Identification of Two Determinants That Attenuate Vaccine-Related Type 2 Poliovirus

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The poliovirus P2/P712 strain is an attenuated virus that is closely related to the type 2 Sabin vaccine strain. By using a mouse model for poliomyelitis, sequences responsible for attenuation of the P2/P712 strain were previously mapped to the 5' noncoding region of the genome and a central region encoding VP1, 2A^{pro}, 2B, and part of 2C. To identify specific determinants that attenuate the P2/P712 strain, recombinants between this virus and the mouse-adapted P2/Lansing were constructed and their neurovirulence in mice was determined. By using this approach, the attenuation determinant in the central region was mapped to capsid protein VP1. Candidate attenuating sequences in VP1 and the 5' noncoding region were identified by comparing the P2/P712 sequence with that of vaccine-associated isolate P2/117, and the P2/117 sequences were introduced into the P2/Lansing-P2/P712 recombinants by site-directed mutagenesis. Results of neurovirulence assays in mice indicate that an A at nucleotide 481 in the 5' noncoding region and isoleucine (Ile) at position 143 of capsid protein VP1 are the major determinants of attenuation of P2/P712. These determinants also attenuated neurovirulence in transgenic mice expressing human poliovirus receptors, a new model for poliomyelitis in which virulent viruses are not host restricted. These results demonstrate that A-481 and Ile-143 are general determinants of attenuation.

The live, attenuated poliovirus vaccine strains developed by A. B. Sabin have been extremely effective in controlling poliomyelitis. These vaccine strains were produced by controlled passage of viruses in animals and cultured cells until variants unable to cause paralysis in primates were obtained (reviewed in reference 25). Since the Sabin strains were isolated, it has been of great interest to determine the molecular and functional basis for their attenuation phenotypes (reviewed in references 2 and 21). This information has provided insight into the biology of poliovirus, permitted better understanding of vaccine-associated disease, and suggested ways to improve the existing vaccines.

The 7.4-kb RNA genome of poliovirus encodes a single long open reading frame preceded by an approximately 0.75-kb 5' noncoding region that is involved in translation initiation and genome replication (reviewed in reference 26). The 5' one-third of the open reading frame encodes the four proteins (VP4, VP2, VP3, and VP1) that make up the icosahedral capsid, and the remainder encodes seven nonstructural proteins, including two proteinases that cleave the primary translation product into functional polypeptides (reviewed in reference 27).

Polioviruses are grouped into three serotypes based on the antigenicity of the capsid, and the live vaccine consists of one representative of each type. To identify determinants of attenuation in each of the three vaccine strains, genomic recombinants have been constructed between the attenuated viruses and closely related neurovirulent strains and the ability of these recombinants to cause paralysis in primates has been assayed. Because the poliovirus genome is an RNA molecule, the recombinants have been constructed by using cloned cDNAs from which virus can be derived by transfection (22). By using this approach, two attenua-

tion determinants have been identified in the type 3 vaccine strain, P3/Sabin: a uridine (U) residue at nucleotide 472 in the 5' noncoding region (U-472), and a phenylalanine (Phe) at amino acid 91 (Phe-91) of capsid protein VP3, which is also responsible for the temperature-sensitive phenotype of the virus (15, 29). Similar studies have revealed that attenuation determinants occur in at least three regions of the type 1 vaccine strain, P1/Sabin, and include a guanine (G) residue at position 480 in the 5' noncoding region (G-480) (4a, 9, 18).

