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Localization of a poliovirus type 1 neutralization epitope in viral capsid polypeptide VP1

(fusion protein/site-directed deletions/nuclease BAL-31/monoclonal antibody)

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ABSTRACT **Poliovirus type 1 cDNA sequences coding for viral capsid polypeptide VP1 were inserted into the β-lactamase sequence of *Escherichia coli* plasmid pBR322. Resulting recombinant plasmid pSW119 expressed in *Escherichia coli* a VP1-β-lactamase fusion protein that reacted with antibodies raised against poliovirus capsid polypeptide VP1 and with a monoclonal poliovirus type 1 neutralizing antibody, C3. Deletions of various lengths were generated within the VP1 sequence. The hybrid proteins expressed by the deleted plasmids did not react any more with C3 when the region of VP1 amino acids 95–110 (poliovirus nucleotides 2,754–2,806) was deleted. Therefore, the C3 epitope responsible for virus neutralization is most probably located in this region of the capsid polypeptide.**

Poliovirus, the causative agent of poliomyelitis, is a picornavirus. These are small viruses with a RNA genome of plus-strand polarity enclosed in a capsid composed of 60 copies each of four structural polypeptides, VP1, VP2, VP3, and VP4. Antibodies that neutralize virus infectivity are the major factor in protection against disease. The purified capsid polypeptides from several picornaviruses have been shown to induce a neutralizing antibody response in animals (1–4). In the case of foot-and-mouth disease virus (FMDV), for example, purified capsid polypeptide VP1 isolated from virions or prepared by DNA recombinant technology (1, 2, 5) was shown to protect susceptible animals against the disease. Moreover, synthetic peptides representing the sequence of the VP1 neutralization epitope have been used successfully as "subunit vaccine" (6, 7).

In the case of poliovirus, however, the induction of neutralizing antibodies and the virus neutralization process are still poorly understood processes. Neutralizing sera raised against native poliovirions (D particles) and neutralizing monoclonal antibodies generated from mice immunized with native virions fail to react with the isolated viral capsid polypeptides (8–10). This suggests that the epitope(s) they recognize result, at least in part, from the three-dimensional arrangement of the proteins in the virion. Attempts have been made to raise neutralizing antisera by using single isolated capsid polypeptides, and a neutralizing antibody response was obtained by inoculating animals with purified VP1 but not with purified VP2 or VP3 (11, 12). In a more recent study, however, another neutralization epitope was identified on VP3 or VP4 (13). The identification of the poliovirus capsid polypeptide recognized by neutralizing antibodies was also approached through chemical crosslinking of the antibodies to their virion binding sites. Two distinct neutralization epitopes were localized in this fashion to VP1 (10).

Recently, we generated a hybridoma cell line producing neutralizing monoclonal antibodies from mice injected with heat-inactivated virions (C particles) (14). In addition to binding to heat-denatured and to native virus, this antibody, C3, was found to selectively immunoprecipitate VP1. The fact that the epitope recognized by C3 is carried by purified VP1 and heat-inactivated virions leads to the assumption that its structure depends directly on its amino acid sequence. The existence of such a continuous antigenic determinant on poliovirions could open the way towards the use of a synthetic peptide in immunization. In order to localize the C3 neutralization epitope and to determine its amino acid sequence, we analyzed the antigenicity of a VP1-β-lactamase fusion protein synthesized in *Escherichia coli* transformed by a recombinant plasmid carrying the poliovirus VP1 sequence inserted in phase in the β-lactamase gene (15). By generating deletions inside the VP1 sequence, we were able to locate the C3 neutralization epitope in the domain between amino acids 95 and 110 of VP1.

METHODS

Preparation of C3 Monoclonal Antibodies and Seroneutralization Assays. Hybridoma cell lines secreting C3 monoclonal antibodies have been described (14). The cells were inoculated into mice to produce ascites fluids containing the monoclonal antibodies. Neutralization tests were performed by a micromethod (9, 14).

