The EMBO Journal vol.10 no.5 pp.1067 - 1074, 1991

Host range determinants located on the interior of the poliovirus capsid

Eric G.Moss and Vincent R.Racaniello

Department of Microbiology, Columbia University College of Physicians and Surgeons, 701 West 168th Street, New York, NY 10032, USA

Communicated by D.Baltimore

The inability of certain poliovirus strains to infect mice can be overcome by the expression of human poliovirus receptors in mice or by the presence of a particular amino acid sequence of the B-C loop of the viral capsid protein VP1. We have identified changes in an additional capsid structure that permit host-restricted poliovirus strains to infect mice. Variants of the mouse-virulent P2/Lansing strain were constructed containing amino acid changes, deletions and insertions in the B-C loop of VP1. These variants were attenuated in mice, demonstrating the importance of the B-C loop sequence in host range. Passage of two of the B-C loop variants in mice led to the selection of viruses that were substantially more virulent. The increased neurovirulence of these strains was mapped to two different suppressor mutations in the N-terminus of VP1. Whereas the B-C loop of VP1 is highly exposed on the surface of the capsid, near the five-fold axis of symmetry, the suppressor mutations are in the interior of the virion, near the three-fold axis. Introduction of the suppressor mutations into the genome of the mouse-avirulent P1/Mahoney strain resulted in neurovirulent viruses, demonstrating that the P2/Lansing B-C loop sequence is not required to infect mice. Because the internal host range determinants are in a structure known to be important in conformational transitions of the virion, the host range of poliovirus may be determined by the ability of virions to undergo transitions catalyzed by cell receptors.

Key words: atomic structure/host range/poliovirus/receptor

Introduction

Picornaviruses are small, naked, plus-strand RNA viruses that cause a wide variety of diseases. The three-dimensional structures of several picornavirus capsids have been solved crystallographically, the cellular receptors for two have been identified, and a number of these viruses can be manipulated genetically by way of infectious cDNA clones (reviewed in Racaniello, 1990). We have used these advances to investigate genetic determinants of poliovirus pathogenesis.

From initial sites of replication in the gut, poliovirus may travel to the central nervous system (CNS) and produce an acute and sometimes fatal paralytic disease, poliomyelitis (Bodian, 1959). In its most severe form, the disease is characterized by extensive and permanent destruction of motor neurons in the anterior horns of the spinal cord, the brain stem and the motor cortex. The virulence of a strain, which is a heritable property, is measured by the efficiency or severity of damage produced upon direct inoculation of virus into the CNS. Strains of all antigenic types may cause paralysis, and highly related strains can vary widely in virulence, indicating that this property is affected by small genetic changes (Sabin, 1955).

The natural hosts for polioviruses are humans, but chimpanzees and certain species of monkeys can be experimentally infected, and strains of poliovirus have been identified that cause paralysis in mice. Intracerebral or intraspinal inoculation of mice with these strains leads to a disease that resembles human poliomyelitis in histopathology and age-dependence of susceptibility (Jubelt *et al.*, 1980a,b). Some strains of poliovirus are naturally virulent in mice (unpublished osbervations), while the virulence of other strains, including P2/Lansing, P1/LS_b, and a variant of P3/Leon, was acquired after a process of adaptation involving serial passage of viruses in mice (Armstrong, 1939; Li and Schaeffer, 1954; Krech, 1954). A number of viral strains are clearly host restricted and cause paralysis in primates but not mice, such as the P1/Mahoney strain (La Monica *et al.*, 1986).

When the gene encoding the human poliovirus receptor (PVR) is introduced into the germline of mice, the resulting transgenic animals can be infected by host-restricted poliovirus strains such as P1/Mahoney (Ren *et al.*, 1990). These results show that the primary block to infection of normal mice by these strains is at the level of cell entry. The molecular analysis of poliovirus host range therefore provides a way of investigating virus–receptor interactions and may shed light on how poliovirus enters cells.

The genetic basis for host restriction of poliovirus has recently been studied using two strains: P2/Lansing, a mouse-adapted strain, and P1/Mahoney. Construction of viral recombinants between these two strains revealed that the capsid-coding region of the P2/Lansing strain is sufficient to confer the mouse-virulent phenotype on the P1/Mahoney strain (La Monica *et al.*, 1986). Monoclonal antibody resistant variants of P2/Lansing containing mutations in neutralization-antigenic site 1 (N-Ag1) have reduced mouse neurovirulence (La Monica *et al.*, 1987). N-Ag1 is one of three sites on the surface of the capsid that together define the three antigenic types of poliovirus (Hogle and Filman, 1989). Amino acids 95–104 of capsid protein VP1, which contributes substantially to N-Ag1, comprise a loop connecting β-strands B and C (the B-C loop) of VP1. This loop is highly exposed on the virion surface near the five-fold axis of symmetry (Hogle *et al.*, 1985). A significant finding concerning poliovirus host range is that variants of P1/Mahoney that carry the VP1 B-C loop of P2/Lansing are neurovirulent in mice (Martin *et al.*, 1988; Murray *et al.*, 1988a).

