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Identification of a Region of the Poliovirus Genome Involved in Persistent Infection of HEp-2 Cells

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Poliovirus mutants were selected during the persistent infection of human neuroblastoma cells. These viruses could establish secondary persistent infections in HEp-2 nonneural cells. We report the identification of a region of the genome of a persistent virus (S11) that was sufficient to confer to a recombinant virus the phenotype that causes persistent infection in HEp-2 cells. This region, between nucleotides 1148 and 3481, contained 11 missense mutations mapping exclusively in the genes of capsid proteins VP1 and VP2. Because recombinant viruses carrying only one of these two mutated genes were not able to cause persistent infection, it seems very probable that two or more mutations in these genes are required for expression of the phenotype that causes persistent infection.

Members of the *Picornaviridae* are important human and animal pathogens. Their genome is single-stranded RNA of positive polarity. A small viral polypeptide is covalently linked to the 5′ end of the genome, and the 3′ end is polyadenylated. A large coding region is flanked by one noncoding (NC) region at either end of the RNA. The viral genome is enclosed in an icosahedral capsid composed of the four structural polypeptides VP1 to VP4. Several members of the *Picornaviridae* are involved in persistent infections in their natural animal hosts, for example foot-and-mouth disease virus in cattle and Theiler's encephalomyelitis virus in mice (4, 7, 9, 26). The hepatovirus human hepatitis A virus induces steady-state infections in cultured cells and can persist for several months in natural or experimental hosts (10, 17, 25). Among the enteroviruses, coxsackievirus B3 can persistently infect the heart muscle of mice, thus inducing myocarditis (15).

Poliovirus (PV) is usually thought to be an extremely lytic virus, and reports of PV persistence in murine models and in immunodeficient children appear as exceptions (8, 12, 19). Recently, however, anti-poliovirus immunoglobulin M antibodies were detected in the cerebrospinal fluid of the majority of patients with the postpoliomyelitis syndrome (24). These results are consistent with the presence of persistent antigenic stimulation, giving rise to an anti-PV immune response in the central nervous system. In agreement with this hypothesis, we showed some years ago that PV can induce a persistent infection in human cells of neural origin, neuroblastoma cells (6). This in vitro cell system appeared to be a good model with which to investigate the ability of PV to induce persistent infections. During the persistent infection of neuroblastoma cells, mutated viruses were selected, and their phenotypic properties differed greatly from those of the parental lytic strains. In particular, the PV mutants isolated from persistently infected neuroblastoma cells had a modified cell tropism. Two kinds of criteria were used to evaluate modifications of cell tropism. The first was the ratio of infectious titers on nonneural HEp-2 and neuroblastoma IMR-32 cells. The second was the size of plaques on nonneural HEp-2 cells (the size of plaques on neuroblastoma cells was not taken into consideration because it was similar for persistent PV mutants and the parental strains [22]). Another remarkable property of the PV mutants was that they could establish a secondary persistent infection in cells of nonneural origin, such as HEp-2 cells (22). We chose the HEp-2 cell system for preliminary investigations of the mechanisms of in vitro PV persistence because, with this cell line, we had both persistent viruses and the corresponding parental lytic strains. In neuroblastoma cells most of the viruses induced persistent infections, thus making the comparison impossible (2). One persistent mutant, S11, was isolated from IMR-32 neuroblastoma cells 6 months after inoculation with the vaccine Sabin 1 (S1) strain. The genome of S11 harbors 31 point mutations (3). There were 3 mutations in the NC regions of the genome, 12 missense mutations (11 in the regions encoding viral capsid proteins and 1 in the 3A region), and 16 silent mutations in the large coding region. Most missense and silent mutations were repeatedly and consistently selected following independent infections of neuroblastoma cells (3). On the basis of these sequence data only, it was not possible to evaluate the relative significance of the various mutated regions in the phenotype of the S11 PV mutant that caused persistent infection in HEp-2 cells.

