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A vector system for introducing foreign HIV-1 *env* genes and pseudotyping of MuLV particles with the recombinant HIV-1 envelope proteins for *anti*-HIV-1 assay

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Abstract

We attempted to incorporate the HIV-1 envelope proteins derived from various HIV-1 strains into MuLV particles for developing a rapid and safe *anti*-HIV-1 screening system. In a previous study, only HIV-1 envelope protein lacking cytoplasmic 144 amino acids has been reported to be able to incorporate into MuLV particles. We designed and constructed a vector, pcKCX, expressing the envelope glycoprotein with cytoplasmic truncation by introducing the partial foreign HIV-1 *env* gene corresponding to the ectodomain of its envelope protein. Three HIV-1 *env* genes of AD8, BaL or 89.6 strains were cloned, and the HIV-1/MuLV pseudotypes were generated in the transfected TELCeB6 cells with all the cloned plasmids. The pseudotypes displayed host specificity depending on their original HIV-1 strains and their infection to the target cells was inhibited by treatment of a potent *anti*-HIV-1 peptide C34. A stable cell clone against the HIV-1_{BaL} strain was found to express the R5 tropic envelope glycoprotein on the cell surface and to produce continuously HIV-1_{BaL}/MuLV pseudotypes. These results suggested that the vector system is useful for cloning of various foreign HIV-1 *env* genes and the recombinant envelope glycoproteins effectively incorporate into MuLV particles. The HIV-1/MuLV pseudotypes may be useful for *anti*-HIV-1 assay.

Keywords: HIV-1 *env* cloning vector; HIV-1/MuLV pseudotypes; *Anti*-HIV-1 assay

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) enters cells through a series of interactions between its envelope glycoproteins and host receptors. The binding of the viral envelope surface glycoprotein gp120 to its primary receptor, CD4, is considered to induce a conformational change in

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gp120 (Sattentau and Moore, 1991), which allows subsequent interaction with a variety of coreceptors (chemokine receptors; CXCR4, CCR5, CCR3, etc.) (D'Souza and Harden, 1996; Littman, 1998; Moore et al., 1997). As a consequence, the transmembrane protein gp41 undergoes conformational changes that mediate the fusion between the viral and the cellular membranes (Chan et al., 1997; Lu et al., 1995; Weissenhorn et al., 1997). Therefore, blocking of the interaction between gp120 and host receptor(s) is a good target to develop *anti*-HIV-1 drugs. However, until now there is no antiviral material to block the interaction against all HIV-1s because of hypervariability of the envelope proteins of HIV-1s. In other words, to identify the broad *anti*-HIV-1 activity of an *anti*-HIV-1 material, its activity should be tested against many HIV-1 strains.

The assay using live HIV-1 is the best way to identify an *anti*-HIV-1 activity. However, it is very difficult to make a molecular clone of HIV-1. The primary HIV-1 cannot maintain for a long time because the amino acid compositions of the viral proteins, especially the envelope glycoprotein, have continuously changed during culture. Moreover, the use of the live HIV-1 has several disadvantages such as bioharzard, requirement of long time to test the *anti*-HIV-1 effect (Shibata et al., 1999), limitation of target cell usage because of cytotoxicity of HIV-1 and so on. The use of the cell fusion assay is one of the alternative methods using recombinant vaccinia viruses (Alkhatib et al., 1997; Lee et al., 1999; Lu et al., 1997), but the infectious live vaccinia viruses cannot be handled in a conventional laboratory.

The use of the HIV-1/murine leukemia virus (MuLV) pseudotype is an alternative method to overcome those problems mentioned above (Schnierle et al., 1997). Moreover, the use of HIV-1/MuLV pseudotype also offers several advantages. First, they are safe; second, infected cells of the pseudotypes can be easily detected within 2 days by 4-chloro-3-indolyl β-D-galactosidase (X-gal) staining; and third, any kind of cells expressing CD4 and coreceptors on their cell surface can be used as host cells because of no cytotoxicity of the pseudotypes to the host cells. The MuLV particles can be pseudotyped with HIV-1 envelope glycoproteins by transfection of a plasmid construct expressing the HIV-1 envelope glycoprotein with cytoplasmic truncation into the MuLV packaging cell line, TELCeB6 (Cosset et al., 1995) or Anjou 65 (Pear et al., 1993). TELCeB6 cells used in this study express a MuLV-derived *gag-pol* gene construct and an MFG-*nls-lacZ* gene containing a MuLV derived packaging signal, nuclear localizing signal and β-galactosidase gene (Cosset et al., 1995). It has been found that the full-length envelope glycoproteins of HIV-1 cannot be incorporated into the MuLV particles, but the mutant proteins with truncation of cytoplasmic 144 amino acids (seven amino acids remain in cytoplasmic region) can be incorporated into the pseudotyped viral particles (Schnierle et al., 1997).

To express the HIV-1 envelope glycoprotein, expression of HIV-1 Rev proteins is also required. The HIV-1 Rev protein plays a critical role to transport the HIV-1 *env* mRNA with the rev response element (RRE) from nucleus to cytoplasm (Heaphy et al., 1990). Therefore, expressions of both Rev protein and the envelope protein with 144 amino acid truncation are required for pseudotyping of MuLV particles with the HIV-1 envelope glycoproteins. Those factors as well as the hypervariability of the HIV-1 envelope protein provide some difficulties to construct a convenient expression vector of the HIV-1 envelope glycoprotein with cytoplasmic 144 amino acid truncation.

