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NOTES

Precise Missense and Silent Point Mutations Are Fixed in the Genomes of Poliovirus Mutants from Persistently Infected Cells

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Poliovirus mutants selected in persistently infected human neuroblastoma cells have a modified cell tropism and can establish a secondary persistent infection in nonneural cells, such as HEp-2c cells. Nucleotide sequence analysis revealed that the genome of a persistent mutant, S11, differed from that of the parental lytic Sabin 1 poliovirus strain by 31 point mutations. Three mutations occurred in the noncoding regions. The other mutations resulted in 12 amino acid substitutions; 1 substitution occurred in a nonstructural protein (3A), while the other 11 substitutions were clustered in the capsid proteins VP2 and VP1. The same missense mutations, as well as many of the silent mutations that we observed in mutant S11, also accumulated in the genome of two other persistent viruses isolated from independent infections. This finding indicates that both missense and silent mutations are selected during the persistent infection of neuroblastoma cells and suggests that the secondary structure of RNA in the coding region may play a role in viral infection.

Poliovirus (PV), the causative agent of paralytic poliomyelitis, is a member of the family *Picornaviridae*. The viral genome is a monocistronic single-stranded RNA molecule of about 7.5 kb, covalently linked to a small polypeptide, VPg, at the 5′ terminus and polyadenylated at the 3′ end. A large open reading frame encoding all of the structural and nonstructural proteins is surrounded by two noncoding (NC) regions. In the virion, the genome is protected by an icosahedral capsid composed of 60 copies of each of the four structural proteins, VP1 to VP4.

Although many picornaviruses produce persistent infections in vivo or in vitro (12, 17, 35), PV is usually considered to be a lytic virus because in vivo persistence has been demonstrated only in animals and in immunodeficient children (10, 25). However, a postpolio syndrome occurring decades after acute paralytic poliomyelitis has been known for more than a century (5, 9), and recent reports of antipolio immunoglobulin M antibodies in the cerebrospinal fluid of patients with the postpolio syndrome may be indicative of a persistent antigenic stimulation (33). Such a hypothesis can be studied at the molecular level with an in vitro model of PV persistence.

We previously developed such a model and showed that PV can induce a persistent infection in human neuroblastoma cells (8). Mutated viruses with altered phenotypic properties were isolated several months after the inoculation of neuroblastoma IMR-32 cells. These mutants had a modified cell tropism and could persistently infect cells of both neural and nonneural origin (30). By experiments in which HEp-2 cells were coinfected with lytic and persistent viruses, we showed that the lytic phenotype is dominant over the persistent phenotype during the establishment phase of persistence (4). From this fact, together with the high mutation frequency of the viral RNA genomes, which is on the order of 10^{-5} to 10^{-3} (36), it seems very probable that several point mutations are responsible for the PV persistent phenotype. To clarify the mechanisms of PV persistence and to determine the viral genes involved in such PV-host cell interactions, we cloned and sequenced the genome of a PV persistent mutant, S11, derived from the lytic attenuated Sabin 1 (S1) strain.

S11 genomic RNA was extracted from purified virions as described previously (30). The NC regions of S11 RNA were directly sequenced by the dideoxynucleotide technique (6, 32), and no mutation was found in the 5′ first 500 nucleotides. Single-stranded cDNA molecules were synthesized to clone the 5′, central, and 3′ parts of the genome, as shown in Fig. 1. The 5′ part of the cDNA between nucleotides 162 and 3560 was amplified by the polymerase chain reaction (PCR), using the Vent polymerase (New England Biolabs) with 100 ng of cDNA and 30 pmol of primers (23). The 5′ PCR-amplified fragment was separated from the primers by electrophoresis through an agarose gel and electroelution. This fragment was repaired with the Klenow enzyme to restore the blunt ends and then inserted into the vector pGEM-7Zf(+) (Promega), which had been linearized by *Sma*I. *Escherichia coli* DH5α was used for transformation. The recombinant plasmid containing the PV DNA corresponding to the 5′ part of the S11 genome was named pS11-5′, and bacterial colonies harboring it were white on plates containing isopropylthiogalactopyranoside (IPTG)–5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