Our approach to identifying attenuation determinants in the type 2 vaccine strain, P2/Sabin, has involved construction of recombinants with P2/Lansing. This type 2 strain is able to cause poliomyelitis in mice, allowing the recombinants to be tested for virulence in mice instead of in primates. For these studies, a cDNA clone of P2/P712, a viral strain that is nearly identical in nucleotide sequence to P2/Sabin, has been used (16, 20). Two regions that attenuate P2/P712 were mapped by using this strategy: the 5' noncoding region and a central region encoding capsid protein VP1, 2A^{pro}, 2B, and part of 2C (16). The rest of the P2/P712 genome does not contain determinants of attenuation. Recently, the nucleotide sequence of a neurovirulent type 2 strain, P2/117, which was isolated from a vaccine-associated case of poliomyelitis was determined (20). Comparisons between attenuated type 2 strains and P2/117 may suggest candidate determinants of attenuation.

In this study, we identified specific determinants that attenuate P2/P712 in mice. These determinants also attenuate neurovirulence of poliovirus in transgenic mice expressing human poliovirus receptors (PVRs), which have been characterized as a new model for poliomyelitis (24). Because of the extensive sequence identity between P2/P712 and P2/Sabin, these determinants are probably responsible for attenuation of the vaccine strain in primates.

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MATERIALS AND METHODS

Cells and viruses. HeLa S3 cells were grown in suspension culture or monolayer as described previously (12). Viruses used in these experiments were derived from cloned genomic cDNAs of the poliovirus strains P2/Lansing, P2/P712, and P2/117 or variants of these generated as part of this study (12, 16, 20). In vitro transcription of cDNAs and transfection of RNA into HeLa cells to derive viruses were performed as described previously (16). Viral stocks were prepared after two plaque purifications of the transfection yield. Viral titers were determined by plaque assay on HeLa cell monolayers as described previously (12).

To prepare high-titer viral stocks for inoculation of mice, we infected HeLa cell monolayers at a multiplicity of infection of 10 PFU per cell in 15-cm dishes and then incubated them in medium at 37°C for 5 to 7 h. Infected cells were collected by centrifugation, resuspended in 1 ml of phosphate-buffered saline (PBS), subjected to three cycles of freeze-thawing, clarified, and stored at -70°C.

Growth at low and high temperatures was measured by plaque assay at 32 and 39.5°C as described previously (11).

Construction of recombinants. Plasmid DNAs were grown in *Escherichia coli* DH5 α and purified by CsCl centrifugation (4). DNAs were cleaved with restriction endonucleases under conditions recommended by the manufacturers (Boehringer-Mannheim Biochemicals and New England BioLabs). Restriction fragments were purified by electrophoresis in low-gelling-temperature agarose gels (4). Ligations were performed as specified by the manufacturer of T4 DNA ligase (Boehringer-Mannheim Biochemicals).

The constitution of each recombinant is diagrammed in Fig. 1. The construction of SRL, SLL, and LP1 has been reported previously (16). SPL, SVL, and 117LP were generated as part of this study. SVL and SPL are the result of a reciprocal exchange between P2/Lansing and SRL at *Pst*I sites introduced into their corresponding cDNAs by site-directed mutagenesis at nucleotides 3413 and 3416, respectively. 117LP was generated by replacing a *Kpn*I-*Nar*I fragment (nucleotides 66 to 751) of P2/Lansing cDNA with a corresponding fragment of P2/117 cDNA (20).

Mutagenesis of recombinants. Oligonucleotide-directed mutagenesis was performed on DNAs subcloned in M13 grown in *E. coli* CJ236 (4). *Pst*I restriction sites were introduced into the P2/Lansing sequence at nucleotide 3413 and into the P2/P712 sequence of recombinant SRL at nucleotide 3416. The antisense oligonucleotide used for site-directed mutagenesis in both cases was 5'-TAGCCTG CAGTGTACACAG-3'. The mutations caused by this oligonucleotide occur at analogous positions in the open reading frames of these strains but do not result in amino acid coding changes.

By using the same methods, the ATT codon at nucleotide 2905 of the P2/P712 sequence in the SVL recombinant was changed to GTT (to generate SVL-val) and to ACC (to generate SVL-thr). The T residue at position 437 of the P2/P712 sequence in the cDNA of recombinant SLL was changed to C to generate the cDNA of SLL437. The A residue at position 481 in the SLL cDNA was changed to G to generate the cDNA of SLL481. The mutant-recombinant LP481 was derived from SLL481, has a G at position 481, and otherwise resembles recombinant LP1.