In this work, the effects of amino acid changes in the VP1 B-C loop on mouse neurovirulence are described. Second site suppressors, which restore virulence to viruses attenuated

© Oxford University Press

Table I. Virulence of P2/Lansing VP1 B-C loop variants

Virus	VP1 amino acid											p.f.u./LD_{50}
	92	93	94	95	96	97	98	99	100	101	102	
P2/Lansing	Val	Asp	Asn	Asp	Ala	Pro	Thr	Lys	Arg	Ala	Ser	1×10^5
P1/Mahoney	Val	Asp	Asn	Pro	Ala	Ser	Thr	Thr	Asn	Lys	Asp	$>1 \times 10^9$
R2-1D-3	Val	Asp	Asn	**Pro**	Ala	**Ser**	Thr	**Thr**	**Asn**	**Lys**	**Asp**	$>1 \times 10^9$ [a]
R2-1D-4	Val	Asp	Asn	**Pro**	Ala	Pro	Thr	Lys	Arg	Ala	**Asp**	$>3 \times 10^9$ [a]
R2-1D-5	Val	Asp	Asn	Asp	Ala	Pro	Thr	**Thr**	**Asn**	**Lys**	**Asp**	2×10^8
R2-1D-6	Val	Asp	Asn	**Pro**	Ala	**Ser**	Thr	Lys	Arg	Ala	Ser	2×10^8
R2-1D-12	Val	Asp	Asn	Asp	Ala	Pro	Thr	**Thr**	**Asn**	**Lys**	Ser	$>2 \times 10^9$ [a]
R2-1D-7	Val	Asp	Asn	**Glu**	**Gln**	Pro	Thr	**Thr**	Arg	Ala	**Gln**	$>4 \times 10^8$
R2-1D-9	Val	Asp	–	–	–	–	–	–	–	–	–	$>5 \times 10^8$
R2-1D-10	Val	Asp	**Thr**	**Gly**	**Arg**	**Gly**	**Asp**	**Ser**	**Pro**	Ala	Ser	$>1 \times 10^9$
R2-1D-11	(below)		Asn	Asp	Ala	Pro	Thr	Lys	Arg	Ala	Ser	$>4 \times 10^8$
	Ser	**Thr**	**Ser**	**Phe**	**Asp**	**Lys**	**Leu**					

Amino acid substitutions are in bold; deletions are indicated by dashes. Amino acids 94–102 are deleted in R2-1D-9. R2-1D-11 has seven amino acids replacing amino acids 92 and 93.
[a]At least one mouse was paralyzed by this virus.

by changes in the VP1 B-C loop, are identified. Like the B-C loop of P2/Lansing, these suppressor mutations broaden the host range of certain polioviruses. The location of the suppressors in the three-dimensional atomic structure of the viral capsid suggests mechanisms by which poliovirus host range is determined.

Results

Generation and virulence analysis of VP1 B-C loop variants of P2/Lansing

A series of P2/Lansing variants were generated to determine the effect of specific changes in the VP1 B-C loop on host range. The virulence of these viruses was assayed by intracerebral inoculation into Swiss–Webster mice.

All P2/Lansing-derived viruses with variant B-C loops showed a dramatic reduction in virulence (Table I). Whereas P2/Lansing had an LD_{50} value of 10^5 plaque-forming units (p.f.u.), all the variants had LD_{50} values $> 10^8$ p.f.u., and some viruses did not paralyze any mice. These observations are consistent with previous work showing that certain single amino acid substitutions in this sequence strongly attenuate virulence of poliovirus strain P2/Lansing in mice (La Monica *et al.*, 1987).

The variant strains fell into two groups: those which paralyzed at least one mouse (1D-3, -4, -5, -6 and -12) and those that did not cause disease (1D-7, -9, -10 and -11). The B-C loop sequence of strain 1D-3 is identical to that of P1/Mahoney, and like P1/Mahoney this variant was highly attenuated in mice. 1D-3 was neutralized by polyclonal anti-type 1 antiserum, suggesting that the P1/Mahoney B-C loop is in its native configuration, like the P2/Lansing loop in a P1/Mahoney background (Martin *et al.*, 1988; Murray *et al.*, 1988a). Four variants possess B-C loop sequences that are recombinants of P2/Lansing and P1/Mahoney (1D-4, -5, -6 and -12); these viruses had high LD_{50} values but could paralyze at least one mouse each. None of these viruses was neutralized by polyclonal anti-P1/Mahoney antiserum, or by anti-N-Ag1 monoclonal antibodies that neutralize P2/Lansing (data not shown).

The four variants that did not cause paralysis have drastic changes in the B-C loop. One virus (1D-7) has the B-C loop sequence of P3/Leon, but is not neutralized by anti-P3/Leon antisera (data not shown), suggesting that the type 3 sequence is not correctly displayed. Other viruses with type 3 VP1 B-C loops in a P1/Sabin or P1/Mahoney background are, however, neutralized by anti-type 3 antibodies (Burke *et al.*, 1988; Murray *et al.*, 1988b). In addition, whereas P3/Leon shows moderate virulence in mice (unpublished results), 1D-7 was avirulent (Table I). Deletion of amino acids 94–102 did not affect viral growth in HeLa cells, but abolished mouse virulence (1D-9, Table I). Insertion of seven amino acids in place of amino acids 92 and 93 also abrogated mouse virulence (1D-11, Table I) but did not produce a phenotype in cell culture (data not shown).

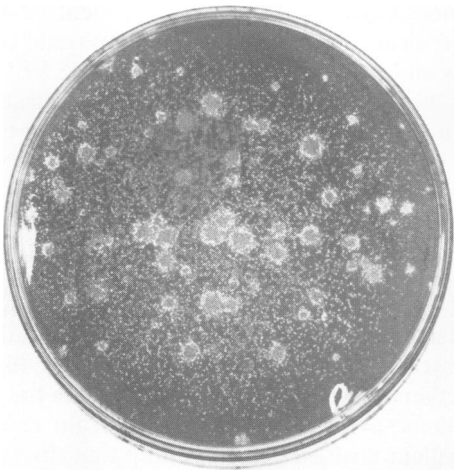

Fig. 1. Plaque phenotype of R2-1D-6. HeLa cell monolayer stained with crystal violet. Large plaque revertants are present.

Only one B-C loop variant had an obvious phenotype in HeLa cells: 1D-6 produced extremely small plaques at 37°C (Figure 1). Large plaque revertants of this virus arise with a frequency expected for a point mutation (10^{-3}–10^{-4}, Figure 1). One-step growth analysis demonstrated that 1D-6 had the same growth rate and yield as P2/Lansing (Figure 2), suggesting that it was not replication defective.

