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Attenuated Poliovirus Strain as a Live Vector: Expression of Regions of Rotavirus Outer Capsid Protein VP7 by Using Recombinant Sabin 3 Viruses

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The ability to express heterologous antigens from attenuated poliovirus strains suggests the potential for use as live vectored vaccines. Full- or partial-length sequences of the gene encoding rotavirus major outer capsid protein VP7 were cloned into the open reading frame of a full-length cDNA copy of poliovirus Sabin type 3. They were inserted either at the 5′ end or immediately after the capsid protein coding region, at the junction between precursors P1 and P2. A protease cleavage site for 3C protease was introduced 3′ to the foreign sequences to enable proteolytic processing of the antigen from the poliovirus polyprotein. Infectious viruses were generated from several of the DNA constructs, and the presence of the foreign gene sequences was confirmed by reverse transcription of the viral RNA and PCR amplification. Viruses with inserts of about 300 bases maintained the foreign sequences during passage in Vero cells. Viruses carrying larger sequences were unstable, and deletions were generated within the foreign sequences. Expression of the VP7 polypeptides was demonstrated by immunoprecipitation with specific antiserum of labeled proteins from cells infected with Sabin 3 recombinant viruses. Comparative studies of RNA synthesis showed similar kinetics for Sabin 3 and the Sabin 3/VP7 recombinants. One-step growth curves showed that production of recombinant viruses was slower than that of Sabin 3 and that the final titers were 1 to 1.5 logs lower. Accumulation of VP7-containing precursors in infected cells suggests that slow cleavage at the engineered 3C protease site may be a limiting step in the growth of these recombinant Sabin polioviruses and may influence the permissible size of foreign sequence to be inserted.

The poliovirus genome is composed of a positive-sense single-stranded RNA molecule that encodes a single open reading frame that is translated into a large precursor polyprotein (19). The polyprotein is subsequently cleaved by virus-encoded proteases (2A, 3C, or 3CD) to form the mature viral structural and nonstructural proteins (41, 43). Intramolecular cleavage of the polyprotein by 2A protease releases the P1 precursor (41), which is subsequently cleaved by 3CD to form the viral capsid proteins VP0, VP3, and VP1 (43). Cleavage of P2 and P3 precursors by 3C protease generates the nonstructural proteins involved in the poliovirus replication process (30). Assembly of viral capsid proteins and packaging of viral RNA result in infectious virus.

The Sabin strains of poliovirus are attenuated and, following infection of the intestinal tract, elicit strong protective immune responses without causing disease. The Sabin strains of poliovirus, therefore, are good candidates for live viral vectors to deliver foreign antigens to the enteric tract when mucosal immunity is desired for protection from disease.

The potential of chimeric polioviruses as an antigen presentation system has been studied in several laboratories for the expression of intertypic or foreign epitopes. In the former case, intertypic poliovirus chimeras have been constructed by replacing the antigenic site 1 of one poliovirus serotype with the corresponding sequence from a different serotype (4, 23, 27, 28). Chimeric polioviruses that carry antigenic sites from other viruses on the surface-exposed loop, such as sequences of human immunodeficiency virus gp41 (9), human papillomavirus type 16 L1 protein (17), hepatitis A virus (22), and foot-and-mouth disease virus (FMDV) (20), have also been designed. Neutralizing antibodies to FMDV were generated in some guinea pigs immunized with poliovirus carrying FMDV epitopes. Protection following challenge with wild-type FMDV was observed in those animals that had developed neutralizing antibodies (20). Chimeric poliovirus containing hepatitis A virus epitopes also generated antibodies, at levels that were low but similar to those reported to be protective; however, this response was seen in only a small fraction of the animals (22). In general, success with chimeric polioviruses has been achieved with linear antigenic determinants which elicit protective immune responses as peptides alone or when conjugated to carrier macromolecules.

An alternative strategy for use of poliovirus as a vector involved replacing the region of the viral genome encoding the capsid proteins with foreign genes, generating a minireplicon. These defective viruses replicate only when cotransfected into cells with helper virus or vaccinia virus expressing the poliovirus capsid proteins (31). In these studies, the recombinant protein is expressed as a fusion with poliovirus proteins.

The work presented here describes another approach in which a rotavirus antigen is expressed from part of the coding region of viable recombinant attenuated poliovirus. A similar experiment in which short sequences of foreign antigens were cloned in the poliovirus genome has been described previously (10). Delivery in this manner allows both endogenous and exogenous introduction of protein or protein fragments to the immune system and greatly increases the potential for induction of universal immune reactivity in a genetically heterogeneous population with both antibodies and cell-mediated immunity being elicited.

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Rotaviruses are pathogens that cause dehydrating diarrhea primarily in infants (up to 2 years of age) and young animals. Antibodies generated to the two outer capsid proteins VP4 and VP7 are able to neutralize the virus. VP7 is the most abundant outer capsid protein that forms the smooth external surface of the rotavirus (7, 32, 42) and is very immunogenic in its native state as part of the viral particle.

Fourteen different rotavirus VP7 serotypes (called G serotypes) have been identified to date (24). Human strains of rotavirus belong to four major G serotypes (G 1 to 4), but some minor strains have been identified as belonging to G serotypes 8, 9, and 12. It has been shown that local intestinal immunity is necessary for protection from rotavirus infection (for a review, see reference 5). Passive protection from rotavirus diarrhea has been achieved in mice with neutralizing monoclonal antibodies to either VP7 or VP4. VP7 has also been shown to induce cross-reactive rotavirus-specific cytotoxic T lymphocytes (14). Although the importance of cell-mediated immunity in protection from rotavirus infections is not yet clear, cell-mediated immunity may play an important role in protection from disease and for virus clearance (6).

