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Toward a Poliovirus-Based Simian Immunodeficiency Virus Vaccine: Correlation between Genetic Stability and Immunogenicity

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Recombinant polioviruses expressing foreign antigens may provide a convenient vaccine vector to engender mucosal immunity. Replication-competent chimeric viruses can be constructed by fusing foreign antigenic sequences to several positions within the poliovirus polyprotein. Artificial cleavage sites ensure appropriate proteolytic processing of the recombinant polyprotein, yielding mature and functional viral proteins. To study the effect of the position of insertion, two different recombinant polioviruses were examined. A small amino-terminus insertion delayed virus maturation and produced a thermosensitive particle. In contrast, insertion at the junction between the P1 and P2 regions yielded a chimeric poliovirus that replicated like the wild type. Eight different chimeras were constructed by inserting simian immunodeficiency virus (SIV) sequences at the P1/P2 junction. All recombinant viruses replicated with near-wild-type efficiency in tissue culture cells and expressed high levels of the SIV antigens. One of the inserted fragments corresponding to gp41 envelope protein was N-glycosylated but was not secreted. Inserted sequences were only partially retained after few rounds of replication in HeLa cells. This problem could be remedied to some extent by altering the sequences flanking the insertion point. Reducing the homology of the direct repeats by 37% decrease the propensity of the recombinant viruses to delete the insert. To determine the immunogenic potential of the recombinants, mice susceptible to poliovirus infection were inoculated intraperitoneally. The antibody titers elicited against Gag p17 depended on the viral doses and the number of inoculations. In addition, recombinants which display higher genetic stability were more effective in inducing an immune response against the SIV antigens, and inoculation with a mix of recombinants carrying different SIV antigens (a cocktail of recombinants) elicited humoral responses against each of the individual SIV sequences.

Sexual transmission is the major route for the spread of the human immunodeficiency virus (HIV) in the AIDS pandemic progression. Changes in sexual behavior may help to contain the spread of the epidemic but will not eliminate the need for an effective HIV vaccine. Although the biology of sexual transmission of HIV is poorly understood, it is clear that an essential first step in the process involves deposition of infectious virus or HIV-infected cells upon mucosal surfaces in the vagina or rectum of the recipient partner. Following introduction into a new host, HIV or HIV-infected cells likely soon encounter susceptible host target cells at the mucosal portal of entry or in nearby lymphatic tissues, initiating an inexorable course of systemic HIV infection (29, 35). Considering the most common acquisition routes for HIV infection, an immune barrier extant at genital mucosal surfaces would have the best chance of interrupting HIV transmission by preventing or rapidly limiting initial viral replication. Unfortunately, little information is available concerning the potential contribution of mucosal immunity to the limitation of transmission of viral pathogens such as HIV (12, 13, 17), and few approaches are available to induce effective mucosal immune responses. In addition, it has been shown that systemic immunization with a live-attenuated simian immunodeficiency virus (SIV), which protects rhesus macaques from systemic challenge with SIV, does not protect from vaginal inoculation with virulent SIV (23). Furthermore, to have a significant impact on the HIV pandemic, a successful HIV vaccine must also be appropriate for use in developing countries, where an estimated 90% of all new HIV infections will occur by the year 2000 and where the World Health Organization estimates that over 300 million individuals may need to be vaccinated during the first years of a mass immunization campaign (26).

Many of these same issues were confronted in the planning and execution of the ongoing World Health Organization campaign to eliminate paralytic poliomyelitis through widespread immunization. The beneficial attributes of the Sabin live-attenuated vaccine have greatly facilitated the conduct of this campaign. The live-attenuated poliovirus vaccine is easy to administer by the oral route, has a low cost for distribution in the developing world, induces both serum antibodies and intestinal mucosal resistance, and confers long-lasting immunity (25, 33). Given the favorable characteristics of the Sabin poliovirus vaccine, a number of investigators have attempted to adapt poliovirus as a vector to express antigens from other pathogens.

The poliovirus genome consists of a single-strand, positive-sense RNA molecule of approximately 7,500 nucleotides. A unique open reading frame encodes a large polyprotein precursor, which must be proteolytically processed by two viral proteases ($2A^{pro}$ and $3C^{pro}$) in order to generate mature structural and nonstructural poliovirus proteins. The major viral protease, $3C^{pro}$, recognizes and cleaves at specific amino acid sequences (AXXQG) within exposed polyprotein domains, whereas the second protease, $2A^{pro}$, cleaves cotranslationally at the junction between the P1 and P2 regions.

Several strategies have been reported for engineering polio-

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virus vectors: small antigenic epitopes were inserted into one of the capsid proteins (8, 11); dicistronic poliovirus RNAs were constructed by duplicating the 5′ noncoding region of the poliovirus genomic RNA (internal ribosomal entry site [IRES]), in which foreign polypeptides were expressed by using one IRES and essential viral proteins were produced by using the other IRES (1, 20); and poliovirus minireplicons were constructed in which poliovirus structural protein genes were replaced by foreign sequences (6, 9, 30). Limitations of these strategies include the small size of the tolerated insert, genetic instability of the inserted sequences, and a requirement for helper virus for viral propagation which potentially would limit minireplicon replication in vivo.

We have used a different method to generate recombinant polioviruses that express genetic sequences of other pathogens and are able to replicate without the need of a helper virus (5). Sequences are inserted at different positions of the poliovirus polyprotein, separated by artificial 3C or 2A protease cleavage sites. In this way, a larger than normal precursor is initially made, but it is appropriately cleaved into the usual array of constituent proteins. The 3C or 2A protease accurately recognizes and cleaves the inserted synthetic proteolytic sites, freeing the exogenous protein sequences from the rest of the poliovirus polyprotein. In this manner, all of the poliovirus proteins are correctly produced and normal viral replication proceeds.

In this report, we describe the construction and characterization of a recombinant poliovirus carrying SIV Env, Gag, and Nef antigenic sequences. We have determined that fusing the exogenous sequences to an internal point within the polyprotein yields recombinant viruses with a phenotype that better resembles that of wild-type nonrecombinant poliovirus. Foreign SIV amino acid sequences were effectively produced in poliovirus chimera-infected cells. The genetic stability of the inserted sequences could be increased by introducing mutations to reduce the homology of the direct repeats created at each side of the inserted sequences. Infection of susceptible mice with the poliovirus recombinants elicited humoral immune responses to SIV proteins in a dose-dependent manner. In addition, administration of a mix of recombinants carrying five different SIV antigens elicited antibodies that recognized each of the SIV proteins.

MATERIALS AND METHODS

Poliovirus recombinant construction and DNA procedures. Restriction enzymes, T4 DNA polymerase, *Taq* polymerase, T7 RNA polymerase, and avian myeloblastosis virus reverse transcriptase were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind.; T4 DNA ligase was obtained from New England BioLabs, Inc., Beverly, Mass.; shrimp alkaline phosphatase came from the United States Biochemical Corp., Cleveland, Ohio. All enzymes and compounds were used as recommended by the manufacturers.

Poliovirus chimeras were constructed by individually cloning eight SIV genome fragments encoding specific antigenic SIV proteins into a molecular clone of Mahoney type 1 wild-type poliovirus vaccine vector MoV-2.1. The Mahoney vector MoV-1.4 has been already described (5). A new vector, MoV-2.1, was constructed by modifying the infectious poliovirus cDNA at the junction of P1 and P2 coding sequences. We inserted an in-frame synthetic polylinker containing *Eco*RI, *Not*I, *Bss*HII, and *Xho*I sites that facilitate the insertion of foreign sequences; an artificial recognition and cleavage site (LTTY/G) for the $2A^{pro}$ poliovirus protease was duplicated at the 5′ border of the polylinker, and the cleavage site was followed by a six-glycine tract.

