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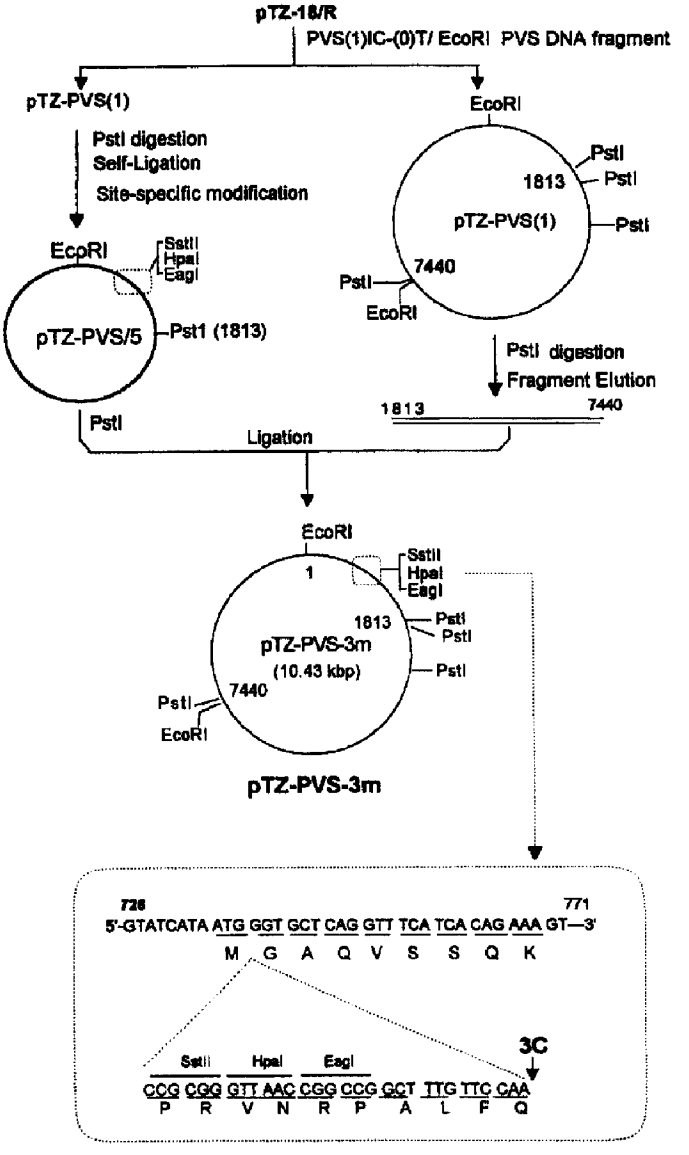
(54) **SOUCHE RECOMBINEE DE POLIOVIRUS, DU TYPE 1 SABIN, A CAPACITE DE REPLICATION**

(54) **REPLICATION-COMPETENT RECOMBINANT SABIN TYPE 1 STRAIN OF POLIOVIRUS**



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(57) L'invention concerne un vecteur recombiné de poliovirus 1 du type Sabin, à capacité de réplication, contenant une séquence codant pour un site de clonage multiple et un site de coupure multiple de protéase 3C. Ce vecteur facilite l'introduction de divers gènes vaccinaux de virus infectieux, dans le poliovirus du type 1 Sabin, et facilite la production de poliovirus chimères du type 1 Sabin, qui sont des vaccins buccaux destinés à être administrés à travers la muqueuse, et dirigés contre les maladies virales infectieuses graves.

(57) A replication-competent recombinant Sabin type 1 poliovirus vector containing a sequence coding for multiple cloning site and 3C-protease cleavage site is provided. This vector makes it easy to introduce various vaccine genes from infectious viruses to the Sabin 1 poliovirus, and facilitates to produce chimeric Sabin 1 polioviruses that are expected to be powerful oral mucosal vaccines against several infectious viral diseases.

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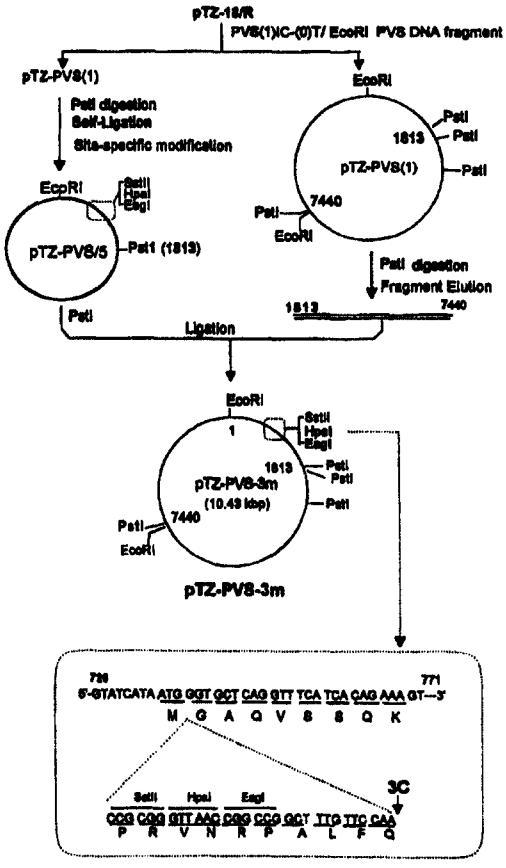
(54) Title: REPLICATION–COMPETENT RECOMBINANT SABIN TYPE 1 STRAIN OF POLIOVIRUS

(57) Abstract

A replication–competent recombinant Sabin type 1 poliovirus vector containing a sequence coding for multiple cloning site and 3C–protease cleavage site is provided. This vector makes it easy to introduce various vaccine genes from infectious viruses to the Sabin 1 poliovirus, and facilitates to produce chimeric Sabin 1 polioviruses that are expected to be powerful oral mucosal vaccines against several infectious viral diseases.



REPLICATION-COMPETENT RECOMBINANT SABIN TYPE 1 STRAIN OF POLIOVIRUS

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

The present invention relates to a vector system useful for developing live viral vaccines. More particularly, it relates to replication-competent recombinant Sabin type 1 strain of poliovirus, which can be used for the development of live viral vaccine capable of inducing mucosal immunity.

2. DESCRIPTION OF THE PRIOR ARTS

Recently it has been reported that various infectious viral disease, which were well known to be spread by blood-mediated routes such as blood transfusions, homosexual intercourse, or sharing of syringes, also may be transmitted by heterosexual intercourse. In the case of AIDS (Acquired Immunodeficiency Syndrome), the number of heterosexual transmission is far greater than that of the blood-mediated cases (Stingl et al., J. Am. Aca. Dermatol., 22, 1210, 1988). In Korea, 363 out of 527 HIV-1 positive patients are heterosexuals while 99 are infected via homosexual routes (National Institute of Health, Korea, Communicable Diseases Monthly Report, April of 1996). These reports strongly suggest that the HIV-1 can be transmitted through mucosal tissues around the genital organs without blood mediation.

Several papers have reported that HIV-1 transmission and spreading are likely to be initiated by the infection of Langerhans cells or dendritic cells (DCs) at the mucosal tissues. Infected cells return to lymph node to deliver antigen, well known as homing property, where the viral replication occurs, resulting in viremia and AIDS

progression. In other words, those who have heterosexual intercourse with HIV-1 infected patients will have Langerhans cells or DCs infected with HIV-1 in mucosal area of the urogenital organs, and the infected DCs return to lymph nodes, activate $CD4^+$ T-cells in lymph nodes and propagate HIV, resulting in depletion of CD4 T-cells followed by AIDS progression (Tschachler et al., J. Inves., Dermator, 88, 238, 1987; Langhoff et al., Proc. Natl.Acad.Aci. USA, 88, 7998, 1991; Patterson & Knight, J. Gen. Virol., 68, 1177, 1987; Patterson et al., Immnol., 72, 361, 1991; Cameron et al., Science, 257, 383, 1992; Embreston et al., Nature, 362, 369, 1993; Fauci, Science, 262, 1011, 1993; Pantleo et al., Nature, 362, 355, 1993; Adema et al., Nature, 387, 713, 1997). These experimental results were substantiated by in vivo experiments by addressing that the Macaque monkeys treated with SIV (Simian immunodeficiency Virus) on their genital organs became infected and then showed AIDS symptom (Miller & Gardner, J. AIDS, 4, 1169, 1991; Miller et al., J. Virol, 63, 4277, 1989). Moreover, since most of the infectious viral diseases are spread by first infection of mucosal tissues at respiratory, digestive or urogenital organs, mucosal vaccine development is highly recommended to prevent infectious viral. In particular, the common mucosal immune system - immunization at one locus can induce identical immunity to the other mucosal areas in the living body, special characteristics of mucosal immunity, encourages many researchers to develop mucosal vaccine (Kott, Science, 266, 1335, 1994; Cease & Verzofsky, Ann. Rev. Immunol., 12, 923, 1994).

Since long ago, smallpox virus has been proposed as a live viral vaccine vehicle. Recombinant vaccinia virus produced by introducing a vaccine gene into smallpox viral genome was reported to induce cytotoxic T lymphocyte in the immunized monkey. But has not yet been allowed to apply it to human because it may cause a vaccinia syndrome to the immunized individuals when overpropagated. To avoid the possibility, an attenuated vaccinia virus was suggested as a vaccine vehicle instead of virulent strain, but it failed to induce an effective immuniity (Cooney et al.,

Lancet, 337, 567, 1991; Tartaglia et al., Virol., 188, 217, 1992).

Adenovirus having a smaller genome (34kbp) than that of vaccinia virus was also proposed as a vaccine vector (Natuk et al., Proc. Natl. Acad. Sci. USA, 89, 7777, 1992; Gallichar et al., J. Infec. Dis., 168, 622, 1993). But the recombinant adenoviruses still have a limitation of side effects, such as conjuctivitis or corneitis, which should be solved for adenovirus to be used as a mucosal vaccine vector.

Poliovirus contains a positive sense single-stranded RNA of 7.4 Kb nucleotides, which encodes an unique open-reading frame of a long polyprotein (Kitamura et al., Nature 291, 547, 1981). Recently, several groups are trying to develop a poliovirus as a vaccine vehicle for its well-known and attractive advantages - safe, easy to administration, economy, and above all having capacity to induce effective life-long mucosal immunity, which is strongly recommended for an ideal vaccine.

Followings are summary of the published vaccine researches in association with poliovirus developed as a vaccine vehicle:

(1) It was proposed to substitute some portion of VP1, major outer capsid protein of poliovirus with presumed vaccine epitopes of HIV such as gp41, PND (Principle Neutralizing Domain) or gp120. The chimeric virus produced from the genetic recombination effectively induced antibody depending on the characteristics of the epitopes (Burke et al., Nature, 332, 81, 1988; Burke et al., J.Gen.Virol. 70,2475, 1989;Evans et al., Nature, 385, 1989; Dedieu et al., J. Virol., 66, 3161, 1992; Rose et al., J. Gen. Virol., 75, 969, 1994). However, this chimeric virus has a size limitation for the Morrow and his introduced vaccine gene. Chimeric poliovirus could not be assembled properly in the infected cells when the inserted vaccine epitope is larger than 25 amino acid residues.

(2) Morrow and his colleagues (Porter et al., J. Virol., 69, 1548, 1993; Ansaradi et al., Cancer Res., 54, 6359, 1994, Porter et al., J. Virol.70, 2643, 1996, Porter et al., Vaccine 15, 257, 1997) have suggested poliovirus minireplicon, in which poliovirus structural genes are replaced by foreign sequences, to develop poliovirus-mediated

mucosal vaccines. In case of poliovirus minireplicon, the replication defective recombinant viral genome must be co-transfected with other capsid protein-expressing vector for packaging of chimeric viral genome (Porter et al., J. Virol., 69, 1548, 1995). Moreover, high titer of minireplicon is required for vaccination to induce effective mucosal immunity because it works as replication-defective target-specific immunogen rather than live viral vaccine.

(3) Recently, a new strategy was suggested for expression of foreign antigens in the replication-competent recombinant polioviruses by Mattion et al.(J. Virol. 68, 3925,1994) and Andino et al(Science, 265, 1448, 1994). They have introduced a new polylinker region and 3C protease-recognition site on the N-terminal end of the polyprotein of poliovirus. According to this system, 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 from the poliovirus polyprotein. The exogenous nucleic acid is incorporated directly into the poliovirus genome. The exogenous sequences are expressed during virus replication as part of the virus polyprotein and subsequently processed by virus-encoded proteases to produce free antigen and mature viral protein. The foreign antigen is not packaged in the virion but released into the cytoplasm. The principle of this method is based on the characteristics of poliovirus-specific 3C-protease published previously. 3C-protease recognizes specific amino acid sequence and then cleaves it at the junction between Glu(Q)/Gly(G) (Hanecak et al., Proc. Natl. Acad. Sci. USA 79, 3793, 1982), and the proteolysis occurs within the intramolecule of long polyprotein (Palmenberg and Reuckert, J. Virol., 41, 244, 1982; Hanecak et al, Cell 37, 1063, 1984). These phenomena are generally observed in picornavirus family (Palmenberg et al., J. Virol. 32, 770, 1979; Palmenberg and Reuckert, J. Virol., 41, 244, 1982). The alanine(A) residue in the P4 position of the Q/G cleavage site (AXXQG) has been confirmed several times to be essential for effective recognition and cleavage by 3C-protease (Nicklinson et al., Biotechnology 4, 33, 1986; Pallai et al., J. Bio.

Chem., 264, 9738, 1989; Cordingley et al., J. Virol., 63, 5037, 1989; Orr et al., J. Gen. Virol., 70, 2931, 1989; Petithory et al., Proc. Natl. Aca. Sci. USA 88, 11510, 1991; Blair and Semler, J. Virol. 65, 6111, 1991). Based on these experimental results, Mattion and his colleagues introduced (J. Virol. 68, 3925, 1994) multiple cloning site and 3C-protease cleavage site into N-terminal of Sabin type 3 strain of poliovirus and constructed chimeric poliovirus expressing rotavirus VP7. Andino and his colleagues (Science, 265, 1448, 1994) constructed recombinant Mahoney vector (MoV-1.4) by introducing multiple cloning site and 3C-protease cleavage site into the N-terminal end of the long polyprotein of poliovirus Mahoney strain. They produced chimeric poliovirus by cloning various HIV-1 subgenomes into the vector (Science, 265, 1448, 1994). Andino group has reported that the chimeric poliovirus expressing HIV-1 nef gene induced effective mucosal immunity in monkey two weeks after immunization through rectum (Andino et al., Science, 265, 1448, 1994). Nevertheless, the recombinant Mahoney poliovirus is not applicable to human without any further detoxification steps since Mahoney strain of poliovirus is a deadly virulent neurotropic virus, which infects central nervous system of primates through the primary infection of digestive organ, resulting in causing paralytic poliomyelitis. Whereas, Mueller and Wimmer (J. Virol., 72, 20-31, 1998) reported that the recombinant virus obtained by cloning green fluorescence protein gene (gfp: 252aa) and HIV-1 gag gene into Andino's Mahoney vector were not so much genetically stable during the passages as reported previously by Andino et al. (Science, 265, 1448, 1994). Their experimental results revealed that most of the recombinant viruses even after a single passage lost the introduced vaccine gene, and none of the progeny virus after 6 passages has been found to have full-length exogeneous insert in RT-PCR experiment.