Bacterial Strains and Plasmids. *E. coli* strain 1106 (803 r_k^- m_k^-) (16) was used for selection and propagation of recombinant plasmids. The minicell-producing strain GC26 (B_1^- thr^- leu^-) was kindly provided by G. Cesareni. Plasmids pPV1-366 and pSW119 have been described (15, 17).

Construction of Plasmids. Plasmid DNA was cleaved by restriction endonucleases following manufacturers' conditions. DNA fragments were recovered from low-melting-temperature agarose gels (18). Protruding 3′ ends were converted to blunt ends by incubating restricted DNA (0.1 mg/ml) with 100 units of *E. coli* DNA polymerase I (Klenow fragment) per ml as described (19). Digestion with nuclease BAL-31 was for 15 min at 30°C in 20 mM Tris·HCl, pH 8.0/1 mM EDTA/0.6 M NaCl/12 mM $CaCl_2$/12 mM $MgCl_2$, with an enzyme-to-DNA ratio of 0.12 unit per μg. Ligation reactions (19) were performed with 1 unit of T4 DNA ligase per μg of DNA. When necessary, linearized plasmids were treated for 30 min at 68°C with bacterial alkaline phosphatase (0.02 unit per μg of DNA) prior to ligation with appropriate fragments.

DNA Sequence Analysis. DNA sequence was determined as described by Guo and Wu (20); 1.3 pmol of plasmid pCW203 DNA was linearized with *Eco*RI, treated with exonuclease III (40 units/pmol of DNA) for 36 min at 23°C, and then digested

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Abbreviations: bp, base pairs; kDa, kilodaltons; VP, viral polypeptide.

with *Xba* I before use as a template in the four sequencing reactions with dideoxynucleotide terminators (20).

Labeling and Immunoprecipitation of Proteins. Conditions for purification of GC26 budding bacteria, labeling of proteins, and preparation of bacterial extracts were as described (15). The extracts were immunoprecipitated by using either nonimmune rabbit serum, anti-VP1 (αVP1) immune serum (11), or C3 ascitic fluid, and the immune precipitates were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis as described (21). ^{14}C-Labeled proteins (Amersham) were run in each gel as size standards.

RESULTS

Properties of C3 Monoclonal Antibodies. The C3 monoclonal antibody was obtained after immunization of mice against heat-denatured poliovirions (C particles). The antibody specifically neutralized homologous type 1 Mahoney virus and, to a lesser extent, the Sabin Lsc2ab strain (Table 1). In addition, it showed the unique property of immunoprecipitating both native and heat-inactivated poliovirions as well as purified capsid polypeptide VP1 (14). Therefore, the neutralization epitope recognized by C3 is carried by and expressed on isolated VP1. In order to confirm its location, we investigated the antigenic properties of a VP1-β-lactamase fusion protein expressed in *E. coli*.

Structure and Antigenicity of a VP1-β-Lactamase Fusion Protein. Poliovirus cDNA sequences representing nucleotides 2,243 to 3,417 of the viral RNA, which encode the COOH-terminal part of VP3 and VP1 and the NH_2-terminal part of NCVP3b (22–24), were subcloned at the *Pst* I site of pBR322 and fused in phase to the upstream β-lactamase sequence of the plasmid after trimming of both β-lactamase and VP3 sequences with nuclease BAL-31 (15). One of the resulting plasmids, pSW119, expressed a VP1-β-lactamase fusion protein, p49, with an apparent mass of 49 kilodaltons (kDa) (15).

The DNA sequence in the region of pSW119, where pBR322 and poliovirus sequences were joined together, is shown in Fig. 1. Ligation occurred between nucleotide 4,126 of pBR322 and nucleotide 2,445 of the poliovirus cDNA. The p49 fusion protein expressed by pSW119 thus comprises nine amino acids from the β-lactamase NH_2-terminal end and a histidine residue resulting from the fusion, followed by the 11 COOH-terminal amino acids of VP3, the 302 amino acids of VP1, the 12 NH_2-terminal amino acids of NCVP3b, and 104 amino acids corresponding to the β-lactamase COOH-terminal half (Fig. 2, map A).

