Mapping of Sequences Required for Mouse Neurovirulence of Poliovirus Type 2 Lansing

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Intracerebral inoculation of mice with poliovirus type 2 Lansing induces a fatal paralysis, while most other poliovirus strains are unable to cause disease in the mouse. To determine the molecular basis for Lansing virus neurovirulence, we determined the complete nucleotide sequence of the Lansing viral genome from cloned cDNA. The deduced amino acid sequence was compared with that of two mouse-avirulent strains. There are 83 amino acid differences between the Lansing and Sabin type 2 strain and 179 differences between the Lansing and Mahoney type 1 strain scattered throughout the genome. To further localize Lansing sequences important for mouse neurovirulence, four intertypic recombinants were isolated by exchanging DNA restriction fragments between the Lansing 2 and Mahoney 1 infectious poliovirus cDNA clones. Plasmids were transfected into HeLa cells, and infectious recombinant viruses were recovered. All four recombinant viruses, which contained the Lansing capsid region and different amounts of the Mahoney genome, were neurovirulent for 18-to 21-day-old Swiss-Webster mice by the intracerebral route. The genome of neurovirulent recombinant PRV5.1 contained only nucleotides 631 to 3413 from Lansing, encoding primarily the viral capsid proteins. Therefore, the ability of Lansing virus to cause paralysis in mice is due to the viral capsid. The Lansing capsid sequence differs from that of the mouse avirulent Sabin 2 strain at 32 of 879 amino acid positions: 1 in VP4, 5 in VP2, 4 in VP3, and 22 in VP1.

Poliovirus was first isolated in 1909, and since then the causative agent of poliomyelitis has been studied extensively. Initially, the pathogenesis of poliovirus infection was studied in the intact animal, and later, with the development of cell culture, the physiology and biochemistry of viral replication was examined. More recently the complete nucleotide sequence of several viral strains has been determined (11, 21, 29, 30, 32), and a complete genetic map of the viral genome has been constructed (11). Despite these advances, little is known about the role of specific viral sequences in the development of paralytic disease. The ability to extensively manipulate the viral genome via cloned, infectious cDNA (22, 27) has now made it possible to study this problem.

We are studying the molecular basis of poliovirus neurovirulence using a mouse model. Although most strains of poliovirus are able to cause disease only in primates, some, such as the type 2 Lansing strain, have been adapted to mice (2). Intracerebral inoculation of mice with Lansing virus leads to a fatal paralytic disease which clinically and pathologically resembles human poliomyelitis (9, 10). To begin to study this mouse model at a molecular level, we recently constructed an infectious, cloned cDNA copy of the Lansing viral genome. Virus produced by transfection of HeLa cells with cloned Lansing cDNA is able to cause paralytic disease in mice (20).

An important question is what enables the Lansing virus to cause disease in mice while most other poliovirus strains cannot. To address this question, we determined the complete nucleotide sequence of the Lansing genome and compared the deduced amino acid sequence with that of other non-mouse-adapted polioviruses. In addition, recombinants between the Lansing virus and the mouse-avirulent type 1 Mahoney virus were constructed by in vitro manipulation of cloned cDNAs. The results of these studies indicate that the Lansing viral capsid is required for mouse intracerebral neurovirulence. A total of 32 amino acid differences distinguish the capsid of the neurovirulent Lansing strain from the capsid of the avirulent Sabin type 2 strain.

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. Two poliovirus type 2 Lansing viral stocks were used in these studies: V667 is an uncloned stock prepared by multiple passage in HeLa cells of a virus obtained from the American Type Culture Collection (20); V676P3 is a plaque-purified virus derived by transfection of HeLa cells with an infectious Lansing cDNA clone, pVR204 (20). The type 1 Mahoney virus, P6a, is an uncloned stock obtained in 1982 from D. Baltimore (Whitehead Institute). Lansing-Mahoney intertypic recombinants PRV1.1, PRV3.5, PRV4.3 and PRV5.1 were constructed as part of this study.

Virus growth and assay. Stocks of V667 (type 2) and P6a (type 1) were prepared in Spinner cultures of HeLa S3 cells. Cells were grown to a density of 4×10^5 cells per ml, centrifuged, washed with phosphate-buffered saline (PBS), and infected at a multiplicity of infection of 10 to 20 PFU per cell. After 30 min of adsorption, cells were diluted to 4×10^6 cells per ml and incubated in a Spinner bottle at 37° C for 5 h. After that time, cells were collected by centrifugation, washed with PBS, and suspended at 4×10^7 cells per ml in serum-free minimal essential medium. The cell suspension

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was subjected to three cycles of freeze-thawing, clarified, and stored at -70° C. All other viruses were plaque purified twice, and stocks were prepared in cell monolayers. Viral plaques were picked by removing the agar above a plaque with a 1.0-ml plastic pipette and suspending the agar in 0.5 ml of PBS. This suspension was then used to infect 4×10^{6} HeLa cells in 6-cm plastic dishes. Infected cells were incubated at 37°C until the monolayer was completely destroyed. Cell fluids were freeze-thawed three times, clarified by centrifugation, and stored at -70° C.

Viral titers were determined by plaque assay on HeLa cell monolayers. Tenfold dilutions of virus stocks were prepared in PBS, and 0.1-ml samples were inoculated per dish and allowed to adsorb for 45 min at 37°C. The monolayers were then covered with 5 ml of 0.9% Bacto-Agar (Difco Laboratories, Detroit, Mich.) in Dulbecco minimal essential medium supplemented with 5% horse serum. Viral plaques were usually visible, without staining, after 2 days of incubation at 37°C. For quantitation, plates were stained with crystal violet as described previously (22).