(4) In 1996, Andino and his colleagues also reported recombinant Mahoney virus expressing preS (118aa, 54aa) and core (155aa) proteins of hepatitis B virus using the same Mahoney vector (Yim et al., Virology, 218, 61, 1996). Even though they have

stated that these chimeric viruses were genetically stable during the passages until the sixth progeny, their RT-PCR experimental result showed that the amounts of recombinant virus maintaining preS gene (118aa) were markedly reduced during the fifth passage. On the other hand, their report revealed that the recombinant virus expressing a small preS protein coding 55aa residues had a markedly reduced replication capacity as compared with that of wild-type or other recombinant Mahoney strains. The result suggests that the replication capacity of recombinant chimeric viruses is not so much tightly associated with the size of the inserted vaccine gene as expected. These experimental results lead us to conclude that the biological characteristics of the recombinant chimeric virus depends on several combined factors like the size of gene inserts, characteristics of the viral vector, introduced vaccine genes, etc.

(5) Andino et al., accepting the problems of their Mahoney vector as mentioned above, developed a new Mahoney vector (MoV-2.1) by inserting a multiple cloning site and 2A-protease cleavage site at P1/P2 junction of Mahoney polyprotein cDNA (Tang et al., J. Virol., 71, 7841-7850, 1997). This MoV-2.1 vector was employed to construct recombinant chimeric virus expressing SIV gag genes (p17 or p27) or env genes (gp130 or gp41). These recombinant chimeric viruses have the similar replication capacity to that of wild type Mahoney, and express the introduced vaccine gene efficiently. However, the MoV-2.1 vector still has a problem of genetic instability. Over 99% of the recombinant chimeric viruses lose the introduced vaccine genes within the third passage. They thought that the sequence deletion during the passages was to be due to the homologous recombination between the repeated sequences at the newly introduced multiple cloning sites. They have reduced the sequence homology by 37% at the repeated sequence of Mov-2.1 vector by performing silent mutation to change the sequence without affecting amino acid sequence, and then named it MoV-2.11. They have constructed the recombinant chimeric virus Sp27(MoV-2.11) by cloning SIV p27 gene into the manipulated

Mahoney vector (MoV-2.11). For the recombinant virus without silent mutation [Sp27(MoV-2.1)], 20-30% of the plaques maintained the cloned vaccine gene after first passage and none of the plaques after third passage carried the cloned gene. On the other hand, for the recombinant virus with silent mutation at the repeated sequence [Sp27(Mo-2.11)], more than 90% of the single-passaged plaques maintained the cloned gene (gag) and 30-50% of the plaques expressed the SIV gene product at third passage. Nevertheless, the Mahoney vector MoV-2.11 still has a problem of genetic instability during the passages, even though they have some progress to increase genetic stability of Mahoney vector by reducing sequence homology. According to their experimental results, the population of insert-maintaining progeny viruses was markedly reduced as passage goes on even within three passages; first (90%), second (50-70%), and third (30-50%). That means that the insert-maintaining recombinant chimeric Mahoney virus will be rapidly diluted out among the total population if passaged little bit further.

Considering several limitations of Mahoney vectors developed by Andino group, the Mahoney vector can not be allowed as a model system for the poliovirus-mediated mucosal vaccine vector.

Therefore, it is highly recommended to develop a new model system for poliovirus-mediated mucosal vaccine vectors, which is able to overcome the limitations shown in Mahoney vectors. For that purpose, the new vector should meet the following requirements: (1) viral vector should be safe to human beings; (2) recombinant virus should be replicable, and have equivalent replication capacity to that of wild type; and (3) the introduced vaccine genes should be stably maintained during the viral passages.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a replication- competent recombinant Sabin 1 poliovirus vector that is useful as a live viral vaccine vector for the development of mucosal vaccines.

Another object of the present invention is to provide a chimeric poliovirus expressing several vaccine genes cloned into the multiple cloning site of the recombinant Sabin 1 poliovirus.

The present invention provides a replication-competent recombinant Sabin 1 poliovirus vector having nongenomic sequences coding for multiple cloning site and 3C-protease cleavage site between the first amino acid and second amino acid of long polyprotein of Sabin 1 poliovirus cDNA.

The present invention further provides a replication-competent chimeric Sabin 1 poliovirus expressing exogenous vaccine genes, respectively, and their recombinant cDNA plasmids, where which have exogeneous vaccine genes, respectively, at the multiple cloning site of the above-mentioned replication-competent recombinant Sabin 1 poliovirus vector.

The objects mentioned above, other features and applications of the present invention would be much more apparent by those of ordinary skills in the art from the following explanation in detail.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation for the construction of a recombinant plasmid pTZ-PVS-3m in order to achieve the present invention.

Figure 2 is a schematic representation of recombinant vectors, pTZ-PVS-3m and pTZ-PVS-4m, derived from Sabin 1 poliovirus cDNA, which have a multiple cloning site and 3C-protease cleavage site, respectively.

Figure 3 is photograph showing that the wild type and recombinant poliovirus cDNAs are able to produce progeny viruses when trsfected into HeLa cells. HeLa cells were tranfected with RNA transcript synthesized from wild-type cDNA (A), with RNA transcript synthesized from recombinant plasmid pTZ-PVS-3m (B), or with RNA transcript synthesized from recombinant plasmid pTZ-PVS-4m (C).

Figure 4 is a schematic representation of the procedure for production of chimeric poliovirus PVS-3m/p24 by inserting HIV-1 p24 gene into the multiple cloning site of recombinant plasmid pTZ-PVS-3m, and expression of HIV-1 p24 protein.

Figure 5 shows one-step growth curve to evaluate the replication capacity of the wild type and recombinant Sabin 1 poliovirus. Titer of virus in the culture supernatant was determined every 3 hrs by TCID50 and plaque assay.

Figure 6 is the western blot of HIV-1 p24 protein expressed in culture supernatant and HeLa cells infected with chimeric poliovirus PVS-3m/p24:

Lane 1 : HIV-1/-tat (tat defective HIV-1 strain; supplied from Dr Sodroski, Dana-Farber Cancer Ins., US)

Lane 2 : Control (wild type Sabin 1 poliovirus-infected HeLa cell lysate)

Lane 3 : Chimeric poliovirus PVS-3m/p24-infected HeLa cell lysate

Lane 4 : Culture supernatant of chimeric poliovirus PVS-3m/p24-infected HeLa cell

Figure 7 is the results of radioimmunoprecipitation to evaluate the antigenecity of HIV-1 p24 protein expressed from the chimeric poliovirus PVS-3m/p24:

Lane 1 : Non-infected cell lysate

Lane 2 : Wild type Sabin 1 poliovirus-infected HeLa cell lysate

Lane 3 : Chimeric poliovirus PVS-3m/p24-infected HeLa cell lysate

Figure 8 represents the PCR analysis of p24 gene, cloned in chimeric poliovirus, up to 12th passage to evaluate the genetic stability of chimeric poliovirus PVS-3m/p24 during the passages:

Lane 1 : PCR product from wild Sabin type 1 poliovirus-infected HeLa cell

Lane 2 : PCR product from recombinant poliovirus PVS-3m-infected HeLa cell

Lane 3 : PCR product from chimeric poliovirus PVS-3m/p24-infected HeLa cell

Lane 4 : PCR product from the second(2nd)-passaged chimeric poliovirus PVS-3m/p24-infected HeLa cell

Lane 5 : PCR product from the fourth(4th)-passaged chimeric poliovirus PVS-3m/p24-infected HeLa cell

Lane 6 : PCR product from the eighth(8th)-passaged chimeric poliovirus PVS-3m/p24-infected HeLa cell

Lane 7 : PCR product from the twelveth(12th)-passaged chimeric poliovirus PVS-3m/p24-infected HeLa cell

Figure 9 is the results of western blot of HIV-1 p24 protein, expressed from the chimeric poliovirus, up to 12th passage to evaluate the expression stability of chimeric poliovirus PVS-3m/p24 during the passages:

Lane 1 : HIV-1/-tat

Lane 2 : HeLa cell lysate

Lane 3 : Wild-type Sabin 1 poliovirus-infected HeLa cell lysate

Lane 4 : Cell lysate of first(1st)-passaged chimeric poliovirus PVS-3m/p24-infected HeLa cell

Lane 5 : Cell lysate of third(3rd)-passaged chimeric poliovirus PVS-3m/p24-infected HeLa cell

Lane 6 : Cell lysate of sixth(6th)-passaged chimeric poliovirus PVS-3m/p24-infected HeLa cell

Lane 7 : Cell lysate of ninth(9th)-passaged chimeric poliovirus PVS-3m/p24-infected HeLa cell

Lane 8 : Cell lysate of twelveth(12th)-passaged chimeric poliovirus PVS-3m/p24-infected HeLa cell

Figure 10 is a schematic representation of the procedures for construction of

chimeric poliovirus PVS-3m/env by cloning HIV-1 envelope glycoprotein gene *env*(125aa) into multiple cloning site of recombinant plasmid pTZ-PVS-3m and its expression of the HIV-1 envelope glycoprotein.

Figure 11 represents the PCR analysis of HIV-1 env gene, cloned in chimeric poliovirus, up to 12th passage to evaluate the genetic stability of chimeric poliovirus PVS-3m/env during the passages:

Lane 1 : PCR product from wild-type Sabin 1 poliovirus-infected HeLa cell

Lane 2 : PCR product from recombinant poliovirus PVS-3m-infected HeLa cell

Lane 3 : PCR product from the third(3rd)-passaged chimeric poliovirus PVS-3m/env-infected HeLa cell

Lane 4 : PCR product from the sixth(6th)-passaged chimeric poliovirus PVS-3m/env-infected HeLa cell

Lane 5 : PCR product from the ninth(9th)-passaged chimeric poliovirus PVS-3m/env-infected HeLa cell

Lane 6 : PCR product from the twelfth(12th)-passaged chimeric poliovirus PVS-3m/env-infected HeLa cell

Figure 12 is the western blot of HIV-1 env protein, expressed from the chimeric poliovirus, up to 12th passage in order to evaluate the expression stability of chimeric poliovirus PVS-3m/env during the passages:

Lane 1 : HeLa cell lysate

Lane 2 : Recombinant poliovirus PVS-3m-infected HeLa cell lysate

Lane 3 : Cell lysate of third(3rd)-passaged chimeric poliovirus PVS-3m/env-infected HeLa cell

Lane 4 : Cell lysate of sixth(6th)-passaged chimeric poliovirus PVS-3m/env-infected HeLa cell

Lane 5 : Cell lysate of ninth(9th)-passaged chimeric poliovirus PVS-3m/env-infected HeLa cell

Lane 6 : Cell lysate of twelveth(12th)-passaged chimeric poliovirus PVS-3m/env-

infected HeLa cell

Figure 13 is a schematic representation of the procedures for construction of chimeric poliovirus PVS-3m/HCVc by cloning HCV core gene into multiple cloning site of recombinant plasmid pTZ-PVS-3m and its expression of the HCV core protein.

Figure 14 shows one-step growth curve to evaluate the replication capacity of chimeric poliovirus PVS-3m/HCVc. Titer of virus in the culture supernatant was determined every 3 hrs by TCID50 and plaque assay.

Figure 15 is the western blot of HCV core protein to check the expression patterns of HCV core protein when HeLa cells are infected with chimeric poliovirus PVS-3m/HCVc:

Lane 1 : HeLa Cell lysate

Lane 2 : Wild Sabin type 1 poliovirus-infected HeLa cell lysate

Lane 3 : Chimeric poliovirus PVS-3m/HCVc-infected HeLa cell lysate

Lane 4 : Pellet of chimeric poliovirus PVS-3m/HCVc

Lane 5 : Culture supernatant concentrate of virus-free HeLa cell

Lane 6 : Pellet of chimeric poliovirus PVS-3m/HCVc-infected HeLa cell lysate

Lane 7 : Supernatant of chimeric poliovirus PVS-3m/HCVc-infected HeLa cell lysate

Figure 16 represents the PCR analysis of the HCV core protein gene, cloned in the chimeric virus, up to 12th passage to evaluate the genetic stability of chimeric poliovirus PVS-3m/HCVc during the passages:

Lane 1 : PCR product from wild-type Sabin 1 poliovirus-infected HeLa cell

Lane 2 : PCR product from recombinant poliovirus PVS-3m-infected HeLa cell

Lane 3 : PCR product from the third(3rd)-passaged chimeric poliovirus PVS-3m/HCVc-infected HeLa cell

Lane 4 : PCR product from the sixth(6th)-passaged chimeric poliovirus

PVS-3m/HCVc-infected HeLa cell

Lane 5 : PCR product from the ninth(9th)-passaged chimeric poliovirus PVS-3m/HCVc-infected HeLa cell

Lane 6 : PCR product from the twelveth(12th)-passaged chimeric poliovirus PVS-3m/HCVc-infected HeLa cell

Figure 17 is the western blot of HCV core protein, expressed from the chimeric poliovirus, up to 12th passage in order to evaluate the expression stability of chimeric poliovirus PVS-3m/HCVc during the passages:

Lane 1 : Cell lysate of first(1st)-passaged chimeric poliovirus PVS-3m/HCVc-infected HeLa cell

Lane 2 : Cell lysate of third(3rd)-passaged chimeric poliovirus PVS-3m/HCVc-infected HeLa cell

Lane 3 : Cell lysate of sixth(6th)-passaged chimeric poliovirus PVS-3m/HCVc-infected HeLa cell

Lane 4 : Cell lysate of ninth(9th)-passaged chimeric poliovirus PVS-3m/HCVc-infected HeLa cell

Lane 5 : Cell lysate of twelveth(12th)-passaged chimeric poliovirus PVS 3m/HCVc-infected HeLa cell

DETAILED EXPLANATION OF THE INVENTION

Poliovirus, belonging to the Picornaviridae family, is a causative agent of poliomyelitis by 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 in vivo and in vitro (Sabin and Boulger, J. Biol. Stand.,1,115 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) was reported to occur every year in the United States after immunization with oral poliovirus (OPV) (Ogra and Faden, J. Pediatr., 108.1031, 1986; Nkowane et al.,JAMA, 257, 1335, 1987). VAP may result from the genetic variation of the Sabin strain, such as recombination (Furione et al., Virology, 196, 199, 1993) or point mutation (Guillot et al.,Vaccine, 12, 503, 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.,J. Virol., 68, 8089, 1994; Friedrich,Acta Virol., 40, 157, 1996). However, VAP has been reported to be most frequently associated with Sabin 2 and 3, but rarely with Sabin 1 (Furione et al., Virology, 196, 199,1993; Otelea et al, Dev. Biol. Stand., 78, 33, 1992). The greater number of attenuating mutations in Sabin 1 is probably reflected in the higher safety of this strain in comparison with 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 protection of infectious disease.

The present invention is based on these advantages of Sabin type 1 strain.

The inventors have made an effort to use Sabin type 1 strain of poliovirus as a mucosal vaccine vehicle. They constructed a recombinant plasmid pTZ-PVS-3m by introducing a multiple cloning site and 3C-protease cleavage site into N-terminal end of the cDNA of Sabin 1 poliovirus. RNA transcript synthesized from the plasmid was infectious when transfected into HeLa cells, resulting in production of recombinant progeny virus (PVS-3m). The recombinant virus has a slightly reduced replication capacity of 1-10 times as lower as that of wild type Sabin. Nevertheless, chimeric Sabin 1 polioviruses, obtained by transfection as mentioned above after cloning various vaccine genes into the vector pTZ-PVS-3m, maintain the foreign genes to be expressed stably at least up to 12 serial passages.