Nucleotide sequencing. Sequencing of recombinant and mutant cDNAs to confirm their identity was performed by using Sequenase as specified by the manufacturer (U.S. Biochemical). The identity of viruses was confirmed by

sequencing of genomic RNA at recombination junctions and mutation sites. Isolation of genomic RNA and chain termination sequencing by using oligonucleotide primers was performed as described previously (10).

Neurovirulence assay. Viruses were tested for neurovirulence in normal Swiss Webster mice and in TgPVR1-17 transgenic mice (24). Groups of eight 3- to 4-week-old mice, four male and four female, were inoculated intracerebrally with 50 μ l of virus. Tenfold dilutions of each virus were made in PBS-0.2% horse serum, and groups of mice each received one dilution, such that each virus was inoculated over a range approximately from 10⁴ to 10⁹ PFU. Mice were observed daily for 21 days for paralysis or death. Paralyzed mice were sacrificed and scored as dead. The amount of the virus which caused paralysis or death in 50% of mice (LD₅₀) was calculated by the method of Reed and Muench (23). All values represent the average of two independent determinations, which did not vary by more than 0.5 log₁₀ from the reported values.

When paralysis resulted, virus was isolated from the spinal cord of at least one infected mouse. The identity of the virus was confirmed by nucleotide sequencing of genomic RNA. By this analysis it was found that all viruses isolated from the spinal cords of paralyzed mice resembled the inoculated virus (data not shown).

RESULTS

Mapping an attenuation determinant in the coding region of P2/P712. By using a strategy of constructing recombinants with the mouse-virulent P2/Lansing strain of poliovirus and testing the recombinants for neurovirulence in mice, an attenuation determinant of the vaccine-related strain P2/P712 has been mapped to a region that encodes capsid protein VP1 and nonstructural proteins 2A^{pro}, 2B, and part of 2C (16). To more precisely map this determinant, we generated recombinants between P2/Lansing and SRL, a strain that contains the attenuating central region of P2/P712 in an otherwise P2/Lansing background (Fig. 1). *Pst*I restriction sites were introduced into the cDNAs of P2/Lansing and SRL near the boundary of sequences encoding VP1 and 2A^{pro}, and two reciprocal recombinant cDNAs were constructed by using the *Pst*I sites, from which viruses SVL and SPL were derived by transfection (Fig. 1). SVL encodes VP1 of P2/P712 in a P2/Lansing background, and SPL encodes part of the P2 coding region (P2') in a P2/Lansing background. The recombinant viruses resembled the parental strains in both plaque size and growth at high and low temperatures (data not shown).

SVL and SPL were inoculated into Swiss Webster mice intracerebrally for determination of LD₅₀ values. SPL was as neurovirulent as P2/Lansing, indicating that the P2' region of P2/P712 carries no attenuation determinants (Fig. 1). However, recombinant SVL was attenuated to the same degree as SRL, indicating that the attenuation determinant is located in VP1 (Fig. 1).

Identification of the major attenuation determinant in capsid protein VP1 of P2/P712. P2/P712 and the neurovirulent strain P2/117 differ at only one nucleotide in the region encoding VP1, which results in a difference at amino acid position 143 (20). P2/P712 encodes an isoleucine (Ile) at this position, and P2/117 encodes valine (Val). The amino acid at this position in P2/Lansing, as well as in several other poliovirus strains, is threonine (Thr).

