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Addition of a foreign oligopeptide to the major capsid protein of poliovirus

(neutralization antigenic site/cDNA mutagenesis/infectious cDNA transfection)

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ABSTRACT **Insertion mutants of type 3 poliovirus (Sabin strain) were constructed that encode additional amino acid sequences at the level of residue 100 of the capsid polypeptide VP1 within the neutralization site 1, corresponding to a loop on the capsid surface. The addition of a tri- or hexapeptide did not hamper virus viability. The antigenic pattern of insertion mutants was only modified locally: all mutants lost reactivity of neutralization site 1 with the corresponding monoclonal antibodies, while the reactivity of sites 2 and 3 was unaffected by the insertion. We have shown for one of the mutants—vFG68—that the antigenic specificity of the neutralization site 1 was replaced by a new one. Although vFG68 differs from its parental Sabin strain only by the addition of three amino acids within VP1, neutralizing antibodies specific for vFG68 were induced by the native virion as well as by the heat-denatured mutated virions. Our results demonstrate that an oligopeptide of three or six amino acids can lengthen VP1 at the level of antigenic site 1 without affecting virus multiplication and that this foreign peptide is exposed on the virion surface.**

Poliovirus (PV), a member of the picornavirus family, has a tight crystalline structure. The icosahedral capsid is composed of 60 capsomeres, each constituted by one copy of the four polypeptides VP1, VP2, VP3, and VP4 (1). The virion contains a single-stranded RNA molecule about 7500 nucleotides long, the 5′ extremity of which is blocked by a small polypeptide, VPg (2, 3). The mutagenesis by oligonucleotide insertion into the noncoding and nonstructural regions of the poliovirus genome has been performed (4-7), but the addition of a foreign sequence to the capsid protein coding region has never been reported to our knowledge. Study of the PV capsid structure has revealed that an amino acid loop in VP1, containing residue 100, is exposed on the virion surface (8) and harbors neutralization antigenic site 1 (9, 10). This site is immunodominant for the mouse in the case of PV types 2 and 3, while it is immunorecessive for type 1 (10). Since the demonstration of the infectivity of PV cDNA in animal cells (11), several groups have constructed intertypic recombinants involving antigenic site 1 borne by the VP1 loop (12–14). These constructions are based on the replacement of a few amino acids of one type by the homologous amino acids of another type. Because of the tight structure of PV capsid, it appears important to know whether foreign sequences could be added to VP1, rather than just substituted, without impairing virus viability. One advantage of such a construction would be that the foreign sequence does not necessarily have to be of picornavirus origin.

As a first step in this study, we recovered infectious virus after insertion of synthetic oligonucleotides within the VP1 coding region. We have selected as site of insertion the codon for amino acid 100 of VP1 located within the VP1 loop harboring the antigenic site 1, so that the insertion would not generate physical constraints impairing capsid structure and would be well exposed on the virion surface. We report here the isolation and biological and immunological characterization of recombinant viruses that have various additional amino acid sequences within the major structural polypeptide of their capsid. Immunological analysis of one of these recombinant viruses revealed that, by inserting a tripeptide within the capsid polypeptide VP1 of PV type 3, we created a new viral immunogen, which is conserved upon heat denaturation of virions.

MATERIALS AND METHODS

Materials. The FLC3 plasmid (15, 16) carrying a full-length copy of the P3/Leon 12 a_1b Sabin strain cDNA was a generous gift from J. Almond (University of Reading, U.K.). The *Escherichia coli* strain HB101 (F^-, *hsdS20* (r_B^-, m_B^-), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*(SM^r), *xyl-5*, *mtl-1*, *supE44*, λ^-) was used for bacterial transformation (SM^r = resistance to streptomycin). The large fragment of DNA polymerase I (Klenow) was from United States Biochemical, Cleveland, and kinase, phage T4 DNA ligase, *Sma* I and *EcoRI* were from Appligène (Illkirch, France). Vero cells were used for transfection and virus cloning. HEp-2 cells were used to prepare virus stocks for RNA sequencing and protein analysis. The monoclonal antibodies (mAbs) IIIo, 3o, 3a, 3b, IIIa, and C3 have been described (9, 17). mAb 204, 877, and 868 were generous gifts from M. Ferguson (National Institute for Biological Standards and Control, Potters Bar, U.K.) (10).