The Sabin type 1 strain of poliovirus was developed in 1961 by Sabin and his colleagues. They developed an attenuated, less neurovirulent and much more immunogenic poliovirus vaccine strain through sequential passages of wild-type Mahoney strain in the established cell line of monkey kidney cells, and then called Sabin 1 poliovirus vaccine strain. Sabin 1 has 57 nucleotide substitutions in comparison with wild type Mahoney strain, 21 of which cause amino acid substitution. The nucleotide sequence of Sabin type 1 strain is shown as SEQ. ID. NO. 1. All of the coding-change alterations are concentrated on the N-terminal half region of VP1 major outer capsid protein (Kitamura et al., Nature, 291, 547-553, 1981; Nomoto et al., Proc. Natl. Acad. Sci. USA, 79, 5793-5797, 1982). Replication capacity of Sabin 1 strain is 2-3 log lower than that of wild-type Mahoney strain. Since it has lost neurotropism during the passages, it does not cause paralytic poliomyelitis in primates. Nevertheless it still maintains the enterotropic capacity enough to induce a potent mucosal and systemic immunity. It was first approved as an oral polio vaccine(OPV) in the United States in 1961 and came into general use by 1963 (Ogra et al., Rev. Inf. Diseases 2, 352-369, 1980).

For the present invention, multiple cloning site and 3C-protease cleavage site are newly introduced between the first and second amino acids of Sabin 1 long polyprotein cDNA by site-specific insertion experiments in order to construct recombinant plasmid pTZ-PCV-3m (Figure 1). The multiple cloning site is designed to have three restriction sites of SstII, HpaI and EagI.

Recombinant plasmid pTZ-PCV-3m was the most effective for production of recombinant progeny virus, when transfected into HeLa cell monolayers with its RNA transcript, among the several recombinant plasmids containing exogeneous multiple cloning site and 3C-protease cutting site at different positions of poliovirus.(Figure 3). One-step growth curve plotted by measuring virus titer through the $TCID_{50}$ assay and plaque assay reveals that the replication capacity of recombinant poliovirus PCV-3m is at most 1 log lower than that of the wild-type

Sabin 1 strain, while is the highest among the various chimeric polioviruses (Figure 5).

In the present invention, three exogenous vaccine genes, HIV-1 p24 (169aa), HIV-1 env (125aa) and HCV core gene (100aa) were successfully introduced into the multiple cloning site of pTZ-PCV-3m, resulting in production of chimeric poliovirus PCV-3m/p24, PCV-3m/env and PCV-3m/HCVc, respectively, when their RNA transcripts were transfected into HeLa cells (Figure 4, Figure 10 and Figure 13).

These chimeric polioviruses effectively express the exogenous protein during the replication in HeLa cells (Figure 6 and Figure 15), and the expressed proteins maintain the original antigenecity (Figure 7). Moreover, these chimeric polioviruses are genetically stable during the serial passages (Figure 8, Figure 11 and Figure 16), and the expression of foreign genes is not damaged during the passages (Figure 9, Figure 12 and Figure 17).

These results strongly suggest that the recombinant plasmid pTZ-PCV-3m can be used as an effective vaccine vector for the development of several mucosal vaccines against several infectious diseases. The recombinant plasmid pTZ-PCV-3m of the present invention has been deposited at the Korea Collection of Type Cultures (KCTC) in Korea Research Institute of Bioscience and Biotechnology (KRIBB) lacated in Taejon on August 6, 1997 with the accession number of KCTC-0365BP.

The exogenous vaccine genes which can be introduced into the multiple cloning site of the recombinant plasmid pTZ-PVC-3m of the present invention may include those derived from various infectious viruses, such as HIV, HBV, HCV, human papillomavirus, rotavirus etc, but not limited thereto.

The recombinant poliovirus plasmids containing several exogenous vaccine genes will be transfected into host cells, such as HeLa cell, by known methods to obtain chimeric polioviruses, which will be used as oral vaccines.

Free Texts in Sequence Listing

SEQ.ID.NO. 2 is an artificial sequence which is a synthetic sequencing primer for DNA amplification having multiple cloning site and 3C-protease cleavage site:

SEQ. ID. NO. 3 is an artificial sequence which is a peptide coded by SEQ. ID. NO. 2:

SEQ.ID.NO. 4 is an artificial sequence which is a primer for DNA amplification having multiple cloning site and 3C-protease cleavage site:

SEQ. ID. NO. 5 is an artificial sequence which is a peptide coded by SEQ. ID. NO. 4:

SEQ.ID.NO. 6 is an artificial sequence which is a primer for DNA amplification having multiple cloning site and 3C-protease cleavage site:

SEQ.ID.NO. 7 is an artificial sequence which is a primer for DNA amplification having multiple cloning site and 3C-protease cleavage site:

SEQ.ID.NO. 8 is an artificial sequence which is a peptide coded by SEQ. ID. NO. 7:

SEQ.ID.NO. 9 is an artificial sequence which is a primer for DNA amplification having multiple cloning site and 3C-protease cleavage site:

SEQ.ID.NO. 10 is an artificial sequence which is a peptide coded by SEQ. ID. NO. 9:

SEQ.ID.NO. 11 is an artificial sequence which is a *Sst*II-sense primer for DNA amplification having multiple cloning site and 3C-protease cleavage site:

SEQ.ID.NO. 12 is an artificial sequence which is a *Eag*I-antisense primer for DNA amplification having multiple cloning site and 3C-protease cleavage site:

SEQ. ID. NO. 13 is an artificial sequence which is a cDNA synthesis primer:

SEQ.ID.NO. 14 is an artificial sequence which is a sense primer for DNA amplification having multiple cloning site and 3C-protease cleavage site:

SEQ.ID.NO. 15 is an artificial sequence which is an antisense primer for DNA amplification covering nucleotide 797-814 of Sabin 1 poliovirus:

SEQ.ID.NO. 16 is an artificial sequence which is a *Sst*II-sense primer for PCR

amplification:

SEQ.ID.NO. 17 is an artificial sequence which is a *Eag*I antisense primer for DNA amplification:

SEQ.ID.NO. 18 is an artificial sequence which is a sense primer for DNA amplification:

SEQ.ID.NO. 19 is an artificial sequence which is an antisense primer for DNA amplification: and

SEQ.ID.NO. 20 is an artificial sequence having multiple cloning site and 3C-protease cleavage site.

Examples

The present invention will be described in more detail by way of various Examples, which should not be construed to limit the scope of the present invention.

Example 1 : Construction of recombinant plasmid pTZ-PCV-3m

Plasmid PVS(1)IC-(0)T (Poliovirus Sabin 1 cDNA plasmid; obtained by courtesy from Dr. Nomoto at Tokyo University) was digested with restriction enzyme EcoRI and cDNA was isolated. The cDNA was cloned into EcoRI site of plasmid pTZ-18/R (Pharmacia) to construct plasmid pTZ-PVS(1).

Plasmid pTZ-PVS(1) was digested with restriction enzyme PstI to reduce the size of cDNA part and subjected to self-ligation to produce plasmid subclone pTZ-PVS(1)/5 having the nucleotide sequence 1-1813 of the poliovirus cDNA.

Plasmid pTZ-PVS(1)/5 was transformed into E. coli CJ236(dut$^-$, ung$^-$, C^{mr}) and the transformant was cultured in LB-amp chloramphenicol medium (LB, 40 μg/ml ampicillin, 30 μg/ml chloramphenicol) supplemented with 0.25 μg/ml of uridine. When the OD600nm of the culture reaches about 0.5-0.6, cells were super-infected with more than 10 moi/bacterium of M13K07 helper phage (Pharmacia), and then

further cultured for about 8 more hours in LB-amp chloramphenicol medium supplimented with kanamycin, thymidine and uridine to obtain uracil-incorporated single-stranded phagemid.

The phagemid was precipitated with PEG/NaCl (20% PEG and 2.5M NaCl in DDW) and extraced with phenol/chloroform to have ss-DNA.

One (1) μg of the ss DNA and 5 pmole of mutagenic primer having the sequence shown as SEQ. ID. NO. 2 were added into 10 ul of buffer (2mM $MgCl_2$, 50mM NaCl in 20mM Tris-HCl: pH 7.4) and incubated at a water bath of 70°C for 10 minutes followed by annealing through gradually cooling to 30°C.

One (1) μl of 10X synthesis buffer (5mM of each dNTP, 10mM of rATP, 100mM of Tris-HCl: pH 7.4, 50mM of $MgCl_2$, 20mM of DTT), 3 units of T4 DNA ligase (Bio-Rad) and 1 unit of T4 DNA polymerase (Bio-Rad) were added and allowed to react on ice for 5 minutes, 25°C for 5 minutes, and then 37 C for 90 minutes, sequentially.

Synthesized ds-DNA was transformed into E. coli MV1190 (dut^+, ung^+, C^{mr}) and screened for those having restriction sites of SstII, HpaI, and EagI by digestion with these enzymes. cDNA fragment (covering nucletotide 1814-7440) obtained by digesting plasmid pTZ-PVS(1) with PstI was ligated into corresponding PstI site of the manipulated plasmid pTZ-PVS(5)/m to produce recombinant plasmid pTZ-PVS-3m. Recombinant vector pTZ-PVS-3m has 3C-protease cleavage site(AXFQ/G) (Dougherty and Semler, Microbiological Rev. 57, 781-822, 1993) and multiple cloning site (SstII- HpaI-EagI) at the junction between the first and the second amino acids of long polyprotein of poliovirus Sabin 1 cDNA. Newley inserted region has the amino acid sequence of SEQ. ID. NO. 3.

Comparative Example 1 : Construction of recombinant vector pTZ-PVS-m

By following the same procedure in Example 1 except using a different mutagenic primer having the sequence of SEQ. ID. NO. 4, recombinant plasmid pTZ-PVS-m

was constructed.

Recombinant vector pTZ-PVS-m has 3C-protease cleavage site and multiple cloning site (SstII- HpaI-EagI) at the junction of the third and the fourth amino acids of N-terminal of poliovirus Sabin 1 cDNA. Newley inserted region has the amino acid sequence of SEQ. ID. NO. 5. This vector was designed to have N-terminal of viral polyprotein begins with GG---, with expecting myristoylation site preserved during the 3C-mediated proccessing.

Comparative Example 2 : Construction of recombinant vector pTZ-PVS-2m

By following the same procedure in Example 1 except using different mutagenic primer having the sequence of SEQ. ID. NO. 6, recombinant vector pTZ-PVS-2m was constructed.

Recombinant vector pTZ-PVS-2m is similar to pTZ-PVS-m of Comparative Example 1 except having one guanine(G) instead of two at N-terminal of viral polyprotein during the 3C-mediated processing.

Comparative Example 3 : Construction of recombinant plasmid pTZ-PVS-2m/1

By following the same procedure in Example 1 except two points, that the recombinant plasmid pTZ-PVS-2m of Comparative Example 2 was used for mutagenic template and different mutagenic primer having the sequence of SEQ. ID. NO. 7 was used, recombinant plasmid pTZ-PVS-2m/1 was constructed.

Recombinant plasmid pTZ-PVS-2m/1 was designed to have a lower G/C content in the multiple cloning site than the plasmid pTZ-PVS-m in Comparative Example 1 or plasmid pTZ-PVS-2m in Comparative Example 2 by replacing some portion of G/C with A/T. And, the multiple cloning site was changed to SstII- HpaI-XhoI and the newley inserted region has amino acid sequence of SEQ. ID. NO. 8.

Comparative Example 4 : Construction of recombinant vector pTZ-PVS-4m

By following the same procedure in Example 1 except using different mutagenic template and mutagenic primer having the sequence of SEQ. ID. NO. 9, recombinant vector pTZ-PVS-4m was constructed.

Recombinant vector pTZ-PVS-4m has 3C-protease cleavage site and multiple cloning site (ApaI- HpaI-XhoI) at the junction site between VP3 and VP1 of long polyprotein of Sabin 1 poliovirus cDNA. Newley inserted exgeneous region has amino acid sequence of SEQ. ID. NO. 10.

Example 2 : Infectivity of the RNA transcripts synthesized from the recombinant poliovirus plasmids

The plasmids in Example 1 and Comparative Examples 1-4 were linealized by SalI digestion and subjected to in vitro transcription to synthesize RNA transcripts as described previously (Bae et al., Nucleic Acid Res., 21, 2703, 1993).

The RNA transcript (0.1 μg) was transfected into monolayered HeLa cells grown in DMEM medium (GIBCO/BRL) supplemented with 10% FCS (Fetal Calf Serum) by DEAE-Dextran method according to the vender's protocol (Stratagene Kit). Transfected cells were cultivated with methylcellulose-DMEM (prepared by adding 1.2 % methylcellulose to DMEM medium supplemented with 10% FCS) at 37°C CO_2 incubator for 2 days. Cells were stained with 0.1% crystal violet to observe plaques. The number of plaques as shown in Table 1 denotes transfection capacity of each RNA transcript. Figure 3 shows the pictures of plaques produced by transfection of each RNA trnscript synthesized from the recombinant plasmid.

Table 1

RNA Transcript	Number of plaques
pTZ-PVS(1)	2.0×10^3
pTZ-PVS-m	0
pTZ-PVS-2m	0
pTZ-PVS-2m/1	< 1

pTZ-PVS-3m	1.9×10^3
pTZ-PVS-4m	2.0×10^2

Table 1 shows that the RNA transcript from the plasmid pTZ-PVS-3m has the most potent transfection capacity among the recombinant plasmids except the wild-type strain. The recombinant plasmid pTZ-PVS-3m was deposited at Korea Collection of Type Cultures on August 6, 1997 under the accession number of KCTC-0365BP.

Example 3 : Construction of chimeric poliovirus plasmid pTZ-PVS-3m/p24 and production of chimeric poliovirus PVSS-3m/p24.

cDNA fragment covering N-terminal 169 amino acid residues of HIV-1 p24 was amplified from the HIV-1 cDNA (HXB2; obtained from NIH AIDS Research and Reference Reagent Program, USA) by PCR with two p24-specific PCR primers: SstII-p24- sense primer (SEQ. ID. NO. 11) and EagI-p24-antisense primer (SEQ. ID. NO. 12) based on the sequence information published by Terwilliger et al (Proc. Natl. Acad. Sci., 86, 3857, 1989). The PCR fragment was extracted from the agarose gel, digested with SstII and EagI and then introduced into the corresponding site of the plasmid pTZ-PVS-3m of Example 1 to construct recombinant plasmid pTZ-PVS-3m/p24 as illustrated in Figure 4.

RNA transcript synthesized from the recombinant plasmid pTZ-PVS-3m/p24 was transfected into the monolayered HeLa cells as described in Example 2. Transfected HeLa cells were further cultured at 37°C CO_2 incubator until full cytopathic effect (CPE) was detected on the monolayered cells. The culture supernatants were harvested as a source of chimeric poliovirus PVS-3m/p24.

Example 4 : One-step growth curve of chimeric poliovirus PVS-3m/p24

In order to evaluate the replication capacity of chimeric poliovirus expressing HIV-1 p24 protein, one-step growth curve was determined by measuring the virus titer of culture supernatants at each time point.