DNA transfection. Approximately 2×10^{6} HeLa cells were seeded in 6-cm plastic dishes and allowed to grow to 80% confluency. The medium was removed, and cells were washed once with 2.0 ml of TS buffer (137 mM NaCl, 5.1 mM KCl, 0.7 mM NaH₂PO₄, 25 mM Tris hydrochloride, 500 mM MgCl₂, 680 mM CaCl₂, pH 7.4). A mixture consisting of 5 µg of plasmid DNA and 500 µg of DEAE-dextran per ml in 0.25 ml of TS buffer was added to each plate. The cultures were left undisturbed for 15 min at room temperature, and then the inoculum was removed and the monolayers were covered with an agar overlay as described for plaque assays. Plaques were usually visible after 2 to 4 days of incubation at 37° C.

Neutralization assay. Approximately 100 PFU of virus in 0.1 ml of PBS were mixed with an equal volume of a 1:5,000 dilution of rabbit anti-poliovirus type 1 antiserum, a 1:500 dilution of rabbit anti-poliovirus type 2 antiserum, or normal rabbit serum. The dilutions of antiserum were found to neutralize 100% of an input of 100 PFU of virus. The virus-antiserum mixtures were incubated at room temperature for 1 h and subsequently assayed for infectious virus by plaque assay on HeLa S3 cell monolayers.

Neurovirulence assay. Sixty 18- to 21-day-old Swiss Webster mice (Camm Research Laboratories, Wayne, N.J.) were used to assay neurovirulence of each virus. Groups of 10 mice (5 male and 5 female) were inoculated in the left cerebral hemisphere (18) with 0.05 ml of PBS containing one dilution of virus. Tenfold increments of virus concentration were used so that each group of 10 mice received from 10¹ to 10⁶ PFU. Mice were observed daily for 21 days for paralysis or death; paralyzed mice were sacrificed and scored as dead. The amount of virus which caused paralysis or death in 50% of mice (LD₅₀) was calculated by the method of Reed and Muench (23).

Construction of recombinants. Plasmid DNAs were grown in *Escherichia coli* DH1 (6) and purified by CsCl sedimentation equilibrium centrifugation (4). The DNAs were cleaved with restriction endonucleases, using 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 (Boehringer Mannheim). Preparation and transformation of competent $E. \ coli$ DH1 was as described previously (6). In the following descriptions, nu-

described previously (6). In the following descriptions, nucleotide numbers refer to the location in the poliovirus type 1 Mahoney (11, 21) or type 2 Lansing (this paper) sequence; the numbering of both sequences differs slightly. Note that nucleotides 1 to 68 of the type 2 cDNA are derived from type 1 (20).

pSV20(N12). The 810-nucleotide PstI-BamHI fragment representing the 5' end of the Lansing genome was purified from pVR204 (20) and subcloned into pSP65 (16), yielding pVN9. This plasmid was cleaved with PstI and BalI (site at nucleotide 629), and the 3.2-kilobase DNA fragment was purified and ligated to a poliovirus type 1 cDNA fragment obtained from the full-length plasmid pVR106 (22), representing the 5' end (PstI site) through nucleotide 629 (BalI site). The resulting plasmid (pVN10) consisted of pSP65 sequences plus nucleotides 1 to 629 of type 1 linked at the Ball site to nucleotides 631 to 810 of type 2 cDNA. The 580-nucleotide BamHI fragment from pVN10 was purified and ligated to pSV20(N11) which had been partially digested with BamHI. pSV20(N11) is a pSV2 derivative (see below) in which the type 1 sequences from nucleotides 1 to 220 have been joined at the BamHI site to type 2 sequences from nucleotide 808 through the 3' poly(A). The insertion of the 580-base-pair fragment derived from pVN10 into pSV20(N11) at the BamHI site at nucleotide 220 resulted in the construction of pSV20(N12). This plasmid contains a full-length cDNA copy of the poliovirus genome consisting of base 1 to 629 from type 1 and bases 631 through the 3th poly(A) from type 2.

pSV20(400). The full-length type 2 Lansing cDNA was removed from its original vector (pVR204 [20]) by PstI cleavage and recloned, using oligonucleotide linkers, into the SstI site of pSV20, yielding pSV20(204). pSV20 is a derivative of pSV2 (19) in which Eco gpt sequences have been removed and replaced with an SstI-XhoI linker, and the pBR322 sequences have been replaced by the Nrul-EcoRI fragment of pAT153 (33) containing the ampicillin resistance gene. pSV20(204) was partially digested with SstI, and linear molecules were isolated and cleaved with XbaI. The DNA fragment lacking type 2 cDNA from nucleotide 4882 (XbaI site) through the 3' poly(A) (SstI site) was purified and ligated to a type 1 cDNA fragment consisting of nucleotides 4886 (XbaI site) through the 3' end of the genome (SstI site). The resulting plasmid pSV20(400) contains nucleotide 1 to 4882 from type 2 (except for the first 68 nucleotides) joined at an XbaI site to type 1 cDNA from nucleotide 4886 through the 3' end of the genome.

pSV20(N15). Viral RNA was extracted from CsCl-purified type 2 Lansing virus as described previously (3). A singlestranded RsaI DNA fragment consisting of nucleotides 3412 to 3568 of the type 1 genome (negative strand) was prepared so that it was phosphorylated with ³²P at the 5' end. This labeled DNA was hybridized to type 2 viral RNA, and the hybrids were treated with reverse transcriptase (14). The resulting cDNA was made double stranded with E. coli DNA polymerase I (31) and was then cleaved with XhoI (site at position 3299 in the type 2 sequence) and cloned into a vector which had been cleaved with XhoI and PvuII. Plasmids were identified which contained a type 2 XhoI-PstI DNA fragment. The 125-base-pair PstI-XhoI fragment was purified and ligated with two other DNAs: one fragment consisted of vector pSV20 cleaved with XhoI and PstI; the other was a fragment of type 2 cDNA from the 5' end (SstI site) to 3299 (XhoI site). The result of this three-part ligation

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FIG. 1. Complete nucleotide sequence of the poliovirus type 2 Lansing genome. U residues are shown as T. The new nomenclature for picornavirus proteins is used (25).

is pSV20(N14), which consists of vector pSV20 containing nucleotides 1 to 3413 of type 2 cDNA with a *PstI* site introduced at nucleotide 3413. A full-length cDNA clone was constructed by ligation of a *PstI* fragment of type I cDNA (nucleotide 3417 through the 3' poly(A) and extending to the *PstI* site in the simian virus 40 transcription termination signal [19]) into the *PstI* site at nucleotide 3413 of pSV20(N14). The plasmid derived from this ligation, pSV20(N15), contains nucleotides 1 to 3413 derived from type 2 (except for the first 68 nucleotides) and nucleotides 3417 to 7440 from type 1.