In the present study, we tried to construct a vector for introducing foreign HIV-1 *env* gene segment corresponding to the ectodomain and transmembrane domain of the HIV-1 envelope glycoprotein and to incorporate the recombinant HIV-1 envelope proteins derived from various HIV-1 *env* genes into MuLV particles. For this purpose, a vector, pcKCX, for introducing foreign HIV-1 *env* genes was constructed. The vector was designed to have full-length HIV-1 *rev* and *vpu* genes and a mutant *env* gene sequence derived from pcIIIBenvD (Fig. 1). The *env* gene in pcKCX has a large deletion from 129 (after the *Kpn*I site) to 2096 (just before a new *Cla*I site introduced for providing a new cloning site) and an early stop codon for cytoplasmic 144 amino acid truncation. The deleted region covers most of ectodomain and transmembrane domain of the HIV-1 envelope

glycoprotein. The foreign DNA covering the deleted region was amplified by PCR using the foreign *env* gene as a template, and the PCR amplified insert DNA was cloned into the *Kpn*I–*Cla*I site of the vector. All the recombinant HIV-1 envelope glycoproteins with cytoplasmic truncation were observed to form infectious HIV-1/MuLV pseudotypes. The infection of each HIV-1/MuLV pseudotype was correlated with the host tropism of the original HIV-1 strain.

2. Materials and methods

2.1. Construction of pcKCX and other plasmids

In order to construct pcKCX, pcIIIBenvD designed to express the Rev protein and the envelope glycoprotein of the HIV-1_{IIIB} strain under the cytomegalovirus (CMV) promotor was initially constructed by inserting a *Sal*I (Klenow)-*Xho*I fragment of pIIIenvCTdel-144 (kindly obtained

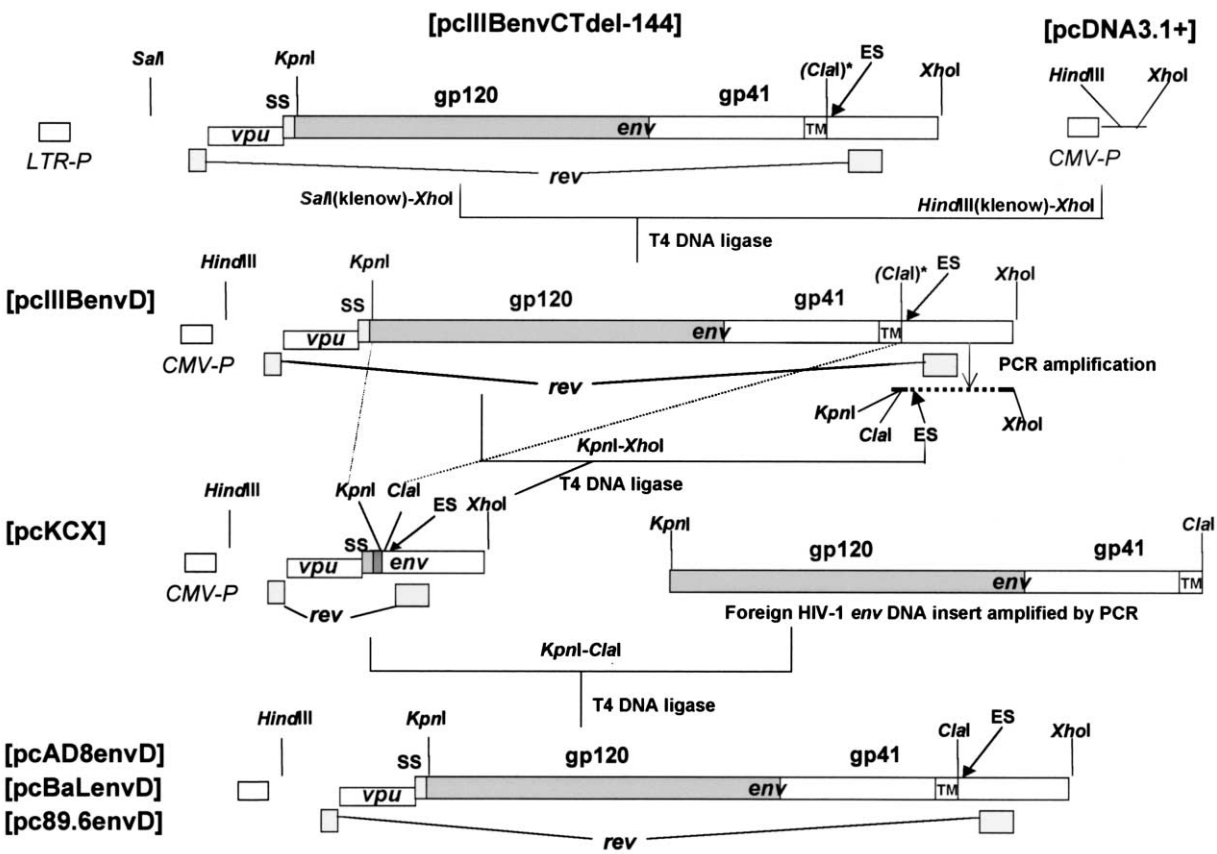

Fig. 1. HIV-1 gene structures of pcIIIBenvD, pcKCX and other plasmids with cloned HIV-1 *env* genes. The pcIIIBenvD was constructed by insertion of the *Sal*I (Klenow)-*Xho*I fragment of pIIIBenvCTdel-144 into *Hind*III (Klenow)-*Xho*I site of pcDNA3.1 + . There is no *Cla*I site in pcIIIBenvD. The *Cla*I site in the parentheses of pcIIIBenvD represents the *Cla*I location in pcKCX introduced newly by base substitution. The other plasmids (pcAD8envD, pcBaLenvD and pc89.6envD) with foreign HIV-1 *env* genes were constructed by inserting the PCR amplified DNAs derived from the foreign HIV-1 strains (AD8, BaL and 89.6, respectively) into the *Kpn*I–*Cla*I site of pcKCX. SS, signal sequence of the HIV-1 envelope protein; TM, transmembrane domain; ES, early stop codon for cytoplasmic 144 amino acid truncation.

