An efficient system for site-directed mutagenesis to make various mutants of the *env* gene of human immunodeficiency virus type 1

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Abstract We developed an efficient system of site-directed mutagenesis for the envelope (*env*) gene of human immunodeficiency virus type 1 (HIV-1). To make a template plasmid for mutagenesis, pS+B/MluI, two independent selection markers, i.e. a unique restriction site, *Mlu*I, and an in-frame termination codon, were introduced into the region encoding the V3 domain of the *env* gene of an HIV-1 strain, NL4-3, which had been cloned in the pUC118 plasmid. When the *env* gene of the pS+B/MluI plasmid was mutated successfully using mutagenic primers such as synthetic oligonucleotides or PCR-amplified DNA fragments longer than 1.5 kbp, the plasmids became resistant to digestion with *Mlu*I and competent *env* genes were formed by suppression of the in-frame termination. Various site-directed mutants of the *env* gene of HIV-1 were accurately constructed in a short time even in the absence of proper restriction sites by this system. The system of site-directed mutagenesis we reported here will be a useful method to analyze the functions of variable genes like the *env* gene of HIV-1 precisely and rapidly.

Key words: Human immunodeficiency virus type 1; *env* gene; Third variable domain; Site-directed mutagenesis

1. Introduction

Site-directed mutagenesis is a useful method to examine the function of genes. Various methods have been developed to introduce artificial modifications into genetic materials and several kinds of kits are commercially available [1–6]. However, it is not yet easy to construct various site-directed mutants in a short time.

Using site-directed mutants, many studies have been done on the biologically divergent properties of human immunodeficiency virus type 1 (HIV-1). The envelope (*env*) protein plays important roles in determination of the phenotypes of HIV-1, although it shows a marked variability in their amino acid sequences [7,9,10]. In particular, the third variable domain (V3) of the *env* protein acts both as a principal neutralizing epitope and as a major determinant of cell tropism of HIV-1 [11,12]. The changes of amino acid sequences in the V3 domain markedly affect these phenotypes [9,10].

We isolated an HIV-1 strain, GUN-1WT, which infected both T-cell lines and macrophages [13]. The GUN-1WT strain has the GPGR (glycine-proline-glycine-arginine) sequence at the tip of the V3 domain. A single amino acid change at the tip sequence confers a crucial effect on cell tropism of the GUN-1WT strain. When proline (P) of the GPGR tip sequence is changed to serine (S), the GUN-1WT strain acquires tropism both for a glioma cell line, U87/CD4, and for BT-3 cells, which are considered to originate from pericytes in brain blood vessels, but not for macrophages [8].

In this study, we made a novel template plasmid for mutagenesis which could facilitate construction of various site-directed mutants of the *env* gene of HIV-1. Two different oligonucleotide primers are generally used in previously reported methods: one is used for mutagenesis and the other for selection of mutants. In our system, a single bifunctional primer was used for mutagenesis and selection of mutants. Moreover, *env* gene fragments longer than 1.5 kbp amplified by PCR could also be used as mutagenic primers. By this system, various mutants of the *env* gene of HIV-1 can be efficiently produced in a short time.

2. Materials and methods

2.1. Plasmids

The pNL4-3 plasmid contains an infectious DNA clone of an HIV-1 strain, NL4-3 (GenBank accession number U26942) [14]. The pS+B plasmid was constructed by subcloning a 2680 bp DNA fragment (S+B fragment) which had been isolated from the NL4-3 plasmid by digestion with *Sal*I and *Bam*HI, into the multi-cloning site of the pUC118 plasmid (Takara Shuzo, Shiga, Japan). The S+B fragment corresponds to nucleotide sites 5785–8465 in the genomic sequence of NL4-3 strain, which contains the Δ*vpr*, *vpu*, *tat*, *rev*, and Δ*env* genes.

The pGW514 and pGW322 plasmids contained the 5′-half and the 3′-half genomic sequences of an HIV-1 clone, GUN-1WT, respectively (GenBank accession number HIVGUNAA) [8]. The pPs-b plasmid contained the 2745 bp *Pst*I fragment of strain GUN-1WT (corresponding to nucleotide sites 5251–7995 of the NL4-3 genomic sequence), which encodes two thirds of the *env* gene.