The central part of the S11 cDNA, between nucleotides 2420 and 5641, was amplified in the same way by PCR, and a 3,131-bp fragment was isolated by *Nhe*I and *Bgl*II cleavage. This fragment was inserted into plasmid pAG0 (7) cleaved with the same enzymes. The 3′ part of the S11 genome, from nucleotide 5522 to the end of the genome, was amplified. Because one mutation (G to A) was found at nucleotide 7441, the complementary dT nucleotide was included in the 3′ primer, together with (i) an oligo(dT) tract

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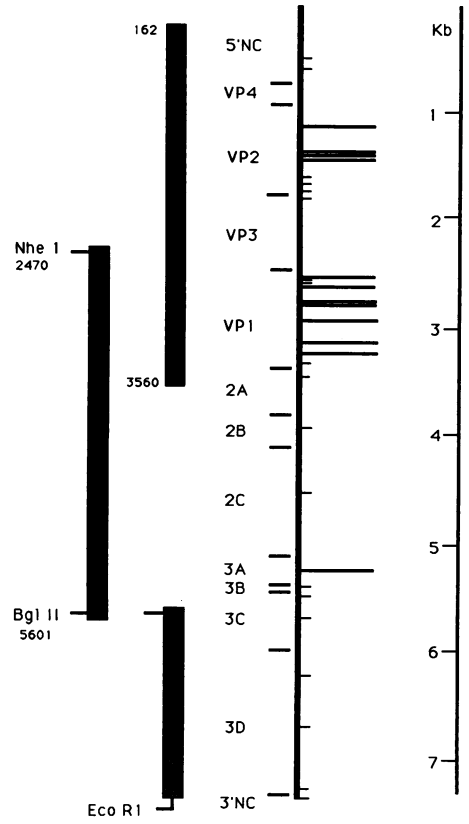

FIG. 1. The PV genome shown diagrammatically with the viral genes and the 5′ and 3′ NC regions. The grey vertical bars represent the cDNA fragments that were cloned and sequenced; they correspond to the 5′, central, and 3′ parts of the genome of the persistent PV mutant S11. The positions of primers used to amplify by PCR the 5′ part are 162 to 182 and 3537 to 3560. The positions of primers used to amplify the central part are 2420 to 2442 and 5618 to 5641. One of the primers used to amplify the 3′ part extends from positions 5522 to 5541. The other primer, IC2-EcoR I, included an *Eco*RI site and hybridized to 10 nucleotides of the poly(A) tail and to the last 6 nucleotides of the S11 genome (7436 to 7441). The preparation of the second primer took into account the mutation at position 7441 that had been found by sequencing directly the NC regions of the S11 genome. PCR was performed with the Vent DNA polymerase (New England Biolabs) according to the manufacturer's directions. The 5′ cDNA fragment was directly inserted into the *Sma*I site of the pGEM-7Zf(+) vector (Promega), while the central and 3′ fragments were first treated with the enzymes indicated before being cloned into pAG0 (7), cleaved with the corresponding enzymes. Mutations resulting in an amino acid substitution are indicated with a large horizontal bar to the right of the genome, while the other mutations are shown with a short bar.

able to hybridize with the viral poly(A) and (ii) an *Eco*RI site for the cloning (see legend to Fig. 1). The 3′ PCR fragment was treated with *Bgl*II and *Eco*RI so that a 1,855-bp fragment could be excised; this fragment was inserted into plasmid pAG0 (7) cleaved with the same enzymes. The recombinant plasmids containing PV DNA corresponding to the central and 3′ parts of the S11 genome were named pS11-c and pS11-3′, respectively. Bacteria harboring these plasmids were identified by their ampicillin resistance and tetracycline sensitivity, and the plasmids isolated from them were characterized by restriction enzyme analysis.