Wild-type Sabin 1 poliovirus (PVS), or recombinant or chimeric poliovirus of PVS-3m, PVS-4m, PVS-3m/p24, or PVS-4m/p24 was inoculated into HeLa cells in 60 mm plate (4.8×10^5 cells) at 10 moi for 1 hour at room temperature so that the virus can be adhered to cells. Cells were washed with PBS and then refed with 3 ml of DMEM, followed by cultivation in 37°C CO_2 incubator. After infection, the culture supernatants were taken every three hours and subjected to titration of the virus from each sample by plaque assay or $TCID_{50}$ assay as described in Virology a Practical Approach (p13 ed. by BWJ Mahy. IRL press, 1985). The results are shown in Figure 5.

As shown in Figure 5, the recombinant poliovirus PVS-3m is maximum 1 log lower than the wild-type Sabin 1 (PVS) in the replication capacity, while the chimeric poliovirus PVS-3m/p24 shows the similar replication capacity to thst of the recombinant poliovirus PVS-3m. Whereas, the recombinant poliovirus PVS-4m shows more than 2 log lower than the wild-type Sabin 1(PVS) in the replication capacity, and the p24-integrated plasmid pTZ-PVS-4m/p24 was unlikely to produce replication-competent chimeric poliovirus PVS-4m/p24.

Example 5 : Expression Pattern of exogenous p24 protein during the replication of chimeric poliovirus PVS-3m/p24

In order to determine whether the chimeric poliovirus PVS-3m/p24 is able to express HIV-1 p24 protein during the replication, chimeric poliovirus PVS-3m/p24 was inoculated into HeLa cells at 10 moi concentration. Six hours later, the cells were harvested, washed with PBS twice, resuspended with cell lysis buffer (80mM NaCl, 5mM $MgCl_2$, 10mM Tris-Cl: pH 7.4, 1mM DTT, 0.5% NP-40), allowed to stand on ice for 5 minutes and then centrifuged to remove nucleus and cell debris.

The supernatant was mixed with the equal volume of 2X sample buffer (50mM Tris:pH 6.8, 1% β-mercaptoethanol, 10% glycerol, 0.03% bromophenol blue), boiled for 3 minutes, and then separated on a 10% SDS-PAGE. Separated samples were transblotted to a nitrocellulose membrane (Millipore) using a semi-dry transblotter (Bio-Rad) and then screened with AIDS patients' sera. The results are shown in Figure 6.

The recombinant p24 protein (169aa) was detected at the lower band (18.4kDa) than that of wild type p24 (24kDa) in western blot experiment, which means that the recombinant p24 was produced by the infection of chimeric poliovirus, not by the contamination of the wild type p24. Therefore, the result shown in Figure 6 clearly elucidates that the chimeric poliovirus PVS-3m/p24 of the present invention is able to express integrated HIV-1 p24 protein effectively.

Example 6 : Maintenance of antigenicity of exogenous vaccine protein expressed by the chimeric poliovirus PVS-3m/p24

In order to determine whether the HIV-1 p24 protein produced by chimeric poliovirus PVS-3m/p24 in Example 4 is able to retain the antigenicity of wild type p24, radioimmunoprecipitation assay was performed with anti-p24 antibody.

Chimeric poliovirus PVS-3m/p24 was inoculated into HeLa cell at moi of 10. Five hours after infection, cells were transferred to a fresh DMEM without methionine/cystein(GIBCO/BRL). After starvation for 1 hr, isotope-labeled methionine/cystein [(L-^{35}S)-Met/Cys, specific activity >1000 Ci/mmole; Amersham] was added to the medium at a final concentration of 50 μCi/mmole,and then cultured for additional 2 hrs. Cells were harvested by trypsinization, washed twice with PBS, and then lysed with 500μl of radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% of NP-40, 0.5% of DOC, 0.1%of SDS, 50 mM Tris-Cl pH 8.0) on ice for 10 minutes. After centrifugation to remove nucleus and cell debris. 3 μl of rabbit anti-p24 antiserum was added to the supernatant and the mixture was allowed to react

at 4°C for 12 hours. 80 μl of 10% protein A-sepharose in PBS was added to the reaction mixture and allowed to react with antibody at room temperature for 1 hour, followed by centrifugation to recover sepharose beads. The antigen-bead complex was washed three times with RIPA buffer, resuspended with 50 μl of 1X sample buffer, boiled for 3 minutes, separated on 10% SDS-PAGE , and then followed by autoradiogram to detect antibody-reactive antigens in the lysate of infected cells. The results are shown in Figure 7.

The results shown in Figure 7 clearly demonstrate that the HIV-1 p24 protein produced by chimeric poliovirus PVS-3m/p24 of the present invention has the similar antigenicity to that of wild type p24 of HIV-1.

Example 7 : Genetic stability of chimeric poliovirus PVS-3m/p24.

In order to evaluate the genetic stability of chimeric poliovirus PVS-3m/p24 of Example 4, the chimeric progeny virus of PVS-3m/p24 was consecutively passaged in HeLa cells and the integrity of the cloned gene was determined by performing RT-PCR from the viral RNA extracted from each passage of virus. Viral particles were precipitated by adding PEG/NaCl at a final concentration of 5%/0.125M, respectively. Standing for 30 minutes at room temperature, the mixture was precipitated by centrifugation for 10 minutes. Viral RNA was obtained by phenol-chloroform extraction and ethanol precipitation.

The extracted RNA (10 μg) was mixed with cDNA synthesis primer (SEQ. ID. NO. 13) (1 μg), and the mixture was subjected to denaturation at 70°C for 10 minutes. The mixture was quickly transferred on ice, and then reverse transcriptase reaction solution (50mM Tris-HCl: pH 8.3, 65mM KCl, 3mM $MgCl_2$, 10mM DTT, 1mM dNTP mixture, 20 units of RNAsin) and 200 units of reverse transcriptase of MMLV (Moloney Murine Leukemia virus; GIBCO/BRL) were added thereto, followed by reaction at 42°C for 60 minutes. The reaction mixture was incubated at 100°C for 3 minutes to inactivate enzymes.

RT-PCR was conducted to amplify the manipulated region containing the cloned exogenous gene by using Sabin type 1 sense primer (SEQ. ID. NO. 14) and Sabin type 1 antisense primer (SEQ. ID. NO. 15). The RT-PCR was performed with Taq polymerase (Bioneer, Korea) for 25 cycles at 94°C for 1 minute, 45°C for 30 seconds, and 72°C for 45 seconds for each cycle. The results are shown in Figure 8.

In Figure 8, the band shown at 666bp consists of the cloned p24 gene (504 bp, 169aa) and parts of the poliovirus genome around the cloning site. Whereas the band at 270bp suggests there are some internal deletions in the cloned p24 gene during the steps of transfection and passages of recombinant chimeric virus. Nevertheless, the result that the band intensity at 666bp was not weakened as passage goes on as shown in Figure 8, strongly suggests that the chimeric poliovirus PVS-3m/p24 of the present invention is very stable to carry the cloned exogenous gene over 12th passage. The genetic stability of the chimeric virus was also confirmed by analysis of the p24 expression patterns of the chimeric virus during the passages.

The expression pattern of HIV-1 p24 protein during the passages of chimeric virus was observed. HeLa cells were inoculated with chimeric progeny virus of each passage at 10 moi. Cells were harvested 8 hours p.i., and the lysates were subjected to electrophoresis on SDS-PAGE, followed by Western blot hybridization with rabbit anti-p24 antiserum. The results are shown in Figure 9.

The results shown in Figure 9 make it affirm that the chimeric poliovirus PVS-3m/p24 is very stable to express cloned p24 protein constantly over 12th passage, indicating that the integrated exogenous gene is efficiently preserved during the passages.

These experimental results substantiate that chimeric poliovirus PVS-3m/p24 of the present invention, obtained by introducing HIV-1 p24 gene (169aa) into the multiple cloning site of recombinant vector pTZ-PVS-3m, can efficiently express cloned p24 protein, which has the similar antigenicity to that of the wild type HIV-1 p24 protein. Moreover, chimeric poliovirus PVS-3m/p24 conserves the sequence of

the cloned p24 gene as well as keeps good expression level of p24 protein over 12 passages, indicating that the chimeric poliovirus PVS-3m/p24 is genetically stable enough to be used as a clone.

Considering that Sabin type 1 strain of poliovirus, which is used as a starting vector for the present invention, has never been reported to cause any adverse side effect to human being up to now, the chimeric poliovirus PVS-3m/p24 of the present invention is highly expected to be used as a powerful CTL-inducible mucosal vaccine candidate against AIDS, as suggested by Walker and his colleagues (Science 280, 825, 1998, Rosenberg et al., Science, 278, 1447, 1997).

For the present invention, the possibility whether the recombinant plasmid pTZ-PVS-3m could be used as a useful vaccine vector to express HIV-1 envelope glycoprotein gp120, especially V3 region, was evaluated. Thus, the gene coding for 125 amino acid residues covering V3 loop region of HIV-1 gp120 was introduced into the multiple cloning site of the recombinant plasmid pTZ-PVS-3m, followed by production of chimeric poliovirus PVS-3m/env. The expression of cloned exogenous env gene and genetic stability of chimeric poliovirus PVS-3m/env were evaluated.

Example 8 : Construction of recombinant poliovirus plasmid pTZ-PVS-3m/env and production of chimeric poliovirus PVS-3m/env

A cDNA fragment (375bp) coding for 125 amino acid residues (amino acid sequence of env protein at 251 - 375) including V3 loop region of HIV-1 gp120 was amplified by PCR and introduced into the multiple cloning site of recombinant vector pTZ-PVS-3m, as described in Figure 10.

Two primers : SstII-env-sense primer (SEQ. ID. NO. 15) and EagI-env-antisense primer (SEQ. ID. NO. 16) having restriction enzyme SstII and EagI-recognition sites at 5`-end and 3`-end, respectively, were employed to amplify the designed region of env(125aa) gene from the HXB2 (obtained from NIH AIDS Research and Reference Reagent Program, US.) by PCR.

PCR product was digested with SstII and EagI, and the gene fragment of 375bp was introduced into the corresponding SstII and EagI sites of the vector pTZ-PVS-3m of Example 1 to produce recombinant plasmid pTZ-PVS-3m/env (Figure 10).

By following the procedures in Example 2, plasmid pTZ-PVS-3m/p24 was in vitro transcribed, and the RNA transcripts were transfected into HeLa cells monolayered in 60 mm culture plate. The transfected HeLa cells were cultivated in DMEM medium supplemented with 10% FCS at 37°C CO_2 incubator for 2 days. When full CPE was observed, the culture supernatants were harvested and used as a source of chimeric poliovirus PVS-3m/env.

Example 9 : One-step growth curve for the chimeric poliovirus PVS-3m/env.

In order to evaluate the replication capacity of the chimeric poliovirus expressing HIV-1 env protein, one-step growth curve was determined by measuring the virus titer of culture supernatants at each time point following the procedures described in Example 4. The experiments were repeated four times and the averages are shown in Table 2.

Table 2

	Virus Titer ($TCID_{50}$/ml) after infection							
	0 hour	3 hours	6 hours	9 hours	12 hours	15 hours	18 hours	21 hours
PVS	0	0	$2.6x10^4$	$4.1x10^5$	$4.1x10^5$	$1.6x10^6$	$6.6x10^6$	$6.6x10^6$
PVS-3m	0	0	$1.6x10^3$	$6.4x10^3$	$2.6x10^4$	$2.1x10^5$	$1.6x10^6$	$1.6x10^6$
PVS-3m/env	0	0	$4.0x10^2$	$1.6x10^3$	$6.4x10^3$	$2.6x10^4$	$1.0x10^5$	$1.0x10^5$

As shown at Table 2, the replication capacity of the chimeric poliovirus PVS-3m/env was maximum 1 log lower than that of wild type Sabin 1(PVS), but was similar to that of PVS-3m.

Example 10 : Expression pattern of exogenous env protein during the replication of

chimeric poliovirus PVS-3m/env.

It was determined whether the chimeric poliovirus PVS-3m/env expresses the cloned env protein during its growth. Chimeric poliovirus PVS-3m/env was inoculated into HeLa cells at 10 moi. Eight hours p.i., infected cells were harvested and subjected to electrophoresis on 10% SDS-PAGE. Then, Western blot hybridization was conducted with AIDS patients' sera, and the results are shown in Figure 11. The results shown in Figure 11 make it affirm that chimeric poliovirus PVS-3m/env of the present invention effectively expresses HIV-1 env protein (125aa, 15kDa).

Example 11 : Genetic stability of chimeric poliovirus PVS-3m/env

In order to evaluate the genetic stability of chimeric poliovirus PVS-3m/env of Example 8, the chimeric progeny virus of PVS-3m/env was consecutively passaged in HeLa cells and the integrity of the cloned gene (375bp) was determined by conducting RT-PCR following the same procedure as described in Example 7. The results are shown in Figure 11. The results shown in Figure 11 elucidate that the chimeric poliovirus PVS-3m/env of the present invention is very stable to carry the exogenous gene over 12th passage.

Moreover, the genetic stability of the chimeric poliovirus was also confirmed by analysis of the env expression patterns of the chimeric virus during the passages.

The expression pattern of HIV-1 env protein during the passages of chimeric virus was determined by the same procedure as described in Example 7 except using AIDS patients' sera for western blot hybridization. The results are shown in Figure 12. The results in Figure 12 make it affirm that the chimeric poliovirus PVS-3m/env expresses cloned env protein (125aa) constantly over 12th passage, indicating that the cloned exogenous gene is efficiently preserved during the passages.

These experimental results substantiate that chimeric poliovirus PVS-3m/env of the present invention, obtained by introducing HIV-1 env gene (125aa) into the

multiple cloning site of recombinant vector pTZ-PVS-3m, can efficiently express cloned env gene. Moreover, chimeric poliovirus PVS-3m/env conserves the intact sequence of the cloned env gene as well as keeps good expression level of env protein over 12 passages, indicating that the chimeric poliovirus PVS-3m/env is genetically stable enough to be used as a clone for vaccine candidate.

Considering that the Sabin type 1 strain of poliovirus, which is used as a starting vector for the present invention, has never been reported to raise any adverse side effect to human being up to now, the chimeric poliovirus PVS-3m/env of the present invention is highly expected to be used as a powerful oral mucosal vaccine against AIDS.

As denoted above, the recombinant vector pTZ-PVS-3m of the present invention has a multiple cloning site and 3C-protease cleavage site so as to make it easy to introduce various exogenous vaccine gene into the recombinant Sabin 1 poliovirus, and facilitate to produce genetically stable chimeric polioviruses which can be used as oral vaccines against several infectious viral disease by taking advantages of the Sabin 1 poliovirus.

For the present invention, in order to evaluate the possibility that the recombinant vector pTZ-PVS-3m can be applicable as a useful vaccine vector to other infectious viral diseases than AIDS, the core protein-coding gene of Hepatitis C virus (HCVc) was cloned into the recombinant vector pTZ-PVS-3m. Thus, a part of core gene coding for 100 amino acid residues of N-terminal was introduced into the multiple cloning site of recombinant vector pTZ-PVS-3m, followed by production of chimeric poliovirus PVS-3m/HCVc. The expression of cloned exogenous HCVc gene and genetic stability of the chimeric poliovirus PVS-3m/HCVc were evaluated.