2.2. Cell and bacterial strains

The human T-cell line C8166 was used to propagate parental and mutant HIV-1 strains [15]. C8166 cells were cultured in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% (v/v) heat-inactivated fetal calf serum.

The *Escherichia coli* strains BMH71-18mutS and HB101 (Takara Shuzo) were used to propagate plasmids. *E. coli* transformed with plasmids were cultured in LB broth [16].

2.3. Oligonucleotide primers for site-directed mutagenesis and PCR

An oligonucleotide, PS+B/MLU (5′-AGTATCCGTATCTAGACGCGTCCAGGGAGAGCATTTG-3′), which corresponded to nucleotide sites 910–946 in the *env* gene of strain NL4-3, was synthesized to introduce an *Mlu*I site and an in-frame termination codon into the V3 domain of the *env* gene in the pS+B plasmid (Fig. 1a). Mutagenic primers and their nucleotide sequences were as follows: NL/BH1 (5′-AGTATCCGTATCCAGAGGGGATCCGGGAGAGC-A-3′, 913–944), NL/Ala (5′-CGTATCCAGAGGGGAGCAGGGAGAGCATTTGTTAC-3′, 916–950), NL/Arg (5′-CGTATCCAGAGGGGACGAGGGAGAGCATTTGTTAC-3′, 916–950), NL/BHI (5′-AGTATCCGTATCCAGAGGGGATCCGGGAGAGC-3′, 910–941), NL/

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Leu (5′-CGTATCCAGAGGGGACTAGGGAGAGCATTTGTTA-C-3′, 916–950), NL/Ser (5′-CGTATCCAGAGGGGATCAGGGAG-AGCATTTGTTAC-3′, 916–950), and NL/Thr (5′-CGTATCCAGA-GGGGAACAGGGAGAGCATTTGTTAC-3′, 916–950). Nucleotides substituted from the original plasmid are indicated by underlines. These primers were used for site-directed mutagenesis of the *env* gene (Fig. 1a). PCR primers and their sequences were as follows: MK603 (5′-CAGAAAAATTGTGGGTCACAGTCTATTATGGG-GTACCT-3′, 92–129), 1590C (5′-CTGTGAGTTGCAACAGATGC-3′, 1683–1702), Bg3 (5′-TAATTAGATCTGCCAATTTCACAGA-CAA-3′, 806–833), Bg4 (5′-ATATCGCCTCCTCCAGGTCTGAA-GATCTC-3′, 1890–1918), P2 (5′-ATGGCAGTCTAGCAGAAGA-A-3′, 779–798), P3 (5′-TTCTGGGTCCCCTCCTGAGGA-3′, 1084–1104), 5′V3NLN (5′-AAAACCATAATAGTACAGCTGAACACA-TCTGTAG-3′, 838–871), and 3′V3NLR (5′-CTGTTTTAAAGTGG-CATTCCATTTTGCTCTAC-3′, 995–1026). These primers were used to amplify DNA fragments of the *env* gene by PCR (Nippon Gene Research Laboratories, Miyagi, Japan).