A derivative of pSW119, pFS119, was constructed by replacing the sequences between the *Bam*HI and *Pst* I sites of pBR322 by the corresponding sequences from pBR327, thus deleting the poison sequences (26, 27). The proteins expressed by plasmid pFS119 in strain GC26 were labeled with [^{35}S]methionine, immunoprecipitated, and analyzed by $NaDodSO_4$/

Table 1. Neutralizing titer of C3 ascitic fluid

Virus		
Serotype	Strain	Neutralizing titer*
1	Mahoney	1,280
	Sabin LSc2ab	40
2	MEF1	<10
	Sabin p712CH2ab	<10
3	Saukett	<10
	Sabin Leon 12a1b	<10

* Titers were expressed as the end-point dilution of C3 ascitic fluid neutralizing 50% of one dose (100 tissue culture ID_{50}) of the indicated poliovirus strains (14).

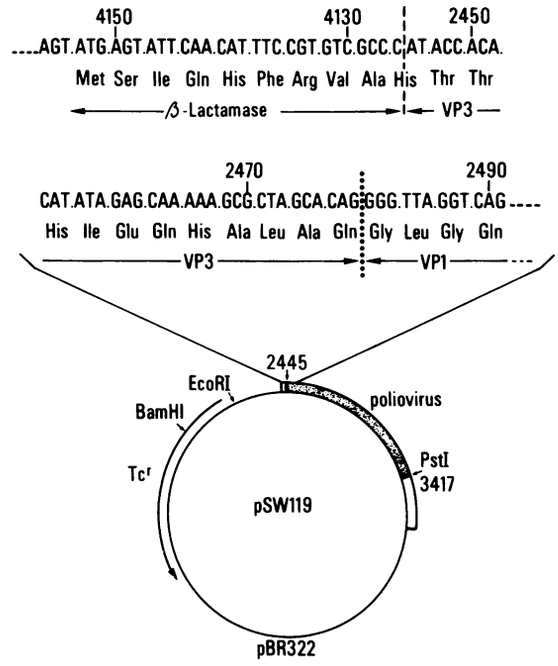

FIG. 1. Diagrammatic representation of pSW119. A 972-bp fragment of poliovirus cDNA containing the sequence of VP1 (▨) was fused within the β-lactamase sequence of pBR322 (□) as described (15). The nucleotide sequence at the junction was determined on a derivative of pSW119, pCW203. Nucleotide numbering followed that of Sutcliffe (25) for pBR322 and of Dorner *et al.* (24) for poliovirus.

polyacrylamide gel electrophoresis. As shown in Fig. 3, pFS119 expressed a p49 fusion protein that was specifically immunoprecipitated with rabbit antiserum raised against purified capsid polypeptide VP1 (VP1 antiserum) and with C3 neutralizing monoclonal antibodies. By contrast, p49 was not immunoprecipitated with control nonimmune serum, nor was it detected in bacterial extracts from transformants carrying plasmid pBR327. pFS119 expressed p49 at a higher level than did pSW119 (data not shown).

Antigenicity of the Proteins Expressed by Derivatives of pFS119. Derivatives of pFS119 were constructed by deleting either the *Xba* I–*Xba* I fragment, encoding VP1 amino acids 23–128, or the *Kpn* I–*Pst* I fragment (VP1 amino acids 196–302). The resulting plasmids, pFS1019 and pFS1023 (Fig. 2, maps