To determine the role of amino acid 143 of VP1 in neurovirulence and attenuation, we performed site-directed

FIG. 1. Constitution and mouse neurovirulence of P2/P712-P2/Lansing recombinant and mutant viruses. The genomic RNA of each virus derived from the recombinant and mutant viral cDNA is represented below a genetic map of viral genomic RNA. The name of each virus is shown at the left, and its corresponding LD₅₀ value in nontransgenic (nTg) mice and in transgenic (Tg) mice expressing PVRs (TgPVR1-17) is shown at the right. Symbols: ■, sequences derived from cDNA of P2/Lansing; □, sequences derived from cDNA of P2/P712. The amino acid and nucleotide substitutions are shown at the relevant position of the viral RNA. nt, Nucleotides.

mutagenesis of SVL cDNA to generate two mutant cDNAs, which, upon transfection into HeLa cells, gave rise to viruses SVL-val and SVL-thr. SVL-val has a Val at VP1-143, as in P2/117, and SVL-thr has a Thr at that position, as in P2/Lansing (Fig. 1). The plaque size and growth at high temperature of the mutant viruses in HeLa cells resembled those of SVL (data not shown). In neurovirulence assays, both SVL-thr and SVL-val were approximately 1,000-fold more paralytogenic than SVL was (Fig. 1). Therefore, Ile-143 is the primary determinant of attenuation in VP1 of P2/P712.

Identification of the major attenuation determinant in the 5' noncoding region of P2/P712. Previous neurovirulence analysis of recombinants between P2/Lansing and P2/P712 indicated the presence of a strong attenuation determinant in the 5' noncoding region of the P2/P712 genome (16). P2/P712 and P2/117 sequences differ in the 5' noncoding region by three nucleotides, at positions 437, 481, and 685 (20). To determine which of these positions are important for the attenuation phenotype, we made mutations in the cDNA of attenuated recombinant SLL, which consists of the 5' noncoding region and a portion of the coding region from P2/P712 in a

FIG. 2. Stereo representation of an alpha carbon model of P1/Mahoney viewed from the outside, down the particle fivefold axis. Only capsid protein VP1 is shown. One of five copies of VP1 is dark blue, the BC loop is green, and the side chain of Thr-143 in the DE loop is orange. Graphics by J. M. Hogle, Research Institute of Scripps Clinic.

P2/Lansing background; the coding region of P2/P712 in this recombinant confers little or no attenuation (16). SLL cDNA was mutagenized to change the nucleotides at 437 or 481 to the corresponding residues in P2/117, and mutant viruses SLL437 and SLL481 were derived by transfection with the altered cDNAs. SLL437 has C at position 437, where SLL and P2/P712 have U. It is no more virulent in mice than is SLL (Fig. 1). SLL481 has G at position 481, where SLL and P2/P712 have A. It is at least 100-fold more paralytogenic than SLL, although it is approximately 100-fold less paralytogenic than P2/Lansing (Fig. 1). To confirm that the coding region of P2/P712 plays no role in the attenuation phenotype, we constructed LP481, which was derived from SLL481 and has the coding region of P2/P712 replaced with that of P2/Lansing. LP481 was as neurovirulent as SLL481 in mice (Fig. 1). These results indicate that A-481 is a major determinant of attenuation in the 5' noncoding region of P2/P712.

To determine the effect on neurovirulence of all three differences between P2/P712 and P2/117 in the 5' noncoding region, we replaced the noncoding region of P2/Lansing with that of P2/117 to generate recombinant 117LP. The neurovirulence of 117LP is slightly higher than that of LP481 (Fig. 1). Therefore, either A-685 or an interaction among the three

nucleotides contributes partially to the attenuation of P2/P712.

Neurovirulence of recombinant viruses in transgenic mice expressing PVRs. To determine the degree of host range restriction caused by Ile-143, we assayed viruses SVL, SVL-val, and SVL-thr for neurovirulence in transgenic mice expressing PVRs. Host-restricted viruses, such as P1/Mahoney, which do not cause disease in mice but are able to induce paralysis in primates, are neurovirulent in transgenic mice expressing PVRs (24; unpublished observations). The LD₅₀ of SVL was about 100-fold lower in TgPVR1-17 mice than in normal mice (Fig. 1). Both SVL-val and SVL-thr were approximately 10-fold more neurovirulent in transgenic mice expressing PVRs than in normal mice, and as virulent as P2/Lansing. These results indicate that Ile-143 is primarily a general attenuation determinant.

