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Poliovirus Sabin 1 as a Live Vaccine Vector: Expression of HIV-1 p24 Core Protein

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The poliovirus Sabin 1 strain has features that make it a particularly attractive live recombinant mucosal vaccine vehicle. Sabin 1 cDNA was manipulated to have multiple cloning sites and a viral specific 3C-protease cutting site at the N-terminal end of the polyprotein. The gene for the N-terminal 169 amino acids of the HIV-1 p24 was cloned into the multiple cloning site of the manipulated Sabin cDNA. A recombinant progeny virus was produced from HeLa cells when it was transfected with the RNA synthesized from the p24-Sabin chimeric cDNA. The recombinant progeny virus expresses substantial amounts of the HIV-1 p24 protein, which was clearly detected in the infected cell lysates and culture supernatants in Western blot experiments with rabbit anti-p24 serum and AIDS patients' sera. Differing from the Mahoney strain, the recombinant Sabin 1 poliovirus maintained the foreign gene stably during the subsequent passages. Replication capacity was about 1 to 1.5 log lower than that of the wild-type Sabin 1. Other physicochemical stability characteristics of the recombinant virus were similar to that of the wild-type Sabin 1. These results suggest that the manipulated Sabin 1 poliovirus can be used as a live viral vaccine vector for the development of mucosal vaccines.

Keywords: Chimeric virus, HIV-1 p24, Mucosal vaccine vector, Poliovirus Sabin 1.

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Introduction

Most human pathogens enter the body at the mucosal surface. Yet to-date, vaccines developed for the vast majority of diseases have been administered so as to elicit systemic immunity. These nonoptimal vaccines have, nonetheless, provided sufficient control of many respiratory and enteric pathogens. Recently, an increased understanding of the importance of mucosal immunity has led investigative efforts to develop mucosal vaccines (Mestecky, 1987; McGhee and Mestecky, 1992; McGhee *et al.*, 1992). The cholera toxin and the related heat labile exotoxin from *E. coli* have been extensively studied as mucosal immunogens and antigens (Munoz *et al.*, 1990; Lehner *et al.*, 1992; Wilson *et al.*, 1993). Microencapsulation technology has been used in mucosal delivery strategies both to protect the antigen and to influence its site and rate of delivery (Marx *et al.*, 1992). Several bacterial (Stover *et al.*, 1991; Fouts *et al.*, 1995) and viral live recombinant vaccine vehicles (Daniel *et al.*, 1992; Yoshida *et al.*, 1997) are being developed to produce a new generation of mucosal vaccines against a broad spectrum of infectious diseases. Among these, the poliovirus Sabin strain has features that make it a particularly attractive live recombinant vaccine vehicle for its well-known advantages — safety, ease of administration, economy, and above all, having the capacity to induce effective mucosal immunity which is strongly recommended for an ideal vaccine.

Poliovirus contains a positive sense ss-RNA of 7.44 kb nucleotides, which encode a unique open-reading frame of a long polyprotein (Kitamura *et al.*, 1981). Two viral-specific proteases, 2A or 3C, which generate mature viral proteins, were used to proteolytically process the long polyprotein. Cleavage of the polyprotein by 2A releases P1 and P2 precursors (Toyoda *et al.*, 1986). P1, P2, and P3 precursors are further cleaved at a specific site (AXXQ/G) into mature viral proteins mostly by 3C and its precursor

protein (3CD) (Ypma-Wang *et al.*, 1988; Palmenberg, 1990). The packaging of the viral RNA into mature capsid proteins results in the production of an infectious progeny virus. The discovery that the poliovirus RNA genome can be transformed into an infectious cDNA (Racaniello and Baltimore, 1981) has provided the means with which to investigate the use of poliovirus as a vector to carry foreign antigens to the enteric tract. Recombinant polioviruses expressing foreign genes, generated in the attenuated vaccine strains, can be easily administered and may provide a safe and efficacious means of controlling a diversity of infectious diseases.

Several laboratories have investigated the use of poliovirus as a vector for the presentation of foreign antigens. Almond and his colleagues (Burke *et al.*, 1988, 1989; Evans *et al.*, 1989; Jenkins *et al.*, 1990; Rose *et al.*, 1994) have constructed chimeric polioviruses expressing foreign antigen on the surface capsid by replacing the surface antigenic site of VP1 with several small antigenic epitopes on the basis of the three-dimensional crystallographic structure of poliovirus type 1 and its attenuated Sabin 1 strain. In this system, only short linear epitopes (shorter than 25 aa) can be expressed as part of the viral capsid. Wimmer's group uses dicistronic poliovirus by duplicating the 5′-noncoding internal ribosomal entry site (IRES) to express foreign antigens together with essential viral proteins (Alexander *et al.*, 1994; Lu *et al.*, 1995). The instability of the inserted sequence, however, remains to be solved. Morrow and his colleagues (Porter *et al.*, 1993; 1996; 1997; Ansaradi *et al.*, 1994) have suggested the use of a poliovirus minireplicon, in which the poliovirus structural genes are being replaced by foreign sequences, to develop poliovirus-associated mucosal vaccines. In the case of minireplicons, the defective recombinant viral genome must be cotransfected with another capsid protein-expressing vector for the packaging of the chimeric viral genome (Porter *et al.*, 1995).

Recently, Andino *et al.* (1994) and Mattion *et al.* (1994) suggested a new strategy for the expression of foreign antigens in the replication-competent recombinant polioviruses. They have introduced a new protease-recognition site at the N-terminal end of the polyprotein. According to this system, the foreign gene, cloned in-frame with the poliovirus open reading frame, is followed by an artificial 3C protease site to allow proteolytic cleavage of the foreign protein by the poliovirus polyprotein. The exogenous nucleic acid is incorporated directly into the poliovirus genome. The exogenous sequences are expressed during viral replication as part of the virus polyprotein and subsequently processed by the virus-encoded proteases to produce free antigens and the mature viral protein. The foreign antigen is not packaged in the virion but is released into the cytoplasm. By this strategy, several HIV-1 subgenomes (Andino *et al.*, 1994) and hepatitis surface antigen (Yim *et al.*, 1996) were reported to be successfully expressed in the poliovirus Mahoney strain and rotavirus VP7 (Mattion *et al.*, 1994); The pre-S2 region of hepatitis B surface antigen (DiMichele *et al.*, 1995) were expressed in poliovirus Sabin 3 attenuated strain.