Exogenous DNA sequences were amplified by PCR (7) with primers that included restriction enzyme recognition sites which were used to insert the PCR-amplified exogenous DNA into the vector polylinker. Coding sequences for the p17 region of SIV *gag* gene was amplified with primers 1 and 2; the p27 region of the SIV *gag* gene was amplified with primers 3 and 4; the N-terminal half of the *nef* gene was amplified with primers 5 and 6; two fragments of the coding sequence of the gp130 envelope protein were amplified with primers 7 and 8 and primers 9 and 10; finally, three fragments corresponding to the N-terminal, central, and C-terminal regions of envelope gp41 protein (gp41.a, gp41.b, and gp41.c) were amplified with primers 11 and 12, 13 and 14, and 15 and 16, respectively. All SIV sequences were amplified by using a plasmid containing pathogenic SIV 239 strain sequences as a template (a gift from Mark B. Feinberg). PCR fragments used in cloning were digested with restriction enzymes *Eco*RI and *Xho*I and ligated to the vector digested with the same enzymes (7). Replication-competent chimeric polioviruses were recovered by transfection of HeLa S3 cells with in vitro-transcribed RNA from recombinant cDNA clones (21).

Cells and virus stocks. HeLa cells, clone S3, were grown in suspension in Joklik's modified minimal essential medium (Sigma Chemical Company, St. Louis, Mo.) supplemented with 10% horse serum (GIBCO Diagnostics, Madison, Wis.), 1% penicillin-streptomycin, and 1% L-glutamine (both from Mediatech, Inc., Herndon, Va.).

HeLa cell monolayers in 100-mm-diameter dishes were grown in a 1:1 mixture of Dulbecco's modified Eagle medium and nutrient mixture F12 (D-MEM/F12) (GIBCO) and transfected with 1 to 10 mg of recombinant viral RNA by a DEAE-dextran procedure (21). Single plaques were isolated and expanded for each poliovirus chimera by standard procedures to generate stocks that were used for further characterization.

Virus infections. In all experiments, 100-mm-diameter dishes containing approximately 3×10^6 cells were used. The dishes were washed once with phosphate-buffered saline (PBS), and the appropriate amounts of virus to yield the desired multiplicity of infection (MOI) were added. Plates were incubated at room temperature for 30 min to allow the virus to adsorb to the cells, then 10 ml of D-MEM/F12 1:1 mixture; Mediatech) supplemented with 10% newborn calf serum (GIBCO), 1% penicillin-streptomycin, and 1% L-glutamine (Mediatech) was added, and the dishes were placed at the appropriate temperature for the specific experiment. At the time of collection, plates were washed once with PBS, and cells were resuspended in 1 ml of PBS. Cells were collected by centrifugation at low speed and stored at −20°C until analysis.

One-step growth curve and virion thermal inactivation. HeLa cell monolayers in 100-mm-diameter dishes were infected with each recombinant, as described above, at an MOI of 10 and incubated at 37 and 39°C. At each time point, infected cells were collected and lysed by freeze-thawing. Viral yield (PFU per milliliter) at each time point was determined by plaque assay (34).

To determine the thermal stability of poliovirus vectors, viral stocks were diluted to 5×10^7 PFU/ml in calcium-magnesium free PBS and incubated at 45°C. At each time point, virus was collected and the titer was determined by plaque assay.

Pulse-chase analysis. HeLa cells were infected as previously described at an MOI of 100 for each of the vectors and wild-type polioviruses. After 30 min of adsorption of the virus at room temperature, the cells were washed with PBS, and methionine-free D-MEM/F12 was added. [^{35}S]methionine (25 μCi/ml; Du Pont NEN Research Products, Boston, Mass.) was added at 2.5 h postinfection. After 10 min of pulse at 37°C, cells were washed with PBS and chased with an excess of unlabeled methionine. Cells were collected at various time points and resuspended in buffer H (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride), and the nuclei were removed by centrifugation (4).

Immunoprecipitations were carried out by incubation for 1 h at 0°C with specific antibodies directed against poliovirus capsid proteins in buffer BDB (100 mM borate buffer [pH 8.2], 10% sodium dodecyl sulfate [SDS], 1% Triton X-100, 0.05% sodium azide, 1% sodium deoxycholate). Protein A-agarose beads (Boehringer Mannheim) were added, and the mixture incubated for 1 h at 4°C. Beads were washed two times with buffer H-BDB (BDB containing 500 mM NaCl) and one time with BDB, resuspended in SDS-loading buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

RT-PCR. Total RNA from cells infected with a recombinant virus was prepared by phenol-chloroform extraction 9 h postinfection and precipitated with ethanol. Reverse transcription (RT) was performed with avian myeloblastosis reverse transcriptase at 42°C for 60 min, using a 6-mer random primer. After the reaction was completed, the enzyme was inactivated by incubation at 100°C for 3 min. PCR amplification was performed with primers 17 and 18 to amplify the region of the poliovirus genome that contained the inserted sequences. PCR was performed for 25 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, using *Taq* polymerase.

Western blot analysis. HeLa cells infected with wild-type and SIV/poliovirus recombinants (MOI of 10) were incubated for 3, 5, and 7 h at 37°C. Cells were harvested and lysed in buffer H, and the nuclei were removed by centrifugation (4). Four-microgram aliquots of proteins of total lysates were subjected to electrophoresis through an SDS–12% polyacrylamide gel and analyzed by immunoblotting. Antisera against SIV proteins were obtained from SIV-infected rhesus macaques (kindly provided by Chris Miller). Antisera directed against poliovirus capsid proteins were obtained by inoculating rabbits with purified poliovirus. Secondary antibodies (both anti-monkey and anti-rabbit) were obtained from Amersham, Arlington Heights, Ill. Immunoblotting was performed as specified by the manufacturer of the ECL chemiluminescence detection kit (Amersham).

Plaque assay immunodetection of recombinant viruses. One milliliter of suspension culture of HeLa cells, containing 10^7 cells, was infected with approximately 100 PFU of recombinant poliovirus. Infected cells were incubated at room temperature for 30 min to allow the virus to adsorb to the cells. Infected cells were mixed with 5 ml of melted 1% agar–D-MEM/F12 supplemented with 10% newborn calf serum (GIBCO), 1% penicillin-streptomycin, and 1% L-

glutamine (top agar). The suspension was poured into plates containing 15 ml of solid D-MEM/F12–1% agar (bottom agar). Afterward, top-agar-solidified plates were inverted and incubated at 37°C for 48 h. When plaques were detected in the top agar, nitrocellulose filters were placed on top, and plaques were transferred to the filters and analyzed by using antipoliovirus and anti-SIV antisera in a protocol similar to the one described for Western blots.

Expression of SIV proteins in *Escherichia coli*. The T7 expression system (32) was used to produce SIV proteins used in enzyme-linked immunosorbent assay (ELISA) determinations. Plasmids were constructed by using a modified version of the original T7 RNA polymerase expression plasmid (pT7-48) in which new restriction sites, on an in-frame synthetic polylinker containing *Eco*RI, *Not*I, *Bss*HII, and *Xho*I sites, were introduced to facilitate the insertion of SIV protein coding regions, and a six-histidine tail was fused in frame at the N terminus. Inserts (encoding p17, p27, Nef, gp130, and gp41) were obtained by digesting recombinant poliovirus plasmids with restriction enzymes *Eco*RI and *Xho*I and ligated to pT7-48 digested with the same enzymes. The proteins were expressed and purified by using an Ni^{2+}-nitrilotriacetic acid resin as recommended by the manufacturer (Qiagen).