Example 12 : Construction of chimeric poliovirus plasmid pTZ-PVS-3m/HCVc and production of chimeric poliovirus PVS-3m/HCVc

HCVc gene coding for 100 amino acid residues of N-terminal core was amplified

by PCR and introduced into the multiple cloning site of recombinant poliovirus pTZ-PVS-3m, as described in Figure 13.

Two primers : SstII-HCVc-sense primer (SEQ. ID. NO. 17) and EagI-HCVc -antisense primer (SEQ. ID. NO. 18) were employed to amplify the designed region of HCV core gene (300 bp, 100aa) from the template of pcDNA/Neo-HCVcore plasmid (obtained by courtesy from Dr. Sung at Postech, Korea) by PCR.

PCR product was digested with SstII and EagI, and the gene fragment of 300 bp was introduced into the corresponding SstII and EagI sites of the plasmid pTZ-PVS-3m of Example 1 to produce recombinant plasmid pTZ-PVS-3m/HCVc (Figure 13).

By following the procedure in Example 2, plasmid pTZ-PVS-3m/HCVc was in vitro transcribed, and the RNA transcripts were transfected into the HeLa cells monolayered in 60 mm culture plate. The transformed HeLa cells were cultivated in DMEM medium supplemented with 10% FCS at 37°C CO_2 incubator for 2 days. When full CPE was observed, the culture supernatants were harvested and used as a source of chimeric poliovirus PVS-3m/HCVc.

Example 13 : One-step growth curve for the chimeric poliovirus PVS-3m/HCVc

In order to evaluate the replication capacity of the chimeric poliovirus expressing HCV core protein, one-step growth curve was determined by measuring the virus titer of culture supernatants at each time point following the procedures described in Example 4. The results are shown at Table 3 and Figure 14. The values in Table 3 are averages from four repeat experiments.

Table 3

	Virus Titer ($TCID_{50}$/ml) after infection							
	0 hour	3 hours	6 hours	9 hours	12 hours	15 hours	18 hours	21 hours
PVS	0	0	$5.0x10^5$	$2.5x10^6$	$7.5x10^7$	$4.0x10^7$	$7.5x10^7$	$1.0x10^8$
PVS-3m	0	0	$2.5x10^5$	$2.0x10^6$	$5.0x10^7$	$5.0x10^7$	$2.5x10^7$	$7.5x10^7$

*rB

PVS-3m/HCVc	0	0	$1.0x10^5$	$5.0x10^6$	$7.5x10^6$	$1.0x10^7$	$2.0x10^7$	$5.0x10^7$

As shown in Table 3 and Figure 14, the chimeric poliovirus PVS-3m/HCVc shows replication capacity of at the most 10 times lower than of that of the wild-type (PVS) at 12 hours p.i. The average replication capacity of the chimeric poliovirus PVS-3m/HCVc is about 5 times lower than that of the wild-teyp Sabin 1 and 3 times lower than that of the recombinant poliovirus PVS-3m.

Example 14 : RNA synthesis of chimeric poliovirus PVS-3m/HCVc

Following the previous report (Mattion et al., J. Virol. 68, 3925, 1994) the experiment was performed. HeLa cells grown in 24-well plates were mock infected or infected with 10 moi of wild type and chimeric poliovirus PVS-3m/HCVc. After adsorption for 1 hr at room temperature, the cells were washed with PBS and fed in DMEM containing Actinomicin D (5μg/ml, Difco). After incubation for 1 hr at 37°C, 25 μCi/ml of [5,6-^{3}H]-uridine (Amersham; specific activty 45Ci/mmole) was added. The cells were harvested every 3 hrs, washed 3 times with PBS, and then lysed by adding 0.5ml of lysis buffer (80mM NaCl, 5mM $MgCl_2$, 10 mM Tris-Cl 8.2, 1mM DTT, 10mM vanadyl ribonuclease complex, and 0.5% of 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 filtrated with glass fiber filters (Whatsman, GF-C filter), and the radioactivty was determined by scintillation counter (Hewlett Packard) and the results are shown in Table 4.

Table 4

	[^{3}H]-uridine (cpm) in viral RNA						
	0 hour	3 hours	6 hours	9 hours	12 hours	15 hours	18 hours
PVS	0	$1.1x10^4$	$4.0x10^5$	$1.6x10^5$	$1.4x10^5$	$1.1x10^5$	$4.8x10^4$
PVS-3m	0	$1.2x10^4$	$3.9x10^5$	$1.6x10^5$	$1.4x10^5$	$1.0x10^5$	$5.0x10^4$

PVS-3m/HCVc	0	$1.2x10^5$	$3.7x10^5$	$1.6x10^5$	$1.3x10^5$	$1.0x10^7$	$4.8x10^4$

As shown at Table 4, the kinetics of RNA synthesis of the chimeric poliovirus PVS-3m/HCVc is almost similar to the pattern of wild-type (PVS) or recombinant poliovirus PVS-3m in the infected HeLa cells except the level at 6 hours after the infection. The results suggest that the lower replication capacity (about 5 times lower) of the chimeric virus in Example 13 is not to be due to reduced RNA synthesis, but seems to be due to the unefficient protein processing of the chimeric virus at assembly steps.

In conclusion, considering the fact that chimeric poliovirus PVS-3m/HCVc has the similar capacity of RNA synthesis to those of wild-type or recombinant virus, the reduced replication capacity (about 5 times lower than that of wild type PVS) of the chimeric poliovirus PVS-3m/p24 may not adversely affect for using the chimeric poliovirus as oral vaccine.

Example 15 : Expression pattern of exogenous HCVc protein during the replication of chimeric poliovirus PVS-3m/HCVc

In order to determine whether the chimeric poliovirus PVS-3m/HCVc expresses HCV core protein during its growth. Chimeric poliovirus PVS-3m/HCVc was inoculated into monolayered HeLa cells at 10 moi for 1 hour, and the unadsorbed viruses were removed. The infected cells were further cultivated at 37°C CO_2 incubator. The cells were harvested 8 hrs p.i, and subjected to electrophoresis on 10% SDS-PAGE. Western blot hybridization was cperformed with a rabbit antiserum (obtained by courtesy of Dr. Hwang at Hallim University, Korea) or monoclonal antibody against HCV core protein (Bio-genesis, Sandown, NH, USA), and the results are shown in Figure 15. The western blot signal shown in Figure 15 (Lane 3) elucidated that chimeric poliovirus PVS-3m/HCVc of the present invention effectively expresses cloned HCV core protein (100aa, 12kDa).

In Figure 15, several bands other than the one at the desired 12kDa are assumed to be due to HCV core protein fused to the hydrophobic cell organells by the hydrophobic residues at the N-terminal end of the HCV core protein. To confirm this hypothesis, recombinant virual pellet (lane 4), virus-free culture supernatant concentrate (lane 5), or precipitant of the cell lysate (lane 6) or supernatant of the cell lysate (lane 7) of chimeric poliovirus PVS-3m/HCVc-infected HeLa cells were analyzed by western blot hybridization using the same antiserum. The precipitate (lane 6) and supernatant (lane 7) of the cell lysates were obtained by treating the infected cells with 1% NP-40. As shown in Figure 15, only the precipitant (lane 6) and supernatant (lane 7) of the infected HeLa cell lysates show the signal of antigen bands when screened with the specific antiserum, suggesting that the signals are not to be due to the non-specific reaction of antiserum. Moreover, the fact that the precipitant fraction (lane 6) shows a more clear and intensive band at 12kDa makes the assumption much more convincing. These results are in part consistent with the previous report that the assembly of HCV occurs in lumen of endoplasmic reticulum, but not in cytoplasm, and forms a membrane-bound vesicles (Dubission et al., 1994). If the HCV core protein-cell organell complex does not affect the replication of the recombinant virus, the complex will be much more effective to induce CTL immunity together with humoral immunity against HCV.

Example 16 : Genetic stability of chimeric poliovirus PVS-3m/HCVc

In order to evaluate the genetic stability of chimeric poliovirus PVS-3m/HCVc of Example 12, the progeny virus of PVS-3m/HCVc is consecutively passaged in HeLa cells and the integrity of the cloned gene (300bp) was determined by conducting RT-PCR following the same procedure as described in Example 7.

The results are shown in Figure 16, in which a strong band at 459bp was clearly appeared at each sample regardless the number of passages. No other band due to the internal deletion as shown in the PVS-3m/p24, or insertion was detected during the

passages

Thus, the results in Figure 16 elucidate that the chimeric poliovirus PVS-3m/HCV of the present invention is very stable to carry the exogenous gene over 12th passage.

Moreover, the genetic stability of the chimeric poliovirus was also confirmed by analysis of the HCVc expression patterns of the chimeric virus during the passages

The expression pattern of HCV core protein during the passages of recombinant virus was determined by the same procedure as described in Example 7 except using monoclonal antibody against HCV core protein (Bio-genesis, Sandown, NH, USA) for Western blot hybridization. The results are shown in Figure 17. Results shown in Figure 17 make it affirm that the chimeric poliovirus PVS-3m/HCVc expresses HCV core protein (100aa) constantly over 12th passage.

These experimental results substantiate that chimeric poliovirus PVS-3m/HCVc of the present invention, obtained by introducing HCV core gene (100aa) into the multiple cloning site of recombinant vector pTZ-PVS-3m, can efficiently expresses cloned HCV core gene. Moreover, it conserves the intact sequence of cloned HCV core gene and keeps good expression level of the HCV core protein over 12 passages, indicating that the chimeric poliovirus PVS-3m/HCVc is genetically stable enough to be used as a clone for HCV vaccine candidate.

Considering that the Sabin type 1 strain of poliovirus, which is used as a starting vector for the present invention, has never been reported to raise any adverse side effect to human being up to now, the chimeric poliovirus PVS-3m/HCVc of the present invention is highly expected to be used as a powerful oral mucosal vaccine against HCV.

As shown above, the recombinant vector pTZ-PVS-3m of the present invention has a multiple cloning site and 3C-protease cleavage site so as to make it easy to introduce various exogenous vaccine gene into the recombinant Sabin 1 poliovirus, and facilitate to produce genetically stable chimeric polioviruses which can be used as oral vaccines against several infectious viral disease by taking advantages of the

Sabin 1 poliovirus.

Although preferred embodiments of the present invention have been described in detail herein above, it should be clearly understood that many variations and/or modifications of the basic inventive concepts herein taught which may appear to those skilled in the art will still fall within the spirit and scope of the present invention as defined in the appended claims.

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<400> 5
Pro Arg Gly Pro Arg Pro Ala Leu Phe Gln Gly Gly Ala
1 5 10

<210> 6
<211> 76
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer for DNA amplification having multiple cloning site and 3C-protease cleavage site

<400> 6
attgtatcat aatgggtgct ccgcgggggc cccggccggc tttgttccaa ggagcacagg 60
tttcatcaca gaaagt 76

<210> 7
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer for DNA amplification having multiple cloning site and 3C-protease cleavage site

<400> 7

atgggtgctc cgcgggttaa cctcgaggct ttgttccaag ga 42

<210> 8
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Peptide coded by SEQ. ID. NO. 7

<400> 8
Pro Arg Val Asn Leu Gly Ala Leu Phe Gln Gly Ala
1 5 10

<210> 9
<211> 63
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence : Primer for PCR amplification having multiple cloning site and 3C-protease cleavage site

<220>
<223> Description of Artificial Sequence: Primer for DNA amplification having multiple cloning site and 3C-protease cleavage site

<400> 9
gcgctagcac aggggcccgt taacctcgag aaggcacttg cgcaaggatt aggtcagatg 60
ctt 63

<210> 10
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Peptide coded by SEQ. ID. NO. 9

<400> 10
Gly Pro Val Asn Leu Gln Lys Ala Leu Ala Gln
1 5 10

<210> 11
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: SstII-sense primer for DNA amplification having multiple cloning site and 3C-protease cleavage site

<400> 11
aggcctccgc ggcctatagt gcagaaacatc 30

<210> 12
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: EagI-antisense primer for DNA amplification having multiple cloning site and 3C-protease cleavage site

<400> 12
aggcctcggc cgatagaacc ggtctacata 30

<210> 13
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cDNA synthesis primer

<400> 13
cgttgccgcc cccaccgt 18

<210> 14
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Sense primer for DNA amplification covering nucleotide 680-697 of Sabin 1 of poliovirus

<400> 14
cattgagtgt gtttactc 18

<210> 15
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Antisense primer for DNA amplification covering nucleotide 797-814 of Sabin 1 poliovirus

<400> 15
ggtagaacca ccatacgc 18

<210> 16
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: SstII-sense primer for DNA amplification

<400> 16
attaatccgc ggattaggcc agtagtatca 30

<210> 17
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: EagI-antisense primer for DNA amplification

<400> 17
attaatcggc cgactgtgcg ttacaatttc 30

<210> 18
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Sense primer

*rB

for DNA amplification

<400> 18
aggcctccgc ggatgagcac aaatcctaaa 30

<210> 19
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Antisense primer for DNA amplification

<400> 19
aggcctcggc cggggtagca ggagcca 27

<210> 20
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Sequence having multiple cloning site and 3C-protease cleavage site

<400> 20
ccgcgggtta accggccggc tttgttccaa 30

CLAIMS

1. A replication-competent recombinant Sabin type 1 poliovirus vector containing a sequence coding for multiple cloning site and 3C-protease cleavage site at the junction of first amino acid and second amino acid of N-terminal of Sabin type 1 strain of poliovirus cDNA.

2. A replication-competent recombinant Sabin type 1 poliovirus vector according to Claim 1, wherein said sequence coding for multiple cloning site and 3C-protease cleavage site is SEQ. ID. NO. 19.

3. A replication-competent recombinant Sabin type 1 poliovirus vector according to Claim 1 or 2, which is vector pTZ-PVS-3m (KCTC-0365BP).

4. A replication-competent chimeric Sabin type 1 poliovirus vector comprising an exogenous vaccine gene and replication-competent vector, wherein said vector is a replication-competent recombinant Sabin type 1 poliovirus vector of Claim 1 and said vaccine gene insert is introduced into the multiple cloning site thereof.

5. A replication-competent chimeric Sabin type 1 poliovirus vector according to Claim 4, wherein said vaccine gene is derived from infectious virus.

6. A replication-competent chimeric Sabin type 1 poliovirus vector according to Claim 5, wherein said infectious virus is HIV-1.

7. A replication-competent chimeric Sabin type 1 poliovirus vector according to Claim 5, wherein said infectious virus is Hepatitis C virus.

8. A replication-competent chimeric Sabin type 1 poliovirus vector according to Claim 4, wherein said vaccine gene is HIV-1 p24 gene.

9. A replication-competent chimeric Sabin type 1 poliovirus vector according to Claim 4, wherein said vaccine gene is an envelope glycoprotein gene comprising V3 loop of the HIV-1 gp 120.

10. A replication-competent chimeric Sabin type 1 poliovirus vector according to Claim 4, wherein said vaccine gene insert is HCV core gene.

11. A host cell transfected with a replication-competent recombinant Sabin Type 1 poliovirus vector of Claim 1.

12. A host cell transfected with a replication-competent chimeric Sabin type 1 poliovirus vector of Claim 4.

13. A host cell according to Claim 12, wherein said vaccine gene is derived from infectious virus.

14. A host cell according to Claim 13, wherein said infectious virus is HIV-1.

15. A host cell according to Claim 14, wherein said infectious virus is Hepatitis C virus.

16. A host cell according to Claim 15, wherein said vaccine gene insert is HIV-1 p24 gene.

17. A host cell according to Claim 16, wherein said vaccine gene insert is an

envelope glycoprotein gene comprising V3 loop of HIV-1 gp 120.