To rule out the possibility that all attenuated polioviruses are nonspecifically more paralytogenic in transgenic mice expressing PVRs, we tested the neurovirulence of recombinants LP1 and LP481 in TgPVR1-17 mice. LP1 was as attenuated in normal mice as in PVR transgenic mice (Fig. 1). As expected, LP481, which contains G481, as in the neurovirulent P2/117, was equally neurovirulent in transgenic and nontransgenic mice (Fig. 1).

DISCUSSION

By using a strategy of constructing recombinants between the poliovirus vaccine-related strain P2/P712 and the neurovirulent P2/Lansing, two regions from P2/P712 that attenuate neurovirulence in mice were previously identified: the 5' noncoding region and a central region of the genome (16). All other regions of the P2/P712 genome cause little or no attenuation. In the present study, the attenuation determinant in the central region was mapped to capsid protein VP1. Candidate nucleotides involved in attenuation were then identified by comparing the sequences of the 5' noncoding region and VP1 of P2/P712 with those of the neurovirulent strain P2/117 (20). By changing residues in the attenuated recombinants to the residues that occur in virulent viruses, it has been possible to identify nucleotide A-481 in the 5' noncoding region and Ile-143 of capsid protein VP1 as the major attenuation determinants in P2/P712.

Reduced poliovirus neurovirulence in mice may be the result of general attenuation determinants, such as those that also attenuate poliovirus in primates (10), or the result of host range restriction, which specifically prevents P1/Mahoney from causing disease in mice (12). Recently, it has become possible to bypass potential host range restriction by testing viruses for virulence in transgenic mice that express PVRs (24). All polioviruses tested to date that are virulent in primates are also virulent in PVR transgenic mice, regardless of their ability to cause paralysis in normal mice, indicating that interaction with the mouse poliovirus receptor is the primary determinant of host range (24; unpublished observations). The three Sabin vaccine strains do not cause disease in transgenic mice, indicating that the attenuation determinants of these viruses function in this animal model (24; unpublished observations). The two viruses described here that carry the attenuating regions from P2/P712, SVL and LP1, are also attenuated in PVR transgenic mice (Fig. 1). Therefore, A-481 and Ile-143 of P2/P712 are primarily general attenuation determinants.

It is interesting that SVL is approximately 100-fold more neurovirulent in transgenic mice than in nontransgenic mice, whereas SVL-val and SVL-thr are 10-fold more neurovirulent in transgenic mice. The difference in neurovirulence in transgenic mice versus normal mice is probably due in part to the 17 amino acid differences in VP1 between P2/P712 and P2/Lansing; these differences probably result in host range restriction. However, it is interesting that the difference in LD₅₀ values is greater for SVL than for SVL-val and SVL-thr. Although this difference is probably too close to draw conclusions, the result suggests that Ile-143 might also impart host range restriction. To test this possibility, it will be necessary to introduce Ile-143 into P2/Lansing and determine the neurovirulence of the virus in normal and transgenic mice. This experiment is currently in progress.

Although A-481 and Ile-143 account for most of the attenuation phenotype of P2/P712, changes at these positions to the sequences found in the virulent P2/117 did not fully restore neurovirulence. Although LP481 and SLL481 are able to cause paralysis, they are 100-fold less virulent than P2/Lansing. In addition 117LP, which carries the 5' noncoding region of P2/117, is 10-fold more virulent than LP481. Therefore, either A-685 or an interaction among U-437, A-481, and A-685 contributes partially to the attenuation of P2/P712. Because SLL437 carries the major attenuation determinant, A-481, it is difficult to assess the minor contribution of U-437 to attenuation. It remains unclear why 117LP is about 10-fold less virulent than P2/Lansing. It is

possible that P2/117 still contains additional weak attenuation determinants in the 5' noncoding region. Studies of other vaccine-associated type 2 isolates suggest that nucleotide 398 might also be a weak determinant of attenuation (1), but in P2/117 this nucleotide is identical to that in P2/Sabin.