Several different approaches have been used to develop a rotavirus subunit vaccine, through the expression of cloned genes (full-length or partial sequences) of the outer capsid proteins VP7 and VP4, with varied results. In the case of VP7, expression in prokaryotic vectors (*Escherichia coli* and attenuated strains of *Salmonella typhimurium*) has had disappointing results, although two of the reports found that neutralizing antibodies on the order of 1/300 (26) to 1/750 (2) were generated for a bovine and a simian VP7, respectively. In most of the studies, only antibodies to denatured VP7 were induced, indicating that while an immunogenic protein was expressed, it did not assume a conformation which elicited antibodies that neutralized or bound virus (11, 18, 35). Immunization with VP7 expressed in insect cells by recombinant baculovirus generated low levels of neutralizing antibodies in laboratory animals (8). Immunization with baculovirus-expressed VP7 was also found to induce $CD8^+$ T lymphocytes that mediated clearance of chronic rotavirus infection in SCID mice (6). Expression of rotavirus in live vaccinia virus vectors gave promising results, when VP7 was expressed at the plasma membrane of infected cells by the addition of the membrane anchor and cytoplasmic domain from influenza virus hemagglutinin. In this case, week-old suckling mice born to mothers vaccinated with this recombinant vaccinia virus were protected from homologous challenge (1). Similar results were recently reported for the same VP7 construct expressed in a recombinant human type 5 adenovirus (3).

In this work, portions of a gene encoding rotavirus VP7 have been introduced into the poliovirus genome in frame with the poliovirus polyprotein. An artificial 3C protease recognition sequence adjacent to the foreign protein sequence has been introduced for the processing of the heterologous protein from the polyprotein. The cleavage site has been designed such that, following proteolytic processing, all viral proteins are unmodified and are capable of assembly into infectious viruses. This work may lead to the development of live attenuated vaccine vectors with the potential to be combined with oral poliovirus vaccines.

MATERIALS AND METHODS

Cells and viruses. Vero cells (monkey kidney cells) grown in monolayers were used to grow the polioviruses (38). Sabin 3 poliovirus is an attenuated strain of poliovirus serotype 3. Vero cells were infected with polioviruses as previously reported (39) or transfected with Sabin 3 or recombinant Sabin 3/VP7 RNAs as described below.

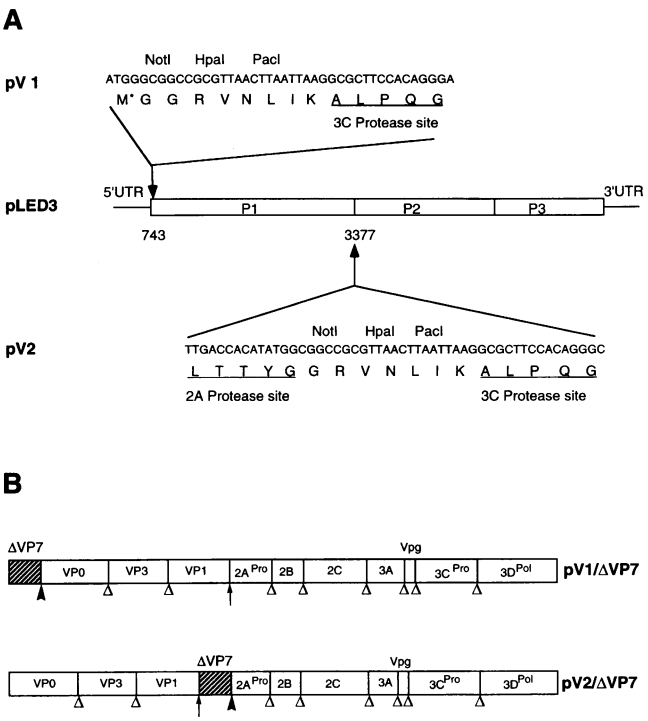

FIG. 1. Poliovirus vectors carrying a multiple cloning site. (A) Identical polylinker cassettes carrying the restriction sites for *Not*I, *Hpa*I, and *Pac*I were inserted either at the 5′ end of the poliovirus open reading frame (pV1) or at the P1-P2 junction (pV2). The vector pV1 encodes an artificial 3C protease cleavage site 3′ to the multiple cloning site. The vector pV2 encodes a 2A and an artificial 3C protease cleavage site 5′ and 3′ to the multiple cloning site, respectively. In both vectors, the open reading frame is maintained for sequences cloned in any of the three sites. UTR, untranslated region; M*, methionine at which the translation of the poliovirus polyprotein starts. (B) Schematic of vectors pV1 and pV2 containing a VP7 insert. Δ, 3C protease sites; vertical arrows, 2A protease site; arrowheads, engineered 3C protease site.

Plasmids. The poliovirus vector used for the studies, pLED3, consists of pBR322 with a full-length cDNA copy of the vaccine strain Sabin type 3 poliovirus genome (39, 45) cloned downstream from a bacteriophage T7 RNA polymerase promoter. Derivatives of pLED3 which contain a polylinker cassette to facilitate gene insertion at different locations within the poliovirus genome were constructed. The polylinker cassette introduces unique restriction enzyme cleavage sites for *Not*I, *Hpa*I, and *Pac*I and the recognition sequence for cleavage by viral protease 3C at the 3′ end (Fig. 1). Vector pV1 contains the polylinker cassette in frame with the poliovirus polyprotein immediately following the initiating AUG at bp 743. Vector pV2 contains the polylinker cassette at the junction between the P1 and P2 regions starting at bp 3377, retaining the 2A protease cleavage site at the 5′ end of the insert. Overlap extension PCR (15) was used to construct the vectors.

The template DNA for rotavirus VP7 was a plasmid clone, pPX1620, containing a full-length VP7 of the C486 bovine strain (G serotype 6) (34). VP7 gene fragments (see Fig. 2) were amplified by PCR with primers designed to incorporate *Not*I and *Pac*I restriction sites at the 5′ and 3′ ends, respec-

tively. The resulting PCR products were gel isolated, digested with *Not*I and *Pac*I (New England Biolabs, Beverly, Mass.), and subcloned into pV1 and pV2. The clones were characterized by restriction digestion analysis (36), and the correct DNA sequence of the insertion sites was confirmed with an ABI 370A DNA sequencer (ABI, Foster City, Calif.). Clones carrying fragments of the VP7 gene are designated by the vector name (pV1 or pV2) followed by the number of amino acids of VP7 encoded.