Recombinant Virus Isolation. Plasmid FLC3 was linearized with *Sma* I, and the extremities were repaired with Klenow enzyme. The oligonucleotide 5′-GGAACAAGC-3′ and its complementary strand were hybridized, treated with kinase, and ligated to the linearized vector in a molar ratio of 100:1. The ligation mixture was used to transform *E. coli* strain HB101, and recombinant plasmids were identified by restriction analysis with *Sma* I and DNA sequencing (18). Recombinant virus was recovered after transfection of Vero cells with recombinant plasmids by using the calcium phosphate technique as described (19, 20). Virus stocks were prepared from a single plaque for each recombinant virus, and the RNA genome was sequenced in the region of the insertion by using the dideoxy termination method of Sanger *et al.* adapted to the RNA (18, 21).

Analysis of Virus Structural Polypeptides. The capsid proteins of recombinant viruses, labeled with [^{35}S]methionine and purified in CsCl density gradient, were analyzed by 0.1% $NaDodSO_4$/12.5% PAGE as described (9, 22). In some

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Abbreviations: mAb, monoclonal antibody; PV, poliovirus.
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experiments, virus was treated with trypsin from bovine pancreas (Sigma) at a concentration of 25 μg/ml for 2 hr at 37°C prior to electrophoresis (23).

RESULTS

Construction of Insertion Mutants of PV. To find out whether the surface loop including amino acid 100 of the Sabin PV type 3 capsid protein VP1 (8, 10) could accommodate an additional foreign amino acid sequence, we constructed several insertion mutants. An oligonucleotide of nine base pairs was inserted into plasmid FLC3 (16) linearized by *Sma* I (at nucleotide 2768 of the PV type 3 cDNA) in the VP1 coding sequence (Fig. 1). The site of insertion corresponds to the codon encoding Arg-100 of VP1, which is sensitive to trypsin digestion in PV type 3 (23). Since the oligonucleotide could be ligated to the vector in either orientation, the foreign oligopeptide inserted into VP1 could be Asn-Lys-Arg, Leu-Phe-Arg, or multimeres of these sequences after reconstitution of the natural Arg-100 residue (Fig. 1). We chose the oligonucleotide 5'-GGAACAAGC-3' and its complementary strand because, in the first orientation, the Asn-Lys sequence corresponds to amino acids 100 and 101 of VP1 of the Mahoney and Sabin 1 strains of PV type 1. These amino acids are part of the continuous neutralization epitope C3 of antigenic site 1 (9). In the first orientation of the insertion (Asn-Lys-Arg), two positive charges are added to VP1. In the opposite orientation (Leu-Phe-Arg), the amino acid insertion adds one positive charge and two hydrophobic amino acids to VP1 of PV type 3.

The two complementary oligonucleotides were hybridized, treated with kinase, and ligated to the FLC3 vector linearized with *Sma* I in the VP1 coding sequences as described. Recombinant plasmids, among which pFG68, pFG27, and pFG13 (Fig. 1) were identified on the basis of their *Sma* I restriction resistance, and the orientation and number of insertions were determined by direct sequencing of the plasmid by the dideoxy termination method (18). Corre-