2.4. Construction of mutagenic plasmid

Twenty μl of the kinase reaction mixture (2 μg of the pS+B plasmid DNA, 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM EDTA, 0.1 mM spermidine, 1 mM ATP, and 10 U of T4 polynucleotide kinase (Takara Shuzo)) was incubated at 37°C for 60 min to phosphorylate the 5′-terminus of the PS+B/MLU primer and then heated at 65°C for 10 min to inactivate the enzyme. 20 μl of the annealing reaction mixture (0.2 μg of the phosphorylated PS+B/MLU primer, 0.2 μg of the pS+B plasmid DNA, 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 50 mM NaCl) was heated at 100°C for 3 min and then cooled on ice for 5 min. 30 μl of the mutagenesis reaction mixture (0.1 μg of the pS+B plasmid annealed with the PS+B/MLU primer, 20 mM Tris-HCl (pH 7.5), 4% (v/v) glycerol, 8 mM DTT, 80 μg/ml bovine serum albumin (BSA), 24 μg/ml single-strand-binding protein (SSB), 25 mM dNTP, 10 mM ATP, 10 U of T4 DNA ligase (Takara Shuzo) and 10 U of DNA polymerase III (Toyobo, Osaka, Japan)) was incubated at 37°C for 60 min to extend DNA strands from the annealed primers and then heated at 70°C for 5 min to inactivate the enzymes. An *E. coli* strain, BMH71-18mutS (Takara Shuzo), was transformed with 0.01 μg of the mutated plasmid DNA and seeded onto LB broth plates containing 50 μg/ml ampicillin. The plasmid DNA was recovered from each ampicillin-resistant colony proliferated on the plate. Introduction of an *Mlu*I site into the pS+B/Mlu plasmids was checked by digestion with *Mlu*I.

2.5. Amplification of the env gene fragments by PCR

The DNA fragments of the *env* gene were amplified by PCR using MK603/1590C, Bg3/Bg4, P2/P3, and 5′V3NLN/3′V3NLR as primer pairs, and the pNL4-3 or pGW322 plasmid as a template (Fig. 4a).

2.6. Site-directed mutagenesis

Site-directed mutagenesis of the V3 domain was done using the mutagenic oligonucleotide primers such as NL/BHI, NL/Ser, NL/Thr, NL/Ala, NL/Leu, and NL/Arg and the pS+B/Mlu plasmid as a template (Fig. 1a,b).

After annealing the pS+B/Mlu plasmid with one of these primers, the primer DNA was extended by DNA polymerase III and digested with *Mlu*I. Then, *E. coli*, BMH71-18mutS, were transformed with one of the mutated plasmids and cultured at 37°C for 16 h.

The plasmids were recovered from *E. coli*, propagated in the medium containing ampicillin and digested with *Mlu*I to select the mutants. This selection cycle was repeated several times to concentrate the mutants. *E. coli* strain HB101 was used as a host of the plasmids after the second selection cycle. Introduction of the site-directed mutation was checked by sequencing the plasmid DNA or by examining their digestion patterns with several restriction enzymes.

2.7. Production of mutant viruses

The DNA fragments of the *env* gene were isolated from the mutated pS+B/Mlu plasmids by digestion with *Bam*HI and *Sal*I and then ligated into the original position of the pNL4-3 plasmid to make infectious DNA clones. The infectious DNA clones of HIV-1 were transfected into C8166 cells using Lipofectamine (Gibco-BRL, USA) following the manufacturer's protocol. Production of HIV-1 from C8166 cells was confirmed by detecting syncytia in C8166 cells or reverse transcriptase activity in the culture supernatants of C8166 cells [17].