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pSV20(N16). Plasmids pSV20(N12) and pSV20(N15) were digested with restriction enzymes *Smal* and *Pvul*. Cleavage of both plasmids yielded two fragments: one extended from the *Pvul* site in the pBR322 ampicillin resistance gene through the 5' end of type 2 cDNA up to the *Smal* site at

nucleotide 1872, and the other fragment extended from the PvuI site in pBR322 through the 3' end of poliovirus cDNA up to the *SmaI* site. Plasmid pSV20(N16) was constructed by joining DNA fragments containing the poliovirus 5' and 3' ends from pSV20(N12) and pSV20(N15), respectively. The result is plasmid pSV20(N16), which contains nucleotides 1 to 629 of type 1, 631 to 3413 of type 2, and 3417 through the 3' poly(A) of type 1.

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Nucleotide sequence analysis. A full-length infectious cDNA clone (pVR204 [20]) was used to determine the nucleotide sequence of the Lansing viral genome. The poliovirus type 2 cDNA was purified from vector sequences, ligated, and then digested with DNase I (1). The digestion products were fractionated on an agarose gel, and DNAs from 1,000 to 2,000 base pairs in length were isolated and cloned into M13 sequencing vectors as described previously

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6A	TGC	TC 2	CA4 770	ACA J	AAG	CG1 27	16C0 78Ø	:Agt	Â	279Ø	TT1	TCA	GTC 28Ø	itge 1ø	iaag	ATA 28		TAC	AAA 2	GAC 2820	ACC	GT	1CA0 283	STT/ SØ	vaga	CG 28	TAAG 34ø	ITG	igag 2	1TC 85Ø	ITT	ACA	286	TCA Ø	AGG	28	GAC.	ATG	igag 2	1TC
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(17). The inserts in recombinant phage were sequenced by the chain termination technique (26). The ends of the full-length type 2 cDNA clone were sequenced by the chemical technique (15). Nucleotide sequence data were analyzed in a VAX 11/780 computer, using database programs developed by Staden (28).

RESULTS

Nucleotide sequence of Lansing viral genome. The complete nucleotide sequence of the Lansing viral genome was determined and compared with that of other mouse avirulent viruses to identify sequences required for mouse neurovirulence. The nucleotide sequence was determined from pVR204, which contains a cloned cDNA copy of the Lansing RNA genome that gives rise to mouse virulent poliovirus after transfection into HeLa cells (20). The complete nucleotide sequence is shown in Fig. 1, together with the predicted translation of the long open reading frame that begins at nucleotide 745 and terminates at nucleotide 7365. Protein assignments have been made based on homology with other published polioviral sequences (11, 21, 29, 30, 32). The deduced amino acid sequence of Lansing viral proteins was compared with the amino acid sequence of two mouseavirulent viruses, type 1 Mahoney (11, 21) and type 2 Sabin (32). There are 83 amino acid differences between Lansing and Sabin 2 and 179 amino acid differences between Lansing and Mahoney 1 (Table 1). These differences are scattered randomly throughout the genome, with the exception of a high concentration at the N terminus of VP1.

The 5'- and 3'-noncoding regions of the Lansing, Sabin 2, and Mahoney genomes were also compared. The Mahoney and Lansing 3'-untranslated regions are identical, while those of Lansing and Sabin 2 differ at two positions. The 5'-untranslated regions of the three viruses show a homology of approximately 85%.

Since the sequence differences between Lansing and other mouse-avirulent strains are scattered throughout the

W Q T S S N P S V F Y T Y G A P P A R I S V P Y V G I A N A Y S H F Y D G F ΑK TGGCAAACGTCATCTAACCCATCAGTGTTTTACACTTACGGGGCACCTCCAGCTAGAATATCAGTGCCCTACGTGGGCATTGCCAATGCATATTCTCATTTTTACGATGGGTTTGCCAAA 3010 3020 3030 3040 3050 3060 3070 3080 3090 3100 3110 3120 V P L A G Q A S T E G D S L Y G A A S L N D F G S L A V R V V N D H N P T K L T GTACCACTAGCCAGGCCCAAGCCTCAACAGAGGGTGACTCGCTGTATGGAGCGGCGCTTCATTGAATGACTTCGGATCACTGGCTGTTCGAGTGGTGAATGACCACAACCCTACGAAACTCACC 3130 3140 3150 3160 3170 3180 3190 3200 3210 3220 3230 3240 SKIRVYMKPKHVRVWCPRPPRAVPYYGPGVDYKDGLAPLP
 TCAAAAATCAGAGTGTACATGAAACCAAAGCACGTCAGAGTGTGGTGTCCCCGCGACCCCCTCGAGCAGTCCCATACTACGGGACCAGGGGTTGACTACAAGGATGGACTAGCCCCACTGCCA