Inoculation of transgenic mice with recombinant poliovirus and determination of antibody titers by ELISA. Transgenic mice expressing the poliovirus receptor (PVR) under the transcriptional control of the β-actin promoter were generated by using established techniques (unpublished data). This strain of transgenic mice proved to be highly susceptible to wild-type Mahoney type 1 infection. Transgenic mice were infected intraperitoneally with 100 μl of SIV/poliovirus recombinant stocks at the concentrations indicated in the figure legends. As a negative control, nontransgenic mice were infected by intraperitoneal injection of 100 μl of each recombinant viral stock. Mice were inoculated every 4 days. Sera were obtained from infected mice at 0, 4, 8, 12, 16, and 20 weeks postinoculation and were analyzed by ELISA (7). Plates coated with purified SIV antigens (p17, p27, Nef, gp41, and gp130, expressed in *E. coli*) at 5 μg/ml were incubated with doubling dilutions of test samples. Bound antibody was detected by incubation with antibodies to mouse immunoglobulin (Ig) antibodies conjugated to horseradish peroxidase (Amersham). Enzymatic activity was determined with ABTS tablets (Boehringer Mannheim). The absorbance was measured at 405 nm. Results are expressed as the reciprocal of the lowest dilution that gave an absorbance of 0.2 to 0.3 units above the background. Background controls included assay plates coated with 5 μg of bovine serum albumin as well as evaluation of preimmune samples. The reproducibility of the ELISA after three repeated assays of the same sample of serum was within one dilution.

Oligonucleotides. The oligonucleotides used were as follows: 1 (CTCCGCCTGACTCGAGGTAATTTCCTCCTCT [P17]), 2 (GGTGGGGGAGGTGAATTCGGCGTGAGAAACTCCGTCTTG [P17]), 3 (CCTCCGCCTGACTCGAGCATTAATCTAGCCTTC [P27]), 4 (ATGGCTGCTCAGGAATTCCCAGTACAACAAATAGG[P27]), 5 (GGGAGGTGAATTCGGTGGAGCTATTTCCAT [NEF.A]), 6 (AGGCGCGCTCCAGCGGCCGCCCCTTCCAGTCCCCCCT [NEF.A]), 7 (GGGGGAGGTGAATTCTATTGTACTCTATAT [130.A]), 8 (GGTCAGATCCTCGAGGTACCAAGTTTCATT[130.A]), 9 (GGGGGAGGTGAATTCATAAAAGAGGTGAAG [130.C]), 10 (GGTCAGATCCTCGAGTGAGGTGCCACCAGT[130.C]), 11 (GGGGGAGGTGAATTCAGAAATAAAAGAGGG [41.A]), 12 (GGTCAGATCCTCGAGTGCATTTGGCCATGG[41.A]), 13 (GGGGGAGGTGAATTCCCATGGCCAAATGCA [41.B]), 14 (GGTCAGATCCTCGAGGGAAGAGAACACTGG[41.B]), 15 (GGTCAGATCCTCGAGCAAGAGAGTGAGCTC [41.C]), 16 (GGGGGAGGTGAATTCCCAGTGTTCTCTTCC[41.C]), 17 (TTGGTATTTCGAACGCCT [NT 3074]), and 18 (AACCTGCAGTGTACA [NT 3468]).

RESULTS

Replicative characteristics of two poliovirus vectors with different insertion sites. We constructed chimeric polioviruses carrying a small insertion either at the amino terminus (MoV-1.4) or at the junction between the P1 and P2 regions (MoV-2.1) (Fig. 1A). MoV-1.4 (N-terminus insertion) replicated more slowly than the parental wild type, exhibited a lag in replication of about 1 to 2 h, and by 6 h postinfection achieved only 10 to 20% of wild-type titers (Fig. 1B). In contrast, MoV-2.1 (with an insertion at the junction between P1 and P2) replicated with kinetics remarkably similar to those of the wild type and at 39°C achieved the same final titers.

Because the foreign sequences in MoV-1.4 are produced as a fusion peptide to the capsid protein VP0, we suspected that a capsid assembly defect might be responsible for the poor replicative efficiency. We examined particle stability by incubating viruses at 45°C for different times and observed a 99% reduction of MoV-1.4 titer by 30 min. Under the same conditions, there was no significant change in wild-type or MoV-2.1 titers (Fig. 1C). In pulse-chase and immunoprecipitation experiments, the capsid formation defect correlated with inefficient proteolytic processing of the foreign sequence at the N terminus (Fig. 1D, lanes 4 to 6, band marked with a dot). Also, we noticed a defect in producing mature capsid, evidenced by a delay in the appearance of the maturation cleavage product VP2 (Fig. 1D, lanes 4 to 6). In contrast, MoV-2.1 produced particles with protein composition and kinetics very similar to those of wild-type particles. Thus, it appears that fusion at the amino terminus alters virus assembly and results in defective capsids. In contrast, the junction between P1 and P2 is permissive. We therefore constructed a new series of poliovirus chimeras carrying SIV antigens at this internal position.

Construction of SIV poliovirus recombinants and expression of foreign proteins. Eight chimeric polioviruses were prepared by inserting nucleotide sequences coding for SIV antigens into MoV-2.1 (Fig. 2A). The antigens include the Gag proteins p17 and p27 (134 and 229 amino acids, respectively), 130 amino acids from the amino terminus of Nef protein, and five fragments corresponding to the envelope proteins gp130 and gp41 (amino acids 21 to 188 [gp130.a], amino acids 354 to 521 [gp130.c], amino acids 522 to 626 [gp41.a], amino acids 622 to 727 [gp41.b], and amino acids 723 to 879 [gp41.c]). With this strategy, the size of the insertion was less than 700 nucleotides, which may contribute to replication fitness of the recombinants (see Discussion).

All chimeric poliovirus cDNAs yielded replication-competent viruses after transfection of HeLa cells with in vitro-synthesized RNA. Seven of eight recombinants produced plaques similar in size to wild-type plaques (data not shown). Recombinant Sp27, in contrast, produced plaques that were 60 to 70% smaller than those corresponding to wild-type virus.

To determine whether recombinant viruses expressed the foreign polypeptides, HeLa cells were infected with wild-type and chimeric polioviruses, and cytoplasmic extracts were obtained at different times following infection and analyzed by immunoblotting. In some cases, more than one band was produced, suggesting that the proteolytic processing was incomplete. For example, extracts from cells infected with recombinants Sp17 and Sgp130.a yielded two major polypeptides that reacted with anti-SIV antibodies (Fig. 2B, lanes 5 to 7 and 14 to 16). We did not attempt to examine in detail the nature of these polypeptides, but based on molecular weight, the larger band probably corresponds to the fusion of the foreign protein with either VP1 or $2A^{pro}$ and the other band probably corresponds to the free foreign protein. Extracts from cells infected with recombinants Sgp130.c, Sp41.a, and Sp41.c displayed only a single immunoreactive polypeptide band corresponding to the free SIV antigenic protein (Fig. 2B, lanes 17 to 19, 20 to 22, and 26 to 28). Extracts from cells infected with poliovirus recombinant Sp27 showed three polypeptides that reacted with anti-SIV antibodies. Based on molecular weight, these bands may be fusions between the SIV core and poliovirus protein $2A^{pro}$, the free SIV p27 Gag protein, and a degradation product (Fig. 2B, lanes 8 to 10). Thus, proteolytic processing at this insertion point appears to be incomplete, but it has a minor effect on viral replication since all of the recombinants showed wild-type replication phenotypes. Perhaps viral proteins at this position fused to foreign polypeptides still retain their normal function.