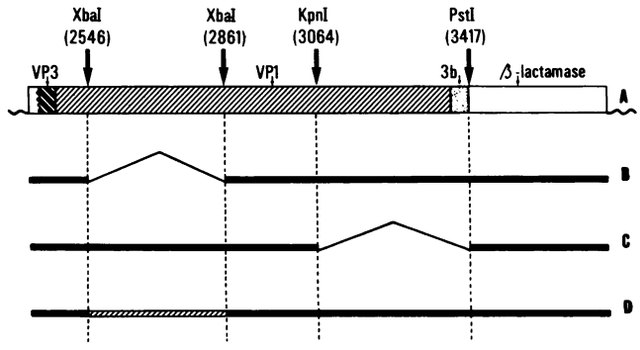

FIG. 2. Organization of the DNA sequences encoding the VP1-β-lactamase fusion protein in pSW119 (map A) and derived plasmids. Plasmid pFS1019 (map B) was derived from pFS119 by digesting the DNA with *Xba* I and recircularizing with T4 DNA ligase. Plasmid pFS1023 (map C) was derived from pFS119 by digestion with both *Kpn* I and *Pst* I. Protruding 3′ ends were converted to blunt ends, and the DNA was recircularized. To construct pCW119 (map D), pFS1019 DNA was linearized with *Xba* I and ligated with the *Xba* I–*Xba* I fragment (nucleotides 2,546–2,861) isolated from pPV1-366 DNA (17).

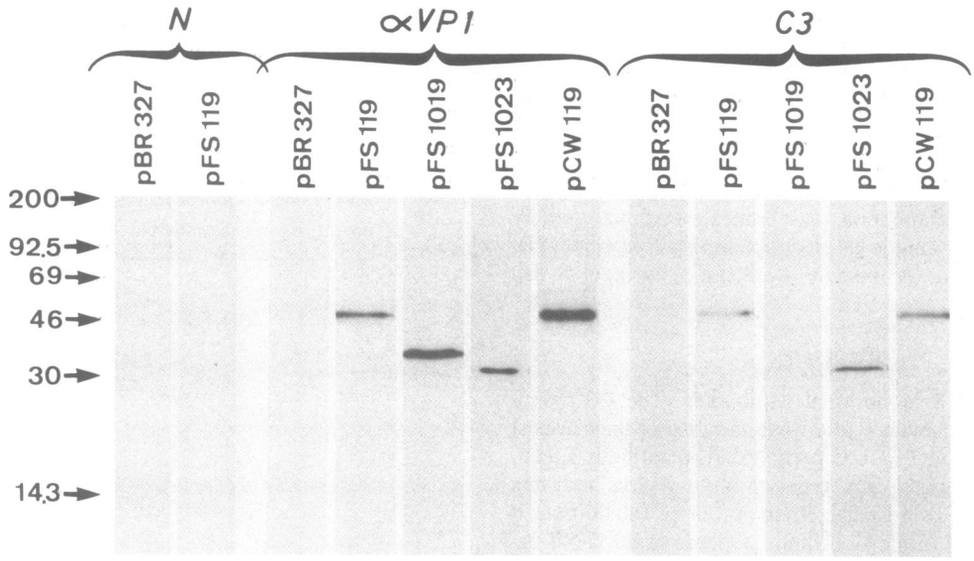

FIG. 3. Immunoprecipitation of VP1-β-lactamase fusion proteins with VP1 antiserum and monoclonal antibodies C3. GC26 bacteria transformed with the indicated plasmids were labeled with [^{35}S]methionine. Proteins were immunoprecipitated with either nonimmune rabbit serum (N), VP1 antiserum (αVP1), or C3, and analyzed on a 20% $NaDodSO_4$/polyacrylamide gel as described. Size is shown in kDa.

B and C), expressed fusion proteins with apparent M_rs of 39 kDa (p39) and 32 kDa (p32), respectively. Both polypeptides were specifically immunoprecipitated with VP1 antiserum (Fig. 3). p32 was still recognized by C3, while p39 could no longer be immunoprecipitated by the monoclonal antibody (Fig. 3). The *Xba* I–*Xba* I fragment was isolated from pPV1-366, another recombinant plasmid cloned independently from those used to construct pSW119 (17), and the fragment was inserted at the *Xba* I site of pFS1019. The new plasmid thus generated, pCW119 (Fig. 2, map D), again expressed in GC26 bacteria a 49-kDa fusion protein that reacted with both the C3 monoclonal antibodies and the VP1 antiserum (Fig. 3). These results suggest that the neutralization epitope recognized by C3 is located in the domain of VP1 between amino acids 23 and 128.