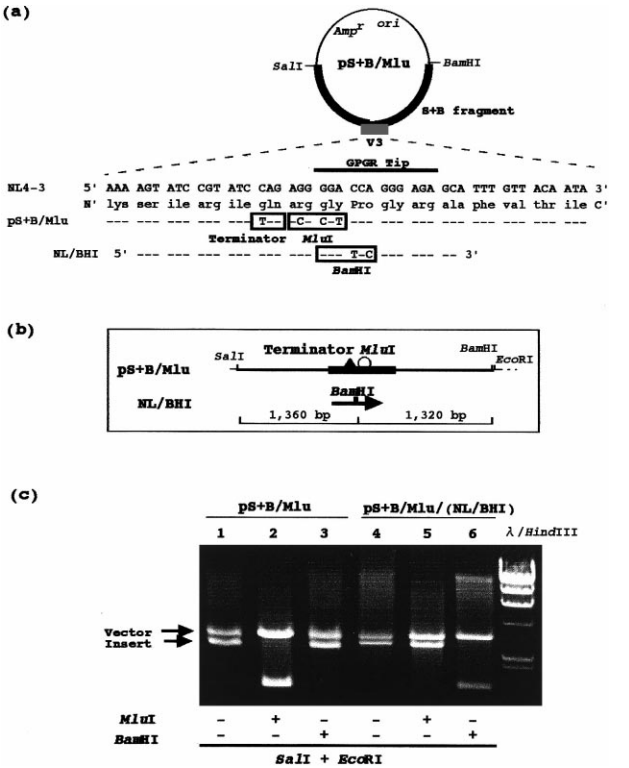

Fig. 1. Site-directed mutagenesis by a single bifunctinal primer. a: Nucleotide sequences of the target region of mutagenesis in the pS+B/Mlu plasmid and the bifunctional NL/BHI primer are shown together with the sequence of the V3 domain of the *env* gene of HIV-1 strain NL4-3. b: Schematic structure of the target region of mutagenesis in the pS+B/Mlu plasmid is shown together with the position of the NL/BHI primer. The NL/BHI primer was designed to suppress both the *Mlu*I site and the termination codon of the *env* gene and introduce the *Bam*HI site into the pS+B/Mlu plasmid. c: Restriction enzyme cutting patterns of the pS+B/Mlu and the pS+B/Mlu/(NL/BHI) plasmids. These plasmids were produced by the mutagenesis of the pS+B/Mlu plasmid using the NL/BHI primer. The insert DNA could be separated from these plasmids by digestion with *Sal*I and *Eco*RI and the *Bam*HI site was used to estimate the efficiency of mutagenesis using the NL/BHI primer. Because the *Eco*RI site was also unique and located close to the *Bam*HI site in the pS+B/Mlu plasmid, *Eco*RI and *Sal*I were used to separate the insert DNA.

3. Results

3.1. Formation of a template plasmid for mutagenesis having two selection markers that function at different steps

We introduced two sequences, an *Mlu*I site and an in-frame termination codon, located close to each other into the V3 domain in the *env* gene of the pS+B plasmid to make a template plasmid for site-directed mutagenesis (Fig. 1a). An *Mlu*I site was used, because the *env* genes of NL4-3 and GUN-1WT strains had no *Mlu*I site. The plasmid obtained was designated pS+B/Mlu. These two sequences act independently as selection markers at different steps in the mutagenesis.

A bifunctional mutagenic and selectable primer such as NL/BHI was designed to suppress the *Mlu*I selection marker and to introduce mutation at the same time (Fig. 1a,b). Loss of an *Mlu*I site in recovered plasmid DNA indicates successful introduction of a specific mutation. Two independent primers, i.e. a selection primer and a mutagenic primer, are necessary

for most kits commercially available now. A selected clone is not always an expected mutant. In our system, a single primer acts bifunctionally, i.e. as a mutagenic and as a selection primer. Therefore, a clone resistant to digestion with *Mlu*I was highly expected to be a clone introduced with the mutation.

The second marker, an in-frame stop codon in the *env* gene, will function in the translation process of the *env* gene (Fig. 1a). The *env* gene having this in-frame stop codon will not lead to production of progeny viruses. When the plasmid is successfully mutated by a mutagenic primer, the stop codon will be suppressed and the competent *env* protein will be translated. Spontaneous nucleotide substitutions at the *Mlu*I site during the mutagenesis process will remove this site without introduction of an expected mutation. The second selection marker will suppress the growth of HIV-1 containing these spontaneous mutations.

3.2. Simple protocol for site-directed mutagenesis using a bifunctional primer

The outline of our system for site-directed mutagenesis using a single bifunctional primer is shown in Fig. 2. We used *E. coli* DNA polymerase III (Pol III) (Toyobo) for this purpose, because Pol III is expected to synthesize DNA strand at a speed of 700 bp or over per second with high fidelity. The