Poliovirus is a cytoplasmic, nonenveloped virus, and none of its proteins are glycosylated. Until recently, it was not known whether this virus could effectively express this type of protein. In fact, our previous experience with insertions at the N terminus suggested that translocation to the endoplasmic reticulum and glycosylation of foreign proteins were inefficient (3). Interestingly, extracts from cells infected with recombinants

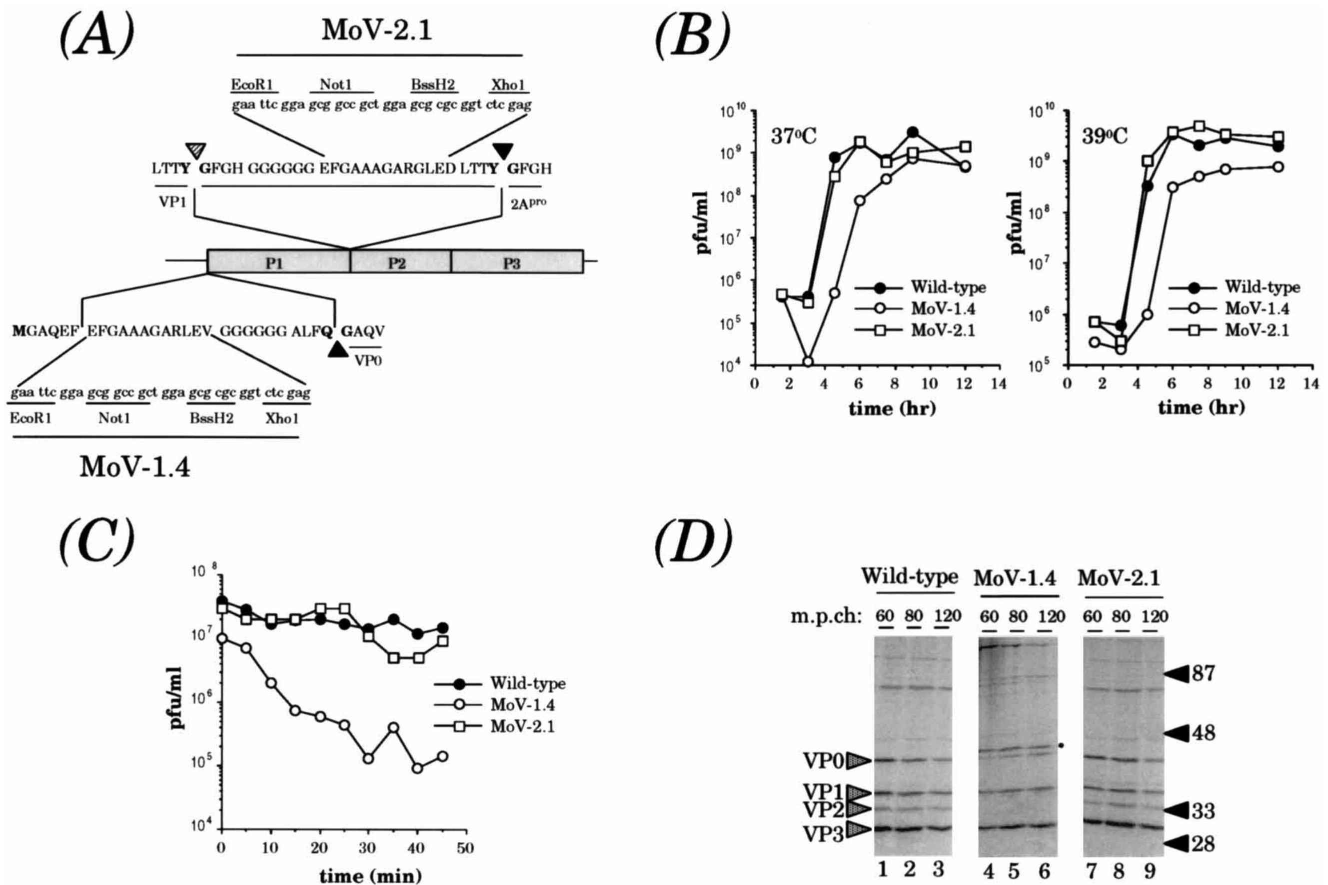
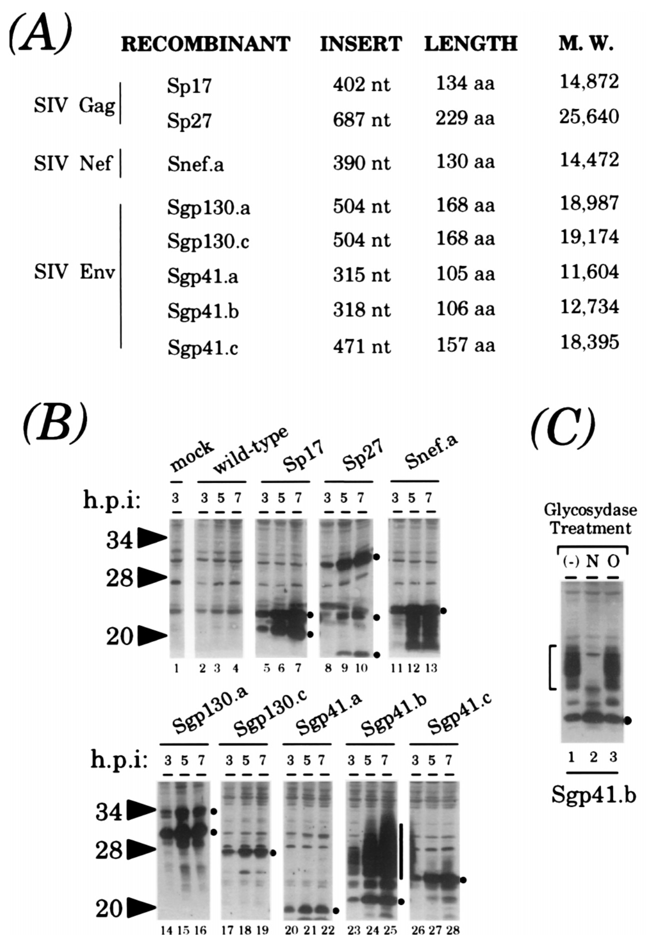


FIG. 1. (A) Schematic diagram of two recombinant poliovirus vectors, MoV-1.4 and MoV-2.1. Viral genes are indicated within corresponding boxes, and the insertion points for exogenous sequences (at the 5′ end of the open reading frame [MoV-1.4] and between P1 and P2 regions [MoV-2.1]) are indicated. Sequences surrounding the insertion points of the exogenous sequence are detailed: the start codon, additional amino acids flanking the exogenous sequence providing a restriction enzyme polylinker, a polyglycine tract, the $3C^{pro}$ artificial cleavage site, and the amino terminus of the viral polyprotein (MoV-1.4) or the carboxy and amino termini of VP1 and $2A^{pro}$ (MoV-2.1). (B) One-step growth curves for wild-type poliovirus and two derived vectors, MoV-1.4 and MoV-2.1, with the insertion between P1 and P2. HeLa cell monolayers were infected (MOI of 10) with wild-type poliovirus, MoV-1.4, and MoV-2.1. Virus production (PFU/milliliter) was determined at each time point by plaque assay. (C) Analysis of the thermostability of poliovirus vector particles. Viral stocks corresponding to the wild type, MoV-1.4, or MoV-2.1 were incubated at 45°C. At each time point, virus was collected and titer was determined by plaque assay. (D) Kinetics of virus formation. HeLa cells were infected at an MOI of 10 for each of vector and wild-type poliovirus. At 2.5 h postinfection, cells were pulse-labeled with [^{35}S]methionine for 10 min and chased with an excess of unlabeled methionine. Cytoplasmic extracts obtained at different time points (minutes) postchase (m.p.ch.) were immunoprecipitated with specific antibodies directed against poliovirus capsid proteins and analyzed by SDS-PAGE. An unprocessed band containing the extra sequences is indicated by a dot. Sizes are indicated in kilodaltons.

Sgp41.b displayed a smear that reacted with anti-SIV antibodies (Fig. 2B, lanes 23 to 25). To test whether SIV protein gp41 was transported into the endoplasmic reticulum and glycosylated, extracts from cells infected with Sgp41.b were treated with glycosidases (Fig. 2C). *N*-glycosidase converted the smear into a single discrete band of the expected molecular weight, while *O*-glycosidase did not modify the migration of the foreign protein. Hence, the fragment of SIV envelope expressed by the Sgp41.b recombinant was N-glycosylated. This result is interesting for two reasons: first, this and similar results obtained very recently by Anderson et al. (2) show that poliovirus, unexpectedly, can indeed produce glycoproteins; second, where production of glycosylated protein is preferable to induce an adequate immunological response, the poliovirus expression system described here would allow the generation of this type of antigen.