Transfection of Vero cells. Plasmid DNAs were linearized with *Pvu*I (New England Biolabs) and purified by extraction with phenol-chloroform followed by ethanol precipitation. One microgram of linearized plasmid DNA was transcribed in vitro with T7 RNA polymerase as previously described (44). Monolayers of Vero cells (4×10^6 cells) were grown in 25-cm^2 flasks. RNAs were transfected into the cells as previously described (39, 44). Cells were incubated until total cytopathic effect was seen, or they were frozen at 7 days posttransfection and a second passage of the supernatants was performed. Alternatively, Vero cells were transfected with Lipofectin (GIBCO-BRL, Gaithersburg, Md.). Titers of viruses in the supernatant of these transfected cultures were subsequently determined by plaque assay on Vero cells (39), and viruses were passaged at a low multiplicity of infection (MOI) (0.1 PFU per cell) in Vero cells.

Viruses were designated V1 or V2 (depending on the location of the insert in the poliovirus genome), followed by the number of amino acids of VP7 encoded. The deleted variants were designated by adding a "Δ" before the original number of amino acids encoded. Plaque-purified viruses were designated by adding a "c" followed by the isolate number.

Detection of VP7 sequences in genomic RNA of recombinant Sabin 3 viruses obtained after transfection of Vero cells. Viral RNA was extracted from virus with phenol-chloroform followed by ethanol precipitation. Reverse transcription (RT) was performed with avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.) at 43°C for 60 min in the presence of a poliovirus type 3 primer. RT was followed by PCR amplification with poliovirus primers flanking the inserted foreign sequences. PCRs were performed with AmpliTaq (Perkin-Elmer, Norwalk, Conn.) for 30 cycles at 94°C for 1 min, 45°C for 1 min, and 72°C for 1.5 min.

One-step growth curves. Vero cells grown in 24-well plates (6×10^5 cells per well) were infected with Sabin 3 or Sabin 3/VP7 recombinant polioviruses at an MOI of 10. Virus was allowed to adsorb to cells for 30 min at room temperature. After 30 min, the cells were washed with phosphate-buffered saline (PBS), and 1 ml of Dulbecco modified Eagle medium (DMEM; GIBCO-BRL) was added. The cells were then incubated at 33.5°C. At different times postinfection (p.i.), cells from duplicated wells were scraped into the supernatant and frozen. The virus titers were determined by plaque assay on Vero cell monolayers after three cycles of freeze-thawing.

Kinetics of viral RNA synthesis. Vero cells grown in 24-well plates were mock infected or infected at an MOI of 10 with Sabin 3 or Sabin 3 recombinants. After a 30-min adsorption period at room temperature, the cells were washed with PBS, and DMEM containing actinomycin D (5 μg/ml) was added. The cells were incubated for 1 h at 33.5°C. The medium was removed, and 0.5 ml of fresh medium containing actinomycin D (5 μg/ml) and 15 μCi of [5, 6-^{3}H]uridine per ml (Amersham; specific activity, 46 Ci/mmol) was added. At different times p.i., the cells from duplicated wells were washed with PBS and scraped into 0.5 ml of buffer NET (50 mM Tris-HCl [pH 7.6], 100 mM NaCl, 1 mM EDTA) containing 0.5% sodium dodecyl sulfate (SDS). Trichloroacetic acid was added to the lysates to a final concentration of 20%, and the lysates were incubated on ice for 30 min. The samples were filtered onto glass microfiber filters, and radioactivity was determined by scintillation counting.

Isotopic labeling of infected cells and immunoprecipitation. Vero cells were infected with Sabin 3 or Sabin 3 recombinants at an MOI of 5. Virus adsorption was allowed for 1 h at room temperature. After the adsorption period, the inoculum was removed, fresh DMEM was added, and cells were incubated at 33.5°C. At 5 h p.i., the cells were starved for 1 h in DMEM without methionine and cysteine. After the starving period, Tran^{35}S-Label (ICN; specific activity, 1,028 Ci/mmol) was added to a final concentration of 80 μCi/ml, and the proteins were labeled for 6 to 8 h p.i. The supernatant was then removed, and the cells were lysed in radioimmunoprecipitation assay (RIPA [25]) buffer. Labeled proteins in the infected cell lysates were separated in SDS-polyacrylamide gels either directly or after immunoprecipitation with polyclonal antisera against either poliovirus type 3, 2A protease, or rotavirus VP7.

Immunoprecipitations were carried out as previously described (25). Briefly, the labeled cell lysates were diluted in RIPA buffer to a final volume of 300 μl and incubated on ice for 3 h with the corresponding antiserum (3 μl of anti-type 3 poliovirus, or 5 μl of anti-2A protease, or 5 μl of anti-VP7 serum). The VP7 antiserum was prepared by immunization of a rabbit with lysates of insect cells infected with baculovirus expressing rotavirus VP7. Protein G-Sepharose was added, and the beads were incubated with the immune complexes for 2 h. The beads were then washed three times with RIPA buffer as previously described (25) before suspension in Laemmli sample buffer. The proteins were separated by electrophoresis in SDS–12.5% polyacrylamide gels.

RESULTS

Construction of pLED3 derivatives carrying rotavirus VP7 sequences. The strategy developed to facilitate cloning of multiple constructs involved the engineering of two separate poliovirus cDNA vectors. Multiple cloning site cassettes were inserted in two different locations in the poliovirus genome (Fig. 1). The two vectors provide a system for cloning foreign genes in frame with the polyprotein either at the 5′ end of the open reading frame (pV1) or at the junction between P1 and P2 (pV2). Both vectors have a sequence encoding the recognition site for 3C protease added at the 3′ end of the cassette region. Different-length rotavirus VP7 gene fragments were generated by PCR, with unique restriction enzyme cleavage sites incorporated at the 5′ and 3′ ends, for direct cloning into both vectors. Figure 2 shows the regions of rotavirus VP7 included in different constructs. The larger constructs include a full-length VP7 beginning at the second (H2) hydrophobic signal sequence (37) starting at methionine 30 (296 amino acids [aa]) and a VP7 lacking both hydrophobic signals and the first 13 aa of the mature protein and starting at methionine 63 (263 aa).