Our work was initiated to develop a safe poliovirus vaccine vehicle with Sabin 1. The Mahoney strain, used by Andino and his colleagues, is a strong neurovirulent strain. In a small number of cases, vaccination with oral polio vaccine (OPV) is associated with vaccine-associated paralytic poliomyelitis (VAPP), and the VAPP is most frequently associated with Sabin 2 and Sabin 3, but rarely with Sabin 1 (Otelea *et al.*, 1992; Furione *et al.*, 1993). Taking advantage of the strategy proposed by Andino *et al.* (1994) and Mattion *et al.* (1994), we have introduced a multiple cloning site and an artificial 3C-recognition site at the N-terminal end and at the VP3/VP1 junction, respectively, of the Sabin 1 open reading frame, resulting in the production of recombinant progeny viruses by the transfection of HeLa cells. The recombinant viruses have a similar replication capacity and physicochemical stability to those of the wild-type Sabin 1. To test the usability of the recombinant Sabin 1 vector, HIV-1 p24 was cloned and expressed in this system.

Materials and Methods

Materials All enzymes used in this experiment were purchased from New England Biolabs (NEB, Beverly, USA), GIBCO/Bethesda Research Laboratory (GIBCO/BRL, Gaithersberg, USA), Bio-Rad, Boehringer-Mannheim Biochemicals (BMB, Mannheim, Germany), Bioneer (Seoul, Korea), and POSCO Chem. (Seoul, Korea). Cell culture media and serum were obtained from GIBCO/BRL. *Escherichia coli* JM109 (Promega, Madison, USA) and XL1-blue (Stratagene, La Jolla, USA) were used for the transformation and propagation of plasmids. CJ236 and MV1190 for site-specific insertion and mutagenesis were purchased from Bio-Rad (Hercules, USA). Plasmid pTZ-18/R (Pharmacia Biotech, Uppsala, Sweden) was mainly used for subcloning and sequencing. Sabin 1 cDNA clone, pVS(1)IC-0(T), was kindly provided by Dr. A. Nomoto (Institute of Medical Science, The University of Tokyo, Minato-ku, Japan). Mahoney cDNA clones, pEV104 and pT7PV1-5, were kindly provided from Dr. E. Wimmer (State University of New York, Stony Brook, USA). Synthetic oligonucleotides were supplied from Bio-Synthesis, Inc. (Lewisville, USA) and Universal DNA, Inc. (Tigard, USA). Isotope-labeled materials were obtained from Amersham Life Science (Buckinghamshire, UK).

Cells and viruses HeLa cells (Human cervical carcinoma cells, HeLa S3 from ATCC) were used for transfection experiments and poliovirus propagation. HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO/BRL) supplemented with 10% fetal calf serum (GIBCO/BRL), 1× penicillin-streptomycin (GIBCO/BRL), 50 μg/ml of gentamicin. Mahoney (wild-type neurovirulent strain) and Sabin 1 (attenuated strain of

Mahoney) polioviruses were produced by transfection of HeLa cell monolayers with viral RNAs, which were transcribed from reconstructed or original cDNAs. Recombinant cDNAs and their RNA transcripts are described below.

Construction of recombinant poliovirus cDNAs The poliovirus vector used for these studies, pTZ-PVS(1), consists of pTZ18R and a full-length cDNA copy of Sabin type 1 poliovirus genome which was taken from the plasmid of PVS-IC-O(T) (Kohara *et al.*, 1996). The cDNA region was cloned into the *Eco*RI site, right after the T7 promoter of a pTZ-18/R plasmid. Parts of the cDNA (1-1813 and 878-3531) was subcloned into pTZ-18/R at corresponding sites (pTZ-PVS/5 and pTZ-VP31) to make it easy to reconstruct the regions of the N-terminal and the VP3/VP1 junction of the open reading frame of Sabin 1. As shown in Fig. 1, multiple cloning sites (*Sst*II-*Hpa*I-*Eag*I and *Apa*I-*Hpa*I-*Xho*I) and an artificial 3C-protease recognition site (AXXQ/G) were newly introduced right after the 743-AUG codon and at position 2480 by site-specific insertion experiments (Kramer and Fritz, 1987) with mutagenic primers (5′-GAC AAT TGT ATC ATA ATG CCG CGG GTT AAC CGG CCG GCT TTG TTC CAA GGT GCT CAG GTT TCA TCA-3′ and 5′-GCG CTA GCA CGA GGG CCC GTT AAC CTC GAG AAG GCA CTT GAG CAA GGA TTA GGT TTA GGT CAG ATG CTT-3′), and the subclones were named pTZ-PVS/5-3m and pTZ-PVS/VP31-4m, respectively. These fragments of manipulated cDNA were rejoined into the corresponding regions of the Sabin cDNA, resulting in the synthesis of two recombinant Sabin cDNA plasmids, pTZ-PVS-3m and pTZ-PVS-4m.