from Dr. Freed E. O., NIH, USA) into the *Hind*III (Klenow)-*Xho*I site of pcDNA3.1 + (Invitrogen, USA) (Fig. 1). Briefly, pIIIenvCTdel-144 with HIV-1 LTR promoter expresses the Rev protein and the envelope glycoprotein with C-terminal 144 amino acid truncation of the HIV-1_{IIIB} strain in the presence of the Tat protein of HIV-1. The *Sal*I (Klenow)-*Xho*I fragment of pIIIenvCTdel-144 contains whole HIV-1 *rev* and *vpu* genes and the *env* gene with an early stop codon for cytoplasmic 144 amino acid truncation (Fig. 1). A vector, pcKCX, with a *Kpn*I site and a *Cla*I site for introducing the foreign *env* gene segment was constructed from this plasmid. An insert DNA was amplified by PCR using a forward primer (GTGTGGTACCAC-T*ATCGAT*AGTGAATAG) with a *Kpn*I site (underlined) and a *Cla*I site (italicized), a reverse primer (TAGACTCGAGATGCTGC) with an *Xho*I site (underlined) and the template DNA (pcIIIBenvD) (Fig. 1). The *Kpn*I-*Xho*I fragment of the PCR amplified DNA was cloned into the *Kpn*I-*Xho*I site of pcIIIBenvD, and named pcKCX. The pcKCX contains a whole *rev* gene, but has the mutated *env* gene with a large deletion from 129 to 2096 of the *env* gene (Fig. 1). To construct the other plasmids (pcAD8envD, pcBaLenvD, and pc89.6envD), the insert DNA was amplified by PCR using a forward primer (ATGGGGTACCT-GTGTGG) with a *Kpn*I site (underline), a reverse primer (CACTATCGATAGTACAGCAAAAA-CTAT) with a *Cla*I site (underlined) and a template DNA (one of pTM-AD8gp140, pTM-BaLgp140 and pTM-89.6gp140 obtained from Dr. Cho, M; NIH, USA), respectively (Fig. 1). The *Kpn*I-*Cla*I fragment of each PCR amplified DNA was cloned into the *Kpn*I–*Cla*I site of pcKCX, and named pcAD8envD, pcBaLenvD and pc89.6envD, respectively (Fig. 1).

2.2. Production of HIV-1/MuLv pseudotypes

The plasmids (pcIIIBenvD, pcAD8envD, pcBaLenvD and pc89.6envD) were transfected into TELCeB6 cells (Cosset et al., 1995) (kindly obtained from Dr Cosset, F. L., Centre National de la Recherche Scientifique, Lyon, France) by the calcium phosphate method. Briefly, TELCeB6 cells used in this study produce the non-enveloped MuLV particles with the MFG-*nls-lacZ* RNA containing a MuLV derived packaging signal, nuclear localizing signal and β-galactosidase gene (Cosset et al., 1995). The MuLV particles can be pseudotyped with viral envelope glycoproteins lacking cytoplasmic 144 amino acids by transfection of a plasmid construct expressing the viral envelope protein into the cells. After an overnight incubation at 37 °C, cells were washed with Dulbecco's modified Eagle's medium (DMEM; Gibco/BRL, USA) containing 10% fetal bovine serum (FBS; Gibco/BRL) and incubated 2 additional days. After the removal of the cell debris by a centrifugation, the culture supernatant containing the HIV-1/MuLV pseudotyped virus particles was collected and directly used for following experiments. The bound cells were used for cell fusion assay as described below. The pseudotyped viruses obtained by transfection of the plasmids (pcIIIBenvD, pcAD8envD, pcBaLenvD and pc89.6envD) were named pvIIIB, pvAD8, pvBaL and pv89.6, respectively.

2.3. Virus infection and neutralization assay

Three $\times 10^4$ of Hos-CD4-CCR5 cells or 2×10^4 of Hos-CD4-CXCR4 cells (Cheng-Mayer et al., 1997) per wells were seeded in 96-well plates and incubated in DMEM containing 10% FBS overnight. Infection of the pseudotypes was carried out for 36–40 h incubation of target cells with 200 μl of diluted culture supernatant in culture medium. The cells expressing β-galactosidase were observed by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining according to the instruction of Gibco/BRL. The titers are expressed in infectious units per mililiter (IU/ml). To test whether this system can be used for neutralization assay, neutralizing activity of a 34mer potent *anti*-HIV-1 peptide (C34; WMEWDREINNYTSLIH-SLIEESQNQQWKNEQELL) (Chan et al., 1997) was analyzed by measuring the infection inhibition of HIV-1/MuLV pseudotypes into the host cells used. The peptide has been reported to elicit the potent *anti*-HIV-1 activity by disrupting the formation of the gp41 coiled coil structure required for HIV-1 infection (Chan et al., 1997).

2.4. Cell fusion assay

TELCeB6 cells transfected with one of pcII-IBenvD, pcAD8envD, pcBaLenvD and pc89.6envD were cultured until they reached 80–90% confluence stage. The cells were trypsinized and mixed with 5- to 10-fold excess of target cells, HOS-CD4-CCR5 or HOS-CD4-CXCR4, and cultured overnight. The cells were stained with X-gal as described above, and the fusion cells were monitored under the microscope.

2.5. Production of stable cell line-generating pseudotypes against HIV-1_{BaL} strain

The pcBaLenvD transfected TELCeB6 cells were cultured in the presence of 1 mg/ml G418 (Gibco/BRL, USA) in DMEM containing 10% FBS. The limiting diluted cells (0.5 cell/well) were split into 96 well plates, and cultured for 10–14 days in the presence of 1 mg/ml G418. The cell clones producing large amount pseudotypes were selected. These steps were repeated five times, and a stable cell line selected was named TELCeB6/BaL20.