Positive clones were obtained for every VP7 fragment cloned in both locations of the Sabin 3 genome. The nomenclature differentiates plasmids on the basis of the place in the poliovirus genome where the foreign fragments were inserted (pV1 or pV2), and of the number of amino acids of VP7 encoded in these fragments.

The plasmid DNAs were sequenced to confirm the presence of the insert into the Sabin 3 genome and the conservation of the correct open reading frame of the poliovirus polyprotein. Full-length RNA transcripts were made from all of these constructs with T7 RNA polymerase after linearization of the

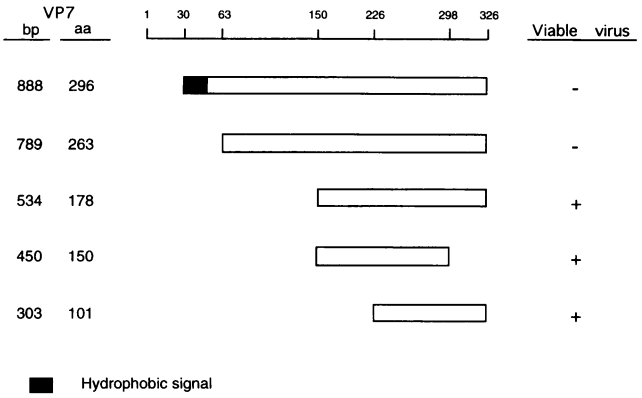

FIG. 2. Full- and partial-length VP7 sequences inserted in poliovirus vectors. A schematic of the VP7 protein is shown at the top, followed by the different constructs. PCR was used to incorporate the restriction enzyme sites for *Not*I (5′-end primer) and *Pac*I (3′-end primer), and the fragments were subcloned into the vectors, pV1 and pV2. These clones are named according to the number of amino acids of VP7 encoded. The shaded region corresponds to the second signal sequence present in VP7 following methionine 30.

plasmid with *Pvu*I. These transcripts were used for transfection into Vero cells in order to generate infectious virus.

Poliovirus RNA carrying rotavirus VP7 sequences is infectious. Different-length VP7 gene fragments were cloned into the poliovirus genome to determine the largest size of foreign sequence that could be inserted and result in infectious virus. Following transfection of RNA into Vero cells, infectious viruses were recovered from constructs containing the three shorter inserts, but not from those containing the two longer ones (Fig. 2).

RT-PCR was used to confirm the presence of inserted VP7 fragments in viruses recovered from transfections of Vero cells with recombinant pLED3/VP7 transcripts. In the RT-PCR assay, the poliovirus region spanning the site of gene insertion was amplified. The PCR product is 250 or 185 bp plus the size of the insert for virus derived from pV1 and pV2, respectively. In some cases, primers corresponding to the 5′ and 3′ ends of the cloned VP7 region were used for confirmation of the VP7 gene (not shown). Several of the recovered viruses were found to be positive by RT-PCR for the presence of inserted VP7 fragments of the correct size (as illustrated in Fig. 3A, lanes 2 and 3, for V1/150 and V1/178, respectively). Other recombinant viruses were found to be negative for the VP7 sequences, or the RT-PCRs showed a band that was only slightly larger than that corresponding to the negative control (Fig. 3A, lane 4 for V1 and lane 11 for V2), suggesting that the insert was totally absent or that only a few nucleotides of the VP7 sequences were present (not shown).

Viruses were serially passaged in Vero cells directly, or were plaque purified, with selected plaques then being serially passaged in Vero cells.

Stability of recombinant polioviruses expressing VP7. To further study gene stability, RT-PCR was performed on viral RNA extracted from the serial passages of the recombinant Sabin 3 viruses with the same primers described previously. The results obtained were the following. (i) Some viruses remained stable, as illustrated in Fig. 3B, for four successive passages of V1/101 and V2/101 (lanes 2 to 5 and 7 to 10, respectively). (ii) Other viruses became negative for the presence of the VP7 sequences at the second or third passage, as illustrated in Fig. 3A (lanes 5 to 7) for V2/150-2. (iii) Other