Localization of the C3 Neutralization Epitope. To localize the C3 neutralization epitope more accurately, a series of deletions were introduced in the VP1 sequence of pCW119. This was done by first linearizing the plasmid DNA at the unique *Kpn* I site (poliovirus nucleotide 3,064) and then digesting away parts of the VP1 sequence with nuclease BAL-31. The resulting DNA molecules were recircularized with T4 DNA ligase and were used to transform *E. coli* strain 1106. Individual clones were picked up and analyzed by restriction endonuclease analysis (Table 2).

Antigenic reactivity of the proteins expressed by the BAL-31-deleted plasmids was examined. All fusion proteins reacted with the VP1 antiserum (Fig. 4*A*). The molecular size of the truncated proteins was found to depend not only on the extent of the BAL-31 deletion but also on whether the remaining sequences on each side of the deletion were in phase with each other (pCW203, -217, -223, and -202) or not (pCW215, -213, -216, and -218) (Table 2). Immunoprecipitation of the proteins with the C3 neutralizing monoclonal antibodies showed that the deleted plasmids fell into two categories: plasmids pCW215, -213, -217, and -202 expressed proteins that still reacted with C3, whereas plasmids pCW216, -203, -218, and -223 expressed proteins that no longer reacted with the antibody (Fig. 4*B*). No obvious correlation could be detected between the overall size of the deletion and the reactivity or lack of reactivity with C3 (Table 2).

To investigate the possibility that nuclease BAL-31 might have digested the plasmid DNAs in an asymmetric fashion, we mapped the various deletions as described in the legend to Fig. 5. We found that plasmids pCW213, -215, and -202, which expressed C3-reactive deleted fusion proteins, had lost the *Sau*3A site corresponding to poliovirus nucleotide 2,806 (amino acid 110 of VP1) but retained the *Alu* I site corresponding to poliovirus nucleotide 2,787 (amino acid 103 of VP1), whereas plasmids pCW203, -218, and -223, which expressed deleted fusion proteins that no longer reacted with C3, had lost both the *Alu* I site at position 2,787 and that at position 2,764 (amino acid 95 of VP1). Plasmid pCW216 was of particular interest, as it had lost the *Alu* I site at position 2,787 but still retained the *Alu* I site at position 2,764. The fusion protein expressed by this plasmid did not react with C3 antibodies. It follows that the C3 neutralization epitope should be included in the domain of VP1 extending from residues 95–103 to 103–110 (poliovirus nucleotides 2,764–2,787 to 2,787–2,806) (Fig. 5).

DISCUSSION

Detailed study of the antigenic and immunogenic properties of isolated poliovirus capsid polypeptides has been much hampered so far by the fact that the polypeptides cannot be isolated free from each other in the native state but only after disrup-

Table 2. BAL-31-generated deletions

Plasmid	Size of deletion, bp	Fusion protein: Apparent size, kDa	Fusion protein: Reactivity with C3
pCW215	510	23.5	+
pCW216	570	17.5	−
pCW203	590	30.0	−
pCW213	590	16.5	+
pCW217	610	29.0	+
pCW218	640	17.0	−
pCW223	650	30.0	−
pCW202	660	28.0	+

The size of the deletions was estimated by restriction enzyme analysis. Properties of the truncated fusion proteins encoded by the plasmids were analyzed as described in the legend to Fig. 3.

tion of the capsid or its 14S assembly subunit by use of drastic denaturation processes (21).

As illustrated in this report, molecular cloning of poliovirus cDNA sequences and expression in *E. coli* can provide a useful alternative approach to this problem. The sequence coding for VP1 was isolated from recombinant plasmids carrying poliovirus cDNA inserts (17) and subcloned within the β-lactamase gene of pBR322. As the VP1 sequence contains no initiator AUG triplet, being located internally in the poliovirus genome (22–24), it was necessary to fuse it in phase behind the AUG of the β-lactamase gene. This was achieved through treatment with nuclease BAL-31 (15). One of the resulting plasmids, pSW119, expressed a VP1-β-lactamase fusion protein that was specifically immunoprecipitated with VP1 antiserum and with the neutralizing monoclonal antibody C3.