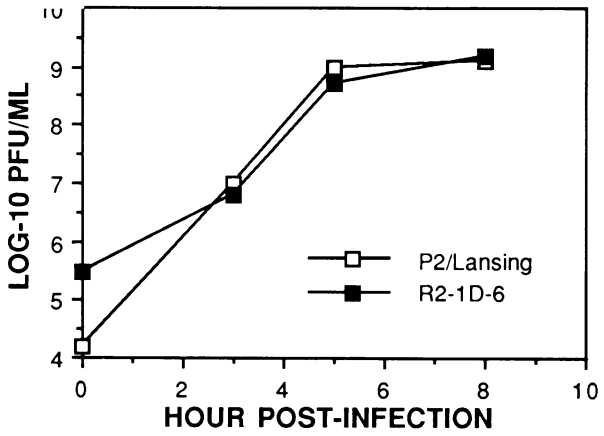

Fig. 2. One step growth curves of P2/Lansing and R2-1D-6. HeLa cell monolayers were infected at a m.o.i. of 10 and total virus titer was determined at the indicated times.

Table II. Effect of trypsin treatment on the virulence of P2/Lansing

Virus preparation	p.f.u./LD_{50}
Untreated	5×10^4
Trypsin-treated	7×10^6
Trypsin + anti-N-Ag1	$>2 \times 10^{7a}$

[a]Some mice were paralyzed by this preparation.

The effect of trypsin treatment on virulence of P2/Lansing in mice

Treatment of certain poliovirus strains with trypsin results in cleavage at amino acid 99 or 100 in the B-C loop (Fricks *et al.*, 1985; Minor *et al.*, 1987; Roivainen and Hovi, 1988). Since P2/Lansing contains a potential trypsin cleavage site within the B-C loop, it was of interest to determine whether cleavage at this site would attenuate P2/Lansing virulence in mice. P2/Lansing virulence was reduced by ~100-fold afer trypsin treatment (Table II). To determine whether residual virulence of the treated preparation resulted from uncleaved virus, trypsin-treated virus was incubated with neutralizing monoclonal antibody directed against anti-N-Ag1. The mAb neutralized infectivity of P2/Lansing in HeLa cells, but not that of trypsin-cleaved virus, as expected (data not shown). The trypsin- and antibody-treated virus preparation had an LD_{50} 1000-fold higher than that of untreated P2/Lansing (Table II), which is comparable to that of a virus heavily attenuated by mutations in the B-C loop (Table I).

Selection in vivo of phenotypic revertants of attenuated B-C loop variants

The results described above demonstrate that a variety of changes in the sequence of the B-C loop of capsid protein VP1 dramatically reduce the virulence of poliovirus strain P2/Lansing in mice. The observation that certain highly attenuated VP1 B-C loop variants caused paralysis in some mice provided a means to identify additional capsid residues involved in host range. The rare paralysis produced after inoculation with an attenuated B-C loop variant may be due to residual ability of the virus to cause disease, or to the successful replication of a spontaneously occurring mutant in which the attenuating mutations in the B-C loop had reverted or were suppressed. If the latter occurs, then serial passages in mice of virus derived from paralyzed animals should result in stocks of virus enriched for the virulent mutants.

To isolate phenotypic revertants, mice were inoculated with high-titer stocks of attenuated variants 1D-3, -4, -5 and -6. Virus was isolated from spinal cords of paralyzed mice, amplified in HeLa cells, and inoculated into mice. After an additional passage in mice, virus was isolated from spinal cords, stocks were prepared in HeLa cells and the LD_{50} was determined. Two spinal cord isolates, scp3 and scp6, had greatly reduced LD_{50} values relative to the parental stocks (Figure 3). These isolates were derived from 1D-3 and 1D-6, respectively (Table I).

Identification of two attenuation suppressors

Sequencing of genomic RNA revealed that isolates scp3 and scp6 retained the VP1 B-C loop sequences of their parent viruses 1D-3 and 1D-6 (data not shown). Therefore the virulence of scp3 and scp6 was due to the presence of a second-site mutation that suppresses the attenuating effects of the B-C loop mutations.

To map the suppressor mutations, regions of the genome from scp3 and scp6 were cloned and exchanged with the corresponding sequence of the parent viruses 1D-3 and 1D-6, respectively. Replacement of nucleotides 2046–2752 of the parent viruses with the corresponding sequences from scp3 and scp6, produced 1D-3,15 and 1D-6,16. These viruses had LD_{50} values in mice comparable to those of scp3 and scp6 (Figure 3), indicating that the suppressors mapped to this region.

Sequence analysis of nucleotides 2046–2752 of 1D-3,15 and 1D-6,16 cDNAs revealed a single mutation in each: the scp3-derived fragment had an A to G change at nucleotide 2597 causing a substitution of Gly for Glu at amino acid 40 of VP1, and the scp6-derived fragment had a C to T change at nucleotide 2638 causing a substitution of Ser for Pro at amino acid 54 of VP1.

Allele specificity of attenuation suppressors

To examine the ability of Gly40 and Ser54 of capsid protein VP1 to suppress the attenuation of other P2/Lansing-derived VP1 B-C loop variants, each was introduced into the variants 1D-4 and -5, as well as reciprocally into 1D-3 and 1D-6. The resulting viruses were tested for virulence in mice (Table III). Only Ser54 increased neurovirulence of another B-C loop variant, 1D-3, to the same degree as the variant in which it was selected (1D-6). However, Ser54 did not increase the neurovirulence of 1D-4 or -5. At the level of inoculum used, Gly40 did not significantly suppress the attenuation phenotype of any strain other than the one in which it was selected (1D-3). Introduction of Gly40 into P2/Lansing slightly attenuated this strain (Table III). Thus, both Gly40 and Ser54 display allele specificity with respect to their ability to suppress the attenuation effects of B-C loop changes.