2.6. Determination of the protein expression of TELCeB6/BaL20 cells

The stable cells were cultured in the presence or absence of 0.5 mg/ml of G418 to 90% confluent stage in a T25 flask. Envelope glycoprotein expression was determined by Western blot and FACS analysis. Western blot analysis was performed by the method as described previously (Lee et al., 2000). The cells grown in T25 flask were washed with Dulbecco's phosphate buffered saline (DPBS; 0.2 g/l potassium chloride, 0.2 g/l potassium phosphate monobasic, 8 g/l sodium chloride and 1.15 g/l sodium phosphate dibasic, pH 7.4) and then solubilized the cells with in 0.5 ml DPBS containing 1% nonidet P40. After removing insoluble materials, 10 μl of the samples were loaded on the wells and 8% SDS-PAGE was done. Proteins were electrically transferred to nitrocellulose membrane and the HIV-1 envelope protein were detected with *anti*-HIV-1 gp160 rabbit antiserum (Obtained from Dr Cho, M. W., NIH, USA) and horse radish peroxidase coupled goat *anti*-rabbit IgG (Sigma-Aldrich, USA) using the enhanced chemiluminescence (ECL) detection kit (Pierce, USA). FACS analysis was done with the same antiserum and FITC coupled *anti*-rabbit IgG under non-penetrating condition. Briefly, the cells were treated with cell disassociation buffer (Sigma-Aldrich, USA) for 5 min at 37 °C, washed in DPBS containing 0.1% BSA and blocked at 4 °C in the same buffer. After staining the cells (5×10^5 per test) with *anti*-HIV-1 gp160 rabbit antiserum for 30 min on ice, FITC conjugated *anti*-rabbit IgG (Dako, USA) was treated for 30 min on ice. The antibody bound cells were analyzed using a FACScan (Becton–Dickinson, USA).

3. Results

3.1. Construction and characterization of pcIIIBenvD

To generate the HIV-1/MuLV pseudotypes, the expressions of the Rev protein and the envelope protein with cytoplasmic 144 amino acid truncation of HIV-1 are required (Schnierle et al., 1997). For this purpose, pcIIIBenvD encoding both HIV-1 Rev protein and envelope glycoprotein with early stop codon (ES) under the CMV promoter was constructed (Fig. 1). The transfected TELCeB6 cells with pIIIBenvCTdel-144 which was used for construction of pcIIIBenvD generated the infectious pseudotyped viral particles in the presence of the HIV-1 Tat protein (data not shown). Whether or not TELCeB6 cells, upon a transfection of pcIIIBenvD, expressed the HIV-1 envelope glycoprotein on the cell surface and formed pvIIIB were investigated.

The TELCeB6 cells transfected pcIIIBenvD were found to form fusion cells by cocultivation with HOS-CD4-CXCR4 cells (Fig. 2A right panel), while no TELCeB6 cells transfected by pcDNA3.1+ formed syncytia with HOS-CD4-CXCR4 cells (Fig. 2A left panel), suggesting that the HIV-1 envelope proteins successfully expressed on the TELCeB6 cell surface. After exposing HOS-CD4-CXCR4 cells to supernatants from

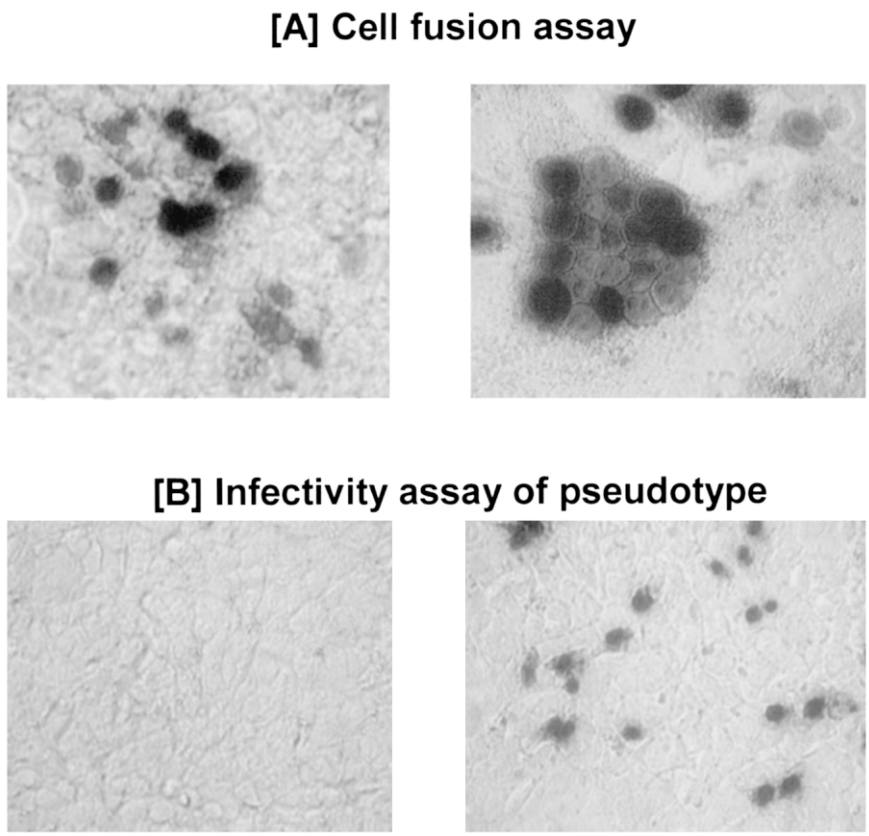

Fig. 2. Demonstration of functional expression of the HIV-1 surface protein by syncytia formation with HOS-CD4-CXCR4 cells (A) and infectivity of pvIIIB to HOS-CD4-CXCR4 cells (B). TELCeB6 cells transfected with pcDNA3.1 + (A, left) or pcIIIBenvD (A, right) were cocultured with HOS-CD4-CXCR4 cells for 24 h before they were photographed. The culture supernatants of TELCeB6 cells transfected with pcDNA3.1 + (B, left) or pcIIIBenvD (B, right) were infected to HOS-CD4-CXCR4 cells for 36-40 h.