To determine which regions of the genome play a role in persistence and cell tropism, we have constructed recombinant viruses, whose genome is composed of S11 and S1 sequences. The plasmid pVS(1)IC-0(T) (abbreviated here as pVS1), kindly provided by A. Nomoto, University of Tokyo, Tokyo, Japan, carries an infectious full-length cDNA copy of the S1 genome (16). The S11 genome has been previously cloned as three subgenomic fragments, plasmids pS11-5′, pS11-c, and pS11-3′, which carry the 5′, central, and 3′ parts of S11 viral cDNA, respectively (3). An *Age*I-*Nhe*I DNA fragment, from nucleotides 339 to 2470 on the PV map, was excised from pVS1 and replaced by the corresponding S11 DNA fragment isolated from plasmid pS11-5′ (3) (Fig. 1). The recombinant plasmid pVS1/11-5′ carries 10 of the mutations from the genome of the persistent virus S11: 2 mutations in the 5′ NC region, 4 missense and 3 silent mutations in the VP2 region, and 1 single silent mutation in the VP3 region. Plasmid pVS1/11-C1 was similarly constructed. A *Ban*II fragment (nucleotides 909 to 3523) was excised from pVS1 and replaced by the corresponding S11 DNA fragment, isolated from plasmid pS11-5′ (3). The recombinant plasmid pVS1/11-C1 carries 19 mutations from

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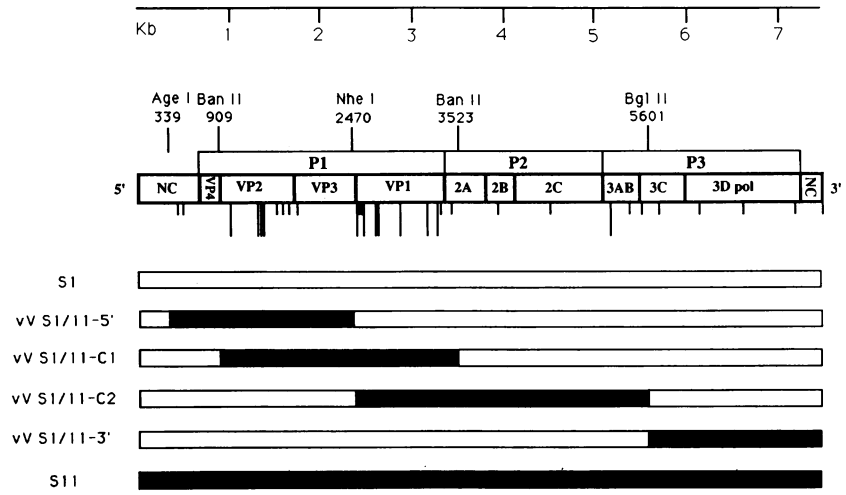

FIG. 1. Genetic maps of recombinants between the lytic S1 and the persistent S11 virus strains. The genomic organization of PV is shown diagrammatically, with the viral genes and the 5′ and 3′ NC regions (14). The restriction sites used for the construction of recombinant cDNAs are indicated. The positions of missense mutations in the VP2, VP1, and 3AB regions of the genome of the persistent virus S11 are indicated, with long vertical bars under the corresponding sites. The positions of silent mutations are indicated by short vertical bars.

S11 (Fig. 1). The 11 missense mutations are only in the VP2 and VP1 genes, and 8 silent mutations are scattered in the regions of VP2 to 2A. To construct pVS1/11-C2, the plasmid pVS1 was cleaved with *Nhe*I and *Bgl*II (nucleotides 2470 and 5601) and the central one-third of the viral cDNA was replaced by the corresponding S11 DNA fragment from plasmid pS11-c (3). The fragment inserted into plasmid pVS1/11-C2 carries the seven missense mutations from VP1, the single missense mutation in the 3AB region, and eight silent mutations in regions encoding VP1 to 3C (Fig. 1). Plasmid pVS1/11-3′ was constructed by replacing the 3′ part of S1 cDNA in plasmid pVS1, between the *Bgl*II site (nucleotide 5601) and a *Cla*I site downstream of the poly(A) tract, with the corresponding S11 DNA fragment (3). The recombinant cDNA in pVS1/11-3′ has no missense mutation, but it does have four silent mutations and the S11 mutation of the 3′ NC region (Fig. 1).