All three Sabin vaccine strains contain strong attenuation determinants in the 5' noncoding region of the viral genome. A mutation from C to U at position 472 is partly responsible for attenuation of the P3/Sabin strain (29), and neurovirulent revertants of P3/Sabin have mutated to C at this position (3, 5). An A-to-G change at position 480 partially accounts for the attenuation phenotype of P1/Sabin (19). On the basis of sequence changes occurring upon passage of P2/Sabin in the human gut, it was suggested that a base change from A to G at position 481 may accompany the acquisition of neurovirulence (14). A neurovirulent revertant of P2/Sabin, P2/117, has three base changes in the 5' noncoding region, including an A-to-G change at base 481 (20). The fact that A-481 attenuates poliovirus P2/P712 in both normal and transgenic mice is consistent with the observation that 5'-noncoding-region determinants that attenuate polioviruses in primates also attenuate these viruses in normal mice (10).

The position of Ile-143 in the structure of the poliovirus capsid may suggest the mechanism by which it attenuates neurovirulence. Amino acid residue 143 of VP1 is exposed on the external surface of the native virion very near the fivefold axis of icosahedral symmetry, in the loop connecting β -strands D and E (DE loop) of VP1 (7) (Fig. 2). Five copies of the DE loop encircle the fivefold axis and, together with five HI and BC loops, form a prominent protrusion on the particle surface. A role for the DE loop in host range has been suggested by examination of the atomic structure of a poliovirus variant in which host range restriction has been overcome. Substitution of the BC loop of P1/Mahoney with that of P2/Lansing confers upon this chimeric virus the ability to infect mice (13, 17). When the structure of the P2/Lansing-P1/Mahoney chimeric virus was solved by X-ray crystallography, in addition to the expected conformational changes in the heterologous BC loop, significant conformational changes in the DE loop were observed although there are no amino acid sequence differences in this area (8). This observation suggests that there may be structural interactions between the BC and DE loops of VP1. This suggestion is supported by the fact that both the BC and DE loops of VP1 make up a discontinuous neutralization antigenic site (30). The BC loop is believed to influence host range through receptor-mediated early events in infection (16a). It is possible that the general attenuation caused by Ile-143 is also receptor mediated, but cannot be completely overcome by the presence of the PVR. Investigation of the interactions between SVL, SVL-thr, and SVL-val with the human receptor *in vitro* should resolve these questions.

The only other structural determinant of attenuation identified in a vaccine strain is VP3-phe-91 in P3/Sabin. This determinant has been extensively characterized with regard to its effect on the temperature sensitivity of the P3/Sabin strain and is believed to act by controlling the structural transitions that virions must undergo at various points in the infectious cycle (6).

P2/P712 and P2/Sabin are highly related in nucleotide sequence and resemble each other phenotypically (16). Nucleotide sequences of two variants of P2/Sabin have been determined. P2/P712 differs from the P2/Sabin described by Toyoda et al. (28) by 22 nucleotides and from the P2/Sabin of Pollard et al. (20) by only 2 nucleotides, which do not affect

amino acid coding. Both variants of P2/Sabin harbor the two attenuation determinants identified here. At least for the P2/Sabin strain described by Pollard et al. (20), the nucleotide differences between it and P2/P712 are not likely to alter the attenuation affects of A-481 and Ile-143 in that strain. Unless the differences suppress the effects of A-481 and VP1-ile-143, the two attenuation determinants identified in P2/P712 are also primary contributors to the attenuation of the Sabin type 2 vaccine strain.

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