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 GKGLTTY GFGHQNKAVYTAGYKICNYHLATQEDLQNAVNI GGGAAAGGCTTGACAACCTATGGTTTTGGCCACCAAAATAAGGCAGTGTACACGGCAGGTTACAAAATTTGCAATTACCACCTCGCCACCCAGGAAGACTTACAAAATGCGGTAAACATT 3370 3380 3390 3400 3410 3420 3430 3440 3450 3460 3470 3480 MWIRDLLVVESKAQGIDSIARCNCHTGVYYCESRRKYYPV ATGTGGATTAGAGACCTTTTAGTAGTGGAATCCAAAGCCCAAGGCATAGACTCAATTGCTAGATGTAACTGCCACACTGGAGTGTACTACTGTGAATCCAGGAGGAGGAAGTACTACCCGGTC 3490 3500 3510 3520 3530 3540 3550 3560 3570 3580 3590 3600 SFTGPTFQYMEANEYYPARYQSHMLIGHGFASPGDCGGIL TCTTITACTGGCCCCACCTTTCAGTACATGGAAGCAAATGAGTACTATCCAGCCCGATACCAATCCCACATGTTAATTGGCCATGGTTTGCATCTCCAGGGGACTGTGGTGGGATTCTC 3610 3620 3630 3640 3650 3660 3670 3680 3690 3700 3710 3720 R C Q H G V I G I I T A G G E G L V A F S D I R D L Y A Y E E E A M E Q N AGGTGCCAACATGGAGTAATTGGAATCATTACAGCTGGAGGAGAAGGCCTAGTCGCTTTCTCGGACATCAGAGATCTGTACGCATACGAGGAGGAGGCTATGGAGCAGGG 3730 3740 3750 3760 3770 3780 3790 3800 3810 3820 3830 Y I E S L G A A F G S G F T Q Q I G N K I S E L T S M V T S T I T E K L L K N L
 TATATTGAGTCCCTTGGGGCTGCATTTGGGAGTGGATTCACCCAGCAAATAGGAAACAAAATTTCAGAACTCACTAGCATGGTCACCAGCACTATAACTGAGAAACTACTAAAGAATCTC

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ILEIPYIMRQ SULKKFTEACNAAKGLEWVSNKISK A C D GCCTGTGACATCTTGGAAATCCCCTACATGCGACAGGGCGATAGCTGGTTGAAGAAGTTTACAGAGGCATGCAATGCAGCTAAGGGATTGGAATGGGTGTCTAATAAAATATCCCAAA 4090 4100 4110 4120 4130 4140 4150 4160 4170 4180 4190 4200 FIG. C

genome, it is not possible to identify which Lansing sequences are required for mouse neurovirulence. However, if the determinants of mouse neurovirulence are located solely in the viral polyprotein, then a small number of amino acid changes would suffice to convert Sabin 2 into a mouse-adapted virus (83 of 2,207; Table 1).

Construction of Lansing-Mahoney recombinant cDNAs. To identify regions of the Lansing genome required for replication in mice, a series of viral recombinants between the Lansing strain and the mouse-avirulent Mahoney type 1 strain were constructed. We took advantage of common restriction enzyme sites in the Lansing and Mahoney sequences to construct recombinant cDNAs containing different amounts of the Lansing and Mahoney viral genomes. The recombinant cDNAs were then transfected into HeLa cells, and the neurovirulence of the resulting viruses was studied in mice.

The recombinants were constructed as described in Materials and Methods and are shown in Fig. 2. The full-length cDNA clones of type 1 (pVR106) and type 2 (pVR204) viruses used in these constructions have been previously described (20, 21). To determine the role of the 5'untranslated region in mouse virulence, pSV20(N12) was constructed, which consists of Lansing cDNA in which the first 629 nucleotides (nearly the entire untranslated region) have been replaced with type 1 sequences. The role of nonstructural protein precursors P2 and P3 in mouse virulence was examined with virus derived from pSV20(N15), which consists of Mahoney sequences into which nucleotides 68 through 3413 of Lansing have been inserted. The construction of pSV20(N15) required the introduction of a PstI site into the type 2 genome at nucleotide 3413. This alteration was accomplished, as described in Materials and Methods, by changing nucleotide 3414 from a G to a T without changing the type 2 amino acid sequence. The contribution to neurovirulence of nonstructural protein precursor P3 was determined with pSV20(400), which consists of Mahoney sequences into which nucleotides 68 through

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3000

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AT	TAA	CAAC 445	TAC Ø	GTA	CAC 44	ittc 6ø	aag	AGC 4	ааа 470	CAC	CGT	ATT 448	gaa Ø	CCA	GTA 44	TGT 9Ø	TTG	TTG 4	gtg 5øø	CAC	GGT	AGC 451	CCA Ø	GGC	ACG 45	iggc. 2ø	aag"	ICA 4	GTT 530	GCC	ACC	AAT 454	TTA Ø	ATT	GCC/ 45	agai 5ø	gcav	ATA 4	gca 56ø
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K	L	F	С	Q	Μ	۷	s	Т	۷	Ε	F	I	Ρ	Ρ	м	Α	s	L	Е	Ε	к	G	T	L	F	Т	S	Ν	Y	۷	L	Α	s	т	Ν	S	S	R	Т
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т	Ρ	Ρ	T	۷	Α	н	S	D	Α	L	Α	R	R	F	Α	F	D	Μ	D	I	Q	I	м	S	Ε	Y	S	R	D	G	к	L	Ν	Μ	Α	Μ	Α	Т	Ε
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м	С	к	N	С	н	Н	Ρ	Α	N	F	к	R	С	С	Ρ	L	۷	С	G	к	Α	1	Q	L	Μ	D	κ	s	S	R	۷	R	Y	S	I	D	Q	I	T
AT	GTG	TAA(493	GAA(BØ	CTGT	CA1 49	icac 94ø	CCA	GC	44AC +950	TTC	CAAG	iaga 496	TG1 Ø	TGC	CCA 49	TTG 7Ø	igtg	TG1	GGC 98Ø	A	GCC	ATC 499	CAG	icte	iato 50	igac 100	AAA	TCT 5	TCC Ø 1Ø	Aga	GTC	aga 5ø2	TAT Ø	Agt	ATA 5Ø	igat 3ø	CAG	ATT 5	ACT Ø4Ø
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E	С	1	N	D	L	L	Q	Α	۷	D	s	Q	Ε	۷	R	D	Y	С	Ε	к	к	G	W	T	۷	D	1	т	s	Q	۷	Q	Т	Ε	R	N	Ι	Ν	R
GA	GTG	CATO 517	CAAT Ø	rgat	TT(51	GCTC 18Ø	CAA	GC/	AGTT 5190	GAT	тст	CAA 52Ø	iga(GTA	AGA 52	GAC 1Ø	TAC	TG	GAG 5220	AAG	GAA(6661 523	TGC Ø	iata	AGT/ 52	AGAC 24Ø	ATC	ACT 5	AGT 25Ø	CAC	igto V P	icaa 526 g	ACC	:GAA	AGA 52	AAC 70	:ATC	AAT 5	AGA
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AA	TGT	CCC0 541	CACC IØ	CATC	AG0 54	act 20	GCC	AA(GTT 5430	CAC	GGC	CCA 544	lGG∕ Ø	TTT	GAC 54	CTAC 150	GCA	GT	GCA 5460 FIC	ATC	GGC(D	CAA4 547	VAGA Ø	VAA(CATT 54	1CTT +8Ø	ACG	GCA 5	act 490	ACC	CAT	FAA0 550	igg/ ØØ	GAC	atto 55	:ACA 1Ø	•ATG	CTO	266/ 552(