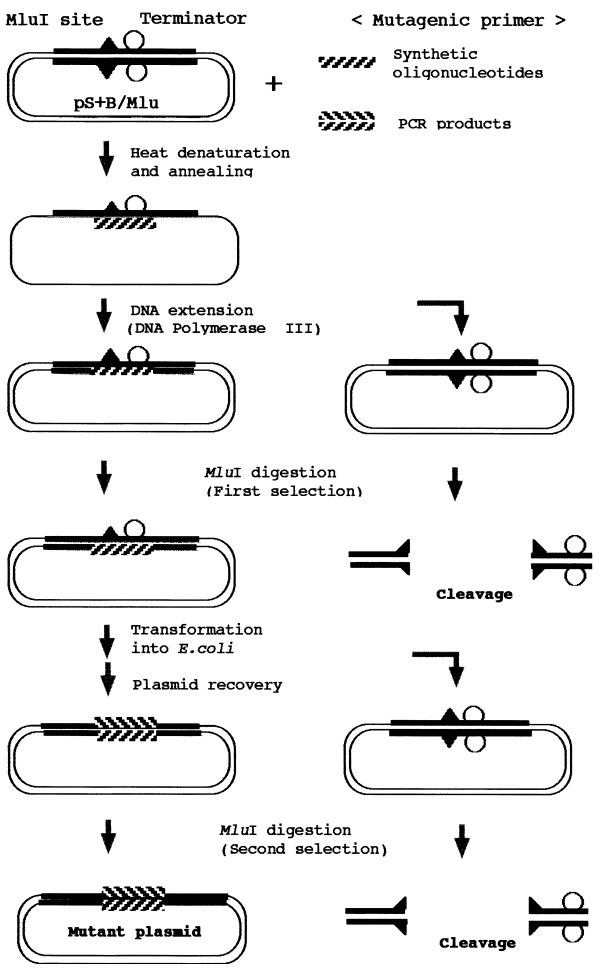

Fig. 2. Schematic explanation of the protocol for the site-directed mutagenesis using a single bifunctional primer. Open circle, the in-frame termination codon of the *env* gene; closed triangle, an *Mlu*I site in the pS+B/Mlu plasmid; striped box, a mutagenic primer.

DNA extension by DNA polymerase III has to be done at 37°C. After heat denaturation of the template plasmid DNA and mutagenic primers, the samples were cooled on ice to anneal them before extension. Therefore, the annealing temperature was possibly not high, although T_m values for all primers used in the mutagenesis were around 65°C. However, immediate cooling of the template plasmid DNA and mutagenic primers after heat denaturation is critical, because gradual cooling will give unexpected mutants which are probably produced by non-specific annealing of the template plasmid DNA with primers [5] and we confirmed this. The SSB (USB, Cleveland, OH) was used in the reaction of DNA extension, because it will maximize the activity of Pol III by blocking the formation of undesirable secondary structures in the template plasmid DNA.

3.3. Easy identification of the mutants

Fig. 1 shows the digestion patterns of DNA of the pS+B/Mlu template plasmid and its mutant plasmid, pS+B/Mlu(NL/BHI), which was produced by mutagenesis using the mutagenic NL/BHI primer, with several restriction enzymes. The 2680 bp insert DNA was produced from the pS+B/Mlu or pS+B/Mlu(NL/BHI) plasmid after digestion with *Sal*I and *Eco*RI (Fig. 1c, lanes 1 and 4). Before mutagenesis the insert DNA was digested with *Mlu*I (Fig. 1c, lane 2), but not with *Bam*HI (Fig. 1c, lane 3). When the pS+B/Mlu plasmid had been mutated by the NL/BHI primer, the insert DNA was cleaved with *Bam*HI but not with *Mlu*I, indicating that the *Mlu*I site was removed from the template plasmid and the *Bam*HI site was introduced into it by the primer (Fig. 1c, lane 5 and 6).

3.4. Efficient introduction of a mutation using a bifunctional primer

We estimated the efficiency of site-directed mutagenesis using a bifunctional primer containing a *Bam*HI site (Fig. 3). After each selection cycle, the plasmid DNA was isolated from *E. coli* transformants, which had proliferated in the liquid medium containing ampicillin, and examined for the presence or absence of the *Mlu*I site in it (Fig. 3a). In parallel, the *E. coli* transformants obtained were seeded onto agar plates containing ampicillin.