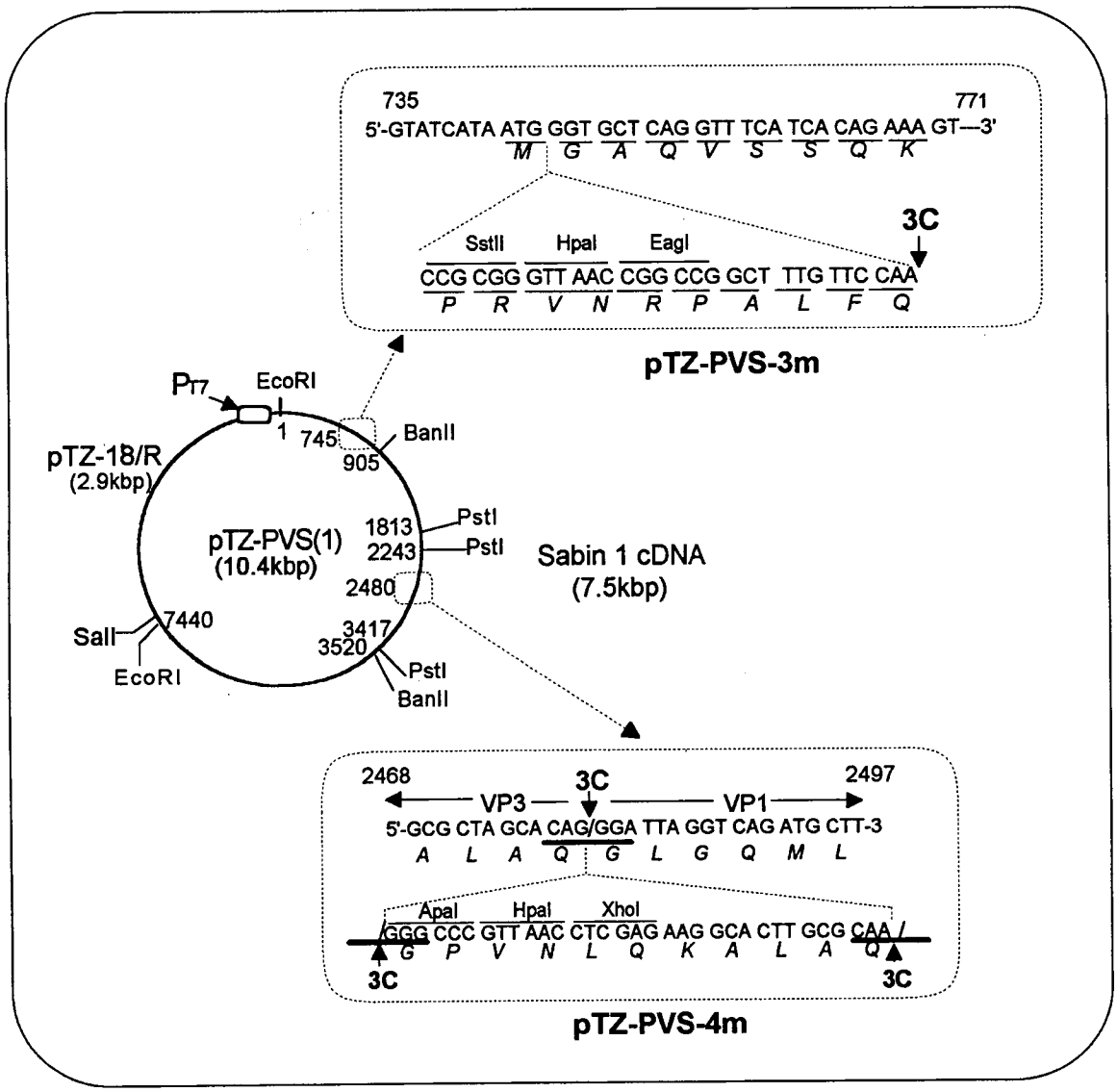

Fig. 1. Schematic representation of recombinant poliovirus cDNAs. Full-length cDNA of poliovirus Sabin 1 was subcloned into pTZ-18/R plasmid. Multiple cloning sites and poliovirus specific 3C-protease cutting site were newly introduced at the N-terminal end (pTZ-PVS-3m) or at the VP3/VP1 junctional site (pTZ-PVS-4m) of poliovirus cDNA as shown in the boxes of dotted line. Sequence insertion was done by site-specific insertion experiments using synthetic mutagenic primers.

The HIV-1 p24 DNA fragment coding N-terminal 169 amino acids was amplified from the template pHXB2 (Wong-Staal *et al.*, 1985) by PCR with primers designed to have *Sst*II and *Eag*I restriction sites at the 5′ and 3′ ends, respectively. The PCR products were purified and digested with *Sst*II and *Eag*I (NEB), and subcloned into the corresponding sites of pTZ-PVS-3m and pTZ-PVS-4m to elucidate the usability of these vectors (Fig. 2). All of these plasmid constructs were tested for their capacity to produce progeny viruses by transfection experiments.

Transfection of HeLa cells Plasmid DNAs were linearized with *Sac*I or *Sal*I (NEB) 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 (NEB or POSCO). Monolayers of HeLa cells (3×10^5) were grown in 60-mm dishes. Less than 1 μg of RNAs were transfected into the cells by a DEAE-dextran procedure (following the Manual of Mammalian Transfection Kit, Stratagene). Cells were incubated until a total cytopathic effect

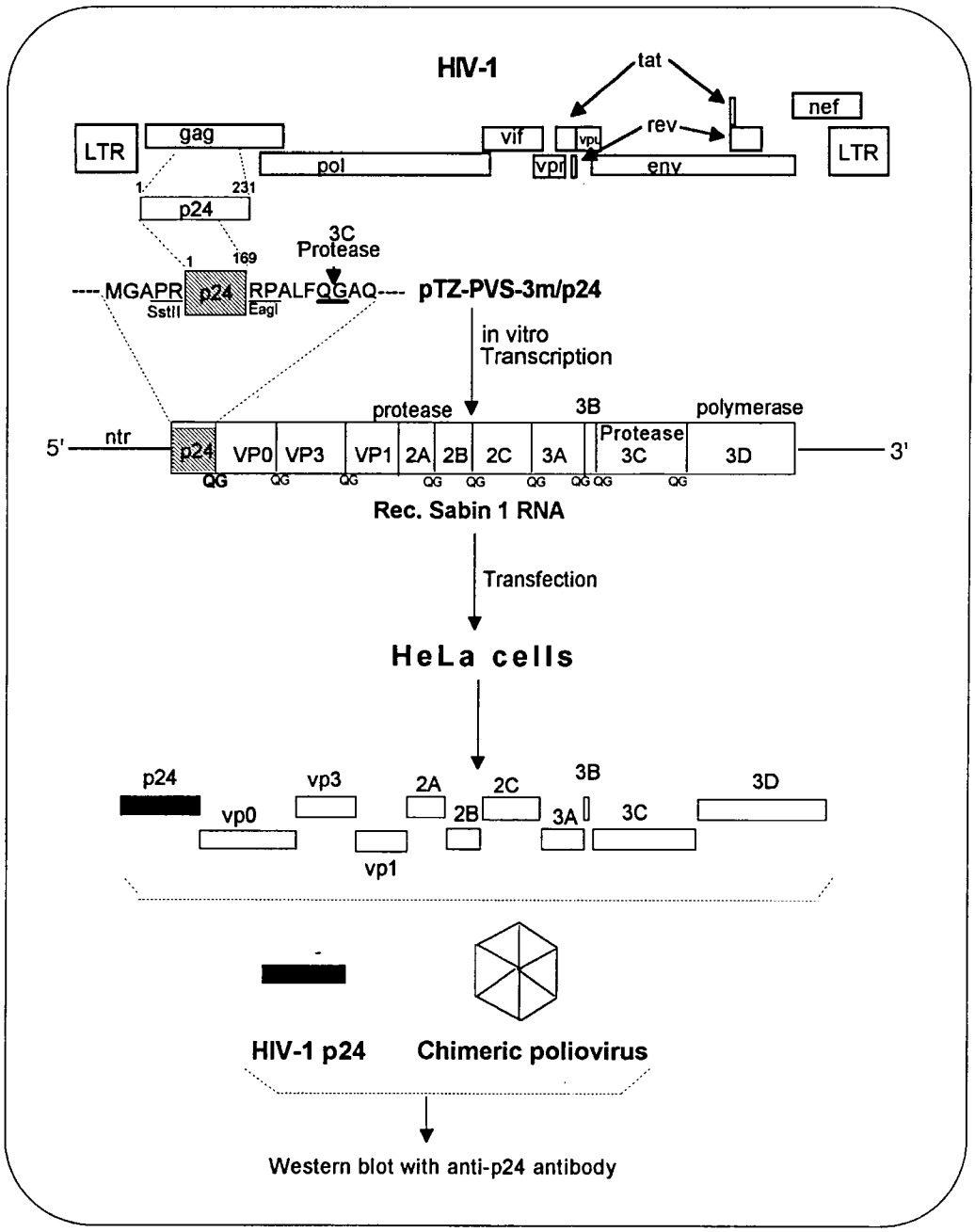

Fig. 2. Experimental flow chart to produce a HIV-1 p24-expressing chimeric poliovirus. The PCR product of HIV-1 p24 (169 aa) was cloned at the N-terminal multiple cloning site, between *Sst*II and *Eag*I, of the pTZ-PVS-3m vector. The RNA transcribed from the recombinant cDNA *in vitro* was introduced into HeLa cells to produce progeny virus by the transfection experiment. The chimeric progeny virus was tested for their capacity to express HIV-1 p24 during replication in the infected cells.

was seen, and a second passage of the supernatants was performed. Titers of viruses in the supernatant of these transfected cultures were subsequently determined by a plaque assay on HeLa cells, and viruses were passaged at a low multiplicity of infection (less than 1 MOI) in HeLa cells.