The effect of different B-C loop sequences and suppressors on the growth of viruses in culture at different temperatures was determined (Table IV). All viruses plated well at 37°C; two were *ts* (1D-3,15 and -2,15) and two were cold sensitive (*cs*) (1D-2,15 and -2,16). Interestingly, 1D-2,15 was both *ts* and *cs*. The degree of *ts* or *cs* of a virus was the result of neither a specific B-C loop nor an attenuation suppressor, but rather depended on particular loop-

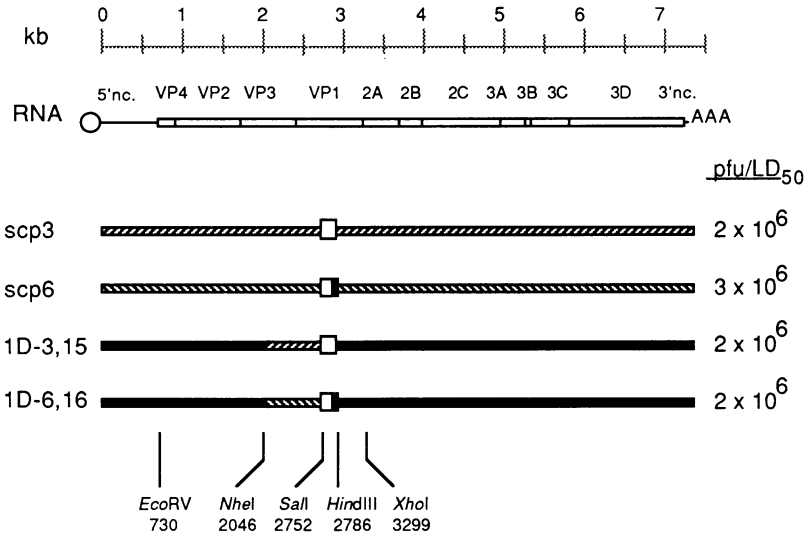

Fig. 3. Constitution and virulence of P2/Lansing-derived viruses. The genome of each strain is represented below a genetic map of poliovirus. The name of each virus is shown at left and its corresponding LD_{50} value is shown at right. Black, sequences from P2/Lansing. Cross-hatched, sequences from virulent revertant stocks of VP1 B-C loop varient viruses. Open box, sequences encoding VP1 B-C loop of R2-1D-3. Half-open box, sequences encoding VP1 B-C loop of R2-1D-6. The positions of the restriction sites used to construct recombinant genomic cDNAs are indicated. Not to scale.

suppressor combinations. There was no correlation between the growth of these viruses at different temperatures and their virulence in mice (Tables III and IV).

Attenuation suppressors are host range determinants

Recently it was shown that transgenic mice expressing human PVR are susceptible to host-restricted strains such as P1/Mahoney (Ren *et al.*, 1990). PVR transgenic mice were used to determine whether the reduced mouse neurovirulence of 1D-3, in which the B-C loop of P2/Lansing is substituted with that of P1/Mahoney, was due to host range effects or general attenuation. This virus was significantly more virulent in PVR transgenic mice than in normal mice, and nearly as virulent in PVR transgenic mice as P2/Lansing is in normal mice (Tables I and V). Thus, the reduced neurovirulence of 1D-3 in normal mice is a result of host restriction. Therefore Gly40 and Ser54 are host range determinants.

It was of interest to determine whether Gly40 and Ser54 could also extend the host range of P1/Mahoney. Introduction of either mutation into the P1/Mahoney background resulted in viruses that were significantly more virulent in normal mice than P1/Mahoney, which is highly attenuated in this host (Table VI).

Discussion

The VP1 B-C loop and host range

When the VP1 B-C loop of P1/Mahoney is substituted with the sequence from P2/Lansing, the resulting chimeric virus is virulent in mice, while the parent Mahoney strain does not replicate in this host (Martin *et al.*, 1988; Murray *et al.*, 1988a). The reciprocal recombinant is attenuated in normal mice and virulent in PVR transgenic mice (Table I). Therefore the B-C loop of VP1 is the primary determinant of the host restriction of P1/Mahoney and the mouse virulence of P2/Lansing.

Alterations in the B-C loop of P2/Lansing reduce the pathogenicity of the virus in mice (Table I; La Monica *et al.*, 1987). Observations similar to those made with P2/Lansing have been made by generating variants of a Mahoney chimera containing the Lansing B-C loop (Girard *et al.*, 1990). These results underscore the importance of the B-C loop in host range. However, VP1 B-C loops of different poliovirus strains can be deleted or substituted by a variety of sequences with no effect on growth in cell culture (Table I; reviewed in Almond and Burke, 1990). Therefore, the B-C loop appears to be non-essential for viral replication in cultured cells. However, the composition of the B-C loop can affect viral growth in culture (Burke *et al.*, 1988; Murray *et al.*, 1988b). The small plaque phenotype of 1D-6 is a striking example of the effect the B-C loop can have on growth of a virus in cultured cells (Figure 2). This virus differs from P2/Lansing by two amino acid substitutions which effectively move a Pro residue from position 97 to position 95 in VP1. The location of this Pro residue appears to have substantial effects on the conformation of B-C loops and neighboring structures (Hogle *et al.*, 1989; Filman *et al.*, 1989). One-step growth experiments show that 1D-6 replicates efficiently, suggesting that its defect may be in spreading from cell to cell, exit from or entry into cells. A virulent variant of this virus which retains the mutant B-C loop sequence (1D-6,16) has the same small plaque phenotype as its parent (data not shown). Thus the defect caused by this loop sequence in cell culture can be uncoupled from its effect on mouse virulence.

Host range determinants on the interior of the capsid

We have identified two amino acid changes that suppress the requirement for the P2/Lansing-specific B-C loop sequence for infection of mice. Like the VP1 B-C loop, each of the two mutations can extend the host range of poliovirus to include mice. The B-C loop and the internal suppressors are therefore host range determinants.

