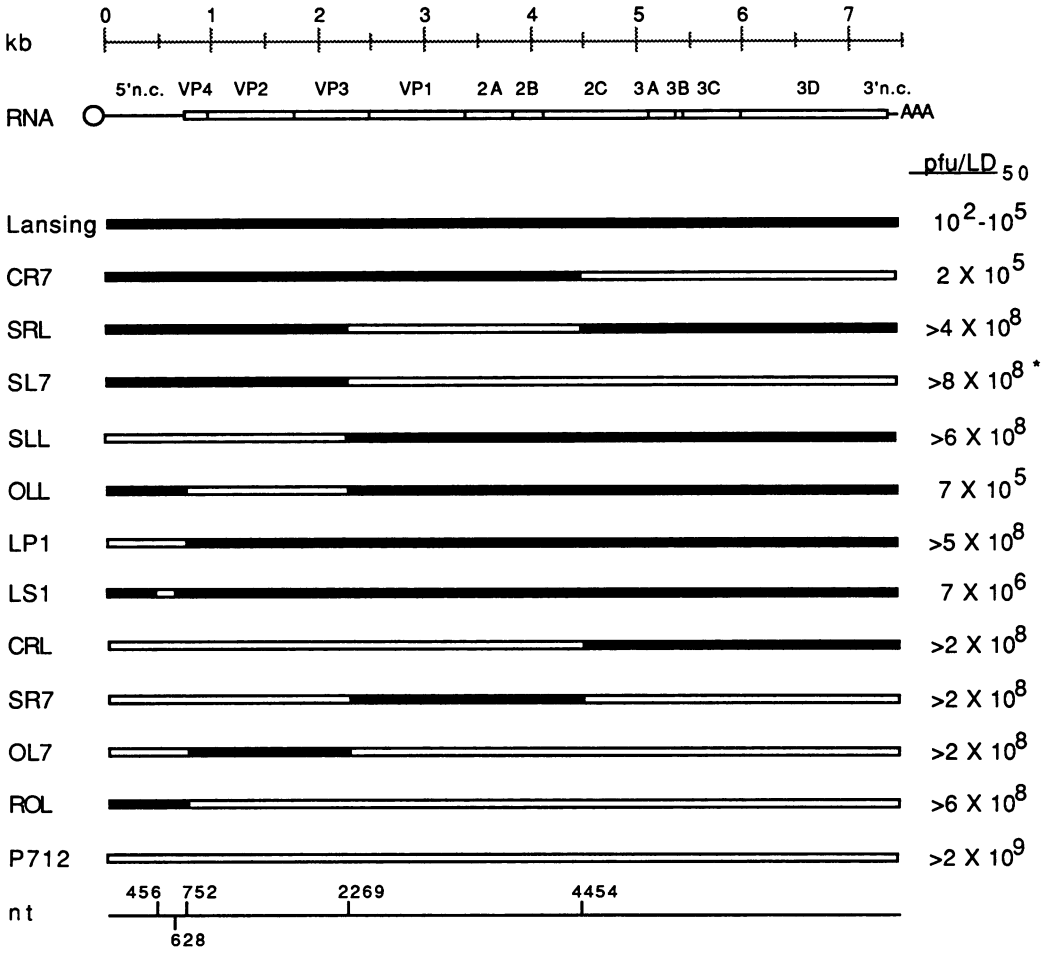

FIG. 2. Constitution and neurovirulence of P2/P712-P2/Lansing recombinant viruses. The genomic RNA of each virus derived from recombinant cDNA is represented below a genetic map of viral genomic RNA. Virus names are shown at the left, and the corresponding LD_{50}s are shown at the right. Bottom line shows the nucleotide (nt) positions of restriction enzyme cleavage sites within poliovirus cDNA used to construct the recombinants; the sites are described in Materials and Methods. Symbols: ▬, sequence derived from cDNA of P2/Lansing; ▭, sequence derived from cDNA of P2/P712; *, value does not reflect the fact that some mice were paralyzed by this virus. kb, Kilobases; n.c., noncoding.

full-length infectious cDNA of the P2/P712 genome was named pT712.