One-step growth curve HeLa cells grown in 60-mm plates were infected with wild-type or recombinant polioviruses at an MOI of 1. The virus was allowed to adsorb to the cells for 1 h at room temperature. After 1 h, the cells were washed with PBS, and 3 ml of DMEM was added. The cells were then incubated at 37°C. The supernatants were harvested every 3 h, and then titrated for the amounts of progeny viruses at each time point after infection. The virus titers were determined by a plaque assay and an $TCID_{50}$ assay.

Detection of exogeneous sequence in recombinant Sabin 1 poliovirus by RT-PCR Viruses or infected cells were harvested at each passage, or after transfection. Viral RNA was extracted from the viral precipitate after ultracentrifugation with phenol-chloroform followed by ethanol precipitation. Cytoplasmic RNA was prepared from cells infected with a recombinant poliovirus by the disruption of cell membrane with a lysis buffer (80 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris, pH 8.2, 1 mM DTT, 10 mM vanadyl ribonuclease complex, and 0.5% NP-40) for 5 min on ice. Clear supernatant was mixed with a same volume of solution D (4 M Guanidium isothiocyanate, 25 mM sodium citrate, 0.5% N-larylsalcosine, and 0.2 M 2-mercaptoethanol), extracted with phenol-chloroform and then precipitated with ethanol. Reverse transcription (RT) was performed with AMV RTase (Promega) at 42°C for 60 min using Sabin antisense primer (complementary to the Sabin 1 sequence of 814-797 for 3m vector-mediated virus and 2545-2528 for 4m-mediated virus). RT was followed by polymerase chain reaction (PCR) amplification with Sabin 1 primers (680-697/sense and 814-797/antisense for 3m-mediated and 2411-2431/sense and 2545-2528/antisense for 4m-mediated viruses). Note that these PCRs were performed with Tao polymerase (Bioneer, Seoul, Korea) for 30 cycles at 94° for 1 min, 45°C for 30 s, and 72°C for 45 s. Amplified cDNA fragments were analyzed in agarose gel.

Immunoblot analysis As described previously (Shin *et al.*, 1998), HeLa cells infected with 10 MOI of wild-type and recombinant poliovirus were harvested after 8 h pi. Cells were washed and resuspended with PBS, and then mixed with the same volume of 2× SDS-PAGE sample buffer (62.5mM Tris-Cl, pH 6.8, 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, 0.03% bromophenol blue, and 0.01 mg/ml Xylene cyanol). After boiling for 10 min, samples were subjected to electrophoresis through a 12% SDS-polyacrylamide gel, and analyzed by immunoblotting. Antisera directed against the Sabin poliovirus were raised from mice by immunizing with large amounts of poliovirus as immunogens. HIV-1 p24 expressed in Sabin recombinant poliovirus was detected by AIDS patients' sera and as rabbit antiserum raised in our laboratory. Alkaline phosphatase-conjugated secondary antibodies were obtained from Promega.

Replication capacity of the recombinant viral RNA Following the previous report (Mattion *et al.*, 1994), the experiment was performed. HeLa cells grown in 24-well plates were mock-infected or infected with 10 MOI of Sabin 1 and its recombinant viruses. After adsorption for 1 h at room temperature, the cells were washed with PBS and fed in DMEM containing Actinomicin D (5 μg/ml). After incubation for 1 h at 37°C, 25 μCi/ml of [5,6-^{3}H]-uridine (Amersham; specific activity, 45 Ci/mmol) was added. The cells were harvested at every 3 h, washed 3 times with PBS, and then lysed by adding 0.5 ml of lysis buffer (80mM NaCl, 5mM $MgCl_2$, 10 mM Tris, pH 8.2, 1mM DTT, 10mM vanadyl ribonuclease complex, and 0.5% NP-40) for 5 min on ice. 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 fiber filters (Whatsman, GF-C filter), and radioactivity was determined by scintillation counting.

Protein labeling and immunoprecipitation HeLa cells were infected with 10 MOI of wild-type or recombinant Sabin 1 virus. The cells were incubated for 1 h in DMEM without methionine for 5 h pi. After starvation, isotope-labeled methionine [(L-^{35}S)Met/Cys, specific activity >1000 Ci/mmole: Amersham] was added to a final concentration of 50 μCi/ml. Cells were harvested after labeling for 2 h and then lysed with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50mM Tris, pH 8.0). Labeled supernatants were separated in 12% SDS-PAGE either directly or after immunoprecipitation with antisera raised in our laboratory against poliovirus and HIV-1p24. Viral proteins were also synthesized *in vitro* as previously described (YPMA-Wang and Semler, 1987) by using a rabbit reticulocyte lysate translation kit (Promega Co.). Immunoprecipitation was carried out as previously described (Mattion *et al.*, 1994). The labeled cell lysates or proteins translated *in vitro* were diluted with RIPA buffer to a final volume of 300 ml and incubated on ice for 3 h with 5 μl of corresponding antisera (anti-poliovirus or anti-p24 serum). Protein A-sepharose (Pharmacia Biotech. Co) was added, and the cell lysates and proteins were incubated 1 h further. The beads were washed 3 times with RIPA buffer, and analyzed in 12% SDS-polyacrylamide gels.