The viruses vVS1/11-5′, vVS1/11-C1, vVS1/11-C2, and vVS1/11-3′, derived from the four recombinant plasmids described above, were isolated by transfection of human neuroblastoma IMR-32 cells by using the calcium technique. Viruses were amplified by a single passage on IMR-32 cells at 34°C before being studied (this cell system was chosen to avoid counterselection of the mutations). The presence of mutations in the insertions of the recombinant viral genomes was verified by direct sequencing of the RNA of the viruses recovered by transfection, as previously described (22). Similarly, the absence of mutations outside the insertions was verified by sequencing the viral RNA in the vicinity of the insertions.

The titers of virus stocks were determined on nonneural HEp-2 cells and on neuroblastoma IMR-32 cells by using a micromethod, and the results are expressed as the ratio of titers on the two cell lines (Table 1). The titers of the recombinant virus vVS1/11-3′ and of the lytic S1 strain were about twofold higher on HEp-2 cells than on IMR-32 cells. In agreement with this, the recombinant vVS1/11-3′, like strain S1, produced large plaques on HEp-2 cells (Table 1). These results suggested that, in vVS1/S11-3′, the four silent mutations of the 3C-3D regions and the mutation of the 3′ NC region did not play a major role in cell tropism. The titers of vVS1/11-5′ and of vVS1/11-C2 were similar on the two cell lines, and these viruses produced small plaques on HEp-2 cells (Table 1). Therefore, the phenotypes of vVS1/11-5′ and vVS1/11-C2 were intermediate between those of the two parental strains S1 and S11, suggesting that the genomic regions derived from S11 in these recombinants harbor determinants of cell tropism. These determinants could be mapped to regions defined by the mutations in the recombinant plasmids (3). The first should be between nucleotides 525 and 1798 (in vVS1/11-5′), and the second should be between nucleotides 2544 and 5470 (in vVS1/11-C2). The titer of vS1/11-C1 was about 18-fold lower on HEp-2 cells than on IMR-32 cells (Table 1). In agreement with this, plaques of vVS1/11-C1, like those of the persistent virus S11 on HEp-2 cells, were extremely small (Table 1). This indicated that, compared with the lytic S1 strain, vVS1/11-C1 had a strongly modified cell tropism. The viruses vVS1/S11-5′ and vVS1/S11-C2 carry different regions of the S11 genome. Both these regions appear to be involved in cell tropism. It is therefore probable that the vVS1/S11-C1 region

TABLE 1. Phenotypic markers related to cell tropism of the recombinant viruses between the lytic S1 and persistent S11 strains[a]

Virus	Ratio of titers on HEp-2 and IMR-32 cells[b]	Avg plaque diam on HEp-2 cells[c]	Persistent infection in HEp-2 cells[d]
S1	1.8	1.6	−
vVS1/11-5′	0.8	0.8	−
vVS1/11-C1	0.056	0.2	+
vVS1/11-C2	1.0	0.3	−
vVS1/11-3′	2.0	1.4	−
S11	0.025	0.2	+

[a] The means of two to four independent experiments are given.
[b] Titers are expressed as ID_{50} per milliliter.
[c] Virus-infected monolayers were stained after 4 days of incubation at 34°C under an agar overlay. Each result is the average of 40 plaque measurements (in millimeters).
[d] Defined as the growth of cells for more than 3 months after infection, with a continuous production of virus (infectious titer > 10^6 ID_{50}/ml).

from VP2 to 2A has at least two mutations involved in cell tropism, with a cumulative effect. These should map between the 5′- and 3′-most mutations of vVS1/S11-C1, i.e., between nucleotides 1148 and 3481.