PV DNA in the plasmids was sequenced by using a Circumvent sequencing kit (New England Biolabs) (1), and

TABLE 1. Differences between the lytic S1 and persistent S11 PV strains[a]

Genome region	Nucleotide			Modified amino acid		
	Position	S1	S11	Position	S1	S11
5′ NC	525	U	C			
	606	U	C			
VP2	1148	G	A	67	Asp	Asn
	1370	A	G	141	Met	Val
	1373	C	U	142	His	Tyr
	1452	C	U	168	Thr	Ile
	1600	G	A			
	1693	A	G			
	1735	A	G			
VP3	1798	U	C			
VP1	2544	C	U	22	Thr	Met
	2566	C	U			
	2593	C	U			
	2607	C	U	43	Ala	Val
	2781	A	G	101	Lys	Arg
	2795	A	G	106	Thr	Ala
	2957	G	A	160	Val	Ile
	3147	C	U	223	Ala	Val
	3234	A	G	252	Lys	Arg
	3361	C	U			
2A	3481	C	U			
2B	3955	U	C			
2C	4558	C	U			
3A	5244	A	G	45	Asn	Ser
3B	5374	A	G			
3C	5470	U	C			
	5710	U	C			
3D	6262	A	G			
	6667	G	A			
	7324	G	A			
3′ NC	7441	G	A			

[a] In the 5′ NC region and in the regions of VP2, VP1, and 3A, all mutations of S11 have accumulated in the genome of two other persistent viruses isolated from independent infections. The mutation at position 7441 was found in only one of the two new isolates.

all of the regions of S11 RNA containing mutations were also sequenced directly, as described previously (6). Thirty-one mutations differentiating the S11 genome from the genome of the parental S1 strain maintained in our laboratory were found (Table 1). All of them were transitions, confirming that the PV polymerase makes this type of nucleotide substitution predominantly (19). Probably because of clonal variation, the sequence of the S1 strain in our laboratory differs from the published S1 sequence (35) at positions 355 (published, T; laboratory, C) and 6734 (published, G; laboratory, A). In the S11 genome, three mutations were found in the NC regions, at positions 525, 606, and 7441. The mutation at position 525 may influence the efficiency of translation initiation and play a role in cell tropism, because this nucleotide is known to interact with another at position 480 (29). It is also known that a mutation at or near the latter position affects viral translation and neurovirulence (21, 34). The mutation at position 606 occurred within a stem-loop structure predicted by models of the secondary structure of the 5′ NC region (34). This loop can be deleted from the S1 genome without altering the time course of virus growth in monkey kidney cells (20). However, this loop may participate in tissue-specific expression of PV, because it binds a cellular factor, p52 (24). The mutation at position 7441, which is the last nucleotide before the poly(A) tail, may be involved in the initiation of minus-strand RNA synthesis.

TABLE 2. Localization on the PV structure of the amino acid substitutions in the capsid polypeptides of S1-derived persistent viruses and possible effects on the antigenic sites of neutralization

Position of amino acid substitution	Localization on PV structure	Possible effect on[a]:	
		Antigenic site 1	Antigenic site 2
VP2 (67)	β strand B	−	−
VP2 (141)	E-F loop	−	−
VP2 (142)	E-F loop	−	−
VP2 (168)	E-F loop	−	+++
VP1 (22)	N terminus	−	−
VP1 (43)	N terminus	−	−
VP1 (101)	B-C loop	+++	−
VP1 (106)	β strand C	+	−
VP1 (160)	β strand E	+	−
VP1 (223)	G-H loop	−	+++
VP1 (252)	β strand I	+	−

[a] −, no likely effect; +, possible interaction between the substituted amino acid and the antigenic site; +++, the substitution is located within the antigenic site of neutralization.

The mutations at positions 525 and 7441 are both also probably implicated in the thermoresistant phenotype of S11, since they have already been found in the genome of thermoresistant mutants derived from the S1 strain (6).

In the coding region, 28 mutations were found, 12 of which are responsible for amino acid substitutions (Table 1). Among the 12 missense mutations, only 1 mutation occurred in the P3 region encoding nonstructural proteins, specifically in 3A (Fig. 1). The 3AB precursor of VPg is involved in the initiation of viral RNA replication (15). This mutation may thus be responsible for the reduced level of viral RNA synthesis detected in HEp-2 cells infected with S11 (4). The other 11 mutations were all found to be clustered in the regions encoding the capsid proteins VP2 and VP1 (Fig. 1). The mutation at nucleotide 2795, altering amino acid residue 106 of VP1, is a reversion to the wild-type Mahoney genotype, and this mutation was correlated to the thermoresistant phenotype of the virus (6). Six of the other capsid mutations were localized within known antigenic sites of neutralization or in regions thought to interact with these sites (27) (Table 2). Thus, as previously shown for foot-and-mouth disease virus, antigenic variation is not necessarily the result of immune selection (12).