Results

Construction of recombinant Sabin 1 plasmids As shown in a previous report (Kapalan *et al.*, 1985), the RNA transcribed *in vitro* from poliovirus cDNA was much more infectious than cDNA itself. To increase the transfection capacity of the recombinant poliovirus cDNA, poliovirus Sabin 1 cDNA, taken from the original pVS(1)IC-0(T) cDNA (Kohara *et al.*, 1986), was subcloned into a pTZ-18/R vector right after the T7 promoter site. Exogeneous sequences, containing multiple cloning sites and a 3C-protease recognition site, were newly inserted in frame into the region of the N-terminal or the VP3/VP1 junction of the Sabin 1 cDNA, and the recombinant vectors were named pTZ-PVS-3m and pTZ-PVS-4m (Fig. 1). The two recombinant plasmids were used to facilitate the cloning of foreign genes into the poliovirus genome, and for processing foreign proteins from the polyprotein by the viral-specific 3C-protease during the replication. The DNA

fragment encoding the N-terminal 169 amino acids of HIV-1 p24 was PCR amplified and cloned into the multiple cloning sites of these two vectors as illustrated in Fig. 2. Positive clones were selected by PCR for the p24 fragment cloned in both of the multiple cloning sites of Sabin 1 vectors, and then named pTZ-PVS-3m/p24 and pTZ-PVS-4m/p24, respectively. These recombinant vectors were sequenced to confirm whether the p24 fragment was cloned in frame in the vectors.

Recombinant RNA synthesized from pTZ-PVS-3m/p24 is infectious Full-length RNA of the p24-integrated recombinant Sabin 1 was synthesized from the p24-integrated Sabin 1 cDNA with T7 RNA polymerase after linearization with *SalI*. These RNA transcripts were introduced into HeLa cells. Following the transfection of HeLa cells with RNA transcripts, both vectors (pTZ-PVS-3m and -4m) produced recombinant viruses. The virion forming capacity of pTZ-PVS-3m RNA transcript was similar to that of the wild-type Sabin 1, but that of -4m was about 1 log lower than that of the wild-type Sabin 1 (Table 1). However, the HIV-1 p24-incorporated 3m (pTZ-PVS-3m/p24) transcript produced a progeny virus (PVS-3m/p24) when transfected. The replication capacity of the recombinant chimeric virus was about 1 log lower than that of the PVS wild-type Sabin 1 (Fig. 3), suggesting that the recombinant progeny viruses produced by transfection experiments possess slightly attenuated cytopathic effects as compared with that of wild-type Sabin 1. On the other hand, pTZ-PVS-4m/p24 transcript did not produce any detectable infectious progeny virus in the same experiments.

PVS-3m/p24 recombinant chimeric virus expresses functional p24 PVS-3m/p24 recombinant chimeric virus was tested for its capacity to express the processed p24 protein during viral replication in HeLa cells. Both the infected HeLa cells and supernatants harvested at

Table 1. Virion forming capacity of RNA transcripts.

RNA Transcript[a]	Number of Plaques/μg[b]
Mahoney	
pT7PV1/5	1×10^4
Sabin 1	
pTZ-PVS/wt	2×10^3
pTZ-PVS-m	0
pTZ-PVS-2m	0
pTZ-PVS-2m/1	<1
pTZ-PVS-3m	1.9×10^3
pTZ-PVS-4m	2×10^2

[a] About 0.1 μg of RNA transcript were introduced into monolayered HeLa cells in the presence of 500 μg/ml of DEAE-dextran.

[b] Plaques were counted 3 d after transfection.

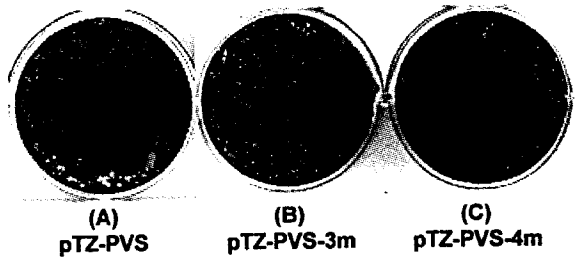

Fig. 3. Plaque formation by transfection of wild type and manipulated RNA transcripts of poliovirus (Sabin type 1). HeLa cell monolayers of 60% confluency were transfected with about 0.1 μg of RNA transcripts synthesized from the wild-type (A) and recombinant (B and C) cDNAs of Sabin poliovirus. Four days after transfection, the cells were stained with 1% Crystal Violet solution to detect the plaques. B and C show the plaques generated by the transfection of RNA transcripts synthesized from the r-cDNAs manipulated at the N-terminal region (B) and the VP3/VP1 junction site (C) of the Sabin cDNA.

18 h p.i. contained substantial amounts of recombinant p24, detected by AIDS patients' sera in Western blot experiments (Fig. 4). In order to see whether the expressed p24 is functionally intact, infected cells were labeled with ^{35}S-labeled amino acids, and the lysates were precipitated with anti-p24 antiserum. As shown in Fig. 5, a clear signal of the p24 band appeared at the autoradiogram of the sample separated on SDS-PAGE. Trace amounts of unprocessed precursor bands were also detected (Fig. 5). These results suggest that the recombinant p24 expressed from the chimeric poliovirus is immunologically similar to the wild-type p24.

Replication capacity of PVS-3m/p24 recombinant chimeric virus PVS-3m/p24 recombinant chimeric virus shows 1 to 1.5 log lower than that of the wild-type Sabin 1 (PVS) in their replication capacity, but was similar to that of the PVS-3m recombinant virus (Fig. 6). The rate of the viral RNA synthesis was measured for Sabin 1, PVS-3m, and PVS-3m/p24 by counting the amounts of [^{3}H]-uridine incorporation into the RNA. As shown in Fig. 7, the kinetics of RNA synthesis were similar for all three viruses. However, the polyprotein processing of the recombinant virus was shown to be relatively delayed in the radioimmunoprecipitation (RIP) assay (Fig. 5) despite the similar kinetics in RNA synthesis. This suggests that the attenuated one-step growth curve of PVS-3m/p24 is likely to be due to the slow processing and assembly of the recombinant viruses. These one-step growth curves were denoted by measuring infectious virus particles. We also titrated the total amount of progeny viruses by measuring the amount of [^{3}H]-uridine incorporated viral RNA in the supernatant culture at each time point to see whether the lower titer of the recombinant viruses is associated with the different ratio of defective interfering (DI) particles. As expected, the one-step growth curves were similar for all

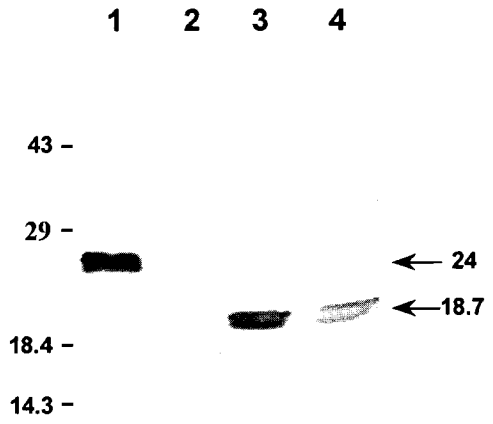

Fig. 4. Western blot analysis of the p24 expressed in the chimeric poliovirus-infected cell. The supernatant culture and cell extracts from HeLa cells infected with chimeric poliovirus carring the N-terminal 169 amino acids of HIV-1 p24 were separated in 10% SDS-PAGE and analyzed by the Western blot analysis with AIDS patients' sera. The lanes shown are HIV-1/⊿ tat (1), control poliovirus-infected HeLa cell extracts (2), chimeric poliovirus-infected HeLa cell extracts (3) and supernatant culture of chimeric poliovirus-infected HeLa cells (4). Arrows indicate the bands of wild-type p24 and the truncated forms of the 169 amino acids which were expressed from the chimeric poliovirus.