VP1 is 302 amino acids long (22, 24). To identify the location of the C3 neutralization epitope in the fusion protein, different types of deletions were generated in the VP1 sequence of pSW119 or of its derivatives, pFS119 and pCW119. Plasmids deleted of the sequence encoding amino acids 23–128 of VP1 (poliovirus nucleotides 2,546–2,831) expressed a truncated fusion protein that was no longer immunoprecipitated by C3, although it was still immunoprecipitated by the VP1 antiserum. Another series of deleted plasmids were obtained by introducing deletions with nuclease BAL-31 within the VP1 sequence. Deletion of the COOH-terminal part of VP1 did not affect reactivity of the fusion protein with C3. Deletion of the central part of VP1 was similarly without effect, as long as the deletion did not reach the upstream limit marked on the DNA by the *Alu* I site at nucleotide 2,787 (amino acid 103 of VP1). Reactivity with C3 was totally lost when the deletion extended further upstream than this limit.

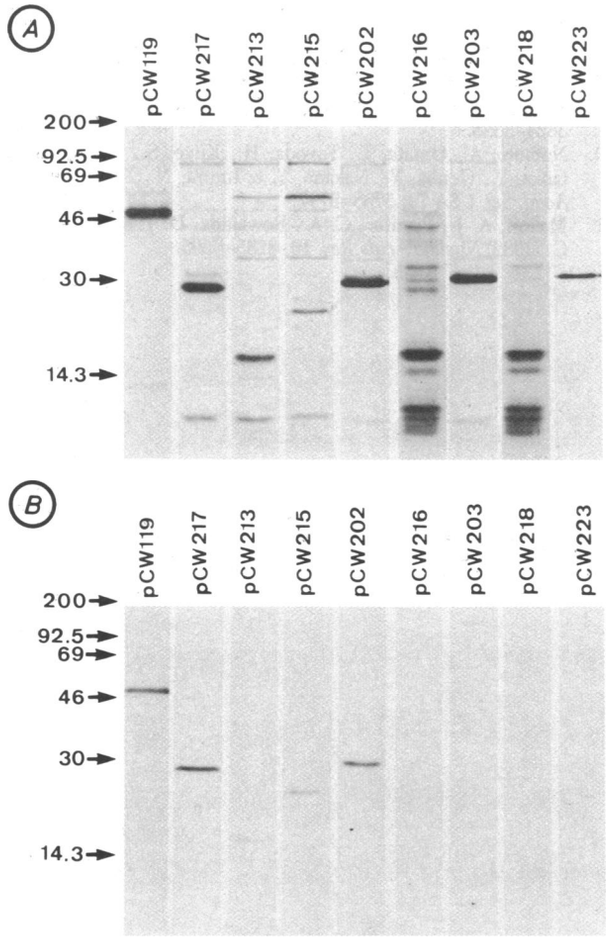

FIG. 4. Immunoprecipitation of truncated fusion proteins coded by the BAL-31-deleted plasmids. [^{35}S]Methionine-labeled proteins were immunoprecipitated with either VP1 antiserum (*A*) or C3 monoclonal antibodies (*B*) as described in the legend to Fig. 3. Size is shown in kDa.