Sequencing. Sequencing of P2/P712 cDNA subclones in M13 sequencing vectors (36) was performed by using 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 (8), using oligonucleotide primers specific for P2/Lansing and P2/P712 viral RNA.

Construction of recombinants. Plasmid DNAs were grown in *E. coli* DH1 (3) and purified by CsCl centrifugation (10). The DNAs were cleaved with restriction endonucleases under conditions recommended by the manufacturers (Boehringer-Mannheim Biochemicals, Indianapolis, Ind., and New England BioLabs, Inc., Beverly, Mass.), and DNA fragments were separated by electrophoresis in agarose gels buffered with 40 mM Tris acetate–5 mM sodium acetate–2 mM EDTA, pH 7.8. A modification of the glass powder method was used to recover DNA fragments from agarose gels (34). Ligations of DNA fragments were performed according to the instructions of the manufacturer of T4 DNA ligase (Boehringer-Mannheim Biochemicals).

Eleven recombinant cDNAs were constructed by the exchange of restriction fragments derived from the cloned cDNAs of P2/P712 and P2/Lansing (20). The constitution of each recombinant is diagramed in Fig. 2. Five restriction sites mark the ends of the exchanged DNA fragments: *Bsm*I at nucleotide 456, *Bal*I at 628, *Nar*I at 751, *Kpn*I at 2268, and *Sna*BI at 4453. Nucleotide numbers refer to locations in the poliovirus cDNAs; numberings of the P2/P712 and P2/Lansing cDNAs differ slightly.

In vitro RNA synthesis and transfection. In vitro synthesis of RNA from DNA templates was performed by using T7 RNA polymerase (Pharmacia). Reaction mixtures (50 μl) contained 2 μg of plasmid DNA [linearized 3′ to the poly(A) tract in the poliovirus cDNA], 1 mM each nucleoside triphosphate, 1 U of RNasin (Promega Biotec) per μl, 0.5 mg of bovine serum albumin (RNase and DNase free; Boehringer-Mannheim Biochemicals) per ml, 5 mM dithiothreitol, 40 mM Tris chloride (pH 8), 15 mM $MgCl_2$, and 30 U of T7 RNA polymerase (Pharmacia). After incubation at 37°C for 30 min, the reaction mixtures were used to transfect HeLa cell monolayers in 6-cm-diameter dishes, using DEAE-dextran as the facilitator (9).

Neurovirulence assay. Groups of eight 21-day-old Swiss Webster 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 daily for 21 days for paralysis or death. Paralyzed mice were sacrificed and scored as dead. The amount of virus that caused paralysis or death in 50% of mice, LD_{50}, was calculated by the method of Reed and Muench (24). At least two LD_{50} determinations were performed on each virus.

RESULTS

Infectious cDNA of poliovirus type 2 strain P2/P712. Since determinants of attenuation have not been identified for a type 2 poliovirus strain, we chose to study strain P712, obtained from the American Type Culture Collection. The passage history of this strain is not known, but its name suggests that it either is the parent of P2/Sabin (known as P712; 26) or is derived from P2/Sabin (the name of the vaccine strain is P2/P712, Ch, 2ab). The P2/P712 strain that we obtained was avirulent in monkeys (Minor, personal communication) and in mice yet replicated as well as neurovirulent viruses in cultured HeLa cells (data not shown). To identify determinants of attenuation in the genome of P2/P712, a full-length infectious cDNA was assembled by using cDNA clones derived from virion RNA (Fig. 1). When transfected into HeLa cells, the cloned, full-length P2/P712 cDNA gave rise to virus that resembled P2/P712 in plaque size and neutralization with a panel of monoclonal antibodies (data not shown).

Both the transfection-derived P2/P712 and the stock used for cDNA synthesis were distinct from the type 2 Sabin vaccine strain in that they were not temperature sensitive, whereas the vaccine strain carries a temperature sensitivity marker (Minor, personal communication; data not shown).