TELCeB6/pcIIIBenvD cells or from TELCeB6/pcDNA3.1 + cells and monitoring expression of β-galactosidase, blue colonies were only observed in the HOS-CD4-CXCR4 cells treated with supernatants from TELCeB6/pcIIIBenvD cells (Fig. 2B), displaying that the HIV-1 envelope proteins were incorporated into the MuLV particles. The pvIIIB titer was observed approximately 16,000 IU/ml culture supernatant (average of three different experiments ranged from 9100 to 22400 IU/ml) (Fig. 3). Unexpectedly a low level (around of 25% of CXCR4) of infection of pvIIIB into the HOS-CD4-CCR5 cells was also found (Fig. 3), even though HIV-1_{IIIB} is known to be strict X4 tropic. This result may be due to the presence of low endogenous levels of CXCR4 on HOS cells as we reported previously (Cho et al., 1998).

3.2. Construction of a vector pcKCX

We designed and constructed a vector, pcKCX, for introducing the foreign HIV-1 *env* gene sequence covering most of ectodomain and transmembrane of the HIV-1 envelope protein from pcIIIBenvD (Fig. 1). The *Kpn*I site at position 129 of pcIIIBenvD *env* gene is a useful because it is highly conserved and locates at just after signal sequence, whereas there is no available restriction enzyme site from the end of ectodomain to the early stop codon of pcIIIBenvD. Therefore we introduced a new enzyme site *Cla*I by changing two bases without amino acid change [TC*T*TC*T*ATA (Lys-Ser-Ile) to TC*A*TC*G*ATA (Lys-Ser-Ile), changed bases are italicized and the new *Cla*I site is underlined]

at the interface between transmembrane and cytoplasmic domain (Fig. 1). To introduce the new *Cla*I site, we designed the forward primer (GTGTGGTACCACT*ATCGAT*AGTGAATAG) with both *Kpn*I (underlined) and *Cla*I sites (italicized), and the reverse primer (TAGACTCGAGATGCTGC) with an *Xho*I site (underlined). A PCR band with approximately 600 bp was generated by PCR using pcIIIBenvD as a template DNA, and the PCR DNA was inserted into the *Kpn*I–*Xho*I site of pcIIIBenvD (data not shown). The pcKCX has the whole *rev* and *vpu* gene sequences, but has the modified *env* sequence with a large deletion from 129 (after the *Kpn*I site) to 2096 and with a new *Cla*I site at position 2100 for cloning and an early stop codon (Fig. 1). The *Kpn*I-*Cla*I site of pcKCX was used for introducing foreign *env* genes amplified by PCR.

3.3. Cloning of foreign HIV-1 env genes into pcKCX and generation of HIV-1/MuLV pseudotypes

We constructed three plasmids (pcAD8envD, pcBaLenvD and pc89.6envD) using pTM-AD8-gp140, pTM-Balgp140 and pTM-89.6gp140 as template DNAs, respectively. All the insert DNAs with around 2000 base pairs were successfully amplified using a set of the primers used (data not shown), digested with *Kpn*I and *Cla*I, and cloned into the *Kpn*I–*Cla*I site of pcKCX. The constructed plasmids were confirmed to contain the insert DNA sequences by restriction enzyme mapping and DNA sequencing (data not shown).

All the TELCeB6 cells transfected with one of the plasmids were observed to form syncytia with the HOS-CD4-CCR5 cells, while those cells transfected with pcDNA3.1 + did not form any syncytia (Fig. 4), indicating that the envelope glycoproteins successfully expressed on the cell surface of TELCeB6 cells. The recombinant HIV-1 envelope glycoproteins were also found to incorporate into the MuLV particles. High titers (9000–15,000 IU/ml) of the HIV-1/MuLV pseudotypes were produced in the TELCeB6 cells transfected with the plasmids (Fig. 3). The pseudotypes showed infectivity to the target cells, HOS-CD4-CCR5 or HOS-CD4-CXCR4, according to their host tropism of the original HIV-1 strains (Fig. 3). The HIV-1/MuLV pseudotypes

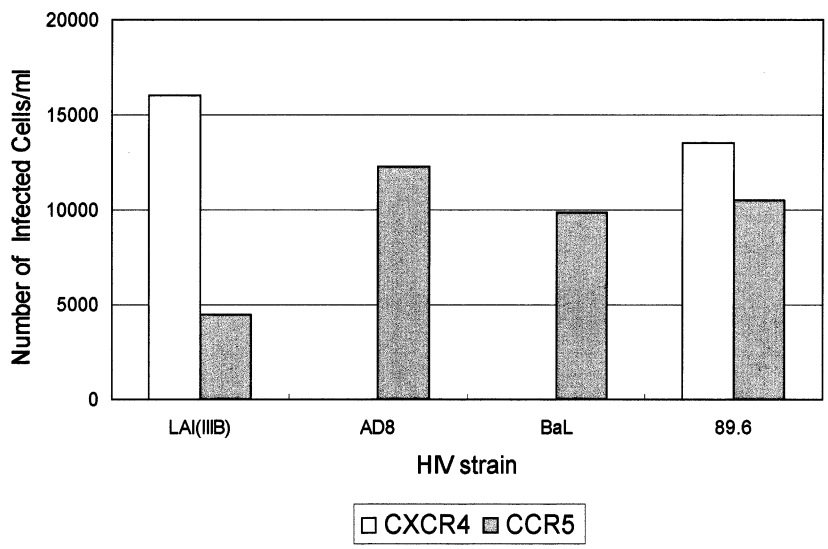

Fig. 3. Titers of the HIV-1/MuLV pseudotypes to HOS-CD4-CCR5 and HOS-CD4-CXCR4 cells. The culture supernatants of TELCeB6 cells transfected with pcIIIBenvD, pcAD8envD, pcBaLenvD or pc89.6envD were infected to HOS-CD4-CXCR4 cells and to HOS-CD4-CCR5 cells for 36–40 h. The stained cells were counted under the microscope. The data were obtained by averaging the values of 2–3 different experiments.