Genetic stability of SIV/poliovirus recombinants. Poliovirus exhibits a high rate of genetic variation (point mutations, deletions, etc.). To examine the genetic stability of the SIV sequences at the P1/P2 junction, we studied all new recombinants constructed at this position by RT-PCR (Fig. 3B). The original genetic structures of Snef.a, Sp17, Sgp130.a, Sgp130.c, Sgp41.a, and Sgp41.b were only partially retained after three rounds of replication in HeLa cells, and smaller bands corresponding to wild-type sequences emerged (Fig. 3B, lanes 5, 6, 8, 9, 10, and 11). Furthermore, recombinants Sp27 and Sgp41.c appeared to be even less stable, since no full-length bands were detected after a few passages (Fig. 3B, lanes 7 and 12).

Because RT-PCR is only semiquantitative, we further examined the genetic stability of chimeric viruses by in situ plaque immunodetection assay using antibodies directed against poliovirus or SIV proteins. Because each plaque represents an independent virus of the viral population, the proportion of plaques (positive spots with antipoliovirus antibodies) that express SIV antigens (positive spots with anti-SIV antibodies) directly measures the reversion rate of poliovirus chimeras. Surprisingly, Sp17 was the only recombinant displaying positive plaques with anti-SIV antibodies, although 50% of the plaques were revertants that no longer express the SIV antigen (Fig. 3C). The rest of the recombinants did not contain any detect-



(A)

	RECOMBINANT	INSERT	LENGTH	M. W.
SIV Gag	Sp17	402 nt	134 aa	14,872
	Sp27	687 nt	229 aa	25,640
SIV Nef	Snef.a	390 nt	130 aa	14,472
SIV Env	Sgp130.a	504 nt	168 aa	18,987
	Sgp130.c	504 nt	168 aa	19,174
	Sgp41.a	315 nt	105 aa	11,604
	Sgp41.b	318 nt	106 aa	12,734
	Sgp41.c	471 nt	157 aa	18,395

FIG. 2. (A) SIV/poliovirus recombinants. The inserted SIV antigens, their lengths (in nucleotides [nt] and amino acids [aa]), and the expected molecular weights (M. W.) are indicated. (B) Expression of the exogenous proteins in cells infected with recombinant polioviruses. Cytoplasmic lysates prepared 3, 5, and 7 h postinfection (h.p.i.) from HeLa cells infected with wild-type poliovirus or various recombinant viruses were analyzed by Western blotting with antibodies directed against SIV proteins. Lanes: 1, mock-infected HeLa cells; 2 to 4, wild-type poliovirus; 5 to 7, Sp17 (SIV Gag p17 recombinant); 8 to 10, Sp27 (SIV Gag p27 recombinant); 11 to 13, Sgp130.a (SIV envelope gp130 amino-terminus recombinant); 14 to 16, Sgp130.c (SIV envelope gp130 COO-terminus recombinant); 17 to 19, Sgp41.a (SIV envelope gp41 COO-terminus recombinant); 20 to 22, Sgp41.b (SIV envelope gp41 membrane-proximal domain recombinant); 23 to 25, Sgp41.c (SIV envelope gp41 COO-terminus recombinant); 26 to 28, Snef.a (SIV Nef amino-terminus recombinant). Bands detected by antibodies directed against SIV sequences are indicated by dots. Sizes are indicated in kilodaltons. (C) Glycosidase treatment of extract of HeLa cells infected with recombinant Sgp41.b. Cytoplasmic lysate from HeLa cells infected with recombinant gp41.b was treated with glycosidases that digest either N-linked or O-linked polysaccharides. The treated extract was analyzed by SDS-PAGE and Western blotting.

able SIV-positive plaques. Thus, for most recombinants, less than 1% of viruses present in the viral stocks retained the inserted sequences after three rounds of replication in HeLa cells.

Silent mutations reduce the rate of homologous recombination and augment the genetic stability of the recombinants. It has been shown that recombination contributes to the high genetic variation of RNA viruses, with up to 10 to 20% of replicating poliovirus molecules being the product of recombination (18). The mechanism of RNA recombination is not fully understood, but it has been suggested to be a copy choice by template switching of RNA polymerase during negative-strand synthesis (19). Similarly, large genome deletions (such as those observed in defective interfering particles) appear to arise through RNA recombination. Since the engineered polioviruses contained potentially recombinogenic direct repeats at each side of the insertion, we tested if we could reduce the rate of deletion by altering sequence of these flanking direct repeats. We used recombinant Sp27 because it forms smaller plaques than the rest (data not shown). Thus, this insert was poorly tolerated, possibly imposing additional pressure to delete the foreign sequences (notice that the RT-PCR analysis showed no full-length band after few rounds of replication in tissue culture [Fig. 3B, lane 7]).

As shown in Fig. 4A, four mutants were constructed. In Sp27(2.11), the nucleotide sequences upstream of the cleavage site was altered without changing the amino acid sequence (silent mutations). In this form of the virus, the direct repeats differ by 37%. To construct Sp27(2.12), the coding sequence for three amino acids near the upstream cleavage site was deleted, reducing the extent of the repeats to 18 nucleotides. A third mutant, Sp27(2.13), was generated by deleting 12 nucleotides encoding four amino acids next to the downstream proteolytic site, reducing the repeats to 15 nucleotides. In a fourth mutant, Sp27(2.14), Sp27(2.12), and Sp27(2.13) deletions were combined. After RNA transfection, all deletion mutants displayed minute-plaque phenotypes, suggesting that amino acid sequences in this region play an important role in viral repli-

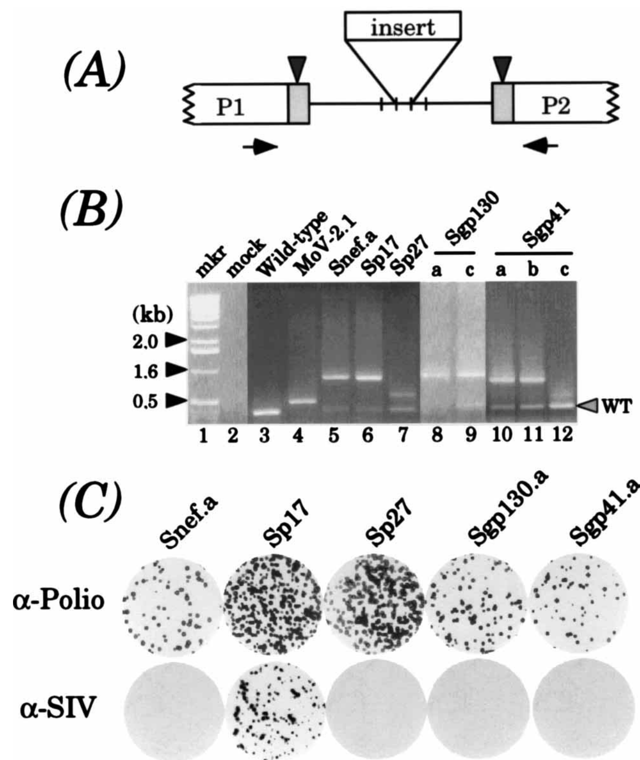
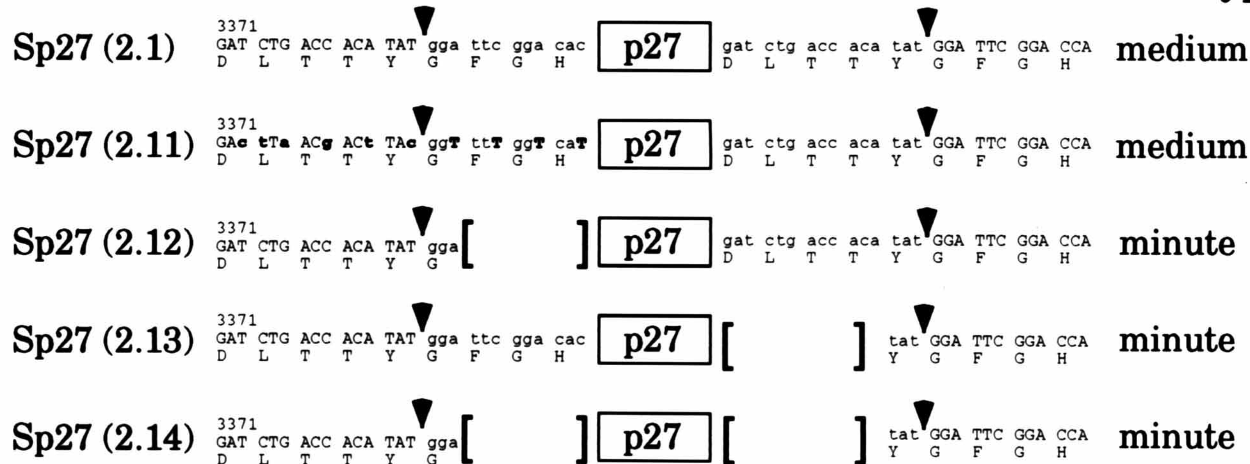
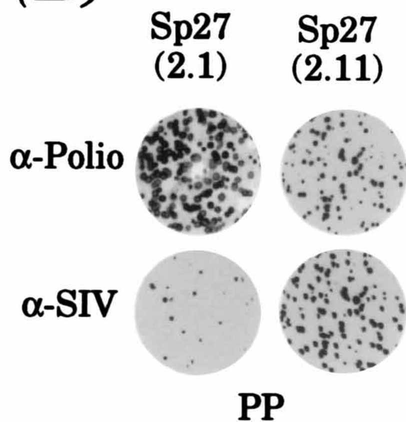
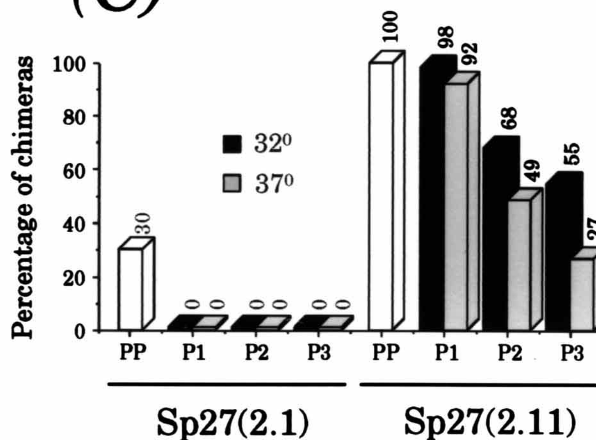