4882 of Lansing have been inserted. Another recombinant, pSV20(N16), contains all Mahoney sequences with the exception of nucleotides 631 to 3413, which are derived from Lansing. This recombinant was constructed to determine whether the Lansing virus capsid would suffice to convert the Mahoney strain into a mouse-virulent virus. The structures of all four recombinant plasmid DNAs were verified by restriction enzyme digestion (data not shown).

Analysis of recombinant viruses. HeLa S3 cell monolayers were transfected with closed circular forms of pSV20(400), pSV20(N12), pSV20(N15), and pSV20(N16), and the corresponding viruses PRV1.1, PRV3.5, PRV4.3, and PRV5.1 were recovered. The plaque size of all the recombinant viruses was identical to that of the parental type 1 and type 2 viruses (data not shown). Furthermore, virus yields of cells infected with the recombinants were the same as those obtained with the parental viruses (approximately 1,000 PFU per cell). These results indicate that the growth characteristics of the recombinant viruses did not differ from those of the parent viruses.

The serological type of the recombinant viruses was determined by a neutralization assay with homotypic antisera (Table 2). The recombinant viruses were neutralized only by the type 2-specific antiserum, demonstrating that all the recombinants possessed the type 2 capsid as expected. The type 1-type 2 junctions in the recombinant viral RNAs were mapped by the nuclease S1 protection technique. All four viral recombinants contained the expected junction sites (data not shown).

Neurovirulence of recombinant viruses. To determine whether the recombinant viruses were capable of causing paralysis and death in mice, Swiss Webster mice were injected intracerebrally with different amounts of either the parental type 1 or type 2 virus or the recombinant PRV1.1, PRV3.5, PRV4.4, or PRV5.1. The amount of virus causing paralysis or death in 50% of the mice (LD_{50}) was calculated.