The plasmid DNA was isolated from colonies proliferated on the plates by the minipreparation method and the ratios of colonies containing the plasmids which had been successfully introduced with the *Bam*HI site were determined (Fig. 3b).

After the first selection cycle, only 10% of colonies formed on an agar plate contained plasmids without the *Mlu*I site (Fig. 3a, lane 1 and Fig. 3b). In the first selection cycle, a mutagenic primer anneal only to a complementary strand of the template plasmid (Fig. 3). Therefore, at least half of the plasmids propagating after the first selection cycle will keep the initial phenotype, even though digestion with *Mlu*I is done perfectly. The second selection cycle produced a markedly increased ratio of colonies containing the mutant plasmids: 70% of the colonies contained the plasmids lacking the *Mlu*I site (Fig. 3a, lane 2, and Fig. 3b). After the third selection cycle, 98% of the colonies contained the mutant plasmids (Fig. 3a, lane 3, and Fig. 3b). All plasmids lacking the *Mlu*I site were introduced with new *Bam*HI sites, indicating that these plasmids were the mutants produced from the pS+B/Mlu plasmids by mutagenesis using the NL/BHI primer. When

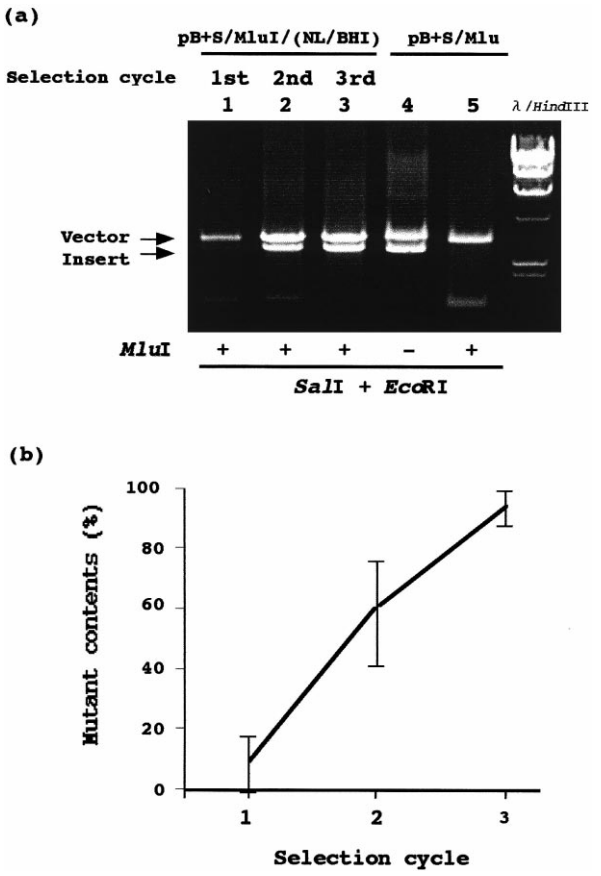

Fig. 3. Efficiency of the site-directed mutagenesis using the NL/BHI primer. a: The plasmids were isolated from *E. coli* cultured in liquid medium after every selection cycle. The plasmids were digested with *Sal*I and *Eco*RI to separate the insert DNA from the vector. The insert DNA was cleaved with *Mlu*I to estimate the ratios of mutants to the parental template plasmids. b: *E. coli* propagated in liquid medium were cultured on LB plates. The ratios of colonies containing the *Mlu*I-resistant but *Bam*HI-sensitive plasmids to those containing the *Mlu*I-sensitive but *Bam*HI-resistant plasmids were determined.

other mutagenic primers were used, the ratios of colonies containing other mutant plasmids were over 98% after three *Mlu*I selection cycles (data not shown). These results indicate that three cycles of the *Mlu*I selection are enough to obtain highly concentrated mutants.