The suppressor mutations are located in the N-terminal extension of VP1, which is on the interior surface of the virus particle, near the three-fold axis of symmetry (Hogle *et al.*, 1985; Filman *et al.*, 1989) (Figure 4). In contrast,

Table III. Virulence of P2/Lansing VP1 mutants

Virus	VP1 amino acid										p.f.u./LD_{50}
	40	54	95	96	97	98	99	100	101	102	
R2-1D-3	Glu	Pro	**Pro**	Ala	**Ser**	Thr	**Thr**	**Asn**	**Lys**	**Asp**	$>1 \times 10^{9}$[a]
R2-1D-3,15	**Gly**	Pro	**Pro**	Ala	**Ser**	Thr	**Thr**	**Asn**	**Lys**	**Asp**	2×10^{6}
R2-1D-3,16	Glu	**Ser**	**Pro**	Ala	**Ser**	Thr	**Thr**	**Asn**	**Lys**	**Asp**	1×10^{6}
R2-1D-6	Glu	Pro	**Pro**	Ala	**Ser**	Thr	Lys	Arg	Ala	Ser	2×10^{8}
R2-1D-6,15	**Gly**	Pro	**Pro**	Ala	**Ser**	Thr	Lys	Arg	Ala	Ser	3×10^{7}
R2-1D-6,16	Glu	**Ser**	**Pro**	Ala	**Ser**	Thr	Lys	Arg	Ala	Ser	2×10^{6}
P2/Lansing	Glu	Pro	Asp	Ala	Pro	Thr	Lys	Arg	Ala	Ser	1×10^{5}
R2-1D-2,15[b]	**Gly**	Pro	Asp	Ala	Pro	Thr	Lys	Arg	Ala	Ser	1×10^{7}
R2-1D-2,16	Glu	**Ser**	Asp	Ala	Pro	Thr	Lys	Arg	Ala	Ser	$<1 \times 10^{5}$
R2-1D-4	Glu	Pro	**Pro**	Ala	Pro	Thr	Lys	Arg	Ala	**Asp**	$>3 \times 10^{9}$[a]
R2-1D-4,15	**Gly**	Pro	**Pro**	Ala	Pro	Thr	Lys	Arg	Ala	**Asp**	2×10^{8}
R2-1D-4,16	Glu	**Ser**	**Pro**	Ala	Pro	Thr	Lys	Arg	Ala	**Asp**	$>2 \times 10^{8}$
R2-1D-5	Glu	Pro	Asp	Ala	Pro	Thr	**Thr**	**Asn**	**Lys**	**Asp**	2×10^{8}
R2-1D-5,15	**Gly**	Pro	Asp	Ala	Pro	Thr	**Thr**	**Asn**	**Lys**	**Asp**	$>2 \times 10^{8}$
R2-1D-5,16	Glu	**Ser**	Asp	Ala	Pro	Thr	**Thr**	**Asn**	**Lys**	**Asp**	$>2 \times 10^{8}$

Amino acid substitutions are bold.
[a]At least one mouse was paralyzed by this virus.
[b]R2-1D-2 is P2/Lansing.

the B-C loop is located on the virion surface, near the five-fold axis. Unlike the B-C loop, whose sequence is quite variable among polioviruses, the amino acids at positions 40 and 54 in VP1 are invariant (Palmenberg, 1989).

If the internal host range determinants act by bypassing a requirement for the P2/Lansing-specific B-C loop sequence, then it would be expected that the suppressors of one B-C loop would suppress the effect of other similar loops. However, our observations suggest that the suppressors are allele-specific.

Host range determinants and receptor-mediated conformational transitions

The host restriction of P1/Mahoney and R2-1D-3 can be overcome in three ways: by substituting the P1/Mahoney-specific VP1 B-C loop with that of P2/Lansing, by the presence of human PVR in mice, or by the intragenic suppressors described here. Because the human receptor can overcome host restriction, it seems likely that polioviruses which cannot infect mice are blocked in one of the events of the virus–receptor interaction.

This idea is supported by the observation that virulence of P2/Lansing in mice can be reduced by trypsin treatment of the virus, which is expected to change the conformation of the B-C loop to the same degree as amino acid substitutions. In both cases the ability of anti-N-Ag1 antibodies to neutralize virus is eliminated. However, trypsin treatment can only affect a single infectious cycle, since after one round of replication, the loop is again intact. If the integrity of the B-C loop were only required in virus assembly, trypsin cleavage of the loop would have no effect on virulence. Therefore, trypsin treated viruses are likely to be blocked in cell entry in mice. By analogy, changes in the B-C loop that prevent polioviruses from infecting mice are likely to block viral entry into cells.

Host range may be determined by receptor recognition, because virus entry, the cellular receptor and the highly exposed B-C loop of VP1 are involved in this property. Because the B-C loop is non-essential for growth in cultured human cells and because host restricted strains can infect

Table IV. Effect of temperature on the growth of P2/Lansing VP1 mutants

Virus	Titer[a] 32°C/37°C	Titer 39°C/37°C
P2/Lansing	1.4	0.5
R2-1D-2,15	0.02	<0.001
R2-1D-2,16	0.02	0.8
R2-1D-3	0.6	0.5
R2-1D-3,15	0.5	0.02
R2-1D-3,16	0.4	0.4

[a]Determined by plaque assay on HeLa cells.

Table V. Virulence of viruses in PVR transgenic mice

Virus	p.f.u./LD_{50}
P1/Mahoney	1×10^{5}
P2/Lansing	1×10^{4}
R2-1D-3	$<1 \times 10^{5}$

PVR transgenic mice, this suggestion implies that this loop is the recognition site for the mouse receptor and is distinct from that used in human cells. Few changes in the B-C loop sequence of P2/Lansing and the Mahoney–Lansing chimera are compatible with infection of mice (Table I; La Monica *et al.*, 1987; Girard *et al.*, 1990). However, when either of the two attenuation suppressors are present, the B-C loop sequences required for mouse virulence are significantly different. It is not clear how internal mutations overcome host restriction if the B-C loop is a receptor binding site.