18. A host cell according to Claim 12, wherein said vaccine gene insert is HCV core gene.

19. A method for producing replication-competent recombinant Sabin type 1 poliovirus vector of Claim 1 which comprises the steps of transfecting host cells with the vector of Claim 1 to give an infected cell; cultivating the infected cell in a cultivation medium to produce progeny virus; and harvesting the progeny virus from a culture supernatant.

20. A method for producing replication-competent chimeric Sabin type 1 poliovirus vector of Claim 4 which comprises the steps of transfecting host cells with the vector of Claim 4 to give an infected cell; cultivating the infected cell in a cultivation medium to produce progeny virus; and harvesting the progeny virus from a culture supernatant.

FIG. 1

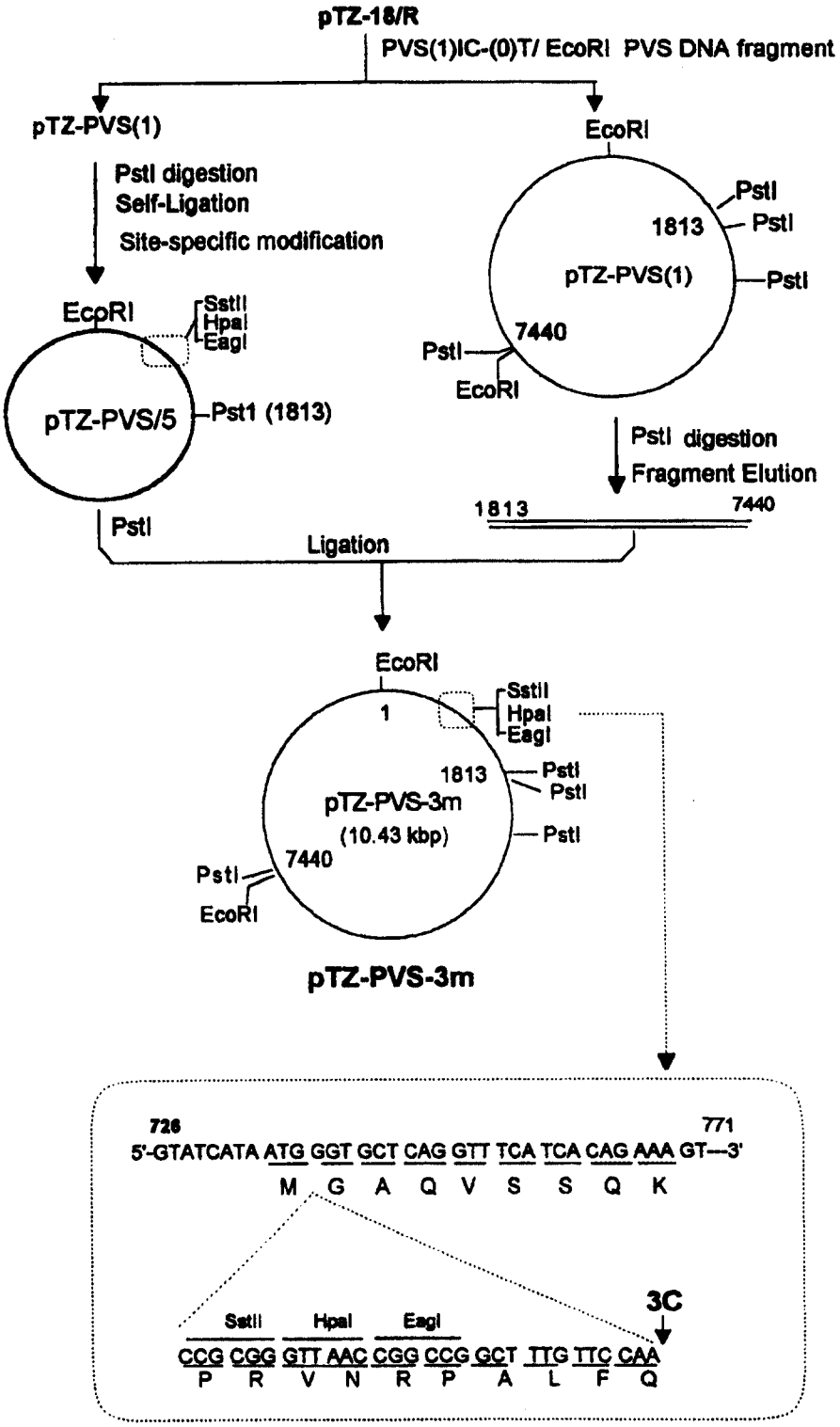

pTZ-18/R
PVS(1)IC-(0)T/ EcoRI PVS DNA fragment
pTZ-PVS(1)
EcoRI
PstI digestion
Self-Ligation
Site-specific modification
PstI
1813
PstI
pTZ-PVS(1)
PstI
7440
PstI
EcoRI
EcoRI
SstII
HpaI
EagI
pTZ-PVS/5
Pst1 (1813)
PstI digestion
Fragment Elution
1813
7440
PstI
Ligation
EcoRI
1
SstII
HpaI
EagI
1813
PstI
PstI
pTZ-PVS-3m
(10.43 kbp)
PstI
7440
PstI
EcoRI
pTZ-PVS-3m
726
771
5'-GTATCATA ATG GGT GCT CAG GTT TCA TCA CAG AAA GT—3'
M G A Q V S S Q K
3C
SstII
HpaI
EagI
CCG CGG GTT AAC CGG CCG GCT TTG TTC CAA
P R V N R P A L F Q

FIG. 2

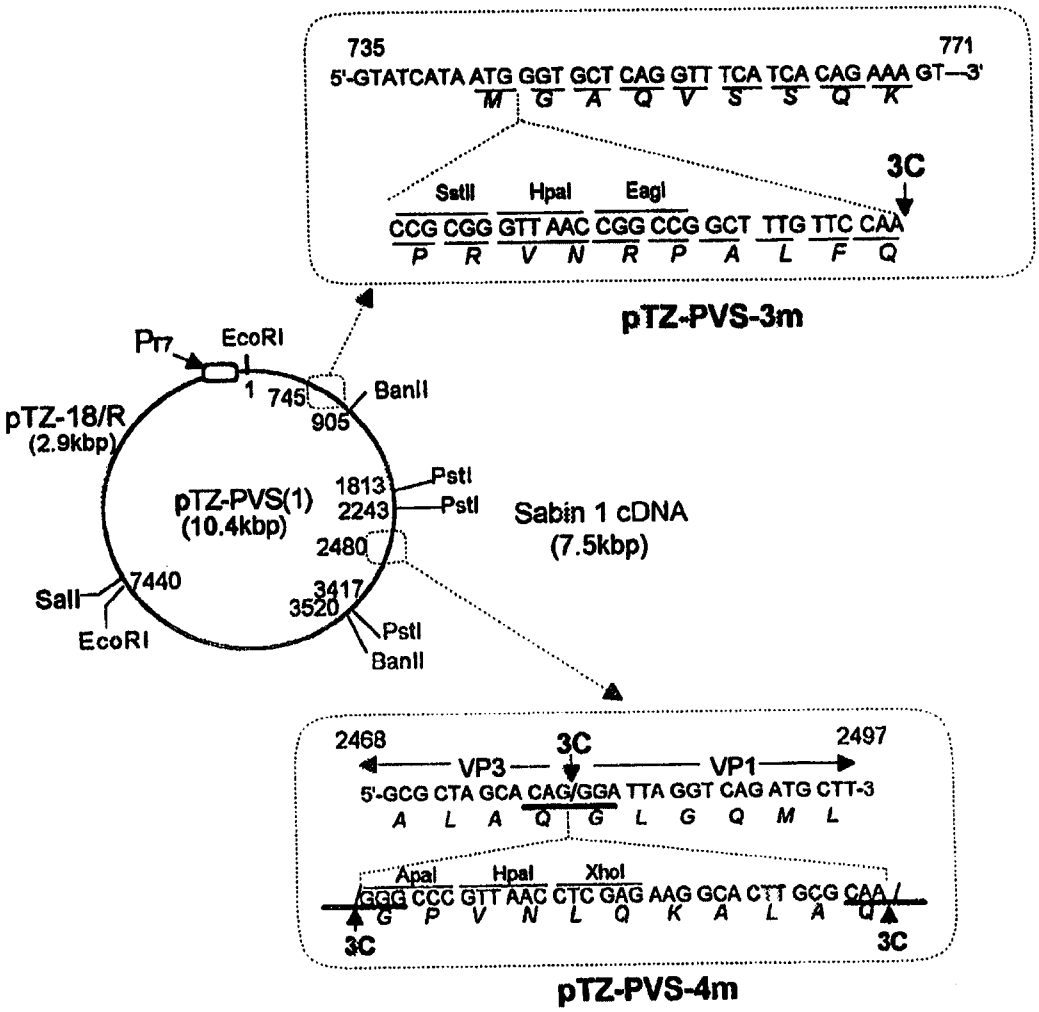

735
5'-GTATCATA ATG GGT GCT CAG GTT TCA TCA CAG AAA GT—3'
M G A Q V S S Q K
771
3C
SstII HpaI EagI
CCG CGG GTT AAC CGG CCG GCT TTG TTC CAA
P R V N R P A L F Q
pTZ-PVS-3m
PT7
EcoRI
1
745
905
BanII
pTZ-18/R
(2.9kbp)
pTZ-PVS(1)
(10.4kbp)
1813
2243
2480
PstI
PstI
Sabin 1 cDNA
(7.5kbp)
SalI
7440
EcoRI
3417
3520
PstI
BanII
2468
3C
2497
VP3
VP1
5'-GCG CTA GCA CAG/GGA TTA GGT CAG ATG CTT-3
A L A Q G L G Q M L
ApaI HpaI XhoI
GGG CCC GTT AAC CTC GAG AAG GCA CTT GCG CAA
G P V N L Q K A L A Q
3C
3C
pTZ-PVS-4m

FIG. 3

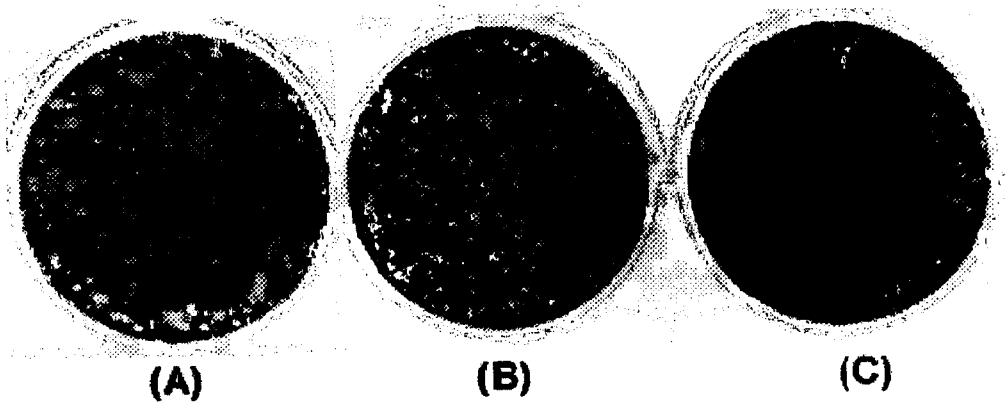

(A)
(B)
(C)

FIG. 4

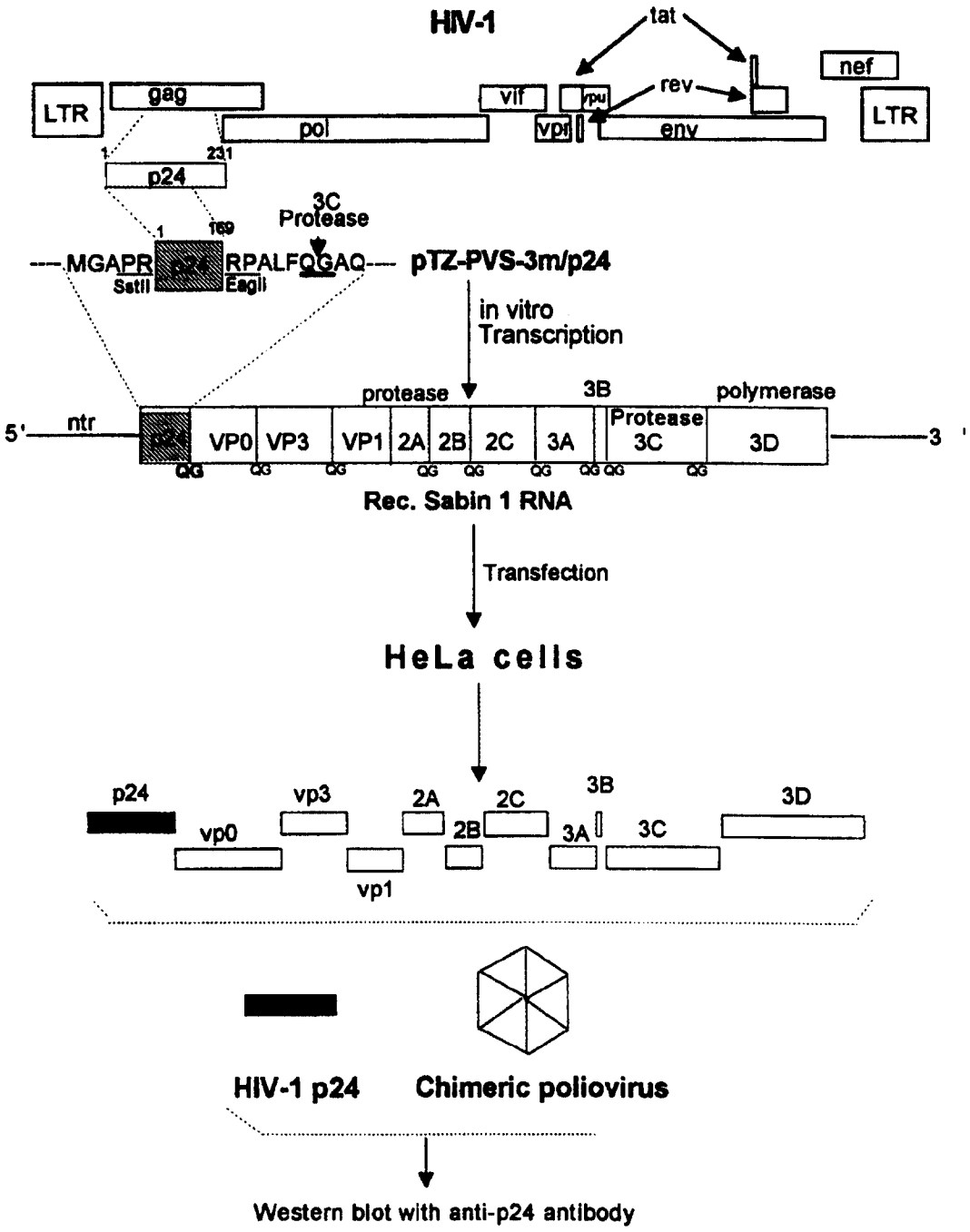

HIV-1
tat
rev
nef
LTR
gag
vif
vpu
pol
vpr
env
LTR
1
231
p24
3C
Protease
1
169
---MGAPR p24 RPALFQGAQ---
SatII
EagII
pTZ-PVS-3m/p24
in vitro
Transcription
protease
3B
polymerase
5'
ntr
p24
VP0
VP3
VP1
2A
2B
2C
3A
Protease
3C
3D
3'
Rec. Sabin 1 RNA
Transfection
HeLa cells
p24
vp3
2A
2C
3B
3D
vp0
2B
3A
3C
vp1
HIV-1 p24
Chimeric poliovirus
Western blot with anti-p24 antibody