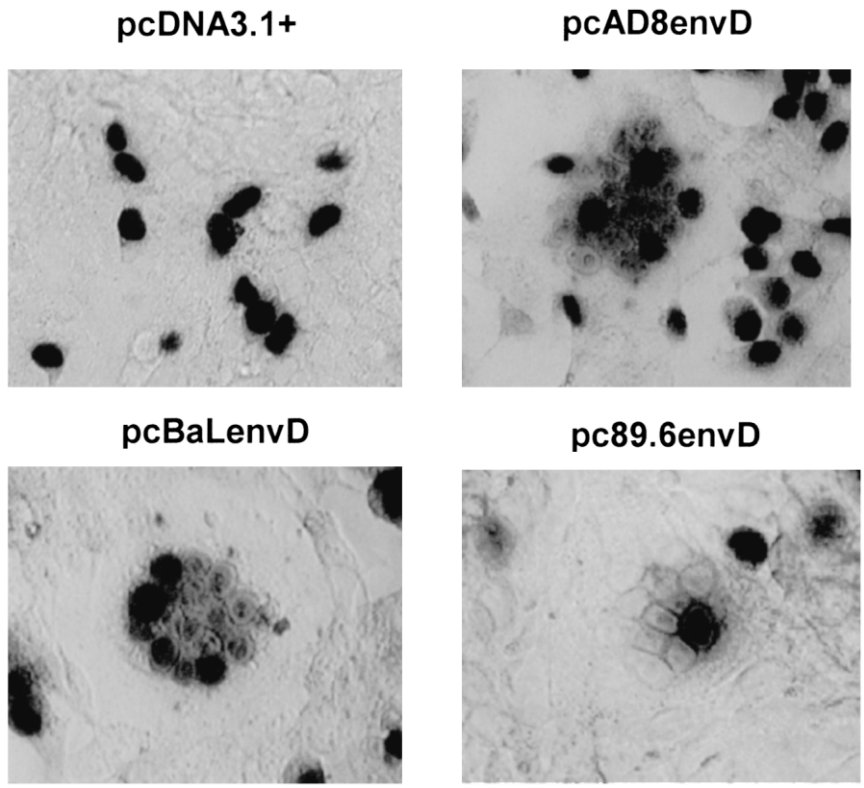

Fig. 4. Demonstration of the functional expression of the HIV-1 surface glycoprotein by syncytia formation with HOS-CD4-CCR5 cells. TELCeB6 cells transfected with pcAD8envD, pcBaLenvD or pc89.6envD were cocultured with HOS-CD4-CCR5 cells for 24 h before they were photographed. TELCeB6 cells transfected with pcDNA3.1 + were used as negative control.

(pvAD8 and pvBaL) derived from R5 tropic HIV-1 strains (AD8 and BaL), respectively, infected to HOS-CD4-CCR5 cells, but showed no infectivity to HOS-CD4-CXCR4 cells. The pseudotyped virus (pv89.6) derived from the R5X4 tropic HIV-1 strain (89.6) (Doranz et al., 1996) infected all the host cells as expected.

3.4. Evaluation of the HIV-1/MuLV pseudotypes for analyzing anti-HIV-1 activity of an anti-HIV-1 peptide, C34

The C34 peptide derived from the C-terminus of the gp41 coiled coil structure of the HIV-1 IIIB strain is known to be the most potent *anti*-HIV-1 activity (Chan et al., 1997). We tested whether the HIV-1/MuLV pseudotypes developed in this study could be used for analyzing *anti*-HIV-1 activity of C34. As shown in Fig. 5, C34 was inhibited all the infections of the pseudotypes according to the dose-dependent manner, but its antiviral activity is various depending on the HIV-1 strains and the chemokine receptors. C34 showed the most potent *anti*-HIV-1 activity to the pv89.6–CXCR4 combination, whereas elicited the lowest *anti*-HIV-1 activity to the pvAD8–CCR5 combination. The result may be due to the differential accessibility of C34 to its gp41-binding region according to the HIV-1 strain and coreceptor.

3.5. Development of a stable cell line, TELCeB6/BaL20

We tried to generate a stable cell line for the HIV-1_{BaL} strain because the stable clone of TELCeB6 packaging cells expressing the truncated HIV-1 envelope protein is useful for generating

continuously the HIV-1/MuLV pseudotypes. The TELCeB6 cells transfected with pcBaLenvD were cultured in the presence of 1 mg/ml G418 in DMEM with 10% FBS. The resistance cells were diluted 0.5 cell/well in the 96 well plates, and the clones were investigated by measuring the titers of the pseudotyped virus particles released. The cell clone TELCeB6/BaL20 released approximately $1.0–2.0 \times 10^5$ IU/ml of pvBaL which was able to infect HOS-CD4-CCR5 cells, but not to infect HOS-CD4-CXCR4 cells (data not shown). The stable cells were found to form syncytia with HOS-CD4-CCR5 cells and the pvBaL infection to HOS-CD4-CCR5 cells was inhibited by C34 (data not shown).

The expression of the HIV-1 envelope protein of the stable clone was further investigated by Western blot and FACS analysis. In Western blot analysis, TELCeB6/BaL20 cells were found to express much more amount of envelope glycoproteins (gp120 + gp140) in the presence of G418 than in the absence of G418 in medium (Fig. 6A, lanes 1 and 2). No envelope glycoprotein was expressed in TELCeB6 cells (Fig. 3A, lane 3). To confirm surface expression of envelope glycoproteins on TELCeB6/BaL20 cells, FACS analysis was also performed using *anti*-HIV-1 gp160 antibody. Significant expression of the HIV-1 envelope protein was detected in the surface of TELCeB6/BaL20 cells but not in TELCeB6 cells (Fig. 6B). These results demonstrated that the HIV-1_{BaL} envelope glycoproteins were stably expressed on TELCeB6/BaL20 cells.