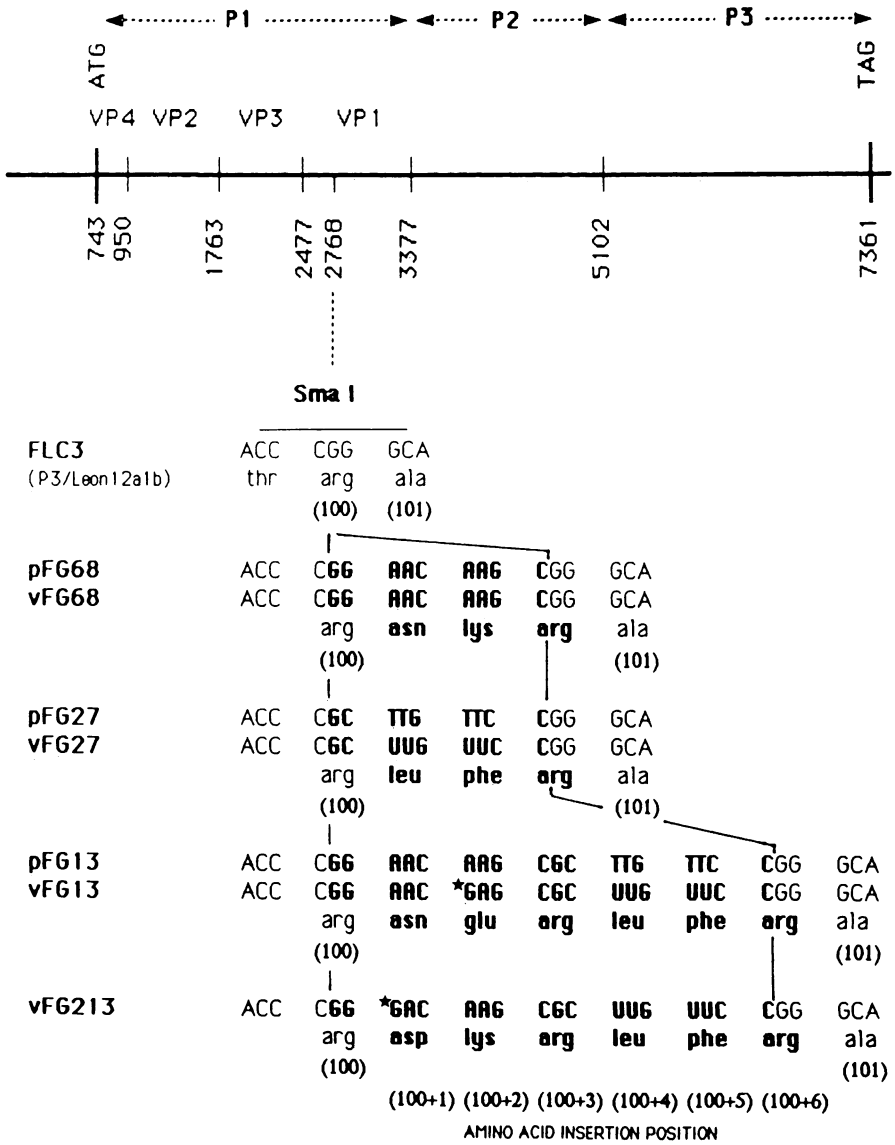

FIG. 1. Construction of PV insertion mutants. The map of P3/Leon $12a_1b$ cDNA (15, 16) is shown with the capsid polypeptides coding region *P1* and the nonstructural viral polypeptides coding regions *P2* and *P3*. Nucleotide numbers corresponding to the beginning of coding regions for these precursor proteins are indicated, as well as those delimiting the capsid proteins VP4, VP2, VP3, and VP1. The unique *Sma* I site at nucleotide 2768 in the coding region for VP1 was used for oligonucleotide insertion (bold face). At the insertion level, the sequences of the recombinant plasmids and of the corresponding RNA genome of insertion mutants are shown. The vFG13 and vFG213 genomes differed from the pFG13 sequence by one nucleotide, indicated with an asterisk. The amino acids encoded by the insertions are given and numbered from 100 + 1 to 100 + 6.

sponding recombinant viruses vFG68, vFG27, vFG13, and vFG213 were recovered 2–6 wk after transfection of Vero cells. All recombinant viruses were cloned by isolation of a single plaque in agarose, and the *in vitro* phenotypic markers were determined for each virus within the five first high-multiplicity passages after cloning.