The apparent allele specificity of the host range determinants with respect to virulence and plating efficiency at different temperatures is evidence for direct interactions between the N-terminal extension and the B-C loop of VP1. However, because the B-C loop and the internal host range determinants are distant from each other in the native structure, it seems unlikely that they directly interact. This conclusion is supported by structural comparison of

Table VI. Virulence of P1/Mahoney VP1 mutants

Virus	VP1 amino acid		p.f.u./LD_{50}
	40	54	
P1/Mahoney	Glu	Pro	$>4 \times 10^{9}$ [a]
R1-1D-1	**Gly**	Pro	7×10^{7}
R1-1D-2	Glu	**Ser**	4×10^{7}

Amino acid substitutions are bold.
[a]At least one mouse was paralyzed by this virus.

P1/Mahoney and the Mahoney−Lansing chimera, which shows that changes in the B-C loop sequence only affect the structure in the vicinity of the five-fold axis (Hogle *et al.*, 1989).

An attractive explanation for the mechanism of action of the internal host range determinants is that they affect the ability of the virion to undergo structural changes early in infection. Interaction between poliovirus and its cell receptor leads to the conformational transitions in the virion that must take place for entry and uncoating (Fricks and Hogle, 1990). Circumstantial evidence that the internal host range determinants affect conformational transitions of the virion comes from the fact that Gly40 and Ser54 are located near sites that may regulate such transitions (Filman *et al.*, 1989; Minor *et al.*, 1989). These sites include an assembly-dependent seven-stranded β-sheet near the three-fold axis of symmetry, and the interface between five-fold related protomers (Filman *et al.*, 1989). Because of the role of the β-sheet in the association of pentamers, mutations that influence the stability of the sheet might regulate conformational transitions in the capsid. The internal host range determinants identified here are likely to affect the conformation or movement of part of the N-terminal extension of VP1 which lies immediately below the interface between five-fold related protomers and constitutes part of the seven-stranded β-sheet.

The B-C loop may also determine host range by effecting conformational transitions of the virion. It is possible that the B-C loop influences the structure or movement of the E-F loop, which forms part of the interface between five-fold related protomers. The E-F loop forms part of N-Ag1, as do the D-E, H-I and B-C loops, and thus clearly interacts structurally with the other loops. Interactions along this interface are likely to play significant roles in the dynamics of the capsid associated with receptor attachment and cell entry. Therefore, by influencing such interactions, the B-C loop and the N-terminus of VP1 might cooperatively regulate conformational transitions in the capsid.

If the virus−receptor interaction is concurrent with early conformational transitions in the virus particle, then different receptors or capsid residues could influence the ability of the virion to undergo such transitions, thereby determining the outcome of infection. Productive interaction between the virus and the mouse receptor may have slightly different requirements than the interaction with the human receptor. As a result, P1/Mahoney might effectively use only the human receptor while P2/Lansing virus might use both receptors.

The analysis of host range determinants presented here suggests that they affect the receptor-mediated conformational transitions of the virion required for entry into cells. This interpretation is complicated by the fact that no structures on the virion that interact with cellular receptors have yet been identified. Further understanding of the virus−receptor interaction and the subsequent events early in infection will be needed to elucidate the functional basis of host range.

Fig. 4. Locations of host range determinants in the atomic structure of P1/Mahoney. The image is an α-carbon tracing of a protomer, 60 of which constitute the virus capsid. VP1 is blue, VP2 is yellow, VP3 is red and VP4 is green. Highlighted are the B-C loop, which is near the five-fold axis, and the two attenuation suppressors, amino acids 40 and 54 of VP1, that are near the three-fold axis. Amino acid 40 is further from the five-fold axis than is amino acid 543. The outside of the particle is at the top of and out of the image and the interior at the bottom and into the image.

Materials and methods

Strains and nomenclature

All viruses used in these experiments were derived by transfection of HeLa cell monolayers with RNA derived from cloned genomic cDNAs of poliovirus strains P1/Mahoney (Racaniello and Baltimore, 1982a) and P2/Lansing (Racaniello, 1984) or variants of these strains generated as part of this study.

Virus variants of defined genotype are named according to the conventions described previously (Bernstein *et al.*, 1986), with slight modification. The virus name consists of a single letter designating its laboratory of origin, the serotype, the name of the genetic locus in which the mutations occur, and an arbitrary allele designation. Viruses in which two mutations in the same gene have been combined retain the original allele designations separated by a comma rather than a new designation. For example, the derivative of R2-1D-3 carrying a suppressor mutation was named R2-1D-3,15. For simplicity, designations of laboratory of origin and serotype are frequently omitted.

Virus growth and assay

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 modified Eagle's medium (DMEM) containing 10% horse serum (La Monica *et al.*, 1986). Viral stocks were prepared in monolayers of HeLa cells, using inocula plaque-purified from the culture

medium of transfected cells. Viral titers were determined by plaque assay on cell monolayers as described (Racaniello and Baltimore, 1981b). Viral stocks of high titer, used for inoculation of mice, were prepared either in suspension cultures or monolayers of HeLa cells as described (Ren *et al.*, 1991).

Growth at low and high temperatures was measured by plaque assay at 32°C and 39.5°C as described (La Monica *et al.*, 1987). One-step growth analysis was performed by infecting HeLa cell monolayers at a multiplicity of infection of 10 p.f.u./cell, and at the indicated times, cells and medium were collected and the cells lysed by freeze–thawing. The titer of each lysate was then determined by plaque assay.