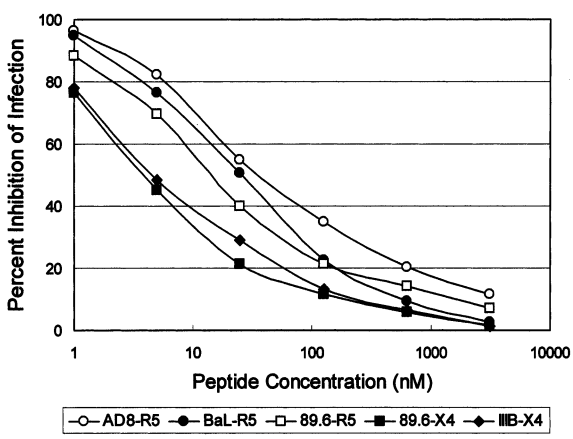

Fig. 5. Neutralization of the infections of HIV-1/MuLV pseudotypes by incubation with a potent *anti*-HIV-1 peptide C34. The culture supernatants containing the pseudotyped virus particles were incubated with the target cells, HOS-CD4-CCR5 or HOS-CD4-CXCR4 for 36-40 h in the presence of serially diluted peptides. One hundred percent infection indicates the pseudotype infection in the absence of C34, and the percent inhibition of infection was calculated by the following equation: percent inhibition of infection = (number of blue cells in the presence of C34)/(number of blue cells in the absence of C34) × 100.

4. Discussion

The HIV-1/MuLV pseudotypes are useful as a safe and rapid assay system to identify the host tropism of the envelope glycoprotein of HIV-1 and to develop *anti*-HIV-1 drugs that interfere HIV-1 infection. Since HIV-1/MuLV pseudotypes are required to test broad *anti*-HIV-1 activity of an *anti*-HIV-1 material because of hypervariability of the HIV-1 envelope glycoprotein, we attempted to create various HIV-1/MuLV pseudotypes comprising MuLV capsid and the HIV-1 envelope glycoproteins in TELCeB6 cells. It has been reported that the HIV-1 envelope glycoprotein with cytoplasmic 144 amino acid truncation can be packaged into the MuLV virus particles, whereas no full-length envelope proteins integrate into the MuLV particles (Schnierle et al., 1997). The HIV-1 Rev protein is also required to transport the nuclear HIV-1 *env* mRNA with the RRE sequence to cytoplasm (Felber et al., 1989; Heaphy et al., 1990; Malim et al., 1989). These factors make difficult to construct a vector for generating the HIV-1/MuLV pseudotypes in TELCeB6 cells after introducing various foreign HIV-1 *env* genes. In addition to these factors, the proper interaction between gp120 and gp41 is also important for HIV-1 infection (Park and Quinnan, 1999; Park et al., 1998). Therefore, we designed a vector to introduce the partial HIV-1 *env* region corresponding to the ectodomain and transmembrane domain of the envelope glycoprotein before the early stop codon for 144 amino acid truncation.

We initially constructed pcIIIBenvD with a *rev* gene and a mutated *env* gene with an early stop

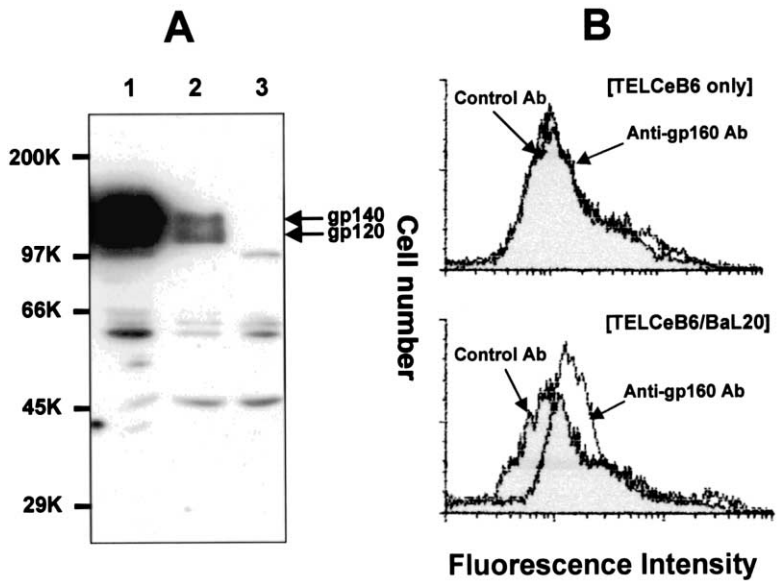

Fig. 6. Detection of expression of the HIV-1 envelope glycoprotein in the stable TELCeB6/BaL20 cells. Nontransfected TELCeB6 cells were used as a negative control (A, lane 3; and B, upper panel). The lanes of panel A represent TELCeB6/BaL20 cells maintained in the presence of G418 (lane 1) and in the absence of G418 (lane 2). Western blot analysis and FACS analysis were performed with the *anti*-gp160 rabbit polyclonal antiserum (Lee et al., 2000), and *anti*-rabbit IgG antibodies conjugated with horseradish peroxidase and FITC, respectively, as secondary antibodies. Horseradish peroxidase activity was detected using the ECL system (Pierse, USA). FACS analysis was done under the nonpermeable condition.

codon under the CMV promotor (Fig. 1). Although those genes of pcIIIBenvD are derived from pIIIenvCTdel-144, pcIIIBenvD has some advantages compared with pIIIenvCTdel-144 controlled by the LTR promotor. No additional transfection of the *tat* gene is required to express the HIV-1 envelope glycoprotein and there is only one *Kpn*I site in pcIIIBenvD (2 *Kpn*I sites in pIIIenvCTdel-144) used for introducing foreign *env* gene in this study. Moreover, the TELCeB6 cells transfected transiently with pcIIIBenvD were found to produce larger amounts of pseudotypes as compared with the previous report (Schnierle et al., 1997). The titers (9100–22,400 IU/ml) of pvIIIB against HOS-CD4-CXCR4 were considered to be high, because it was reported that the TELCeB6 cells transiently transfected with pTr712 (Krausslich et al., 1993; Wilk et al., 1992) produced 200–1000 IU/ml pseudotypes (Schnierle et al., 1997). Since the pIIIBenvCTdel-144 has been cloned from pTr712 (Freed and Martin, 1995), there is no difference of the HIV-1 *env* gene between pTr712 and pcIIIBenvD. These results demonstrate that our system might be more effective than the previous system for producing the HIV-1/MuLV pseudotypes.