3.5. Site-directed mutagenesis using PCR-amplified DNA fragments longer than 1 kbp

DNA fragments longer than 1 kbp were used in the site-directed mutagenesis we had developed. As shown in Fig. 4a, eight DNA fragments of the *env* gene, fragments a (1611 bp), b (613 bp), c (326 bp), d (189 bp), e (1592 bp), f (608 bp), g (320 bp), and h (185 bp), were amplified by PCR using the pNL4-3 or pGW322 plasmid DNA as a template. The *Nhe*I site in the *env* gene of the pNL4-3 template plasmid was used as a marker to assess the efficiency of the introduction of mutations. DNA fragments a, b, and c from the *env* gene of this plasmid had *Nhe*I sites, while fragment d did not have an *Nhe*I site, because this DNA fragment was amplified from the 5′-region upstream of the *Nhe*I site in the *env* gene of the NL4-3 plasmid. The DNA fragments e, f, g, and h amplified from the *env* gene of the pGW322 plasmid had no *Nhe*I site. The pPs-b/Mlu plasmid, which contained the *env* gene of strain GUN-1WT, was constructed by introducing an *Mlu*I site as a selection marker into the V3 domain. The presence and absence of the *Nhe*I site in the insert DNA of the pS+B/Mlu or pPs-b/Mlu plasmid indicated successful and unsuccessful mutagenesis, respectively using these PCR-amplified DNA fragments. The insert DNA fragments were cleaved from the vector DNA by digestion of the pPs-b plasmid with *Pst*I or the pS+B/Mlu plasmid with *Sal*I and *Bam*HI (Fig. 4b, lanes 5 and 12). When the pPs-b/Mlu plasmid was mutated using fragments a, b, or c, the insert DNA became sensitive to digestion with *Nhe*I, indicating that the PCR-amplified DNA fragments having the *Nhe*I site were successfully introduced into the *env* sequence of the pPs-b/Mlu plasmid (Fig. 4b, lanes 1–3). The pPs-b/Mlu plasmid mutated by fragment d, which had no *Nhe*I site, produced the insert DNA without an *Nhe*I site (Fig. 4b, lane 4). When the pS+B/Mlu plasmids were mutated using fragments e, f, and g, which had been amplified from the GUN-1WT DNA lacking an *Nhe*I site, the plasmids lost the *Nhe*I site (Fig. 4b, lanes 8–10). The