Nucleotide sequence of the P2/P712 viral genome. The complete nucleotide sequence of the viral genome of P2/P712 was determined from a full-length infectious cDNA and subgenomic cDNA clones used to construct the full-length cDNA. There were 22 nucleotide differences between P2/P712 and P2/Sabin (Table 1).

Construction and analysis of P2/P712-P2/Lansing recombinants. To identify attenuating sequences in the P2/P712 genome, a set of 11 recombinants between the infectious cDNAs of P2/P712 and P2/Lansing were constructed. Homologous fragments were exchanged by using restriction sites common between the two cDNAs (see Materials and Methods). Viruses were derived by transfection of HeLa cell monolayers with RNA transcribed in vitro from the cloned recombinant cDNAs. The identity of the viral recombinants was confirmed by sequencing of viral RNA at the junctions between the two sequences. The genomes of the P2/P712-P2/Lansing recombinants and the parental strains are shown in Fig. 2. P2/P712 and P2/Lansing were found to differ considerably in their nucleotide sequences; Table 2 compares the sequences of the restriction fragments that were exchanged between P2/P712 and P2/Lansing.

To ensure that the juxtaposition of sequences from the two strains did not introduce new phenotypes, the growth characteristics of the recombinants in culture were compared with those of P2/P712 and P2/Lansing. The recombinants showed no greater degree of temperature sensitivity when grown at 39.5°C than did either of the parental strains (data not shown). In addition, the plaque size of each recombinant resembled that of the parental strain (data not shown).

TABLE 1. Nucleotide and amino acid differences between P2/Sabin and P2/P712

Nucleotide position	P2/Sabin[a]		P2/P712		Amino acid of viral protein
	Nucleotide	Amino acid	Nucleotide	Amino acid	
28	T	—[b]	C	—	
72	T	—	C	—	
309	G	—	A	—	
355	T	—	C	—	
1298	T	Phe	C	Ser	115 of VP2
1299	C		A		
1670	T	Val	C	Ala	239 of VP2
1952	G	Arg	A	Lys	62 of VP3
1953	A		G		
2502	A		G		
2567	G		A		
2568	A	Gly	C	Asp	29 of VP1
2569	C		A		
2570	A	His	C	Thr	30 of VP1
2571	C		A		
4117	G	Glu	A	Lys	96 of 2B
4147	G	Ala	A	Thr	9 of 2C
4290	G		T		
5802	A		G		
6435	T		C		
6744	C		A		
7329	G		A		

[a] From reference 29.
[b] —, Nucleotide difference in the noncoding region of the genome.

Neurovirulence of recombinant viruses. To determine the capacity for neurovirulence of each of the recombinant viruses, eight 21-day-old Swiss Webster mice were inoculated intracerebrally with 50 μl of a high-titer stock of each recombinant and parental strain and with 10-fold dilutions of each stock. The LD_{50} of each virus was calculated by the method of Reed and Muench (24) (Fig. 2). In this assay, inoculation of mice with up to 2×10^9 PFU of P2/P712 did not result in disease; in contrast, we previously noted that as little as 10^3 to 10^5 PFU of transfection-derived P2/Lansing is sufficient to induce paralysis in 50% of mice (8); this range was confirmed in this study. It should be noted that when endpoints are not reached, it is not possible to compare LD_{50} values of different recombinants. When paralysis resulted, virus was isolated from the spinal cords of infected mice, and the nucleotide sequence across junctions was determined to establish that disease was induced by the virus that was inoculated. By this analysis, it was found that all viruses isolated from the central nervous systems of paralyzed mice resembled the inoculated virus (data not shown).