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GTTATCAC 58	CCTGC. 89ø	ACTO	igca 590	Aggt(Ø	CAT	CGGG 5910	ATG	CAT	GTT(592)	GGT(6GG/	ACG 593	GT1 Ø	CAC	ATC 59	6661 94Ø	TCC	6CA	GCA0 5950	jCCO	CTG/	4AG0 596	GA1	LCC.	TAT 5	TTC/ 97Ø	ACTO	CAG	Agt(598)	CAA Ø	GGT	GAA4 599		CAG	TGG 6	atg ØØØ
RPS	sк	Е	v	GΥ	Ρ	۷	T	Ν	Α	Ρ	s	к	Т	к	L	Е	Ρ	s	A	F	н	Y	v	F	Е	G	۷	к	Е	Ρ	Α	۷	L	Т	к	S
AGACCATO	CAAAA Ø1Ø	GAAC	atgg 6ø2	GCTA(Ø		GTT. 5ø3ø	ATT	AAT	GCT(6Ø4(CA1	ICT4	6Ø5	CT4 Ø	AAC	tge 60	1440 160	CCA	AGT	GCA1 6Ø70	тс	CAT	TAT(6Ø8	GTG SØ	TTT	GAA 6	ggt Ø9ø	GTCA	VAG	GAA(61Ø(CCA Ø	GCT	GTGC 611	CTC/	ACCA	444 6	agt 12ø
DPI	RL	к	T	DF	Ε	Ε	Α	I	F	s	к	Y	٧	G	N	к	I	Т	Ε	٧	D	E	Y	м	к	Ε	A	۷	D	н	Y	A	G	Q	L	м
GACCCCA	gattg 13ø	aag/	ACAG 614	ATTT Ø	TGA	Agag 515ø	GCT	ATC	6161	TCC/ Ø	4AG1	617	TG(Ø	GAV	ATA 61	\AG∕ 18Ø	ATTA	ACT	GAA(619(jTG(GAT	GAG 620	rac <i>i</i> ØØ	ATG/	AAA 6	GAA1 2 1 Ø	GCTO	STC	Gat(622)	ÇAT	TAC	GCAC 623	GCC Ø	CAG	CTC 6	atg 24ø
SLI	DI	N	Т	EQ	М	С	L	Ε	D	Α	м	Y	G	Т	D	G	L	Ε	Α	L	D	L	s	т	S	A	G	Y	Ρ	Y	۷	A	м	G	к	к
TCACTAG/ 62	ACATC 25Ø	AAC	ACAG 626	AACA Ø	AAT(GTGC 527Ø	СТТ	GAG	igati 628/	gca/ Ø	ATG	629	igc/ Ø	АСТО	ACC 63	GT (CTCO	6AA	GCT(6310	TA(GAC	CTC/ 632	AGT/ 2Ø	ACC.	AGT 6	GCT 33Ø	GGG1	TAT	CCC 634(TAT Ø	GTG	GCA4 635	ATG(GGG/	444 6	aag 36ø
KR	DI	L	N	ĸQ	T	R	D	Т	Κ	Е	Μ	Q	R	L	L	D	Т	Y	G	I	N	L	Ρ	L	۷	T	Y	۷	Κ	D	Е	L	R	S	к	Т
AAAAGAGA 63	ACATT 37Ø	TTG	4ATA 638	agca Ø	AAC(CAGA 5390/	GAC	aca	aagi 64Øi	gaav Ø	atgo	641	GG(Ø	CTTO	CTGO 64	GAC/ +2Ø	ACCI	TAT	GGT∕ 6430	TT/	AAT.	TTA(644	CCT +Ø	TTA	GTC 6	acc' 45ø	TAT	GTG	AAA 6461	GAT Ø	GAG	CTT/ 647	AGA Ø	TCC	AAG 6	ACC 48Ø
ΚVΕ	EQ	G	ĸ	S R	L	١	Ε	Α	S	S	L	Ν	D	S	۷	A	Μ	R	Μ	Α	F	G	Ν	L	Y	Α	Α	F	Η	к	Ν	Ρ	G	۷	۷	Т
AAAGTGGA	AACAG 490	GGC/	AAGT 65Ø	CCAG Ø	GCT/	4ATT 551Ø	GAG	igcc	TCA 652	AGT(Ø	CTC	ATC 653	ACT Ø	гсто	atco 65	3CC/	ATG/	AGG	atgo 6550	jCT'	TTT	GGC/ 656	AAC 50	TTG	TAC 6	GCA 57Ø	GCA	ттс	CAC 658	aag Ø	AAC	CCA(659	GGT(GTA	GTG 6	aca 6øø
GS/	A V	G	C	DΡ	D	L	F	W	S	к	I	Ρ	۷	L	M	E	Ε	к	L	F	A	F	D	Y	Т	G	Y	D	Α	s	L	S	Ρ	Α	W	F
GGATCGGC 66	CTGTT 61Ø	GGC1	igtg 662	accc. Ø	AGA1	17TG 563Ø	TTT	TGG	agt/ 6641	aaav Ø	ATA	CA0 665	itco Ø	CTC	ATGO 66	Gago 66ø	3AA/		CTC1 6670	TT(gca'	TTT(668	GAT BØ	TAC	ACG 6	GGT 69Ø	TAT	GAT	GCT 67Ø	TCA Ø	СТА	AGC(67		GCC	TGG 6	TTT 72Ø
ΕΑL	LK	Μ	V	LΕ	к	I	G	F	G	D	R	۷	D	Y	I	D	Y	L	Ν	Η	s	Н	Н	L	Y	к	Ν	К	Т	Y	С	۷	к	G	G	Μ

V H D N V A I L P T H A S P G E T I V I D G K E V E V L D A K A L E D Q A G T N

GAGGCTCTCAAGATGGTTCTAGAGAAAATTGGTTTGGTGACAGAGTGGATTACATTGATTATCTGAATCACTCGCACCATCTATATAAAAATAAGACATATTGTGTTAAGGGCGGCATG 6730 6740 6750 6760 6770 6780 6790 6800 6810 6820 6830 6840 FIG. E

Neurovirulence tests showed that all the recombinants caused paralysis and death in mice (Table 3). In contrast, inoculation of 5×10^8 PFU of Mahoney virus did not produce disease. The ability of PRV5.1 to cause paralysis in mice demonstrates that the mouse neurovirulence of Lansing virus is due to sequences within the capsid region.

DISCUSSION

We are using infection of mice by the Lansing strain of poliovirus as a model for addressing questions on the molecular basis of neurovirulence. As a first step it was important to determine why the Lansing virus is able to cause disease in mice, in contrast to most other poliovirus strains, which cannot. The Lansing strain, which was isolated originally from a case of fatal poliomyelitis, was adapted to rodents by a series of passages (2). It would be important to determine which areas of the Lansing genome have undergone change during the adaptation process in mice. Unfortunately, the original mouse-avirulent Lansing isolate is no longer available, and therefore it was necessary to compare Lansing to other viral strains. The complete nucleotide sequence of the Lansing viral genome was determined, and this sequence and its deduced translation product were compared with those of other mouse-avirulent viruses. From these analyses it was not possible to determine which viral sequences are required for mouse virulence. Therefore, a series of Lansing-Mahoney intertypic recombinants were constructed by in vitro manipulation of infectious cDNAs to test the role of various portions of the Lansing genome in mouse virulence. Neurovirulence assays of the recombinant viruses indicated that the Lansing capsid region contains sequences which impart mouse neurovirulence. A similar approach has been used to map the genomic location of in vitro markers which are used to assess the monkey neurovirulence of the type 1 Mahoney strain (12).