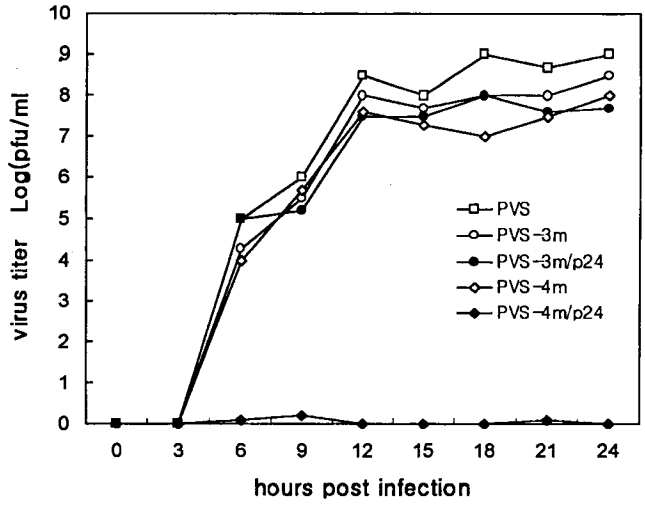

Fig. 6. One-step growth curves of wild-type and recombinant Sabin 1 polioviruses. HeLa cell monolayers were infected with viruses at an MOI of 10 for 1 h, washed twice with PBS, and then fed with fresh medium. The supernatant cultures were harvested every 3 h after infection. The virus titer of each supertanant was determined by $TCID_{50}$ and plaque assays. Each graph described wild-type Sabin 1 (-□-), recombinant Sabin 1 PVS-3m (-○-), PVS-4m (-◇-), p24-cloned recombinant Sabin 1 PVS-3m/p24 (-●-), and PVS-4m/p24 (-◆-).

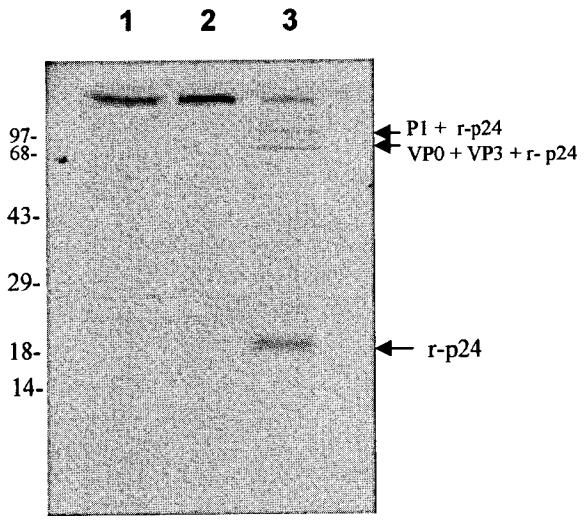

Fig. 5. Radioimmunoprecipitation of HIV-1 p24 expressed in the chimeric virus-infected cells. HeLa cells were labeled for 2 h with ^{35}S-Met/Cys (Amersham Life Science) right after 1 h of starvation at 5 h post-infection and analyzed in SDS-PAGE after precipitation. Proteins in the cells infected with PVS-3m/p24 Sabin 1 recombinant virus were immunoprecipitated with rabbit anti-p24 antiserum. Each lane shows the radioactive signal of the precipitin bands from the uninfected HeLa cell lysate (1), wild-type Sabin 1-infected HeLa cell lysates (2), and PVS-3m/p24 recombinant Sabin 1-infected HeLa cell lysates (3). Arrows indicate the completely processed protein bands or its precursor protein bands as described right beside the arrows.

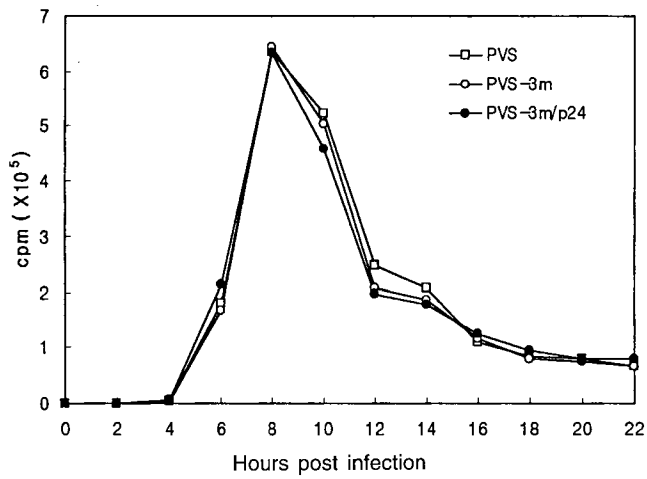

Fig. 7. Kinetics of viral RNA synthesis in the cytoplasm of infected cells. HeLa cells were infected with wild-type or recombinant Sabin 1 viruses at an MOI of 10 for 1 h. Infected cells were fed in the media supplimented with 5 μg/ml of Actinomycine D for 1 h and then followed by adding 20 μCi/ml of [^{3}H]-uridine. Cells were harvested, washed, and lysed every 2 h. Cell lysates were then precipitated with 20% trichloroacetic acid on ice for 30 min. Radioactivity incorporated into acid-precipitable material was measured by scintillation counting (Hewlett Packard). The values for mock-infected cells have been subtracted from each corresponding value. The symbols show wild-type Sabin 1 PVS (-□-), recombinant PVS-3m (-○-), and PVS-3m/p24 (-●-).

of these three viruses when denoted by amounts of total viruses at each time point (data not shown). It reveals that the discrepancy of the replication capacity between the recombinant virus and the wild-type Sabin is likely to be due to the different amounts of defective virus particles.