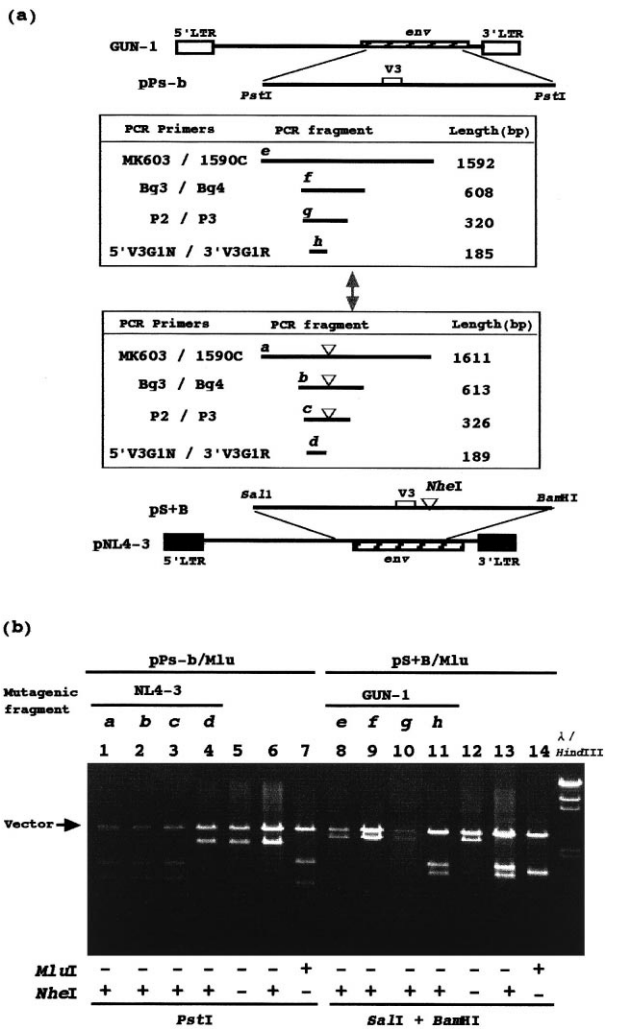

Fig. 4. Site-directed mutagenesis using the PCR-amplified *env* gene fragments. a: PCR-amplified DNA fragments used as the mutagenic primers are shown with their length, templates, and primers. b: Restriction enzyme cutting patterns are shown for the pS+B/Mlu and pPs-b/Mlu plasmids and the plasmids obtained by mutagenesis using PCR-amplified DNA fragments.

*Nhe*I site in the pS+B/Mlu plasmid could not be removed by mutagenesis using fragment h, because this fragment did not contain an *Nhe*I site (Fig. 4b, lane 11). These results indicated that not only oligonucleotides but also long PCR-amplified DNA fragments could be used as mutagenic primers for the site-directed mutagenesis. Spontaneous formation of an *Nhe*I site in the template plasmids was not detected by mutagenesis using PCR-amplified DNA fragments having no *Nhe*I site. Also in the case of mutagenesis using PCR-amplified DNA fragments as mutagenic primers, the efficiencies of introduction of these fragments into template plasmids were similar to those observed when oligonucleotides were used as mutagenic primers (data not shown). Using this system, precise DNA recombinations can be done, even though there are no suitable restriction sites for recombination.

The insert DNA fragments of all mutants obtained here were ligated into the original positions of the pNL4-3 plasmid to form infectious DNA clones of HIV-1. When these ligated DNA clones were introduced into C8166 cells by DNA transfection, the production of progeny viruses was confirmed by the detection of the reverse transcriptase activities in the culture supernatants and syncytia in C8166 cells. The pNL4-3 plasmid which had been reconstituted with the *env* sequence from the pS+B/Mlu template plasmid for mutagenesis could not result in the production of progeny viruses, suggesting that the in-frame termination codon of the *env* gene in the pB+S/Mlu plasmid could inhibit its translation. These findings suggest that the in-frame termination codon of the *env* gene in the pB+S/Mlu plasmid acts as another selection marker for mutants, because the competent *env* protein could not be translated from this plasmid unless the in-frame termination codon of the *env* gene is suppressed by the mutagenesis.

4. Discussion

In this study, we have developed a novel system for site-directed mutagenesis. We introduced two independent selection markers at the target region of mutagenesis. One of them, the *Mlu*I site, acts to select successfully mutated plasmids (Figs. 1 and 2). The other marker, the in-frame termination codon of the *env* gene, acts in the translation step of the gene. We made these two selection markers to reduce spontaneous mutants as much as possible, because the probability that the spontaneous mutations of nucleotides of the template plasmids suppress both of these selection markers is expected to be low enough. Therefore, the clones obtained in this site-directed mutagenesis are highly expected to be mutants. Actually, no spontaneous mutant was detected in all mutants obtained in the mutagenesis in this study.

This system will be effective to achieve a high efficiency of mutagenesis. The possibility that selected clones are not mutants will be higher in other methods using two primers than in our system where the introduction of a mutation and the selection of a mutant were performed at the same time by a single bifunctional primer. Because the two markers were located close enough to be covered by a single primer, a clone selected in this system was highly expected to be a mutant. As shown in Fig. 3, a few rounds of selection of plasmid DNA by digestion with *Mlu*I were enough to obtain highly concentrated mutants.

The system for site-directed mutagenesis using a single oligonucleotide primer which contains two markers is easily applicable to genes other than the *env* gene of HIV-1. For these genes, a restriction site that is absent in a template plasmid should be adopted as a selection marker.

As shown in Fig. 4, DNA fragments longer than 1 kbp could be used as mutagenic primers in this system. Therefore, this system is a useful method to analyze precisely the functions of various genes, because recombination of precise regions of the genes can be performed even in the absence of suitable restriction sites.

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