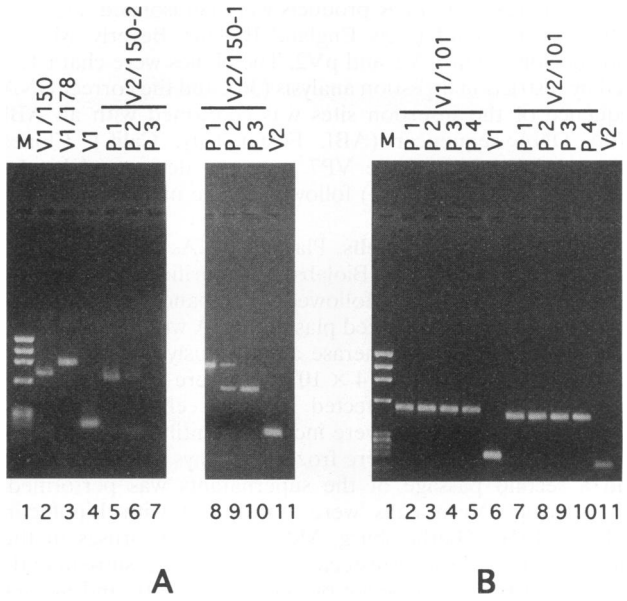

FIG. 3. (A) VP7 sequences are present in infectious recombinant Sabin 3 viruses. RT followed by PCR amplification was carried out on viral RNA with poliovirus primers that amplify the region between bases 613 and 797 (for pV1, lanes 2 to 4) or the region between bases 3305 and 3433 (for pV2, lanes 5 to 11). Lanes 4 and 11 are the negative controls (V1 and V2, respectively) lacking the VP7 sequences. Lanes 2, 3, 5, and 8 show RT-PCR products of the expected sizes in crude transfections. Lanes 5 to 7 illustrate loss of insert after passage for V2/150-2. Lanes 8 to 10 illustrate appearance of a subpopulation for V2/150-1 with a deleted insert at second passage which overgrows culture by third passage. P, passage; M, ΦX174/*Hae*III digest. The numbers 1 and 2 following the names of the viruses refer to viruses generated with the same construct in different transfection reactions. (B) A 303-bp VP7 insert cloned either at the 5′ end of the poliovirus polyprotein (V1) or at the P1-P2 junction (V2) was stably maintained in the Sabin 3 genome over four passages. RT-PCR was performed as described for panel A. Lanes 6 and 11 are the negative controls as described above. Lanes 2 to 5, V1/101, passages 1 to 4; lanes 7 to 10, V2/101, passages 1 to 4.

reactions showed the presence of mixed viral populations carrying inserts of different sizes, as illustrated in Fig. 3A for V2/150-1 (lane 9). Following further passages, the viruses carrying smaller inserts overgrew the others (lane 10).

Stability was also checked for the plaque-purified viruses. The viruses that remained stable after at least four passages were those with inserts of about 300 bp or smaller. Taken together, these results suggested that the VP7 gene inserts were not all stably maintained in the Sabin 3 genome and that populations with shorter or deleted inserts might overgrow less favored populations carrying larger inserts.

Characterization of stable recombinant viruses recovered after plaque purification shows partial deletions in the VP7 gene. The PCR products obtained after amplification with poliovirus primers were sequenced to determine the nature of the deletions, which may provide information about the mechanism by which deletions were produced and selected. Different deletions were observed. Table 1 shows a summary of the size of inserts, obtained from sequencing PCR products for several recombinant Sabin 3/VP7 viruses.

Two constructs carrying 303 bp of VP7 sequences (aa 226 to 326) cloned in pV1 and pV2 (pV1/101 and pV2/101) resulted in more-stable recombinant viruses. The sequence of the

TABLE 1. Viable stable viruses after plaque purification[a]

Virus	No. of VP7 bp[b]	Titers (PFU/ml)
V1/101	303	6.3×10^6
V2/101	303	5.0×10^6
V1/Δ101c4[c,d]	90	ND[e]
V2/Δ101c9	210	1.9×10^7
V2/Δ150c13	237	6.3×10^6
V2/Δ150c7	90	4.2×10^6
V2/Δ178c14	144	7.5×10^6

[a] The sizes of the foreign inserts were determined by sequencing the products amplified from viral RNA.
[b] All sequences also have an additional 30 bp (not included in the table) that codes for the multiple cloning site and the artificial 3C protease site.
[c] Δ indicates stable viruses isolated from crude transfections of originally larger DNAs.
[d] Also has a deletion of 21 bp of the poliovirus noncoding region preceding the initial AUG.
[e] ND, not done.

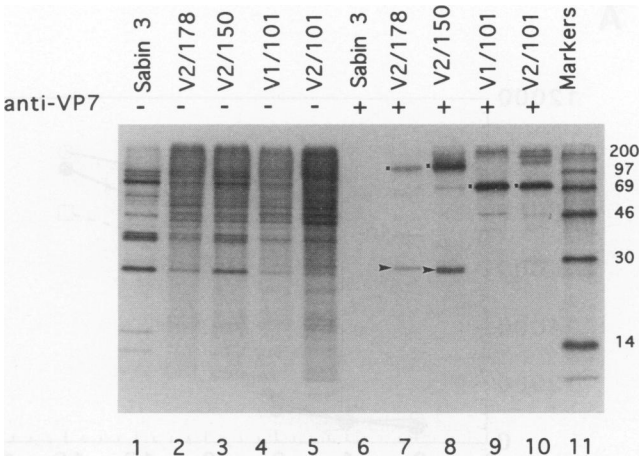

FIG. 4. Expression of the truncated VP7 polypeptides in cells infected with supernatants from recombinant Sabin 3 transfections. Fresh cells were infected with Sabin 3 or Sabin 3/VP7 recombinant polioviruses obtained after transfection of infectious RNA. Proteins were metabolically labeled with Tran^{35}S-Label for 2 h (6 to 8 h p.i.). Cells were lysed in RIPA buffer and immunoprecipitated with anti-VP7 antibodies. The labeled proteins were separated in an SDS–12.5% polyacrylamide gel either directly (lanes 1 to 5) or after immunoprecipitation (lanes 6 to 10). Polypeptides migrating approximately in the region expected for the VP7 inserts are observed (arrowheads), as well as several high-molecular-weight uncleaved precursors (solid squares). Lane 11, ^{14}C-labeled molecular weight markers. Numbers at right indicate molecular weight in thousands.

RT-PCR products from these two viruses confirmed the expected sequence without deletions. Viruses from both constructs were positive for the presence of a 303-bp insert after four passages in Vero cells (Fig. 3B, lanes 2 to 5 and 7 to 10). Simultaneously, plaque purification was done after transfection. Only 2 of 20 plaques showed deletions in the insert (V1/Δ101c4 and V2/Δ101c9 [Table 1]).

The sequence of one of the plaque-purified viruses, V2/Δ150c13, showed a deletion of 213 bases within the VP7 insert, leaving an insert of 237 bases coding for 79 aa (aa 150 to 161 plus 230 to 298) of VP7 in frame with the poliovirus polyprotein. The sequence of the PCR product from virus derived from another plaque-purified virus (V2/Δ178c14) also showed a deletion in the middle of the insert of 390 bases. In this case, 144 bases were left, coding for 48 aa of VP7 (aa 150 to 182 plus aa 312 to 326). One interesting virus is V1/Δ101c4, in which the deletion started at the Sabin 3 nucleotide 722 in the noncoding region and continued into the *Not*I site and the VP7 insert. In this case, the translation of the polyprotein possibly starts at the AUG codon for methionine 310 of the VP7 sequence. In other cases, the deletions are just at the 5′ end (not shown) or at the 3′ end of the VP7 inserts (V2/Δ150c7 and V2/Δ101c9).