Stable expression of cloned p24 during the replication of PVS-3m/p24 chimeric virus The PVS-3m/p24 chimeric virus was tested for its sequence integrity at the HIV-1 p24-cloning site by PCR and sequencing. As reported previously with the Sabin 3 vector (Mattion *et al.*, 1994) we could detect shortened DNA fragments together with intact fragments in the RT-PCR as the number of passages increased (Fig. 8). However, we could not find any sequence changes in the intact PCR fragments (642 bp) of the cloned p24 in any of the chimeric progeny viruses during the passages. On the other hand, the shortened PCR fragments (270 bp) shown in Fig. 8 were found to have an N-terminal 57 aa of p24, and 54–56 nucleotides of an in-frame or frame-shifted VP2 region, in the sequence analysis. Also, the hybridisations shifted the reading frame of the downstream polyprotein of the poliovirus, causing premature chain termination. This is discussed in the following section. Particularly noteworthy is that the cloned p24 (18.4 kDa) was stably expressed in the chimeric viruses even after 12 passages in the HeLa cells (Fig. 9). However, small p24 peptides (about 57 aa) shortened by an internal deletion could not be detected in

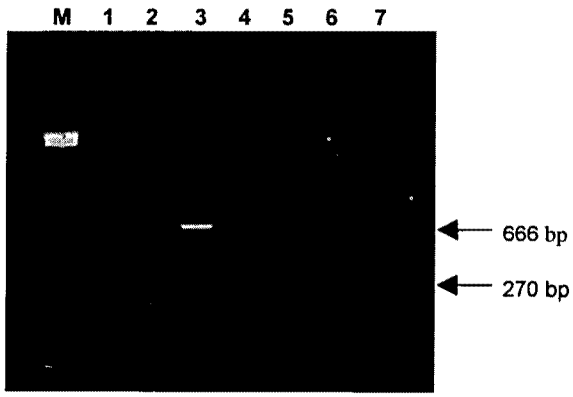

Fig. 8. Cloned p24 sequence in the recombinant poliovirus Sabin 1 is stably maintained during the passages of progeny viruses. Cells infected with serially passaged recombinant viruses were harvested at 8 h pi, and RT-PCR was performed with the cytoplasmic RNA extract with the PCR primer set covering the genomic region of 680-814 of Sabin 1 poliovirus. Each lane shows the PCR bands of RNAs extracted from the cells infected with wild-type Sabin 1 (1), PVS-3m (2), pTZ-PVS-3m/p24 plasmid (3), and second-passaged (4), 4th-passaged (5), 8th-passaged (6), and 12th-passaged (7) PVS-3m/p24 recombinant viruses. The upper arrow indicates the band containing the original size of cloned p24 (169 aa), and the lower arrow indicates the PCR fragments of cloned p24 having internal deletion during the replication of the recombinant viruses.

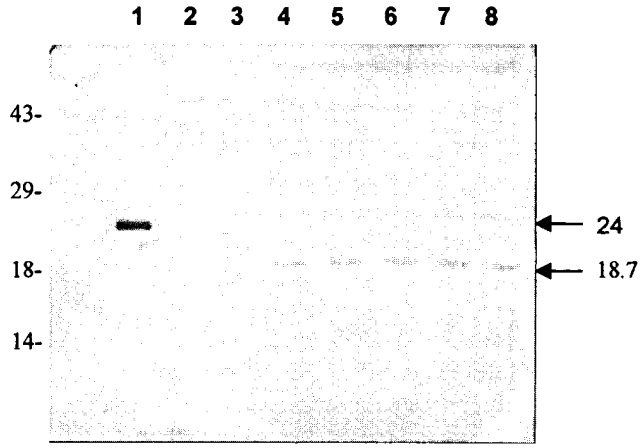

Fig. 9. Stable expression of r-p24 protein from the serially passaged PVS-3m/p24 recombinant Sabin 1 poliovirus in infected HeLa cells. HeLa cell monolayers were transfected with manipulated RNA transcripts (pTZ-PVS-3m/p24). The recombinant progeny virus (PVS-3m/p24) were harvested when full CPE was detected. This viral supernatant was used for the first passage of the recombinant virus by infection of HeLa cells at an MOI of 10. Reproduced progeny virus was reinfected for the next passage to fresh HeLa cells at the same MOI. The procedure was repeated for 12 passages. Finally, HeLa cells were infected with each virus passaged at an MOI of 10, and harvested 8 h post infection. Infected cell lysates were applied to a SDS-PAGE and analyzed by Western blot with rabbit anti-p24 antisera. Each lane shows HIV-1(1), HeLa cell lysate (2), wild type Sabin 1-infected HeLa cell lysate (3), first passaged (4), 3rd passaged (5), 6th passaged (6), 9th passaged (7) and 12th passaged (8) recombinant virus-infected HeLa cell lysates. Arrows indicate wild-type (24 kDa) and recombinant (18.7 kDa) p24, respectively.

any experiments, such as the Western blot and the radioimmunoprecipitation assay (RIA) with anti-p24 polyclonal antibodies. We could only detect the cloned size of the p24 as shown in Fig. 5. All of these data strongly support the assumption that the shortened PCR bands might have originated from the defective virus particles during the replication.

Discussion

Poliovirus, belonging to the *Picornaviridae* family, is the causative agent of poliomyelitis, infecting and destroying the central nervous system (Bodian and Howe, 1955; Couderc *et al.*, 1989). Poliomyelitis has been effectively controlled by the use of inactivated or live attenuated vaccines. Three serotypes of attenuated strains have been selected by numerous passages of wild-type strains in monkey tissues both *in vivo* and *in vitro* (Sabin and Boulger, 1973). These strains (Sabin 1, 2, and 3), which replicate in the primate gut and induce a strong mucosal and systemic immunity, have shown a good safety record. However, 5–10 cases of vaccine-associated

poliomyelitis (VAP) have been reported to occur every year in the United States after immunization with oral poliovirus (OPV) (Ogra and Faden, 1986; Nkowane *et al.*, 1987). VAP may result from the genetic variation of the Sabin strain, such as in recombination (Furione *et al.*, 1993) or point mutation (Guillot *et al.*, 1994). Indeed, vaccine-derived neurovirulent strains are found in the gut of healthy vaccines and in the central nervous system of patients with VAP (Georgescu *et al.*, 1994; Friedrich, 1996). However, VAP has been reported to be most frequently associated with Sabin 2 and 3, but rarely with Sabin 1 (Otelea *et al,* 1992; Furione *et al.*, 1993). The greater number of attenuating mutations in Sabin 1 is probably reflected in the higher safety of this strain in comparison to type 2 and 3 strains. The Sabin 1 strain of poliovirus, therefore, is the best candidate for a live viral vector to deliver foreign antigens to the enteric tract when mucosal immunity is desired for the protection of infectious disease.