FIG. 3. Analysis of the stability of recombinant poliovirus genomes. (A) Schematic representation of the relative positions of the oligonucleotides used for RT-PCR analysis. (B) HeLa cells were infected with wild-type and recombinant viruses obtained after three successive passages in HeLa cells. The presence of the foreign sequence insertion was analyzed by RT-PCR, using total cytoplasmic RNA of infected cells as a template for RT. Molecular weight markers (mkr) indicate relative mobilities. Bands with electrophoresis mobility corresponding to wild-type (WT) sequences are indicated by an arrowhead. (C) Genetic stability of the inserted sequences as determined by in situ immunodetection of SIV proteins in poliovirus plaques. Wild-type and recombinant virus plaques were transferred to nitrocellulose filters and analyzed by using anti-poliovirus capsid (α-Polio) and anti-SIV (α-SIV) antisera.







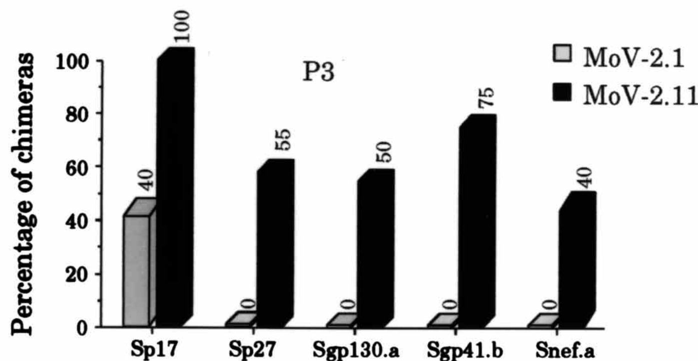


FIG. 4. Reducing the homology between the direct repeats increases the genetic stability of the inserted sequences. (A) The amino acid and nucleotide sequences corresponding to the direct repeats surrounding the insertion point of poliovirus vectors carrying the SIV Gag p27 coding region are shown. The cleavage sites for $2A^{pro}$ are indicated by arrowheads. Silent mutations which changed the nucleotide sequences by 37% but maintained the wild-type amino acids are in boldface [Sp27(2.11)]. Deletion of amino acids are depicted by brackets. The name of each plasmid is shown at the left, and the plaque phenotype of the virus generated after transfection with the RNA made from that plasmid is indicated at the right. For all constructs, the first amino acid of the sequence corresponds to amino acid number 877 of the poliovirus polyprotein. (B) In situ immunodetection of SIV proteins in poliovirus plaques. The original recombinant [Sp27(2.1)] and mutant carrying silent mutations [Sp27(2.11)] were cloned from individual plaques (plaque purified [PP]). HeLa cells were infected with cloned viruses and incubated for 2 days

cation (perhaps for proteolytic processing of the fusion protein). Therefore, we did not attempt to characterize these viruses further. In contrast, Sp27(2.11), in which silent mutations were introduced, displayed a plaque phenotype very similar to that of the original Sp27(2.1) recombinant (not shown).

To evaluate the effect of flanking repeat mutations on genetic stability, the original recombinant Sp27(2.1), with intact repeats, and Sp27(2.11), with silent mutations, were cloned by plaque purification, and the proportion of recombinants in the isolated population was determined by plaque assay immunodetection, which permits the examination of hundreds of independent viruses simultaneously. HeLa cells were infected in suspension, and infected cells were mixed with tissue culture medium containing 1% agar and poured onto plates. After plaques were formed, they were transferred to the filters and analyzed by using antipoliovirus and anti-SIV antisera. Because each plaque originates from independent viruses of the viral stock, the proportion of plaques that express SIV antigens (plaques positive with anti-SIV antibodies) must represent the proportion of poliovirus/SIV recombinants present in the viral stock, even though we cannot be sure of the proportion of recombinants in a given plaque. Therefore, we use this technique to determine the reversion rate of poliovirus chimeras.

Only 20 to 30% of the virus isolated from Sp27(2.1) plaques retained the insert. In contrast, virtually all plaques in the Sp27(2.11) stock produced proteins that immunoreacted with anti-SIV antibodies (Fig. 4B). The genetic instability of Sp27(2.1) was further evidenced after serial passage on HeLa cells. Cells were infected with purified viruses and incubated at 32 or 37°C; viruses obtained after each passage were examined by plaque assay immunodetection. None of the plaques obtained after the first passage of Sp27(2.1) retained the inserted sequences. Conversely, the mutant Sp27(2.11) displayed a much higher genetic stability, and after the third passage, 30 to 50% of the plaques of the mutant still expressed SIV antigenic sequences (Fig. 4C). Interestingly, passaging the recombinant virus at low temperature (32°C) increased the genetic stability of the insert. This finding suggests that the production of viral stocks should be done at low temperature.

We have confirmed and extended these results to the rest of the SIV/poliovirus chimeras described in Fig. 2. A higher proportion of viruses from recombinant stocks constructed in vector MoV-2.11 carried and expressed the foreign sequences for more rounds of replication (Fig. 4D). The immunogenic potential of each of these new and more stable recombinant viruses was evaluated in mice (see below).

These results show that homologous recombination is an important contributor to the genetic instability of recombinant poliovirus and that this tendency to delete the inserted sequences after few rounds of replication can be partially alleviated. However, for recombinant Sp27(2.11), less than 1% of viruses present in the viral stocks retained the inserted sequences after three additional rounds of replication in HeLa cells (passage 6), suggesting that chimeric viruses constructed in MoV-2.11 are still able to delete the SIV sequences, although at lower frequency. Because the stability of the foreign insert likely contributes to the immunological strength of the vaccine (see below), we are further exploring the basis of this phenomenon and other methods to further reduce such instability.