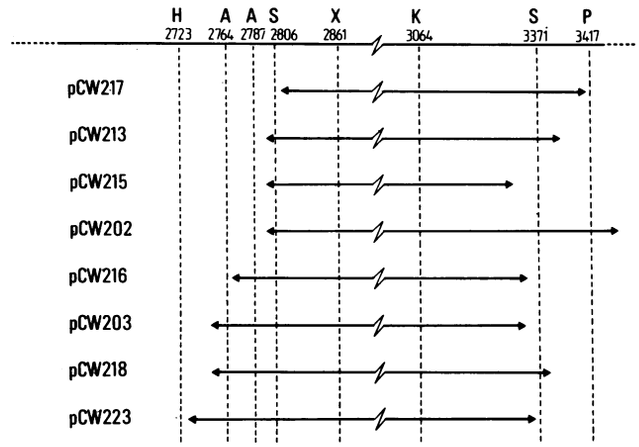

FIG. 5. Location and extent of deletions in BAL-31-deleted plasmids. The deleted sequences are indicated by the arrows. The limits of the deletions to the left were determined by linearizing the plasmids' DNA (1 μg) with *Xba* I and labeling with the Klenow enzyme for 1 hr at 25°C in the presence of [α-^{32}P]dATP (10 μCi; 1 Ci = 3.7 × 10^{10} Bq) and of dGTP, dCTP, and dTTP (0.2 mM each). Labeled DNA was then digested with the indicated enzymes (partial digestion conditions were used in the case of *Alu* I). Labeled restriction fragments were separated on a 5% polyacrylamide gel and visualized by autoradiography. The limits of the deletions to the right were deduced from the overall size of the deletions (Table 2) and confirmed by the presence or absence of the *Pst* I and *Sau*3A sites. X, *Xba* I; H, *Hha* I; A, *Alu* I; S, *Sau*3A; K, *Kpn* I; P, *Pst* I. Numbers refer to the poliovirus nucleotide sequence (24).

A certain domain of VP1 must be present, therefore, in the fusion protein for it to be immunoprecipitated by the C3 neutralizing monoclonal antibodies. From the data presented here, the upstream limits of the longest deletions that did not affect reactivity of the protein with C3 can be localized between nucleotides 2,787 and 2,806 on the poliovirus map (VP1 amino acids 103–110). Similarly, the limits of the shortest deletions that suppressed reactivity of the protein with C3 can be mapped between nucleotides 2,764 and 2,787 (VP1 amino acids 95–103). Therefore, the C3 neutralization epitope most probably is located in the domain of VP1 between amino acids 95–103 and 103–110. Alternatively, the epitope could be located in another part of VP1 but would be masked after deletion of the 95–110 amino acid domain. Site-directed mutagenesis of the VP1 sequence in plasmids such as pSW119 and analysis of the antigenicity of the resulting proteins should allow these hypotheses to be tested.

The localization of the C3 neutralization epitope in the region of amino acids 95–110 in VP1 is strengthened, however, by several observations. First, a major neutralization epitope was identified recently in the same region of VP1 of poliovirus type 3 by Minor *et al.* (28), who analyzed a series of type 3 poliovirus mutants resistant to neutralizing monoclonal antibodies. Three of the mutations could be mapped to VP1 amino acids 98, 99, and 100, respectively. Second, VP1 contains a marked hydrophilic peak between amino acids 93 (Asp) and 103 (Lys) in the very region recognized by C3, as revealed by computer search for hydrophilic domains (29) (unpublished data). As hydrophilic regions are more likely to be exposed on the surface of the protein and therefore to be antigenically relevant, it is tempting to predict that the neutralization epitope recognized

by the C3 antibodies lies in this region. Third, comparison of the nucleotide sequence of the attenuated poliovirus Sabin 1 strain (30) with that of the wild-type Mahoney strain (22) shows that five out of the seven amino acid changes in VP1 occurred at positions 88, 90, 95, 98, and 106—i.e., in the near vicinity or inside the region of the C3 epitope. A similar variability in sequences has been noted in foot-and-mouth disease virus serotypes and subtypes in the near vicinity of the major neutralization epitope (7, 31). Interestingly, C3 antibodies exhibited a much lower neutralizing efficiency for Sabin type 1 virus as compared to wild-type Mahoney virus (Table 1). All these observations point to the location of the C3 epitope in the 95–110 amino acid domain of VP1. It will be of major interest to study the antigenicity and immunogenicity of a synthetic peptide corresponding to this region of VP1.

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