The RNA genome of each recombinant was sequenced at the level of the insertion (Fig 1). vFG68 had one insertion in the first orientation (Asn-Lys-Arg). The vFG27 recombinant had one insertion in the opposite orientation. For both vFG68 and vFG27, the plasmid and the respective viral genomic sequence were similar (Fig. 1). Plasmid pFG13 carried two copies of the insertion in opposite orientations. Two viruses, vFG13 and vFG213, were isolated by transfection of pFG13. In both cases, the sequence of the viral RNA differed from the plasmid sequence by one nucleotide, shown with an asterisk in Fig. 1. These point mutations induced a lysine > glutamic acid substitution (insertion position 100 + 2) and an asparagine > aspartic acid substitution (insertion position 100 + 1) in the insertions of vFG13 and vFG213. Except where mentioned, vFG13 and vFG213 had similar biological properties, so the results are given for the vFG13 virus clone only.

Phenotypic Analysis of Insertion Mutants. The mean plaque size of vFG68 was almost identical to that of the parental Sabin strain in Vero cells (Table 1) and HEp-2 cells (not shown). vFG27 and vFG13 formed plaques smaller by a factor of 6 (Table 1), which may correlate with the addition of two hydrophobic amino acids into the VP1 polypeptide of these viruses. This difference in the plaque diameter under agarose might be due rather to a slower cell-to-cell diffusion of the virus than to a lower growth rate or virus yield per cell, as suggested by a standard one-step growth experiment (Fig. 2). vFG213 had a small plaque phenotype like vFG13, although the plaque diameter was slightly more heterogeneous.

Virus inactivation at 45°C was determined as this gives an indication of capsid stability of the insertion mutants. Although the logarithmic infectivity of the parental strain dropped by about 0.5 $\log_{10}$ in 10 min at 45°C (Table 1), the logarithmic infectivity of the recombinants decreased by 1–2 $\log_{10}$, indicating that their capsid is less heat stable than the parental one. As expected, the multiplication of all four viruses was restricted at supraoptimal temperature: the three insertion mutants are negative for reproductive capacity at supraoptimal temperature (*rct*$^-$) like the parent P3/Leon12a$_1$b strain (24, 25) (Table 1).

Under denaturing conditions, the migration of VP1 in 0.1% $NaDodSO_4$/PAGE was delayed for vFG68 and vFG13 as compared to vFG27 and the parental strain (Fig. 3). For all three recombinants, as for P3/Leon12a$_1$b, VP1 was sensitive to trypsin treatment of intact virions without affecting viral infectivity. Trypsin cleaves VP1 of the Sabin strain at Arg-100, generating two peptides of about 11 and 23 kDa (23), the latter only being labeled with [^{35}S]methionine. In the recombinants, the codon for Arg-100 was reconstituted, and arginine residues were inserted in one (position 100 + 3 in vFG68 and vFG27) or two (position 100 + 3 and 100 + 6 in vFG13 and vFG213) positions in VP1 (Fig. 1). One or several of these residues are thus a likely target for trypsin cleavage of VP1. Since the apparent molecular weight of the larger peptide generated by trypsin cleavage varied relative to the foreign oligopeptide present (Fig. 3), the latter was most probably not excised by trypsin cleavage of VP1.

Table 1. *In vitro* phenotypic markers of PV type 3 insertion mutants

Virus	Mean plaque diameter,* mm	Logarithmic virus inactivation after 10 min at 45°C†	rct: titer at 34°C − titer at 40°C‡
P3/Leon 12a$_1$b	7.9	0.47	5.6
vFG68	6.9	1.73	5.4
vFG27	1.1	1.10	5.3
vFG13	1.1	1.23	5.1

*Virus-infected Vero cell monolayers were stained after 4 days of incubation at 34°C under an agar overlay. Each result is the average of 10–20 plaque measurements.
†Data are means of three independent experiments.
‡Reproductive capacity at supraoptimal temperature (rct) (24) is the difference in titer in ID_{50} per ml in HEp-2 cells after 7 days of incubation at permissive (34°C) and supraoptimal (40°C) temperatures.