All deletions resulted in the open reading frame being maintained, which was expected in order to obtain viable viruses. Deletions in the inserted sequences might stabilize the recombinant Sabin 3 viruses, and these populations might be subsequently selected in cell culture. The deletions seemed to be at random. So far, we were not able to find results indicative of specific mechanisms of deletion or specific sequences at which the deletions were produced. The only common feature was the selection of an insert size under 300 bp.

Expression of rotavirus VP7 in cells infected with recombinant polioviruses. Infectious recombinant viruses were studied to determine whether the truncated VP7 proteins were expressed and correctly processed from the polyprotein in cells infected with recombinant Sabin 3 polioviruses.

Vero cells were infected with transfection supernatants and metabolically labeled with Tran^{35}S-Label for 2 h (from 6 to 8 h p.i.). The labeled proteins were immunoprecipitated with antiserum against rotavirus VP7 and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 4). Sabin 3-infected cells were also labeled and immunoprecipitated with the same antiserum.

In Fig. 4, lanes 1 to 5 correspond to lysates from cells infected with Sabin 3, V2/178, V2/150-1, V1/101, and V2/101, respectively. Apparently, the recombinants are not as efficient as Sabin 3 in the shutdown of host protein synthesis. Lanes 6 to 10 of Fig. 4 show labeled lysates immunoprecipitated with anti-VP7 antiserum. Several high-molecular-weight uncleaved precursors are seen, as well as polypeptides running in the region of the gel corresponding to the expected molecular weights of the VP7 inserts (lanes 7 and 8). These polypeptides are not seen in Sabin 3-infected labeled lysates (lane 6).

The kinetics of RNA synthesis are similar, but virus assembly is slower for the Sabin 3/VP7 recombinants. The rate of viral RNA synthesis was measured for Sabin 3 and for two recombinant viruses carrying the same foreign insert in different regions of the Sabin 3 genome (V1/101 and V2/101). Incorporation of [^{3}H]uridine into RNA was measured by trichloroacetic acid precipitation, and the results are shown in Fig. 5A. The kinetics of RNA synthesis were similar for the three viruses studied, although V2/101 had a slightly slower rate of incorporation and smaller final amounts of RNA. Initiation of the exponential phase of viral RNA synthesis at about 6 h p.i. in Vero cells is consistent with other studies done with Vero cells for attenuated Sabin viruses (40).

Despite the similar RNA kinetics, one-step growth curves of viral production showed that infectious Sabin 3 viruses were assembled more rapidly than the Sabin 3/VP7 recombinants, which presented an average of a 2-h delay (Fig. 5B). Virion assembly of Sabin 3 started at about 6 h p.i., whereas that of the recombinants started at about 8 h p.i. The fact that the cycles were slower for the recombinants is also demonstrated by the final titers obtained after overnight incubation of infected cells. The titers of Sabin 3 after 20 h of incubation remained the same as the titers at 12 h p.i., while the final titers at 20 h p.i. were three- and twofold higher than at 12 h p.i. for V1/101 and V2/101, respectively (Fig. 5B). The final yield of plaque-forming particles in cells infected with V1/101 and

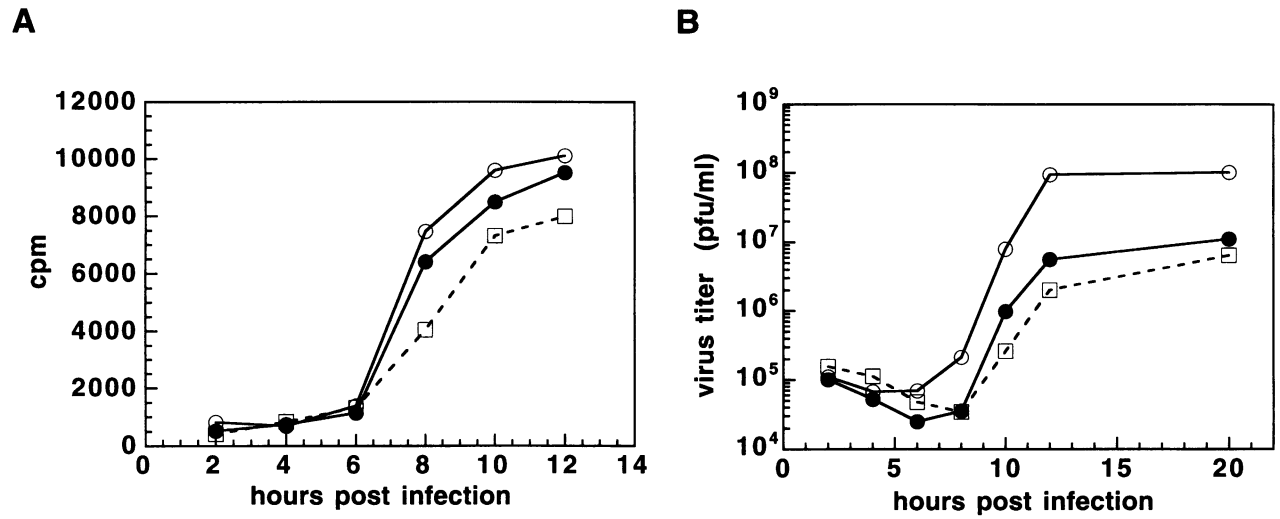

FIG. 5. (A) Kinetics of viral RNA synthesis. Vero cells were mock infected or infected with Sabin 3 or Sabin 3/VP7 recombinants at an MOI of 10 and incubated in the presence of 5 μg of actinomycin D per ml and 15 μCi of [^{3}H]uridine per ml. Infections were stopped at different times, starting at 2 h p.i. Cells were washed and scraped into buffer NET containing 0.5% SDS. The lysates were then precipitated with trichloroacetic acid on ice for 30 min. Radioactivity incorporated into trichloroacetic acid-precipitable material was measured by scintillation counting. For the graphic, the values for mock-infected cells have been subtracted from each of the corresponding values shown. ○, Sabin 3; ●, V1/101; □, V2/101. (B) One-step growth curves. Vero cells were infected as described above with the same viruses. After adsorption, the inoculum was washed, fresh medium was added, and infections were stopped at different times p.i. The cells plus the supernatants were freeze-thawed three times before titration on Vero cell monolayers. Virus titers are expressed as PFU per milliliter on a logarithmic scale. ○, Sabin 3; ●, V1/101; □, V2/101.