Because of the large number of mutations in the genome of persistent viruses and of the probable contribution of several mutations to the persistent phenotype in HEp-2 cells, it is difficult to determine the relative importance of specific mutations in this phenotype. However, our results suggest that the majority of these determinants are localized in the regions encoding capsid proteins VP2 and VP1. Host range determinants of PV were found at the amino terminus of VP1, near the threefold axis of symmetry of the capsid (26). It has been proposed that the amino terminus of VP1 is externalized after interaction with the host cell (14) and that the same region is involved in RNA release and RNA packaging (16). Other host range determinants were mapped in the region of neutralization antigenic site 1 in the B-C loop of VP1, near the fivefold axis of symmetry (22, 28). Thus, some of the mutations inducing amino acid substitutions in VP1 at positions 22, 43, and 101 are probably involved in the modified cell tropism of S11 and in the capacity to establish a persistent infection in HEp-2 cells. In agreement with this hypothesis, a locus situated in the VP1 region of Theiler's virus was found to be involved in persistence in vivo (37).

To determine whether the same or different mutations would be selected in independent persistent infections of neuroblastoma cells, two other persistent infections were established by infecting IMR-32 cells at multiplicities of 10 and 10^{-2} 50% infective doses per IMR-32 cell with a different S1 strain, recovered by transfection with plasmid pVS(1)IC-O(T) (18). Ten weeks after infection, when cytopathic effects had disappeared, persistent viruses were isolated and their genomes were sequenced in the 5′ and 3′ NC regions and in the regions of VP2, VP1, and 3A, where mutations had been found in S11. The genome of the new isolate recovered from IMR-32 cells infected at the highest multiplicity was identical to that of S11 in all regions sequenced: 3 mutations were found in the NC regions, and 12 missense mutations and 6 silent mutations were found in the VP2, VP1, and 3A regions. In the genome of the other new isolate recovered from cells infected at the lowest multiplicity, all of these mutations except the one at position 7441 were also found. Some supplementary mutations (at positions 1365, 1455, 1467, 1757, and 2596), not present in the genome of S11, were observed. Nucleotide changes in other regions of the genome (in addition to those identified) could formally be contributing to the phenotype of the new isolates. However, these results indicated that precise missense and silent mutations had been consistently selected in the viral genomes during the persistent infection of neuroblastoma cells. Because of the extremely heterogeneous nature of RNA genomes, the sequence of S11 could be considered to represent a kind of consensus sequence (13). The selection of precise silent mutations in the PV genome has been reported (11), but our results emphasize the importance of the host cell in this selective process. The silent mutations may play a role in altering the secondary structure of viral RNA. Such a role, which may have been underestimated for the coding region, is well documented for the PV 5′ NC region (2, 34).

The organ-specific selection of viral variants during chronic infection has been demonstrated in vivo in carrier mice infected with lymphocytic choriomeningitis virus (3, 31). Similarly, because the persistent PV mutants isolated from neuroblastoma cells have a modified cell tropism, we propose that the selection of mutants adapted to cells of neuronal origin is correlated to their reduced lytic potential in nonneural HEp-2 cells and thus to their capacity to establish a persistent infection in these cells. The work reported here provides a basis for the construction of recombinant viruses to identify, among the mutations that were found in the genome of the PV mutants selected in cells of neural origin, those responsible for the persistent phenotype in HEp-2 cells.

In conclusion, we have shown that the majority of missense mutations selected during a PV persistent infection of human cells of neural origin is localized in the regions encoding capsid proteins VP2 and VP1. Our results also underline the importance of precise silent point mutations in viral infection of these cells.

Biohazards associated with the experiments described in this publication have been examined previously by the French Commission de Génie Génétique, and the experiments were carried out according to the rules established by this committee.

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