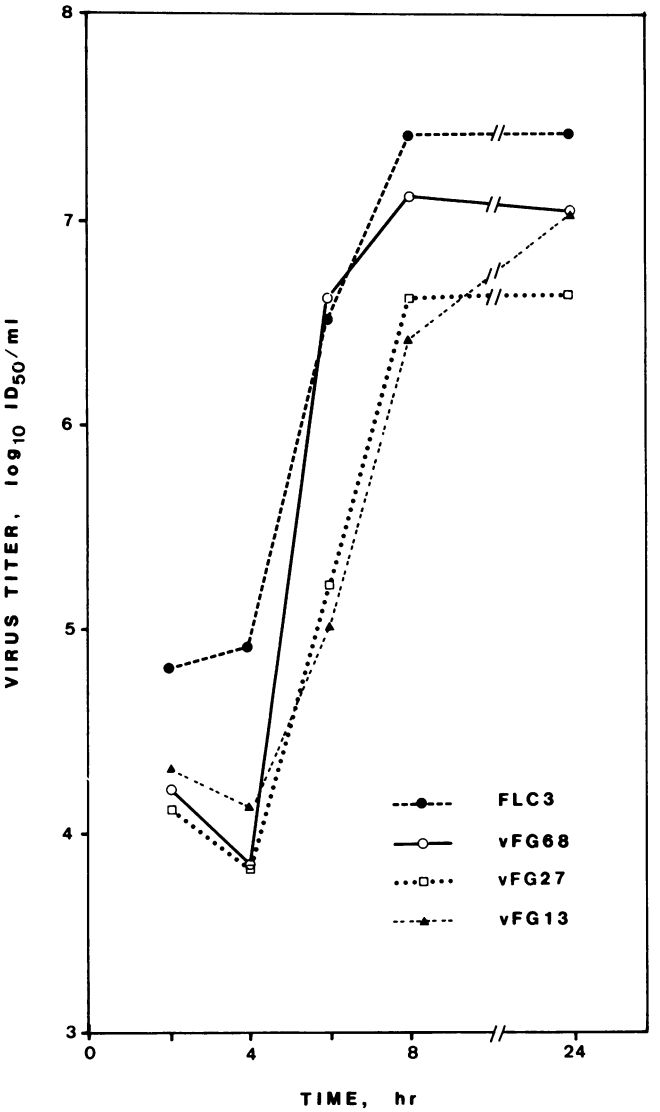

FIG. 2. One-step growth experiment with PV insertion mutants and the parental strain P3/Leon12a$_1$b (FLC3). HEp-2 cells were infected at a multiplicity of 20 ID_{50} per cell. Virus was recovered from infected cells after freezing and thawing at the times indicated after infection and was titered.

Antigenicity and Immunogenicity of the Insertion Mutants. The neutralization epitope map of the mutants was established with mAb against the three known antigenic sites involved in neutralization of poliovirus type 3 (10, 17). The three insertion mutants had a Sabin epitope carried by VP3 (amino acid 59) and were neutralized by an anti-type 3 (Saukett) antiserum (Table 2). The antigenic site 1 carried by VP1 (amino acids 89–100) (10) was destroyed by the insertion of three or six foreign amino acids. On the contrary, sites 2B and 3A–3B (10) were, as expected, not modified by the