V2/101 was 1 and 1.5 logs lower, respectively, compared with Sabin 3, despite the longer growth cycles of the former two viruses. Both recombinants also showed smaller-size plaques than Sabin 3.

Inefficient cleavage of the VP7 protein may be a determinant of stability of Sabin 3/VP7 recombinants. The expression of VP7-containing precursors was examined in cells infected with the viruses containing stable VP7 inserts V1/101 and V2/101. In this experiment, the accumulation of precursors observed was dependent on the type of fusion generated and the antiserum used (either anti-polio, anti-VP7, or anti-2A).

V1/101, which contains 303 bp of VP7 (12.4K) cloned at the 5′ end of the poliovirus polyprotein, showed accumulation of a 50K precursor (Fig. 6A, lane 1) when immunoprecipitated with anti-poliovirus type 3 serum. This precursor corresponds to the expected molecular weight of [ΔVP7+VP0] (12.4K+37K) and was not present in the Sabin 3 (lane 3) or mock- (lane 4) infected cells. Since the anti-polio serum is specific for the capsid proteins, larger precursors are not expected to be seen in viruses derived from pV2 clones, which contain the VP7 inserted between P1 and P2, when infected cells are immunoprecipitated with anti-polio serum (V2/101, lane 2). VP0 was clearly visible in lane 2 while it was almost absent in lane 1. In order for V1/101 to be viable, cleavage of [VP7+VP0] must occur. The presence of a faint band at the VP0 position and the intense band for the [ΔVP7+VP0] precursor suggest that cleavage is slow (Fig. 6A, lane 1).

In Fig. 6B, immunoprecipitation of the same labeled lysates with anti-VP7 serum demonstrates the accumulation of several precursors. Precursors that correspond in size to [ΔVP7+P1] (109K), [ΔVP7+VP0+VP3] (76K), and [ΔVP7+VP0] (50K) are present in V1/101 (lane 1). The free ΔVP7 polypeptide (12.4K) is also seen. In lane 2, precursors that correspond in size to [ΔVP7+P2] (78K) and [ΔVP7+2A] (29K) are observed for V2/101, but free ΔVP7 is not. A higher-molecular-weight precursor that corresponds in size to [VP1+ΔVP7+P2] (111K)

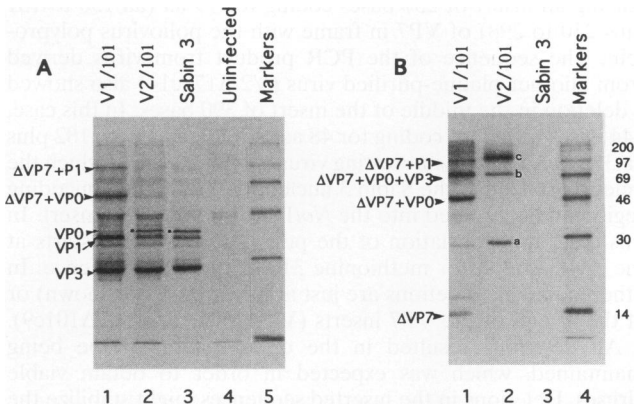

FIG. 6. Slow cleavage of the VP7 fragments determines accumulation of uncleaved intermediate precursors in infected cells. Sabin 3 recombinant viruses carrying VP7 sequences were used to infect Vero cells. Proteins were labeled with Tran^{35}S-Label at 6 to 8 h p.i. and analyzed in a 12.5% polyacrylamide gel after immunoprecipitation. (A) Proteins were immunoprecipitated with anti-poliovirus type 3. A precursor of about 50,000 in molecular weight that represents [ΔVP7+VP0] accumulates in cells infected with V1/101 (lane 1). The amount of free VP0 present in the V1/101 lysate is also smaller than the amount present in V2/101 and Sabin 3 lysates (indicated by solid squares in lanes 2 and 3, respectively). (B) Proteins in cells infected with Sabin 3 or the recombinants V1/101 or V2/101 were immunoprecipitated with anti-VP7 antiserum. Different kinds of precursors are observed in the infected cells. For V1/101 (lane 1), the precursors correspond in sizes to [ΔVP7+P1], [ΔVP7+VP0+VP3], and [ΔVP7+VP0] and are indicated by arrowheads to the left of the figure. Free ΔVP7 (12.4K) is also indicated by an arrowhead. For V2/101 (lane 2), a larger precursor corresponds in size to [VP1+ΔVP7+P2] and the two smaller correspond to [ΔVP7+P2] and [ΔVP7+2A]. These precursors are indicated by the letters c, b, and a, respectively. No free ΔVP7 is detected in this case. Numbers at right indicate molecular weight in thousands.

is also seen in lane 2. Immunoprecipitation with anti-2A protease antibody resulted in the same precursors for V2/101, and a very small amount of free 2A (results not shown), which confirms that the cleavage at the 3C protease site for V2 vectors is highly inefficient.

DISCUSSION

The use of an attenuated strain of poliovirus for expression of a foreign antigen was investigated in this work. Incorporation of up to 534 bp of the rotavirus VP7 gene in the genome of Sabin 3 poliovirus gave rise to infectious viruses that expressed portions of rotavirus VP7 in infected cells. However, only viruses carrying inserts of about 300 bp or smaller were able to maintain the foreign sequences in their genomes without further deletions upon passage in tissue culture. These initial results are encouraging and indicate the possibility of using Sabin strains as live vectors, although more work is needed to improve the system, specifically with regard to the size and the nature of the foreign sequences to be inserted.