Immune responses to the SIV sequences. To determine the optimal infection regimen for immunization, we used a poliovirus recombinant carrying SIV p17. Mice were infected intraperitoneally on one, two, three, or four occasions with 5×10^7 PFU of recombinant Sp17(2.1). Serum antibody titers recognizing SIV p17 polypeptide correlated directly with the number of inoculations. For example, 10 weeks after the first inoculation, we observed 2- to 10-times-higher anti-Gag p17 antibody titers in mice that were inoculated four times than those inoculated one, two, or three times (Fig. 5A). The amount of virus in the inoculum also influenced the immune response, with 5×10^7 PFU eliciting a stronger humoral response than 5×10^4, 5×10^5, or 5×10^6 (Fig. 5B).

To determine whether genetic stability is important for the immunogenic potential of recombinants, we compared the levels of seroconversion elicited by recombinants based on MoV-2.1 (unstable) with those elicited by recombinants based on MoV-2.11 (stable). Groups of six mice were intraperitoneally inoculated with individual recombinants expressing five different SIV antigenic sequences (Gag p17 and p27, Env gp130 and gp41, and Nef), and 6 weeks postinoculation, the level of anti-SIV antibodies produced was determined by ELISA. A clear difference in the potential to generate antibodies was observed between the two types of vectors. From MoV-2.1 vectors, Sp17(2.1) was the only recombinant able to elicit an immune response against the SIV antigen (note that this is the most stable recombinant from this series [Fig. 3C]). The rest of the MoV-2.1 recombinants did not elicit any detectable antibody response (Fig. 6A), even though they expressed the antigens (Western blotting [Fig. 2B]). In contrast, all recombinants with markedly higher genetic stability (based on vector MoV-2.11) elicited an antibody response (Fig. 6B), suggesting that the ability of the recombinant to retain the insert for several rounds of replication is an important factor that determines the level of immune response against the foreign protein in mice. This result is consistent with the observation that the dose of inoculated recombinants has a major effect on antibody induction (Fig. 5B).

Recombinant poliovirus may permit the development of a vaccine strategy that would enable simultaneous vaccination against multiple antigenic determinants through the preparation of a cocktail of poliovirus recombinants carrying different antigenic proteins. To test this possibility, we inoculated a group of six susceptible mice with a mix of recombinant polioviruses carrying five different SIV proteins. Antibodies recognizing each of the SIV proteins were detected by ELISA 6 weeks after the first inoculation in all cocktail poliovirus-infected animals (Fig. 6C). Thus, poliovirus recombinants provide an effective method for the simultaneous in vivo expression of multiple antigens derived from SIV and the induction of immune responses against the heterologous proteins.

DISCUSSION

Foreign sequences can be inserted at several positions within the poliovirus genome (5, 24); however, the location of the

at 37°C. Plaques were transferred to nitrocellulose filters and analyzed by using antipoliovirus capsid (α-Polio) and anti-SIV (α-SIV) antisera. (C) The original recombinant [Sp27(2.1)] and a mutant carrying silent mutations [Sp27(2.11)] were serially passaged in HeLa cells one, two, or three times (passage 1, P1; passage 2, P2; passage 3, P3) at 32 or 37°C. The virus stocks obtained after each passage were analyzed as described for panel B. (D) Poliovirus/SIV recombinants (Sp17, Sp27, Sgp130.a, Sgp41.b, and Snef.a) constructed in vector MoV-2.1 and those constructed in vector MoV-2.11, carrying silent mutations, were serially passaged in HeLa cells three times at 32°C as in for panel B. The virus stocks obtained were analyzed as described for panel B.

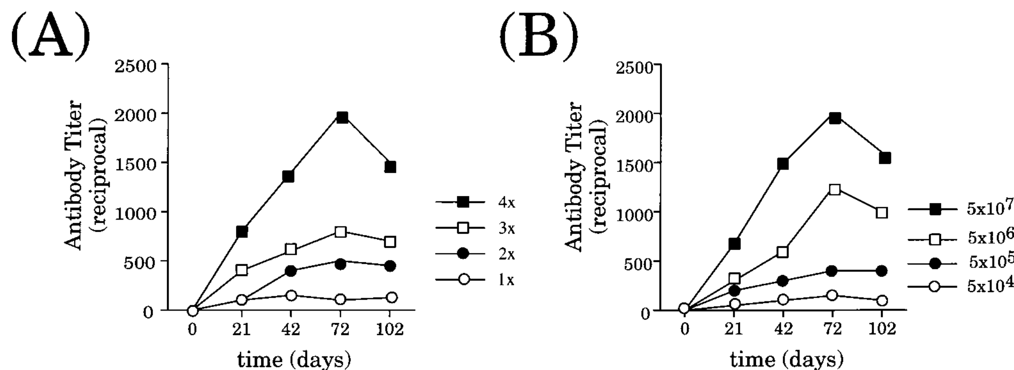


FIG. 5. Transgenic mice exhibit a dose-dependent immune response to recombinant poliovirus. Groups of six PVR-transgenic mice were infected with SIV Gag p17 recombinant virus (Sp17) constructed in vector MoV-2.1. (A) Animals were inoculated intraperitoneally every 4 days one, two, three, or four times with 5×10^7 PFU of recombinant virus. (B) Mice were inoculated four times every 4 days with 5×10^4, 5×10^5, 5×10^6, or 5×10^7 PFU of recombinant Sp17. Antibody titers elicited against Gag p17 in mouse sera were determined by ELISA. Antibody titers were measured every 3 weeks and are expressed as the reciprocal of the antibody dilution.

insertion site can have a major impact on the replication potential of the virus. For example, insertion of a few nucleotides at the junction between 3AB and 3CD yielded noninfectious viral RNA (3); insertion of the same sequences at the N terminus of the polyprotein gave rise to a thermosensitive mutant, defective in particle formation; and insertion at the junction between P1 and P2 produced a virus that replicated with kinetics indistinguishable from that of wild-type poliovirus.