FIG. 5

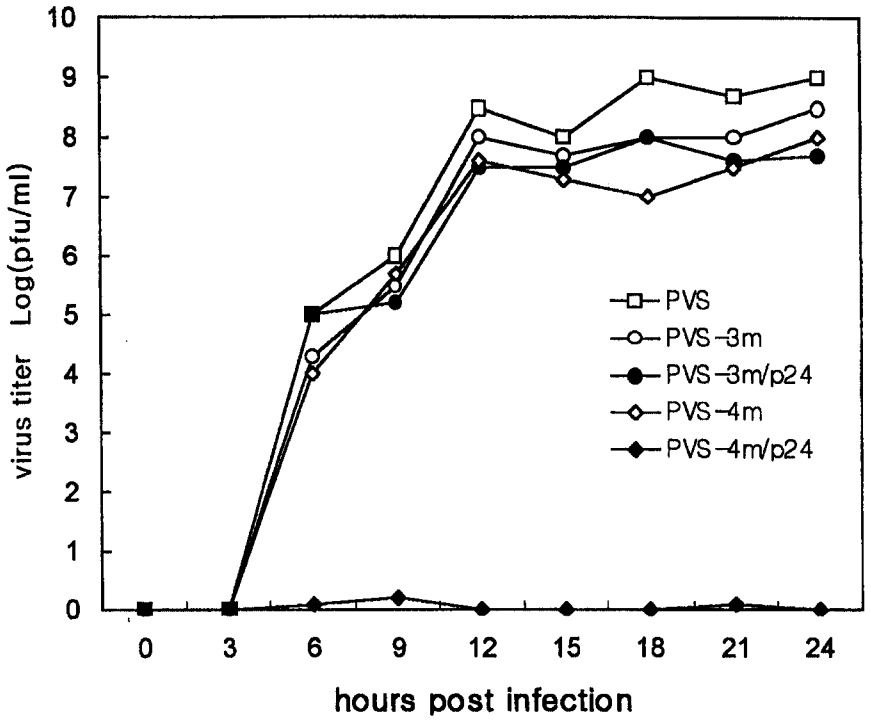

virus titer Log(pfu/ml)
hours post infection
PVS
PVS-3m
PVS-3m/p24
PVS-4m
PVS-4m/p24

FIG. 6

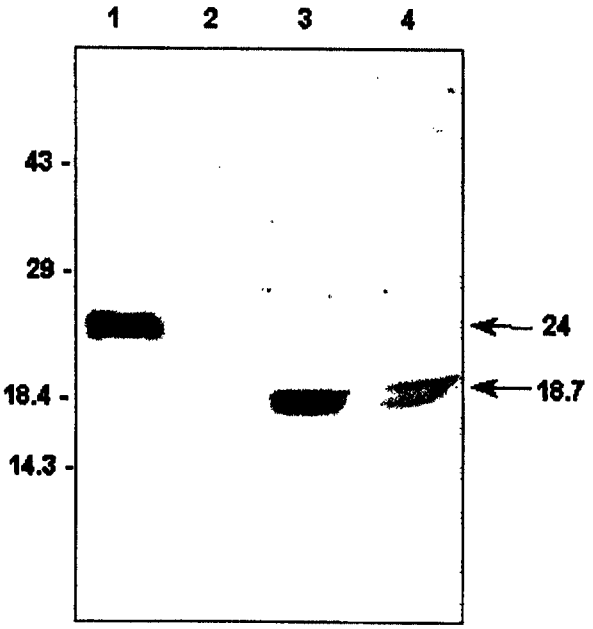

1
2
3
4
43
29
18.4
14.3
24
18.7

FIG. 7

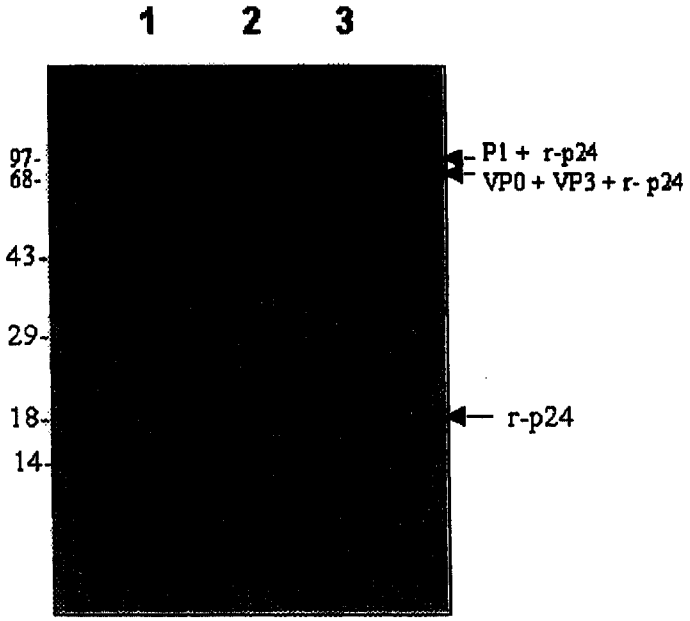

1
2
3
97-
68-
43-
29-
18-
14-
P1 + r-p24
VP0 + VP3 + r- p24
r-p24

FIG. 8

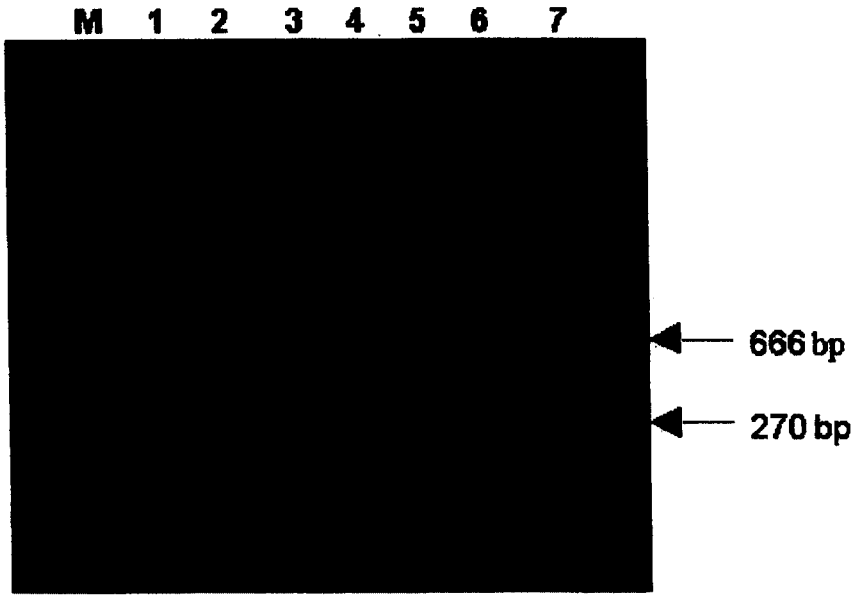

M
1
2
3
4
5
6
7
666 bp
270 bp

FIG. 9

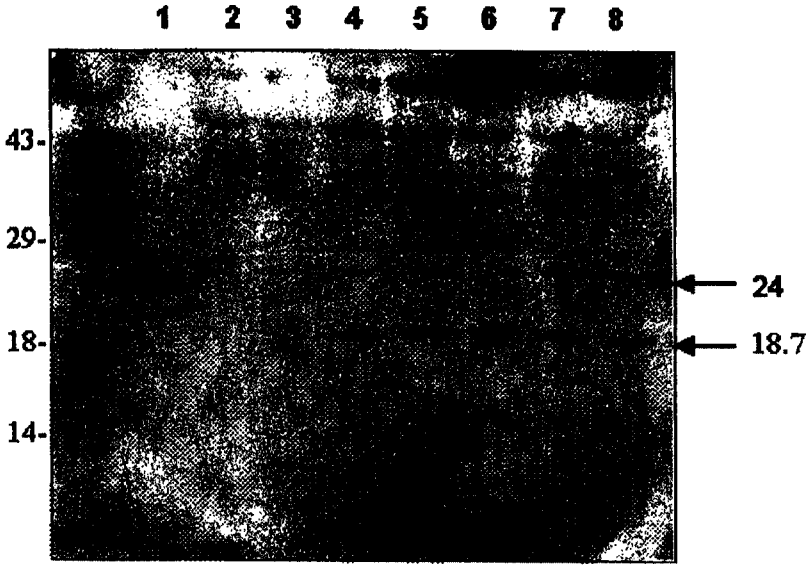


FIG. 10

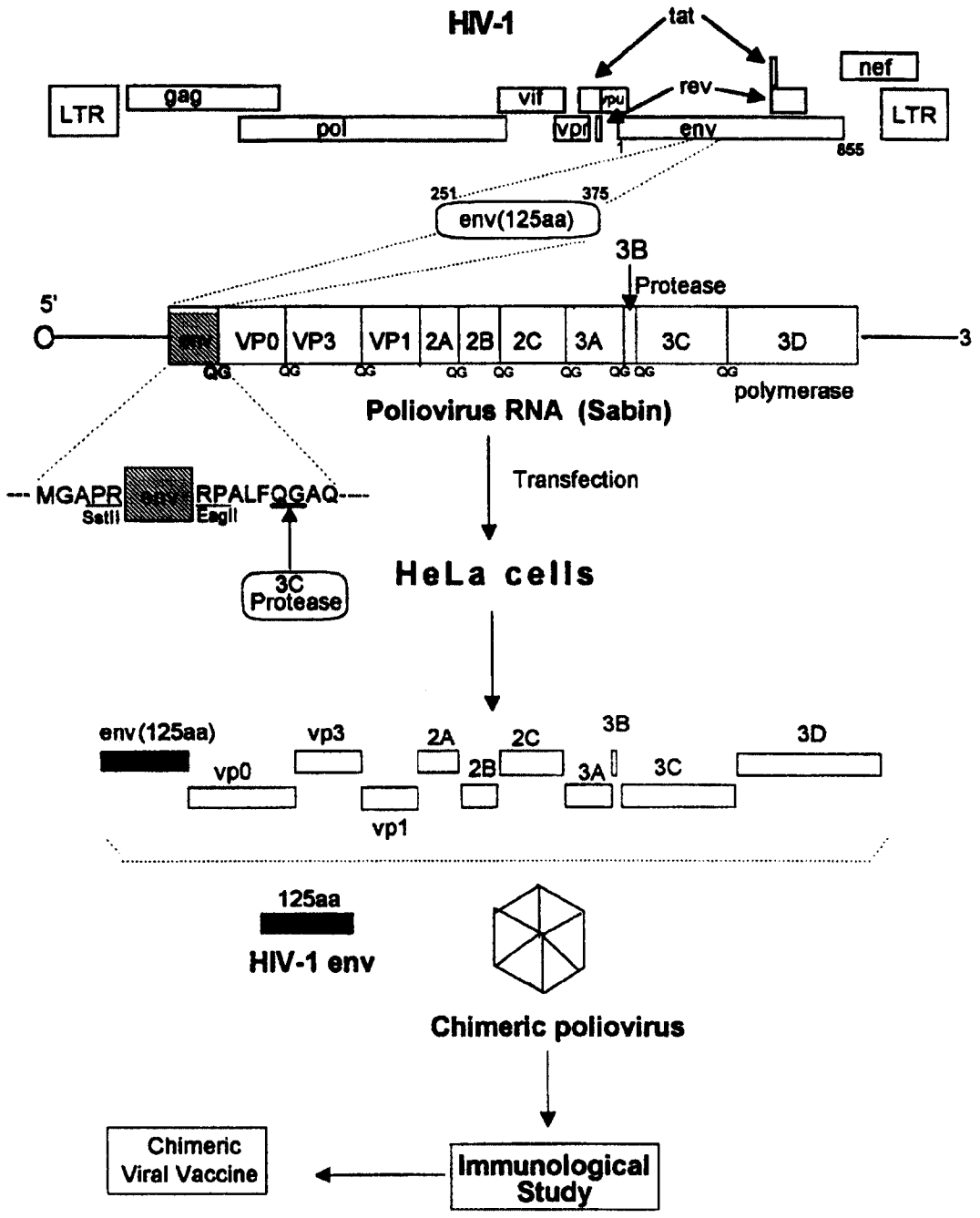

HIV-1
tat
rev
nef
LTR
gag
vif
vpu
pol
vpr
env
LTR
1
855
251
375
env(125aa)
3B
Protease
5'
3
VP0
VP3
VP1
2A
2B
2C
3A
3C
3D
polymerase
Poliovirus RNA (Sabin)
Transfection
--- MGAPR
SstII
RPALFQGAQ----
EagII
3C Protease
HeLa cells
env(125aa)
vp3
2A
2C
3B
3D
vp0
2B
3A
3C
vp1
125aa
HIV-1 env
Chimeric poliovirus
Chimeric Viral Vaccine
Immunological Study