Recombinant clones containing larger VP7 inserts failed to produce infectious viruses. There are several reasons why this may occur: (i) a deleterious effect on RNA replication or translation, (ii) failure of proper protease cleavage due to conformational constraints, (iii) spatial relationship between artificial protease sites and the protease if the cleavage is made in *cis*, (iv) influence of the nature of the foreign sequences on the efficiency of protease cleavage, and (v) the presence of the VP7 signal peptide in some of the constructs. Work done with other viral antigens in our laboratory (not shown) strongly supports the idea that the success of the system is dependent on the nature of the sequences inserted.

A comparison of the Sabin 3/VP7 recombinants with their Sabin 3 parent showed no significant differences regarding the kinetics of viral RNA synthesis in infected cells, but the recombinants were found to be slower in the assembly of infectious virus. The recombinant viruses showed smaller plaques, and titers were usually 1 to 1.5 logs lower than Sabin 3.

One of our working hypotheses is that slow assembly of the recombinant Sabin 3/VP7 viruses results from slow cleavage at the artificial 3C protease sites. This is suggested by the accumulation of specific precursors in infected cells. In addition, a deficient or slow cleavage might render these viruses unable to compete in the presence of mixed populations (i.e., revertants). If some virus populations carrying smaller pieces or no foreign sequences were selectively amplified, they might overgrow the others. This might be more likely in the case of large foreign inserts. On the basis of the sequence of several deleted recombinant viruses (some not shown), it appears that deletions occur at random and that the type of recombinants isolated may represent only those with selective advantages that also have maintained the correct open reading frame for the poliovirus polyprotein. Obviously, the VP7 insert represents the region of the genome most tolerant to deletions that result in viable viruses.

The 3C protease cleavage site introduced 3′ to the foreign antigen has been designed on the basis of the optimal sequence reported for cleavage of the poliovirus polyprotein (29). However, cleavage is dependent not only on this sequence but also on the three-dimensional conformation of the substrate. When the VP7 sequences are inserted at the 5′ end of VP0, cleavage by the 3C protease is required in order to produce viable viruses. The accumulation of precursor ΔVP7+VP0 suggests that this cleavage may occur slowly, although expression of the free VP7 insert can clearly be detected when it is cloned at the 5′ end of the poliovirus polyprotein (pV1 vectors). Similar conclusions have been drawn for 2A protease, which also has a strict substrate preference. 2A protease cleaves at its own amino terminus autocatalytically (41); however, in the viruses studied here, there are VP7 sequences intervening between 2A and its natural cleavage site. The 2A-mediated cleavage at the VP1-VP7 junction appears to be unaffected, as demonstrated by the ability to recover viable viruses. However, we were not able to detect free VP7 when the fragments are cloned at the junction of P1 and P2 (pV2 vectors).

It was observed that for duplicate short inserts engineered separately in pV1 and pV2, those in pV2 resulted in infectious viruses more readily. We have demonstrated that 2A-mediated cleavage occurs in the presence of intervening sequences; however, we speculate that viruses that did not process the VP7 insert from the 2A protease through the artificial 3C protease site (or processed it very slowly) would still be viable. In this case, either very small amounts of 2A may be necessary for the viral replication or the functions of 2A can still be carried out by a 2A-VP7 fusion protein. Even when free VP7 was not detected in V2 viruses, free 2A was detected with anti-2A antiserum. The size of the insert between the 2A protease sequences and the 2A cleavage site may also influence the efficiency of the cleavage.

These results suggest that if the efficiency of cleavage at the artificial sites can be increased, the viability of the poliovirus recombinants might also improve. It is also possible that larger-size inserts may be accommodated, especially at the 5′ end of the polyprotein. This may be dependent on the nature of the sequences inserted and on the secondary and tertiary structure that they adopt in the polyprotein.

Our viable Sabin 3/VP7 constructs include the carboxyl-terminal region of VP7. Current work includes the construction of recombinant Sabin 3 viruses carrying shorter amino-terminal fragments of VP7 with and without the signal peptide. Although the generation of viable viruses may correlate with the nonfunctionality of the VP7 signal peptide, the feasibility of such constructs is of interest because the only cytotoxic T-lymphocyte epitope found in VP7 overlaps the second signal sequence (12). While the amino-terminal regions of VP7 are highly variable and have conformation-dependent serotype-specific epitopes, the carboxy-terminal region has been found to be structurally conserved in the three antigenic rotavirus groups, A, B, and C (aa 192 to 231 and aa 278 to 309) (24). Moreover, portions of this region may be exposed, as suggested by computer analysis and by the finding of a neutralization escape mutant with a mutation at aa 291 (21). In another report, a peptide made to aa 275 to 295 and a monoclonal antibody to this peptide blocked virus adsorption to cells in culture (13, 34). However, as described in the Introduction, other attempts at using recombinant VP7 as a vaccine were not very successful (for reviews, see references 5, 7, and 24). Another potential antigen of interest is the outer capsid protein VP4. Promising results have been obtained with VP4 peptides and recombinant VP4 expressed in different systems (16, 24). We are presently cloning selected regions of rotavirus VP4 in newly designed vectors.

There are several advantages to using Sabin strains of poliovirus as vaccine vectors for the development of multivalent vaccines combined with oral polio vaccine. Work in progress in our laboratory includes cloning of other antigens in both Sabin 3 and Sabin 1 backgrounds and immunogenicity studies in transgenic mice that express the human poliovirus receptor (33). Conclusions based on rotavirus VP7 may not hold true for all other antigens to be expressed in Sabin vectors, but the accumulated understanding of this system will

be very useful for future applications with other viral or bacterial antigens for which mucosal immunity is critical for protection from disease.

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