All four recombinant viruses, like their parental strains, induced persistent infections in neuroblastoma IMR-32 cells (data not shown). On HEp-2 cells, at a multiplicity of infection of 10^{-2} 50% infectious dose (ID_{50}) per cell, vVS1/11-3′, like the lytic S1 strain, generated cytopathic effects and provoked cell death within 4 days. Both vVS1/11-5′ and vVS1/11-C2 had a phenotype intermediate between those of the lytic S1 and the persistent S11 strains: the cytopathic effects of the recombinant viruses were delayed compared with those of S1, and cultures were not entirely lysed before 8 to 10 days. However, under the conditions that we used, we did not succeed in establishing a persistent infection in HEp-2 cells with either of these viruses (Table 1). Of the four recombinant viruses, vVS1/11-C1 was the least lytic for HEp-2 cells. No cytopathic effects were detected before 3 to 4 days after infection, and cells survived infection for more than 3 months in the presence of virus at high titers ($>10^6$ ID_{50}/ml). Persistent infections were established as efficiently with this recombinant virus as with the persistent S11 strain (Table 1).

Because virus vVS1/11-C1 was the only recombinant capable of establishing a persistent infection in HEp-2 cells, it seems highly probable that the determinants of cell tropism, responsible for the reduced lytic potential on HEp-2 cells, are also related to its mutant phenotype allowing it to cause persistent infections in the same cells. The relation between the tissue-specific selection of mutant viruses and a persistent infection has already been described with another virus, lymphocytic choriomeningitis virus (1, 23).

We have previously shown that particular missense and silent point mutations were repeatedly selected in the viral genomes during persistent infections of neuroblastoma cells. This indicated that there was some sort of selective pressure for silent mutations in neuroblastoma cells, perhaps as a result of effects in the secondary structure of the viral RNA (3). There is no evidence that silent mutations are necessary for persistence in HEp-2 cells. If one assumes that silent mutations do not play a major role in the establishment of persistent PV infections in HEp-2 cells, the determinants of persistence in vVS1/S11-C1 map between nucleotides 1148 and 3234, in the regions coding for VP2 and VP1. These two regions contain 11 missense mutations. Because the amino-terminal part of VP1 is thought to play a role in early steps after adsorption (11, 20), release of viral RNA, and encapsidation (13), it is possible that mutations at nucleotides 2544 and 2607, responsible for the substitution of amino acids 22 and 43 of VP1, modify cell tropism and contribute to persistence by affecting one of these steps. Another region of VP1, the neutralization antigenic site 1, contains host range determinants (18, 21). Therefore, mutation at position 2781, modifying amino acid 101 of VP1 in neutralization antigenic site 1, may play a role in cell tropism and persistence in HEp-2 cells. The mutation at nucleotide 2795, modifying amino acid 106 of VP1, is a true reversion toward the Mahoney wild-type genotype; it has been observed in several type 1 PV strains that are fully lytic for HEp-2 cells (5). Therefore, this mutation is presumably not directly involved in the persistent phenotype of vVS1/11-C1. However, the individual role of each missense mutation, the potential role of silent mutations, and their possible synergistic effect remain to be defined.

In conclusion, we have shown that the genome of a PV persistent mutant, S11, has at least two determinants of cell tropism. A region of the genome of S11, between nucleotides 1148 and 3481, containing 11 missense mutations mapping only to the VP1 and VP2 regions (3), was sufficient to confer to a recombinant virus the phenotype allowing it to cause persistent infections in HEp-2 cells. Because the recombinant viruses carrying only one of these two mutated genes did not display this phenotype, it seems very probable that mutations in both of these genes are required.

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

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