The neurovirulence results indicated that the 3′ end of P2/P712 RNA does not contain attenuating determinants, since the neurovirulence of recombinant CR7 was similar to

TABLE 2. Sequence comparison of restriction fragments exchanged between P2/P712 and P2/Lansing cDNAs

Exchanged fragment[a]	No. of differences[b]
1–751	139 nucleotides
456–628	17 nucleotides
752–2268	7 amino acids
2269–4453	41 amino acids
4454–7439	25 amino acids, 2 nucleotides

[a] Numbers refer to nucleotide positions in the P2/P712 genome of restriction sites used to generate the fragments.
[b] Nucleotide differences in coding regions are not listed.

that of P2/Lansing. The central region of the genome, encoding capsid protein VP1 and $2A^{pro}$, 2B, and part of 2C, contained strong attenuating determinants. Recombinant SRL, which contained this region of P2/P712 from bases 2269 to 4453 in a P2/Lansing background, was dramatically attenuated compared with P2/Lansing (note that the LD_{50} of P712 was the highest because virus stocks of very high titer were used). As expected, recombinant SL7, in which nucleotide 2269 to the 3′ end of the Lansing genome was exchanged with P712 sequences, was also attenuated.

The 5′ noncoding region of P2/P712 was also found to contain a strong attenuating determinant. Recombinant LP1, which contained P2/P712 sequences from the 5′ end of the genome to the initiating methionine at base 748 and the rest of the genome from P2/Lansing, was unable to induce paralysis in mice even at the highest concentration of virus inoculated. The sequences from the start of the coding region to base 2268 of P2/P712, which encodes VP4, VP2, and part of VP3, attenuated recombinant OLL to an LD_{50} of 7×10^5, just slightly higher than the highest value observed for P2/Lansing. As expected, recombinant SLL was also attenuated, as it contained bases 1 to 2268 of Lansing exchanged with those of P712.

To further localize attenuating sequences within the 5′ noncoding region of P2/P712, recombinant LS1 was constructed. LS1 contained sequences from bases 456 to 628 from P2/P712 in an otherwise P2/Lansing background. The LD_{50} of LS1 was 7×10^6, which indicated that this virus was less attenuated than recombinant LP1.

Recombinants CRL, SR7, OL7, and ROL all contained two or more of the above-identified P712 attenuating regions exchanged for the Lansing sequences. As expected, all four recombinants were attenuated.

DISCUSSION

Our approach to mapping attenuating sequences in an avirulent strain of type 2 poliovirus is based on several observations. The mouse-adapted strain P2/Lansing induces poliomyelitis in mice after intracerebral inoculation (4). When the 5′ noncoding region of the type 3 Sabin vaccine strain, known to carry a strong attenuating mutation at base 472, was substituted for that of P2/Lansing, the recombinant was dramatically attenuated for neurovirulence in mice (7). In contrast, when the 5′ noncoding region from a virulent type 3 strain was substituted for that of P2/Lansing, there was no effect on the ability of this recombinant to cause disease in mice (7). It is therefore possible to use the mouse model to identify attenuating sequences in the genomes of avirulent viruses by constructing recombinants between attenuated strains and P2/Lansing. Here we have used this approach to identify attenuating sequences in the avirulent strain P2/P712, a strain that our sequence analysis indicates is closely related to the type 2 Sabin vaccine strain.

The results of neurovirulence tests in mice indicate that two regions of the genome of P2/P712 contain strong determinants of attenuation: the 5′ noncoding region from nucleotides 1 to 751 and a central region from nucleotides 2269 to 4453, encoding part of capsid protein VP3, and all of VP1, $2A^{pro}$, 2B, and part of 2C. A third region of the genome, from nucleotides 752 to 2268, encoding VP4, VP2, and part of VP3, appears to contain a weak determinant of attenuation. There appear to be no determinants of attenuation in the 3′ 2,990 bases of the P712 genome. It appears that bases 752 to 2268, replaced in recombinant OLL, contain a weak attenuating mutation. However, given the small numbers of mice used, the LD_{50} of 7×10^5 may not be significantly higher than the highest value observed for P2/Lansing.