Nucleotide sequencing

Sequencing of recombinant and mutant cDNAs to confirm their identity was performed using Sequenase™ according to the manufacturer's directions (US Biochemical). The identity of viruses was confirmed by sequencing of genomic RNA at recombination junctions and mutation sites using reverse transcriptase (Pharmacia). Isolation of genomic RNA and chain termination sequencing using oligonucleotide primers was performed as described (La Monica *et al.*, 1987).

Construction of recombinants

Plasmid DNAs were grown in *Escherichia coli* DH5α and purified by CsCl centrifugation (Ausubel *et al.*, 1987). 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 (Ausubel *et al.*, 1987). Ligations were performed according to the instructions of the manufacturer of T4 DNA ligase (Boehringer-Mannheim Biochemicals).

cDNAs of VP1 B-C loop variants were derived from a modified P2/Lansing clone in which a *Sal*I restriction site at position 2752 and a *Hin*dIII site at position 2786 were introduced by oligonucleotide-directed mutagenesis (see below). Annealed complementary oligonucleotides encoding the amino acids between 94 and 103 of VP1 listed in Table I were inserted between these two sites.

All type 2 recombinants in Figure 3 and Table III were constructed by replacing a *Nhe*I–*Sal*I fragment of the cDNAs of the parental viruses (R2-1D-3, R2-1D-6, etc.) with the corresponding fragment from PCR-amplified cDNA of scp3 (indicated by 15) or scp6 (indicated by 16).

Oligonucleotide-directed mutagenesis

Oligonucleotide-directed mutagenesis was performed on DNAs subcloned in pBluescript (Stratagene) and grown in *E.coli* CJ236 as described (Ausubel *et al.*, 1987). Enzymes used were T4 polynucleotide kinase and T4 DNA polymerase (New England Biolabs).

Nucleotide changes were introduced into P2/Lansing cDNA to create *Sal*I and *Hin*dIII restriction sites at nucleotides 2752 and 2786 respectively; these mutations caused no change in amino acid coding. The virus derived from this clone containing P2/Lansing B-C loop sequence, named R2-1D-2, resembles P2/Lansing in plaque size and antigenicity (data not shown).

A single nucleotide change was introduced into the P1/Mahoney sequence to cause an amino acid substitution of Glu for Gly at amino acid 40 of VP1, resulting in the cDNA of virus R1-1D-1. Similarly, a single change was made to cause a substitution of Pro for Ser at amino acid 54 of VP1, resulting in the cDNA for virus R1-1D-2.

In vitro amplification of cDNA

Stocks of scp3 and scp6 were each used to infect three monolayers of HeLa cells in 15 cm plates. Virus was purified by CsCl density gradient centrifugation and RNA was extracted as described (Racaniello and Baltimore, 1981b). cDNA was generated using MMLV reverse transcriptase (BRL) and oligo$(dT)_{12-18}$ as a primer in the buffer for *Taq* DNA polymerase (Perkin–Elmer Cetus) in a volume of 10 μl. Amplification was performed according to the directions of the manufacturer of *Taq* DNA polymerase (Perkin–Elmer Cetus) in a volume of 100 μl. The following P2/Lansing-specific primers were used for amplification: P2-735+ (plus-sense; nt 718–735), P2-2266 (plus-sense; nt 2247–2266) and P2-329 (minus-sense; nt 3439–3457). Amplified fragments were cleaved with restriction endonucleases, extracted from agarose gels and cloned into plasmids.

RNA synthesis and transfection

In vitro synthesis of RNA from DNA templates was performed 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/μl RNasin (Promega Biotech), 0.5 mg/ml bovine serum albumin (RNase and DNase free; Boehringer-Mannheim Biochemicals), 5 mM dithiothreitol, 40 mM Tris–Cl, 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 dishes using DEAE–dextran as facilitator (La Monica *et al.*, 1986).

Virulence assay

Viral virulence was assayed in normal Swiss–Webster mice and in TgPVR1-17 transgenic mice (Ren *et al.*, 1990). Groups of eight 3- to 4-week-old mice, four male and four female, were inoculated intracerebrally with 10-fold dilutions of virus in 50 μl, over the range $\sim 10^4 - 10^9$ p.f.u. Mice were observed daily for 21 days for paralysis or death. Paralyzed mice were killed and scored as dead. The amount of virus which caused paralysis or death in 50% of mice, LD_{50}, was calculated by the method of Reed and Muench (1938). When paralysis resulted, virus was isolated from the spinal cord of at least one infected mouse per virus, and the identity of the virus was confirmed by nucleotide sequencing of genomic RNA (data not shown).

Trypsin treatment of virions

P2/Lansing was purified by density gradient centrifugation as described previously ((Racaniello and Baltimore, 1981b). Approximately 1×10^9 p.f.u. of virus in 50 μl were mixed with 50 μl of trypsin–EDTA (10 mg/ml trypsin) (Gibco) and incubated at 25°C for 20 h. Cleavage of VP1 into two smaller fragments was monitored by SDS–PAGE (data not shown). To neutralize uncleaved virus, the preparation was incubated with anti-type 2 N-Ag1 monoclonal antibody 433 (Minor *et al.*, 1986). Virus was first diluted 1:100 in PBS plus 0.2% horse serum to inhibit trypsin, then mixed with an equal volume of a 1:500 dilution of ascites fluid. Trypsin-treated virus appeared resistant to neutralization as measured by plaque assay (data not shown).

Viral neutralization with antibodies

Equal amounts of virus in PBS plus 0.2% horse serum were mixed with a dilution of monoclonal or polyclonal antisera, incubated at 37°C for 60 min, and titrated by plaque assay. The anti-poliovirus type 2 monoclonal antibodies used were gifts from Drs F.Horaud and P.Minor and have been described (Minor *et al.*, 1986; Fiore *et al.*, 1987).