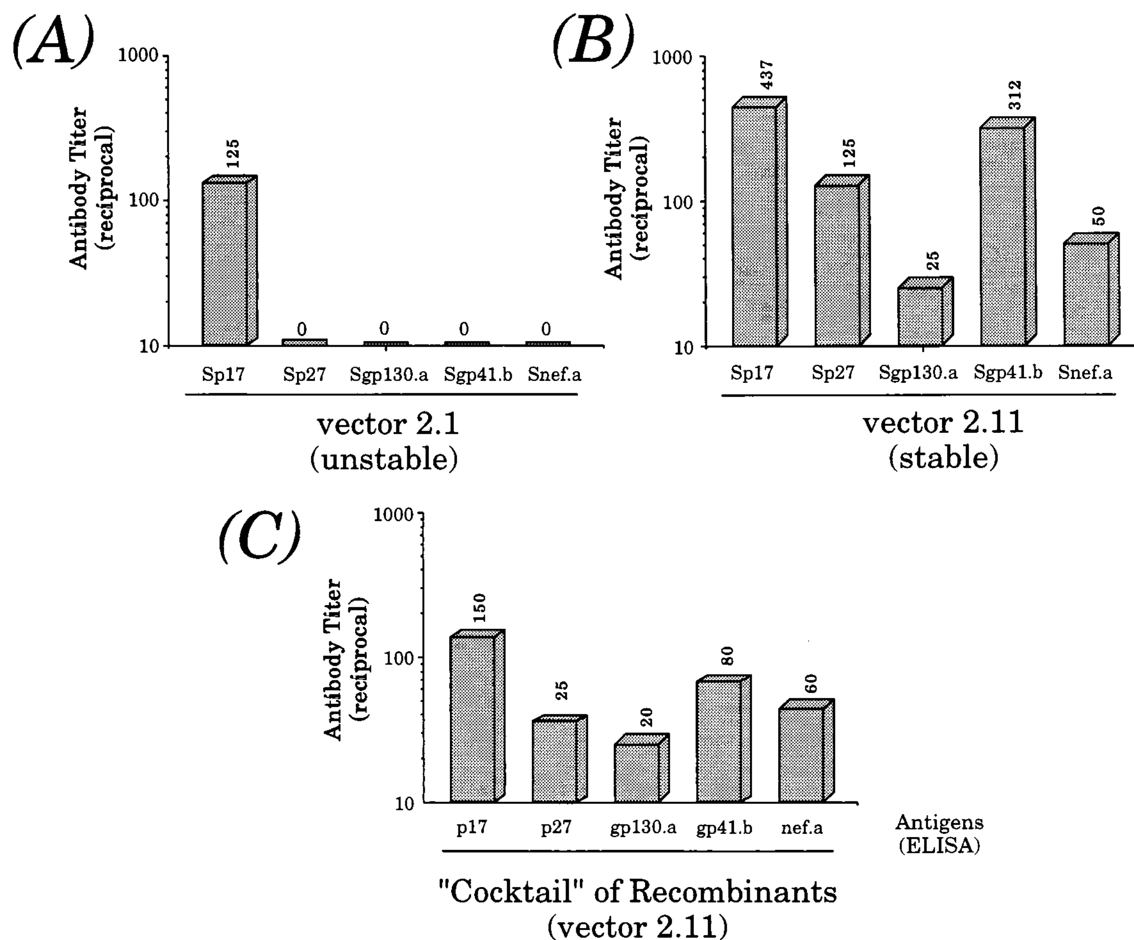


FIG. 6. Inoculation of PVR-transgenic mice with the SIV/poliovirus recombinants elicits a humoral response against the SIV inserted proteins. Groups of six PVR-transgenic mice were infected with the following poliovirus recombinants carrying SIV sequences: Sp17, Sp27, Sgp130.a, Sgp41.b, and Snef.a. Animals were inoculated with SIV/poliovirus recombinants based on vector MoV-2.1 (A) or vector MoV-2.11 (B). (C) Simultaneous inoculation of a cocktail of six different recombinants (Sp17, Sp27, Sgp41.b, Sgp130.a, and Snef.a). Animals were inoculated three times (once every 4 days) by intraperitoneal injection with 100 μl of viral stock (10^8 PFU per ml). At 6 weeks after the first inoculation, serum samples were obtained and analyzed by ELISA, using purified SIV proteins produced in *E. coli*. Antibodies reacting with the SIV proteins were visualized with anti-mouse total Ig-specific second-stage antibodies conjugated to horseradish peroxidase.

The N-terminal insertion induced a delay in viral assembly, which correlates with a delay in the proteolytic processing of the fusion between the foreign sequences and VP0 (Fig. 1D). Myristylation of the N terminus of VP0 occurs cotranslationally, and it is essential for assembly of mature viral particles. In MoV-1.4, VP0 myristylation would only occur after removal of the foreign sequences. Thus, inefficient cleavage would cause inefficient myristylation and capsid formation defects. We did not attempt to determine the fate of unprocessed fusion polypeptides, but they could be targeted for degradation, assemble with other capsid proteins to form nonfunctional capsids, or be utilized in the formation of unstable viral particles (Fig. 1C). We were unable to determine whether the thermally unstable nature of the MoV-1.4 particle is due to undetectable amounts of foreign sequences or of nonmyristylated VP0 present in the virus. However, in both cases, the incorporation of few unmyristylated VP0 molecules into virions could determine the unstable character of the particle by disrupting interactions between the myristic moiety and VP4 (27). Furthermore, foreign sequences fused to VP0 can be readily detected in the empty capsid assembly intermediate but not in mature MoV-1.4 viral particles, suggesting that the fusion polypeptide is inefficiently incorporated into the mature particle (33a). Not only does the site of insertion have an effect on the phenotype of the recombinant virus, but so does the nature of the insert. For example, SIV Gag p17 was better tolerated than others similar in size (i.e., SIV nef.a and SIV gp41.a) (Fig. 3C). However, the insert length may also influence viral replication. Of all recombinants constructed at the P1/P2 junction, Sp27 was the only recombinant showing a smaller-plaque phenotype. This insert is larger than all of the others, and so it is tempting to speculate that in this case, the size of the insert may be the determining factor. The upper size limit of tolerated sequences at the P1/P2 position remains to be determined.

Poliovirus exhibits a high rate of homologous recombination, with 10 to 20% of replicating molecules in an infected cell undergoing this process (14). In addition, picornaviruses also undergo less frequent nonhomologous recombination events, such as those thought to be responsible for the generation of defective interfering particles. Foreign sequences inserted in poliovirus provide no contribution to the viability of the virus; therefore, there is no selective pressure for the insert to be retained, and, in some cases, it can exercise a negative effect in viral replication, which may result in complete deletion of the insert.

Foreign sequences can be detected after three rounds of replication by Western blotting and RT-PCR. In contrast, in situ immunodetection analysis suggested that most of infectious viruses present in recombinant stocks are revertants (Fig. 3C). This may be due in part to the higher sensitivity of RT-PCR and Western blotting and the fact that these assays were done by infecting cells at a high MOI (MOI of 10); thus, many cells would become infected with recombinant viruses even if the proportion of chimeric virus is low. Furthermore, in situ immunodetection requires that a virus undergo several additional rounds of replication to form plaques, allowing for an unstable recombinant virus to delete the inserted sequences before assaying. In any case, the immunogenicity of the recombinants correlated better with results obtained with in situ immunodetection than with results of either RT-PCR or Western blotting.

The reduction of homology of the direct repeats flanking the insert greatly enhanced the stability of the foreign sequences and led to a dramatic effect on the ability of recombinant viruses to elicit immune responses in transgenic mice (Fig. 6A). Presumably, the recombinant virus must be able to replicate in vivo and express the foreign protein in sufficient amounts and for several rounds of replication in order to elicit a strong and long-lasting immune response. This might be especially important if effective replication is required before a potent mucosal immune response can be elicited by poliovirus.

Simultaneous vaccination against different SIV antigens (including a glycoprotein) was achieved by using a cocktail of several recombinants (Fig. 6C). This result suggests that a vaccine formulation could be prepared to obtain protective immunity against multiple pathogens by using a cocktail of polioviruses, each expressing different antigens.

In addition, vaccination of cynomolgus monkeys with live-attenuated Sabin poliovirus elicited an IgA response readily detected in vaginal secretions of immunized animals (33b). Taken together, these results suggest that poliovirus recombinant vectors can offer an effective strategy to deliver antigens in adequate inductive sites to elicit local mucosal immunity.

A concern with the use of poliovirus as a vaccine vector is the potential reversion of the attenuated vaccine strain to a more neurovirulent virus. In fact, a small number of vaccine-associated cases of poliomyelitis have been reported over the years. However, most of these cases appear to be associated with Sabin 3, suggesting that this strain has a higher tendency to revert to neurovirulent forms. Therefore, we have chosen to build recombinant polioviruses based on the Sabin 1 and Sabin 2 strains with the hope that they will better maintain their attenuated phenotype. Nevertheless, it will be important to design approaches and methods to preserve the attenuation and fully evaluate neurovirulence of the recombinant polioviruses.

In addition, it has been argued that preexisting immunity against poliovirus in human populations may constrain the use of poliovirus as vaccine vector. However, the limited published data suggest that 50% of previously immunized individuals will show at least a fourfold increase in antibody titers after administration of an oral poliovirus vaccine booster. Such response was more commonly seen in persons with lower prebooster titers (15, 22, 28, 31). It is thus conceivable that at higher doses, recombinant polioviruses might be able to establish infection and elicit immune responses against heterologous antigens in individuals previously immunized against poliovirus. It is further possible that even limited replication in a relatively immune host might induce immunologic priming to the inserted antigenic proteins at mucosal sites that could then be boosted by subsequent delivery of HIV type 1 antigens via another form or route, as previously reported for vaccinia virus recombinants (10, 16). Furthermore, an HIV vaccine effective in immunizing children would be extremely worthwhile, and if poliovirus recombinants are effective in experimental models and in children, it would strengthen the argument to adapt other picornaviruses as HIV vaccines to overcome limitations imposed by prevailing antipoliovirus immunity. Studies are ongoing to further develop and fully evaluate the safety and immunogenic and protective potential of these recombinant viruses.

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