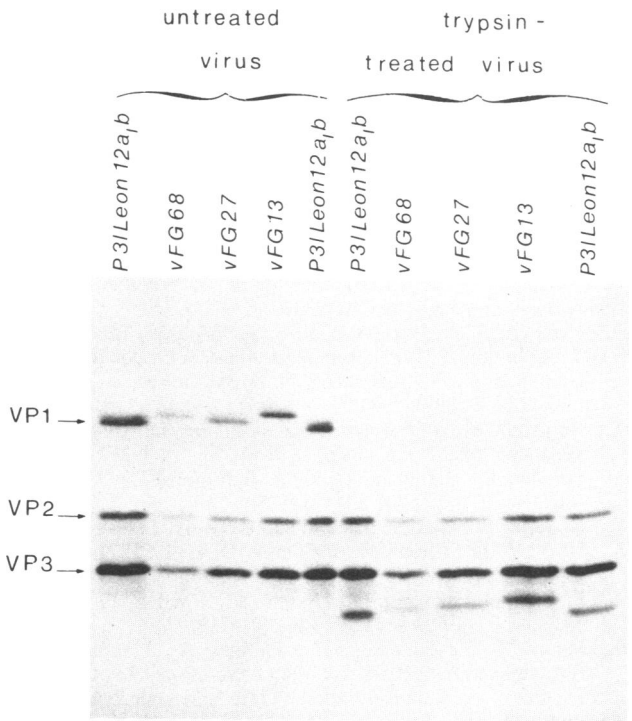

FIG. 3. Analysis of capsid polypeptide encoded by insertion PV mutants. After purification on CsCl density gradients, capsid polypeptides of [^{35}S]methionine-labeled virions were analyzed by 0.1% $NaDodSO_4$/12.5% PAGE before and after trypsin treatment as described (22, 23). Capsid proteins VP1, VP2, and VP3 are indicated by arrows.

insertion. The C3 epitope (9), which is part of the antigenic site 1 of PV type 1, includes the dipeptide Asn-Lys at positions 100 and 101. In vFG68, the dipeptide Asn-Lys occupies similar positions (100 + 1 and 100 + 2, see Fig. 1), but the C3 neutralization epitope was not found on vFG68 (Table 2). C3 mAb also did not immunoprecipitate vFG68 (not shown).

These results prompted us to investigate whether a new specificity for antigenic site 1 had been generated by insertion of an oligopeptide in VP1 and whether the modified site would be immunogenic. We tested the immunogenicity of vFG68 in rabbits, using as reference the parental PV type 3 Sabin strain (Table 3). Rabbits were hyperimmunized with native virions (bearing the D antigenicity) or heat-denatured empty virions [C antigenicity (26)]. In the case of the native D virions, rabbits 1–3 immunized with the mutant vFG68 virus had higher neutralizing titers against vFG68 than against the P3/Leon12a$_1$b Sabin strain (Table 3). For rabbits 2 and 3, there was a 4 $\log_2$ difference in favor of the recombinant virus. Rabbits 4 and 5 immunized under the same conditions with the native D virus of the Sabin strain did not have a higher neutralizing titer for the homologous virus than for vFG68.

Table 3. Immunogenicity in rabbits of insertion mutant virus vFG68 as compared with that of the parental strain

Immunizing virus	Rabbit	Neutralization titers with test viruses* P3/Leon12a$_1$b	vFG68
vFG68 (D)†	1	11	12.5
	2	11.5	15.5
	3	11	15
P3/Leon12a$_1$b (D)	4	16.5	16
	5	14	16
vFG68 (C)‡	6	6.5	8
	7	1.5	14.5
	8	<1.5	12
P3/Leon12a$_1$b (C)	9	5.5	2.5
	10	5.5	2.5

*Rabbits were immunized with three i.m. injections of 10^8 plaque-forming units at 1-wk intervals and were administered a booster 3 wk later by an i.v. injection with the same dose of virus. Sera were tested 1 wk after the booster. Titers are expressed as the logarithm (base 2) of the serum dilution able to neutralize half of a challenge virus dose (100 ID_{50}). The titers of preimmune sera were ≤2.5.
†D particles are native virions purified on CsCl density gradient.
‡C particles are heat-denatured (1 hr at 56°C) purified virions (the structure of which was verified by electron microscopy).

The phenomenon was even more striking when heat-denatured C virions were used for immunization. C particles of the Sabin strain induce little neutralizing antibodies (rabbits 9 and 10 in Table 3) as shown earlier (9, 26). On the contrary, C particles of vFG68 were highly immunogenic, and for two of three immunized rabbits (rabbits 7 and 8 in Table 3), neutralizing antibodies were strictly specific for vFG68, attaining neutralizing titers of 12–14.5 $\log_2$. Neutralization of trypsin-treated vFG68 with sera from rabbits 7 and 8 was diminished in neutralization index tests (see the legend to Table 2) by 0.73 and 1.93 logarithms, respectively, as compared with intact virions (not shown). A new specificity for neutralization antigenic site 1 was thus created on PV type 3, and the modified site 1 was highly immunogenic for rabbits and was conserved upon heat-denaturation of virions.