Like the attenuated type 1 and type 3 vaccine strains, the avirulent P2/P712 strain contains strong attenuating sequences in the 5′ noncoding region of the genome (16, 35). It has been shown that in the type 3 vaccine strain this is due to a base change at position 472, whereas a base change at 480 in the type 1 vaccine strain has been suggested to partially account for the attenuation phenotype. On the basis of sequence changes observed in stool isolates of vaccinated infants, it was recently suggested that a base change from A to G at position 487 of the Sabin 2 strain may accompany acquisition of neurovirulence (12). This nucleotide is also an A in the P712 strain. To determine whether a sequence around 480 of P712 contained an attenuating determinant, recombinant LS1 was constructed. It contains only a portion of the P2/P712 5′ noncoding region, from bases 456 to 628, in an otherwise P2/Lansing genome. The sequences of P2/P712 and the P2/Sabin vaccine strain are identical in this region (Table 1). LS1 has an LD_{50} of 7×10^6 and thus is clearly more attenuated than Lansing but not as attenuated as LP1. To determine whether an A at base 487 is important for the attenuation phenotype, we are currently changing this base to a G in recombinant LP1.

Our observation that recombinant LS1 is less attenuated than LP1 suggests that attenuating sequences other than those between nucleotides 457 and 630 are present in the 5′ noncoding region of P2/P712. Alternatively, it is possible that RNA structure in the 5′ untranslated region is important for the attenuation phenotype, and the juxtaposition of sequences in LS1 may disrupt this structure.

It has been suggested that attenuation of the type 3 Sabin vaccine strain caused by mutations in the 5′ noncoding region results from reduced translation efficiency of the viral RNA, thereby limiting the replication capacity of the virus (27). Indeed, examination of the function of the 5′ noncoding region by mutagenesis indicates a major role in translation of the viral RNA (17–19, 30, 31). A similar mechanism may also explain attenuation of P2/P712 by sequences in the 5′ noncoding region.

The functional basis for attenuation conferred to P2/Lansing by exchange of bases 2269 to 4453 of P2/P712 is also not known. This region encodes part of capsid protein VP3, and all of VP1, $2A^{pro}$, 2B, and part of 2C. Since P2/Lansing and P2/P712 do not differ in the VP3 portion of this region, any or several of these proteins except VP3 might contain the attenuating determinants. Experiments are currently in progress to determine on which side of the VP1-$2A^{pro}$ border the attenuating sequences are located. Attenuation determinants have been identified in the capsid proteins of two Sabin strains. A mutation in VP3 of the Sabin 3 strain confers both the temperature sensitivity and attenuation phenotypes (35). The Sabin 1 strain also appears to contain attenuating determinants in this region (16).

It was recently determined that the ability to infect the central nervous systems of mice may be conferred to P1/Mahoney by substitution of six amino acids of neutralization antigenic site I in VP1 with those of P2/Lansing (11, 13). The six amino acids in P2/P712 are identical to those of P2/Lansing, and therefore failure of P2/P712 to infect mice is probably due not to lack of this sequence but rather to the presence of other attenuating determinants. However, there is a single amino acid difference between Lansing and P712 (arginine in P712, lysine in Lansing) at amino acid 103 of VP1, just outside the eight-amino-acid sequence of neutral-

ization antigenic site I, and this difference may affect the host range of P712. We are currently testing this hypothesis.

The identification of precise base changes that control the attenuation phenotype in the type 1 and 3 Sabin vaccine strains has been aided by comparison of the nucleotide sequences of these strains with those of their virulent parents (16, 35). Since the parent of the Sabin 2 vaccine strain is naturally avirulent, such a comparison would not be informative (26). We selected the P2/P712 strain for study on the assumption that it would be closely related to P2/Sabin and its attenuated parent strain. Indeed, our sequence analysis of P2/P712 shows that it differs very little from the vaccine strain: by 22 base changes, 4 of which are in the 5′ noncoding region and 6 of which lead to amino acid differences (Table 1). The five consecutive base differences from 2567 to 2571 might have resulted from an insertion in P2/P712 of a G at position 2567 and a deletion from P2/P712 of an A at position 5271. One or more of the coding differences must be responsible for the temperature sensitivity phenotype, which is carried by the vaccine strain but is not present in P2/P712.

These studies show that the avirulent poliovirus strain P2/P712 contains strong attenuating sequences in the 5′ noncoding region of the genome and in a central region encoding both structural and nonstructural proteins. Because of the high degree of sequence identity between P2/P712 and P2/Sabin, it is likely that strains used for vaccine production also carry these determinants of attenuation.

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