Several investigators have tried to develop poliovirus as a mucosal vaccine vector by means of epitope substitution (Burke *et al.*, 1988, 1989; Evans *et al.*, 1989, Jenkins *et al.*, 1990; Rose *et al.*, 1994), dicistronic IRES (Alexander *et al.*, 1994; Lu *et al.*, 1995), poliovirus minireplicon (Porter *et al.*, 1993; 1996; 1997; Ansaradi *et al.*, 1994), or autoprocessing replication-competent recombinant poliovirus (Andino *et al.*,1994; Mattion *et al.*, 1994; Yim *et al.*, 1996; Tang *et al.*, 1997). We took advantage of the strategy of an autoprocessing recombinant poliovirus, to produce Sabin 1 poliovirus as a mucosal vaccine vector. The multiple cloning site (MCS) and the viral specific 3C-protease cutting sites were newly introduced into the Sabin 1 cDNA followed by the production of recombinant Sabin 1 poliovirus which has a slightly reduced replication capacity as compared with that of the wild-type Sabin 1. We have tried to introduce the MCS and 3C-protease recognition sites into several positions at the N-terminal end, such as, between the 2nd and 3rd amino acids (G/A) and between the 3rd and 4th amino acids (A/Q), but have failed to produce recombinant progeny virus by the transfection experiment with the RNA transcript synthesized from recombinant cDNA plasmids. To confirm if the failure of progeny virus production is due to the disruption of the myristylation signal (Ansaradi *et. al.*, 1992), we redesigned the mutagenic primer to preserve the myristylation signal (GXXXS/T) after processing by the 3C-protease, but there was no change in results. The recombinant virus was produced only when the gene was manipulated between the 1st and 2nd amino acids (M/A), and at the VP3/VP1 junction site as shown in Fig. 1.

The N-terminal 169 aa of HIV-1 p24 was cloned into the multiple cloning sites of pTZ-PVS-3m and -4m recombinant vectors, and their RNA transcripts (PVS-3m/p24 and PVS-4m-p24) were introduced into HeLa cells to generate the chimeric recombinant poliovirus. The RNA transcript synthesized from pTZ-PVS-3m/p24 produced chimeric virus when transfected, but those synthesized from pTZ-PVS-4m-p24 did not (Table 1). Cloning of the p24 into PVS-4m seemed to worsen its transfection capacity due to inefficient processing at the VP3/VP1 junction site. However, the 3C protease accurately recognizes and cleaves the inserted synthetic proteolytic site in PVS-3m/p24, freeing the exogenous HIV-1 p24 protein from the rest of the poliovirus polyprotein. The recombinant p24 was detected at a lower band (18.4 kDa) than that of the wild-type p24 in the Western blot hybridization (Fig. 4). This means that the recombinant p24 was produced by the infection of chimeric virus, not by the contamination of the wild-type p24. The recombinant p24 processed from the long polyprotein turned out to be similar in its antigenicity and structural integrity to those of wild-type p24 in the Western blot experiment and radioimmunoprecipitation assay with AIDS patients' sera (Fig. 4 and Fig. 5). It means that the manners of expression and processing are expected to be applicable to any vaccine gene which is introduced into the same MCS and expressed in the recombinant Sabin 1 live vaccine vehicle.

The Mahoney vector (Andino *et al.*, 1994) was reported recently for the instability of its inserted foreign gene during the passages. The rapid deletion of the inserted sequence during the passages was explained to be due to the high rate of homologous recombination in picornavirus (Yim *et al.*, 1996; Tang *et al.*, 1997; Mueller and Wimmer, 1998). Differing from the Mahoney vector, our Sabin 1 vector has been shown to maintain the inserted p24 sequence even after 12 passages. In our case, small PCR fragments were also detected at each passage together with the full-length clone of p24, as shown in Fig. 8, when the progeny viruses were tested by RT-PCR with poliovirus primers (680-697/sense and 814-797/antisense). Upper bands (642 bp) of PCR fragments in Fig. 8 maintain the sequence integrity of the cloned p24 region, regardless of the number of passages. However, the lower band of the PCR fragments turned out to be very heterogeneous in their sequences even though it looks like a single band, due to the genomic deletion between the internal sequences of p24 and the poliovirus genome, probably by the homologous recombination. Some of the deleted regions of the poliovirus covers the sequence of the antisense PCR primer (814-797) which was used to select the recombinant clones. That means that some of the small PCR fragments are artifacts, which were produced by nonspecific annealing of the antisense-PCR primer. Truly, the 3′-end portion of the antisense PCR primer shows sequence homology to the poliovirus genome at the position about 2300. Therefore, the defective poliovirus with the internal deletion could be detected by the short fragments in the PCR experiment as an artifact band in all passages. This artifact band of PCR fragments enables us to explain the

reason why the chimeric viruses have a reduced replication capacity as compared with that of the wild-type Sabin 1. During the replication of the chimeric viruses, homologous recombination occurs to some extent in the sequences between the p24 and poliovirus, causing internal deletion, and the intact capsid proteins produced from the normal chimeric viral genome are packed in the same cell as the replication-defective viral genomes. Therefore, the chimeric virus-infected cells produce substantial amounts of defective viruses together with replication-competent chimeric viruses, reducing the replication capacity of the chimeric viruses as shown in Fig. 6. Actually, the total amount of viral RNA synthesized in the infected cells and the amount of the progeny viruses produced from the chimeric virus-infected cells were shown to be similar to those of the wild-type Sabin 1 infected cells (Fig. 7). The initiation of the exponential phase of viral RNA synthesis is at about 6 h pi in HeLa cells, and is consistent with other studies done with *Vero* cells for attenuated Sabin 3 viruses (Mattion *et al.*, 1994).

Other explanations for the reduced replication ability of the chimeric virus, at least partly, is that the N-terminal insertion at the polyprotein sequence may induce inefficient myristylation of the polyprotein, resulting in the delay of viral assembly, followed by slow replication of the chimeric viruses.

The recombinant poliovirus constructed from the Mahoney vector rapidly deleted parts of the inserted foreign sequences during the passages, resulting in the production of unwanted, but replication-competent, recombinant polioviruses (Tang *et al.*, 1997; Mueller and Wimmer, 1998), and these unwanted recombinant viruses were relatively stable and exhibited improved replication capacity (Mueller *et al.*, 1997). Therefore, the internally deleted recombinant polioviruses would become dominant shortly during the passages and the expected recombinant virus having relatively lower replication capacity was assumed to be diluted out within a couple of passages. That seems to be the reason why the expected cDNA band could not be detected in the RT-PCR with progeny viruses even after a couple of passages. On the contrary, our Sabin 1 vector-derived recombinant poliovirus causes internal deletion to a certain extent during the replication by homologous recombination, but the internal deletion of the recombinant Sabin 1 results in the production of replication-defective polioviruses. On the other hand, our Sabin vector-derived chimeric virus produced substantial amounts of replication-competent progeny virus, which maintains sequence integrity. That is thought to be the reason why the chimeric virus was able to maintain the expression of p24 during the passages as shown in Fig. 9.

All of our experimental results strongly suggest that the recombinant poliovirus Sabin 1 vector, developed in our laboratory, can be used as a live mucosal vaccine vector. Further studies are underway to investigate the probability of using our viral vector for the development of live mucosal vaccines.

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