Table 2. Neutralization epitope map of PV type 3 insertion mutants

Virus	Rabbit anti-PV type 3 (Saukett) antiserum AP3	mAb and antigenic site (capsid polypeptide) Anti-PV type 3 mAb Site 1 (VP1) 3a	3b	IIIa	204	877 site 2B (VP2)	868 sites 3A–3B (VP1–VP3)	3o site 3B (VP3)	Anti-PV type 1 mAb C3 site 1 (VP1)
P3/Leon12a$_1$b	+	+	+	+	+	+	+	+	−
vFG68	+	−	−	−	−	+	+	+	−
vFG27	+	−	−	−	−	+	+	+	−
vFG13	+	−	−	−	−	+	+	+	−
PV type 3 Saukett	+	+	+	+	+	−	−	−	−
PV type 1 Mahoney	−	−	−	−	−	−	−	−	+

The neutralization index (NI) was defined as the difference of the logarithm of the virus titer in the absence and presence of each mAb (10, 17). NI ≥ 2 indicates the presence (+), while NI < 2 indicates the absence (−) of the neutralization epitope defined by the respective mAb. The antigenic site to which each epitope maps and the corresponding capsid polypeptide(s) are indicated (9, 10).

DISCUSSION

Addition of a foreign oligopeptide to a capsid protein of a virus from eukaryotes has never been described to our knowledge. We report here the isolation and partial characterization of mutated polioviruses that have three or six additional amino acids within the major capsid protein, VP1. Our results show that the insertion of a short oligopeptide at the level of amino acid 100 of VP1, within the loop harboring antigenic site 1, does not impair virus viability. Recombinant virions were slightly more heat labile than the parental virus, but their growth cycle was similar.

In this study, the longest foreign peptide that was added to the VP1 loop had six amino acids, but the maximum length of an insertion probably depends on the nature of the amino acids inserted, as suggested by the fact that some *in vitro* phenotypic markers vary according to the insertion (see Table 1, plaque diameter). Two viruses, vFG13 and vFG213, were obtained after transfection of recombinant plasmid pFG13, and carry six additional codons within the VP1 coding region. Both of these viruses have a point mutation within the insertion. This could be either due to the high mutation frequency of the transfected DNA and/or of the viral RNA (27, 28) or due to both high mutation frequency and the nonviability of a virus having the exact pFG13 insertion. Additional viral clones derived from pFG13 (Fig. 1) and another recombinant plasmid (pFG22), which had nine additional codons and generated infectious virus, may help to answer this question.

In the three groups of insertion mutants that we isolated, the antigenic structure of PV was changed only locally at the level of the neutralization antigenic site 1 [VP1, amino acids 89–100 (10)], the site altered by insertion. Sites 2 and 3 were not antigenically modified. In the case of vFG68, which was studied in greater detail, a new specificity of antigenic site 1 was created. This new site, highly immunogenic for rabbits, is necessarily well exposed on the virion surface, since it is implicated in virus neutralization. The fact that the new antigenic site is conserved upon heat denaturation of virions suggests that the corresponding neutralization epitopes are sequential rather than conformational. Only some epitopes were destroyed by trypsin cleavage of VP1, which might be explained by the fact that trypsin treatment did not excise the foreign oligopeptide (Fig. 3). However, the sequential character of the epitopes should be confirmed by generating mAb against this new immunogen.

In conclusion, it is clear that, although the PV has a very tight crystalline structure, it is possible to construct viable mutants that harbor an additional foreign amino acid sequence on the surface of the capsid. This sequence could be viral or cellular. It should be useful for studying either cellular receptors or the interaction of an oligopeptide with such receptors or simply for building viruses harboring an antigenic site with a new specificity.

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