It is interesting that the LD_{50} of the transfection-derived

Ρ	S	G	С	S	G	Т	S	1	F	N	S	М	1	Ν	Ν	L	I	I	R	Т	L	L	L	Κ	Т	Y	Κ	G	ł	D	L	D	Н	L	Κ	Μ	1	Α	Y
CC/	тст	GGC 685	TGC Ø	тст	GGC 68	acc 6ø	TCA	ATT 6	111 87Ø	AAT	TCA	ATG 688	ATT Ø	AAT	AAT 68	CTA 90	ATA	ATC 6	agg. 9øø	ACT	стс	CTTA 691	CTG Ø	AAA	ACC 69	tac. 2ø	aag	GGC/ 69	ata 93ø	Gat	TTA	GAC 694	CAC Ø	CTG	AAG 69	atg. 5ø	ATA	300 6	TAT 96Ø
G	D	D	۷	I	Α	s	Y	Ρ	н	Ε	۷	D	A	S	L	L	Α	Q	s	G	к	D	Y	G	L	т	м	Т	Ρ	Α	D	к	S	Α	T	F	Е	T.	۷
GG1	GA1	GAT 697	GTA Ø	ATT	GCT 69	TCC 8Ø	TAC	000 6	CAT	GAG	GTT	GAT 7ØØ	GCT Ø	AGT	CTC 7Ø	CTA 1Ø	GCC	CAA 7	TCA Ø2Ø	GGA	AAA	GAC 7Ø3	TAT Ø	GGA	CTA 7Ø	acc. 4ø	ATG.	ACAI 71	CCA 050	GCT	GAC	AAA 7Ø6	TCA Ø	GCC.	ACC 7Ø	TTT 7Ø	GAA	ACA 7	gtc Ø8Ø
Т	W	Ε	Ν	۷	Т	F	L	κ	R	F	F	R	Α	D	Ε	к	Y	Ρ	F	L	۷	н	Ρ	۷	м	Ρ	Μ	к	Ε	1	н	Ε	s	I	R	W	Т	к	D
ACA	AT GO	igag 7ø9	iaat Ø	GTA	ACA 71		TTG	AAA 7	VAGA 1110	TTC	TTT	aga 712	gca Ø	GAT	GAA 71	AAG 3Ø	TAT	CCC 7	TTT 14Ø	CTG	igta	CAT 715	CCA Ø	GTG	ATG 71	CCA 6Ø	ATG	AAA 7	GAA 17Ø	ATT	CAC	GAA 718	TCA Ø	ATT.	AGA 71	tgg. 9ø	ACT	444 7	gat 2øø
Ρ	R	Ν	T	Q	D	н	۷	R	S	L	С	L	L	Α	W	Н	N	G	Е	Ε	Ε	Y	Ν	к	F	L	Α	к	I	R	S	۷	Ρ	T	G	R	Α	L	L
CCO	CAG/	VAAC 721	act Ø	CAC	igat 72	CAT 220	GTT	CGC 7	:TCA 230	CTG	itgc	TTA 724	TTG Ø	GCT	TGG 72	icac 5ø	AAT	GGC 7	GAG 26Ø	gaa	GAG	itac 727	aat. Ø	AAA	71 72	TTA 8Ø	GCT	AAG 7	ATT 29Ø	Aga	Agt	GTG 73Ø	CCA Ø	ATC	GGA 73	Aga 1ø	GCA'	TTA 7	CTG 32Ø
L	Ρ	Е	Y	S	T	L	Y	R	R	W	L	D	S	F	۵	۵																							
CTO	CCCI	GAC 733	iTAC	TCC	ACA	ATTG 840	itac	CGC	CGT	TGG	icto	GAC	ТСА	TTT	TAG	iTAA	CCC	TAC		AGT	CGA	ATT 739	GGA	TTG	iGGT	CAT.	ACT	GTT(GTA	GGG	GTA	AAT	TTT	TCT	TTA	ATT 30	CGG	AGG	

FIG. F

type 2 virus was about 40 times higher than the LD_{50} of the parental type 2 stock (Table 3). There are several possible reasons for this difference in neurovirulence. Virus V667 is an uncloned stock which had been passaged 99 times in mice before passage in HeLa cells (20). This virus may therefore consist of a relatively homogeneous population with respect to the ability to cause disease in mice. In contrast, transfection-derived virus V676P3 is plaque purified and furthermore is derived from a single DNA sequence cloned from all the RNA sequences in the V667 stock. This virus may therefore be a variant which does not have the optimum nucleotide sequence for mouse virulence. Virus V676P3 may also contain mutations introduced during the molecular cloning process which alter its virulence. However, it is not likely that the neurovirulence of virus V676P3 is altered simply because it is a temperature-sensitive mutant or is impaired in replication, since this virus grows as well as V667 in HeLa cells at all temperatures tested (data not shown). The first 68 nucleotides of V676P3 are derived from type 1 (20). There are four nucleotide differences between Lansing and Mahoney viruses in this region which might affect mouse neurovirulence.

There is also some variation in the LD_{50} values of the recombinants. This variation in neurovirulence might also be the result of mutations acquired during the molecular cloning process or may reflect plaque-to-plaque variation, as dis-

 TABLE 1. Comparison of capsid protein sequence of Lansing 2, Sabin 2, and Mahoney 1 viruses

Vinal anatain	No. of amino acid di (% hor	fferences/total residues mology)
virai protein	Lansing 2 vs Sabin 2	Lansing 2 vs Mahoney 1
VP4	1/69 (98.6)	2/69 (97.1)
VP2	5/271 (98.2)	32/272 (88.2)
VP3	4/238 (98.3)	29/238 (87.8)
VP1	22/301 (92.7)	70/301 (76.7)
P2	30/575 (94.8)	31/575 (94.6)
P3	21/753 (97.2)	15/753 (98.0)
Overall	83/2,207 (96.2)	179/2,207 (91.9)

cussed above. We have not determined whether the recombinants are temperature sensitive; however, all recombinants replicated in HeLa cells to the same titers as the parental viruses. Alternatively, noncapsid sequences may play a role in modulating neurovirulence. For example, recombinant PRV1.1 shows approximately the same neurovirulence as transfection-derived type 2. However, replacement of the Lansing P2 and P3 proteins with the Mahoney counterparts caused a decrease in neurovirulence (PRV4.3). Replacement of the Lansing P2, P3, and 5' end noncoding sequences with Mahoney counterparts resulted in a virus with the lowest neurovirulence, PRV5.1. In addition, replacement of only the Lansing 5' end resulted in virus PRV3.5, which is less neurovirulent than transfectionderived type 2. We do not know what role nonstructural proteins or noncoding sequences might have in production of disease, but examination of mutant viruses containing lesions in these regions would enable us to address this question.