Although the HIV-1_{IIIB} strain is known to be strict X4 tropic (Lee et al., 1999; Lu et al., 1997), pvIIIB was also found to infect to HOS-CD4-CCR5 (Fig. 3). The similar result was also found in our previous study using live HIV-1 (Cho et al., 1998). This result may be caused by low endogenous expression of CXCR4 and is consistent with the observation that the envelope glycoproteins of some X4-tropic HIV-1 isolates (HXB2 and SF2) exhibit low levels of infectivity in HOS-CD4 cells (Cheng-Mayer et al., 1997; Deng et al., 1996). This supports that the infection of the HIV-1/MuLV pseudotype mimics that of the live HIV-1.

From pcIIIBenvD, the vector, pcKCX, was constructed by deletion and introduction of a new *Cla*I site in the *env* gene (Fig. 1). The *env*

deleted region (between the *Kpn*I site and the *Cla*I site) of pcKCX contains most of the ectodomain and the transmembrane domain of the HIV-1 envelope protein. Both restriction enzyme sites are rare (0–2 sites in most of HIV-1 *env* genes), and the nucleotide sequences around the restriction enzyme sites are highly conserved within the HIV-1 major (M) group (subtypes A to J) (see review Leitner et al., 1998). Therefore the insert DNAs can be amplified by PCR using several primer combinations and templates derived from various HIV-1 *env* gene sources such as HIV-1 molecular clones, recombinant HIV-1 *env* genes, HIV-1 particles and HIV-1 patient T-cells.

Three plasmids (pcAD8envD, pcBaLenvD and pc89.6envD) were successfully cloned using pcKCX and the PCR amplified inserts derived from HIV-1 molecular clones for AD8, BaL and 89.6 strains, respectively. The constructed plasmids were found to express the functional envelope glycoproteins on the cell surface in TELCeB6 cells and to generate HIV-1/MuLV pseudotypes with high titers (9000–15,000 IU/ml) (Fig. 3), and all the pseudotypes were found to have the host tropism in accordance with their original HIV-1 strains. These results demonstrate that pcKCX is useful for one step cloning of foreign HIV-1 *env* genes and the TELCeB cells transfected with the cloned plasmids generated the infectious pseudotypes.

To test whether the pseudotypes are able to use for measuring *anti*-HIV-1 activity, the potent *anti*-HIV-1 peptide, C34 (Chan et al., 1997), was used. The peptide is derived from the C-terminal sequence of the gp41 coiled coil structure of the HIV-1_{LAI} strain, and known to have the broad and potent *anti*-HIV-1 activity (Chan et al., 1997). In this study, C34 was also found to inhibit the infections of the pseudotypes tested with different activities Fig. 5. Interestingly R5 or R5X4 tropic pseudotype infection to HOS-CD4-CCR5 cells was more resistance to the inhibition by C34 than X4 or R5X4 tropic pseudotype infection to HOS-CD4-CXCR4 cells. The similar results have been reported that R5 or R5X4 tropic virus using CCR5 for entry is more resistance to the inhibition by the gp41 derived peptides than X4 or R5X4 tropic virus using CXCR4 (Xu et al., 2000). Even though it is not clear why different coreceptor usage influences susceptibility of HIV-1 to inhibition by the gp41 derived peptides, the results suggest that gp120 binding to CD4 and CXCR4 induces more susceptible conformation for the peptide to interact with the counterpart domain (N-terminus of the gp41 coiled coil structure) than that to CD4 and CCR5.

The TELCeB6 cell clone expressing the HIV-1_{BaL} envelope protein with cytoplasmic truncation is a powerful tool for preparation of the pseudotyped virus particles. We found a stable cell clone generating high titer of pvBaL ($1–2 \times 10^5$ IU/ml). The best result was obtained when the cells were cultured in the medium with G418 because the pcDNA3.1 + used for vector construction contains neomycin resistant gene. The virus titer was correlated well with the envelope protein expression (Fig. 6A). We also found the cell surface expression of the envelope protein (Fig. 6B). The envelope expression could be maintained over 4 months under the culture condition, suggesting that the cell clone is useful for continuously producing high titer of pvBaL.

In conclusion, we constructed the vector (pcKCX) for introducing directly the foreign *env* genes covering the most of gp120 and the ectodomain and transmembrane domain of gp41. Various HIV-1 gene sources such as HIV-1 molecular clones, T-cells of HIV-1 carriers, primary HIV-1s and so on can be used as template DNAs (or RNAs). The cloned HIV-1 *env* inserts were found to express the envelope glycoproteins with cytoplasmic truncation, and the expressed recombinant envelope glycoproteins were found to incorporate into the MuLV particles. The HIV-1/MuLV pseudotypes with high titers were produced using our system and they showed the same coreceptor usage in accordance with their original HIV-1 strains. The cell clone for the HIV-1_{BaL} strain was useful for generating continuously pvBaL with high titer. The HIV-1/MuLV pseudotypes were useful for analyzing *anti*-HIV-1 activity of C34, providing that the HIV-1/MuLV pseudotypes will be useful as a safe and rapid assay system for development of drugs that interfere with the HIV-1 infection.

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