Acknowledgements

We thank D.Filman and J.Hogle for helpful discussions, S.J.Silverstein for comments on the manuscript, J.Hubbard for assistance with graphics, and R.Ren for providing PVR transgenic mice. This work was supported by Public Health Service grant AI-20017 to V.R.R. from the National Institute of Allergy and Infectious Diseases. Computer graphics were produced in the Molecular Modeling Facility, supported by National Science Foundation grant DIR-8720229.

References

Almond,J.W. and Burke,K.L. (1990) *Sem. Virol.*, **1**, 11–20.

Armstrong,C. (1939) *Pub. Health Rep.*, **54**, 2302–2305.

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Smith,J.A., Seidman,J.G. and Struhl,K. (eds) (1987) *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.

Bernstein,H.D., Sarnow,P. and Baltimore,D. (1986) *J. Virol.*,**60**, 1040–1049.

Bodian,D. (1959) In Rivers,T.M. and Horsfall,F.L. (eds), *Viral and Rickettsial Infections of Man*. Lippincott, Philadelphia, pp. 479–498.

Burke,K.L., Dunn,G., Ferguson,M., Minor,P.D. and Almond,J.W. (1988) *Nature*, **332**, 81–82.

Filman,D.J., Syed,R., Chow,M., Macadam,A.J., Minor,P.D. and Hogle,J.M. (1989) *EMBO J.*, **8**, 1567–1579.

Fiore,L., Pierangeli,A., Lombardi,F., Santoro,R., Crainic,R., Venuti,A. and Perez-Bercoff,R. (1987) *Intervirology*, **27**, 196–204.

Fricks,C.E. and Hogle,J.M. (1990) *J. Virol.*, **64**, 1934–1945.

Fricks,C.E., Icenogle,J.P. and Hogle,J.M. (1985) *J. Virol.*,**54**, 856–859.

Girard,M., Marc,D., Martin,A., Couderc,T., Benichou,D., Candrea,A., Crainic,R., Horaud,F. and van der Werf,S. (1990) In Brinton,M.A. and Heinz,F.X. (eds), *New Aspects of Positive-strand RNA Viruses*. American Society for Microbiology, Washington, DC, pp. 319–327.

Hogle,J.M. and Filman,D.J. (1989) *Phil. Trans. R. Soc. Lond. B*, **323**, 467–478.

Hogle,J.M., Chow,M. and Filman,D.J. (1985) *Science*, **229**, 1358–1365.

Hogle,J.M., Syed,R., Yeats,T.O., Jacobson,D., Critchlow,T. and Filman,D.J. (1989) In Notkins,A.L. and Oldstone,M.B.A. (eds), *Concepts in Viral Pathogenesis III.* Springer-Verlag, New York, Vol. 3, pp. 20–29.
Jubelt,B., Gallez-Hawkins,B., Narayan,O. and Johnson,R.T. (1980a) *J. Neuropathol. Exp. Neurol.*, **39**, 138–148.
Jubelt,B., Narayan,O. and Johnson,R.T. (1980b) *J. Neuropathol. Exp. Neurol.*, **39**, 149–158.
Krech,U. (1954) *Proc. Soc. Exp. Biol. Med.*, **54**, 344–346.
La Monica,N., Meriam,C. and Racaniello,V.R. (1986) *J. Virol.*, **57**, 515–525.
La Monica,N., Kupsky,W. and Racaniello,V.R. (1987) *Virology*, **57**, 429–437.
Li,C.P. and Schaeffer,M. (1954) *Proc. Soc. Exp. Biol. Med.*, **87**, 148–153.
Martin,A., Wychowski,C., Couderc,T., Crainic,R., Hogle,J. and Girard,M. (1988) *EMBO J.*, **7**, 2839–2847.
Minor,P.D., Ferguson,M., Evans,D.M.A., Almond,J.W. and Icenogle,J.P. (1986) *J. Gen. Virol.*, **67**, 1283–1291.
Minor,P.D., Ferguson,M., Phillips,A., Magrath,D.I., Huovilainen,A. and Hovi,T. (1987) *J. Gen. Virol.*, **68**, 1857–1865.
Minor,P.D., Dunn,G., Evans,D.M.A., Magrath,D.I., John,A., Howlett,J., Phillips,A., Westrop,G., Wareham,K., Almond,J.W. and Hogle,J.M. (1989) *J. Gen. Virol.*, **70**, 1117–1123.
Murray,M.G., Bradley,J., Yang,X.F., Wimmer,E., Moss,E.G. and Racaniello,V.R. (1988a) *Science*, **241**, 213–215.
Murray,M.G., Kuhn,R.J., Arita,M., Kawamura,N., Nomoto,A. and Wimmer,E. (1988b) *Proc. Natl. Acad. Sci. USA*, **85**, 3203–3207.
Palmenberg,A.C. (1989) In Semler,B.L. and Ehrenfeld,E. (eds), *Molecular Aspects of Picornavirus Infection and Detection.* American Society for Microbiology, Washington, DC, pp. 211–242.
Racaniello,V.R. (1984) *Virus Res.*, **1**, 669–675.
Racaniello,V.R. (1990) (ed.) *Picornaviruses.* Springer-Verlag, Berlin.
Racaniello,V.R. and Baltimore,D. (1981a) *Science*, **214**, 916–919.
Racaniello,V.R. and Baltimore,D. (1981b) *Proc. Natl. Acad. Sci. USA*, **78**, 4887–4891.
Reed,L.J. and Muench,H. (1938) *Am. J. Hyg.*, **27**, 493–497.
Ren,R., Costantini,F.C., Gorgacz,E.J., Lee,J.J. and Racaniello,V.R. (1990) *Cell*, **63**, 353–362.
Roivainen,M. and Hovi,T. (1988) *J. Virol.*, **62**, 3536–3539.
Sabin,A.B. (1966) *Ann. NY Acad. Sci.*, **61**, 924–938.

Received on January 21, 1991