Recombinant PRV5.1 derives only nucleotides 631 to 3413 from Lansing virus. Since the capsid region is encoded by nucleotides 745 to 3381, this recombinant has 114 nucleotides of 5'-noncoding region and 36 nucleotides encoding part of protein 2A from Lansing as well as the Lansing capsid. Since the 12 amino acids of protein 2A are identical in Lansing and Mahoney viruses, they cannot play a role in Lansing neurovirulence. We feel it is unlikely that the 114 nucleotides of 5'-noncoding sequence are responsible for the virulence of Lansing virus in mice. This particular region of the genome (nucleotides 650 to 745) is very different from the rest of the 5'-untranslated region since the sequences are poorly conserved from one strain to another, suggesting that this region may serve only as a spacer in the genome (32).

Since the capsid appears to be responsible for the unique ability of Lansing virus to cause disease in mice, it seems likely that most poliovirus strains do not cause disease in the mouse because they cannot enter the appropriate cells. This block to virus entry is probably caused by the inability of mouse-avirulent strains to attach to receptors on cells of the mouse central nervous system. However, the block to virus entry might also occur at the penetration or uncoating steps, which are probably mediated in part by the viral capsid.

A comparison of the Lansing capsid sequence with that of



FIG. 2. Maps of full-length poliovirus cDNAs used to generate recombinant viruses. The viral RNA is shown at the top (thin line, untranslated regions; thick line, open reading frame) with the locations of the poliovirus proteins. The DNA inserts of different plasmids are shown below, as well as the three restriction enzyme sites used in the constructions. The bracketed *PstI* site in the Lansing cDNA is not present in the original clone pVR204 but was introduced as described in Materials and Methods. The name of each plasmid is shown at left, and the name of the virus derived by transfection with that plasmid is shown at right. Single lines, Mahoney sequences; double lines, Lansing sequences.

other nonvirulent viruses might identify regions of the capsid which are involved in either binding to the cell receptor or penetration or uncoating. The Sabin 2 capsid was chosen for closer examination since it was more related to Lansing virus than Mahoney virus (Table 1). The observed differences between the Lansing and Sabin 2 capsids are depicted in Fig. 3. There are 32 amino acid differences, about half of which are scattered throughout all four capsid proteins and about half of which are concentrated in the first 41 residues of VP1. None of the amino acid changes from Sabin to Lansing virus result in alteration of the overall hydropathy or secondary structure of the capsid proteins when calculated by published methods (5, 8, 13). In most cases, the changes in the Lansing sequence do not involve amino acids of different charges. At three positions, all in VP1, the charge of the Lansing amino acid differed significantly from that of the Sabin amino acid. Position 29 is glycine (polar) in Sabin virus and aspartic acid (negatively charged) in Lansing virus; position 30 is histidine (positively charged) in Sabin virus and threonine (polar) in Lansing virus; and position 31

TABLE 2. Serological characterization of transfection-derived polioviruses

N/:	Antiser	rum"
virus	Anti-1	Anti-2
PRV1.1	_	+
PRV3.5	-	+
PRV4.3	-	+
PRV5.1	_	+
type 1 $(M)^b$	+	_
type 2 $(L)^b$	-	+

^{*a*} +, 100% reduction in plaque number by a 1:5,000 dilution of antipoliovirus type 1 or a 1:500 dilution of anti-poliovirus type 2 antisera; -, no effect of the same dilution.

^b M, Mahoney; L, Lansing.

is lysine (positively charged) in Sabin virus and glutamine (polar) in Lansing virus. Since these amino acid changes are clustered, their overall effect might be to produce a negative charge in a small region of the Lansing capsid which is positively charged in the Sabin capsid. Such a charge difference might be consistent with the ability to bind a different cellular receptor in the mouse central nervous system. In influenza virus, single amino acid changes in the hemagglutinin, the receptor-binding protein, can alter receptorbinding specificity (24). However, the real importance of these amino acid changes in Lansing virus is not clear, particularly in light of the observation that the N terminus of VP1 is the most variable region of the poliovirus genome (32). To determine which amino acids play a role in pathogenesis, we are assaying the mouse neurovirulence of recombinants in which small portions of the Lansing virus capsid sequences have been inserted into the corresponding region of the Sabin genome.

It was previously observed that inoculation of type 1 RNA into mouse brain does not cause disease but results in limited replication of virus, while inoculation of type 1 virus does not lead to replication. On the basis of these results, Holland

TABLE 3. Neurovirulence of transfection-derived polioviruses

Virus	LD ₅₀ (PFU)
	4.00×10^{3}
PRV3.5	4.25×10^4
PRV4.3	1.40×10^4
PRV5.1	4.50 × 10 ⁵
V667"	1.60×10^2
V676P3 ^b	6.25×10^3
P6a ^c	>5.00 × 10 ⁸

" Uncloned type 2 Lansing virus.

^b Transfection-derived type 2 Lansing virus.

^c Uncloned type 1 Mahoney virus.



FIG. 3. Location of amino acid differences between the capsid proteins of Lansing and Sabin 2 viruses. Line shows the map location of each capsid protein and its length in amino acids. Both the Lansing and Sabin 2 amino acids are displayed at points where the two viruses differ. Numbers refer to the amino acid residue within the individual capsid protein. The N terminus of VP1 is expanded above the line for clarity.

et al. (7) suggested that type 1 virus cannot cause disease in mice because it cannot bind the mouse brain receptor. However, from these studies it was not possible to determine whether other type 2 sequences were also required for mouse neurovirulence. Our results show that the ability of the Lansing virus to cause disease in mice lies solely within the capsid region. Furthermore, it appears that other Lansing sequences may play a role in mouse neurovirulence as well. The ability to manipulate the Lansing genome through cloned infectious cDNA coupled with the convenience of a small animal model will make it possible to further study the role of specific viral sequences in the production of disease.

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