FIG. 11

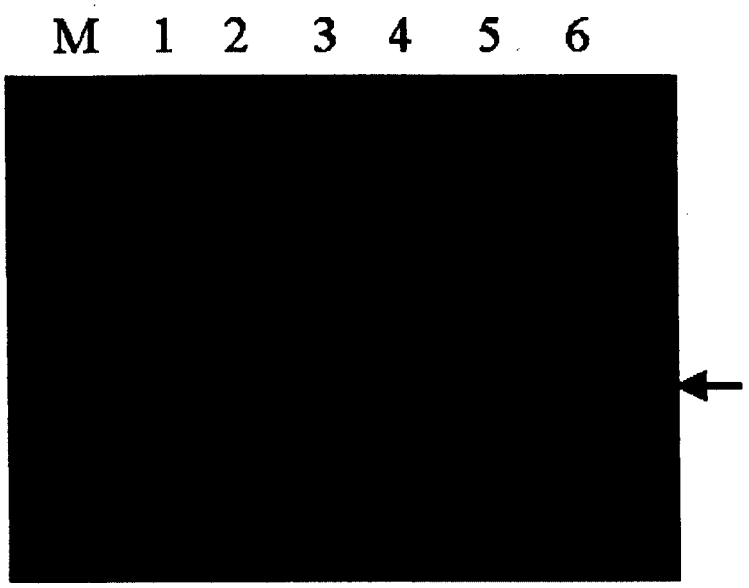


FIG. 12

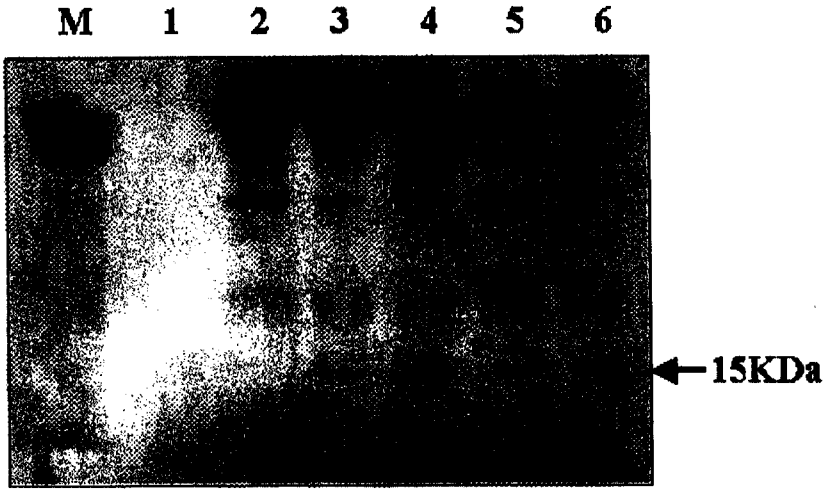


FIG. 13

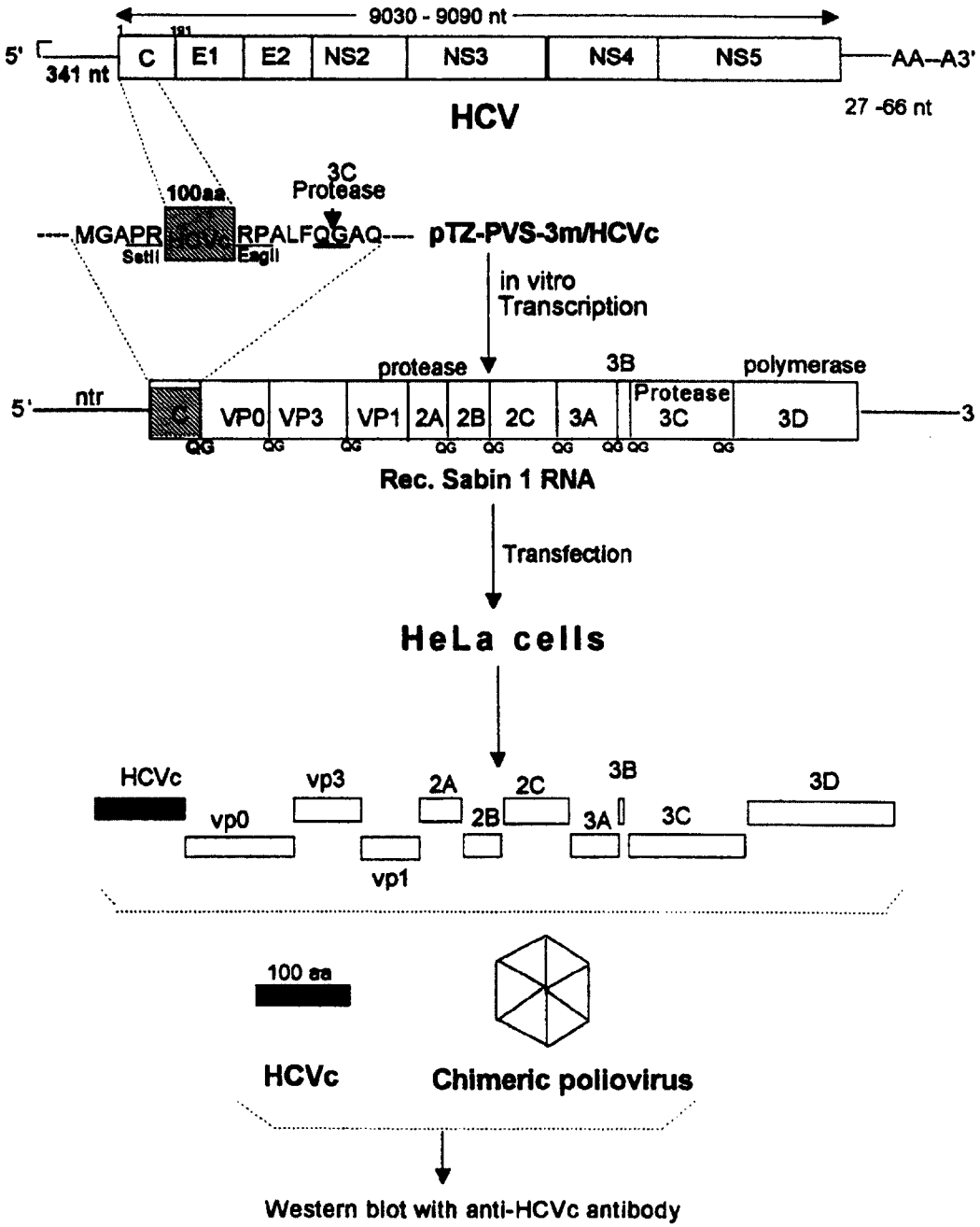

9030 - 9090 nt
5'
341 nt
C
E1
E2
NS2
NS3
NS4
NS5
AA--A3'
27 -66 nt
HCV
100aa
3C
Protease
MGAPR
RPALFQGAQ
SetII
EagII
pTZ-PVS-3m/HCVc
in vitro
Transcription
protease
3B
polymerase
5'
ntr
C
VP0
VP3
VP1
2A
2B
2C
3A
Protease
3C
3D
3
Rec. Sabin 1 RNA
Transfection
HeLa cells
HCVc
vp3
2A
2C
3B
3D
vp0
2B
3A
3C
vp1
100 aa
HCVc
Chimeric poliovirus
Western blot with anti-HCVc antibody

FIG. 14

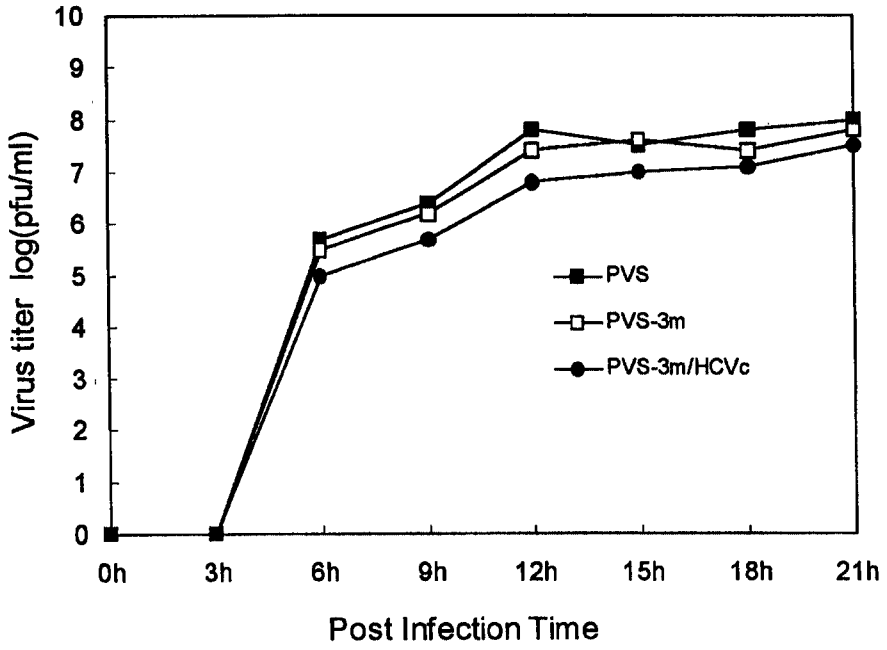

Virus titer log(pfu/ml)
10
9
8
7
6
5
4
3
2
1
0
0h
3h
6h
9h
12h
15h
18h
21h
Post Infection Time
PVS
PVS-3m
PVS-3m/HCVc

FIG. 15

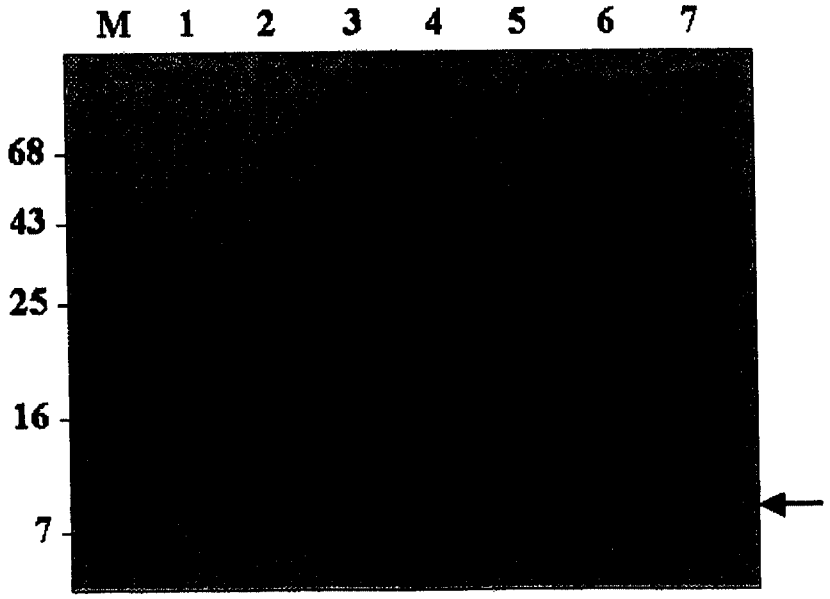

M
1
2
3
4
5
6
7
68
43
25
16
7

FIG. 16

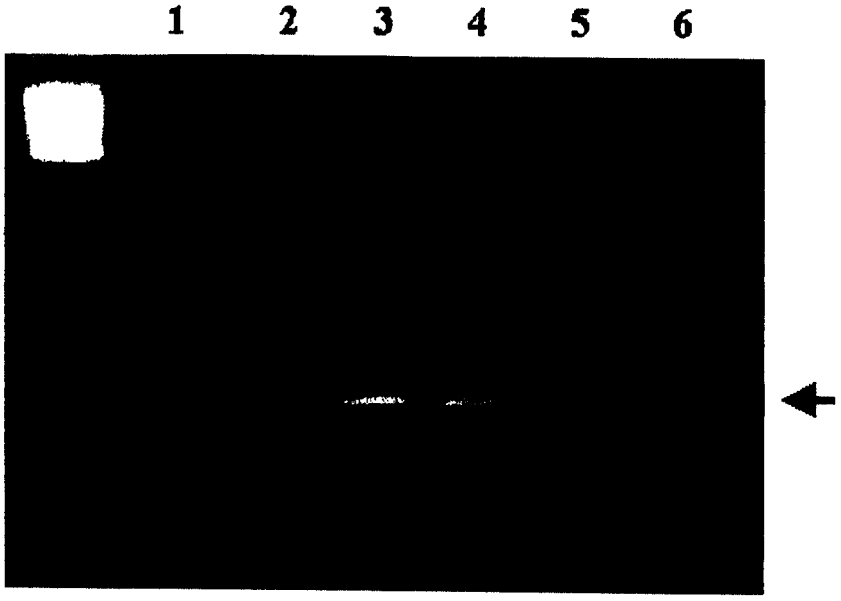
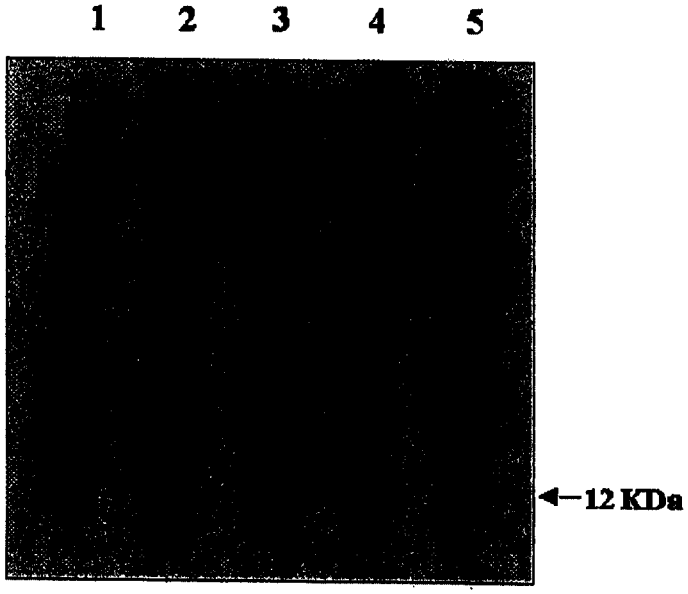
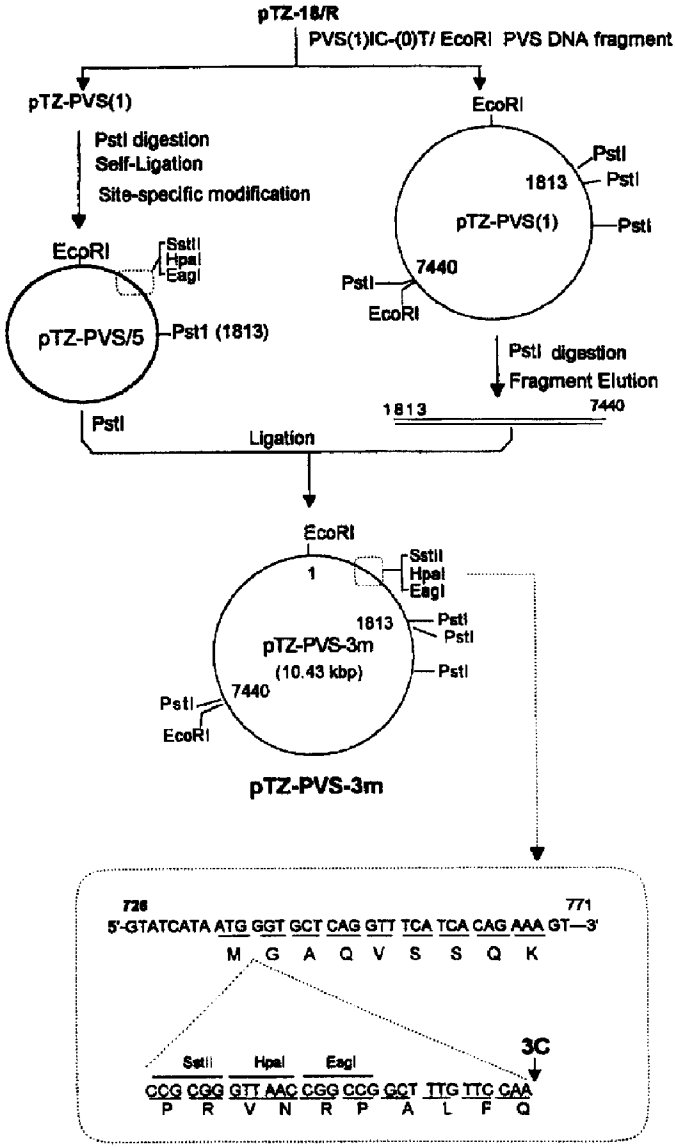


FIG. 17




pTZ-18/R
PVS(1)IC-(0)T/ EcoRI PVS DNA fragment
pTZ-PVS(1)
PstI digestion
Self-Ligation
Site-specific modification
EcoRI
SstII
HpaI
EagI
pTZ-PVS/5
Pst1 (1813)
PstI
EcoRI
PstI
1813
PstI
pTZ-PVS(1)
PstI
7440
PstI
EcoRI
PstI digestion
Fragment Elution
1813
7440
Ligation
EcoRI
1
SstII
HpaI
EagI
1813
PstI
PstI
pTZ-PVS-3m
(10.43 kbp)
PstI
7440
PstI
EcoRI
pTZ-PVS-3m
726
771
5'-GTATCATA ATG GGT GCT CAG GTT TCA TCA CAG AAA GT—3'
M G A Q V S S Q K
3C
SstII
HpaI
EagI
CCG CGG GTT AAC CGG CCG GCT TTG TTC CAA
P R V N R P A L F Q