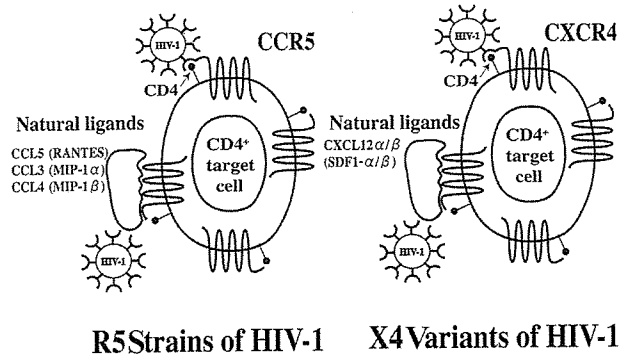

Figure 1. Host factors involved in the entry of human immunodeficiency virus 1 (HIV-1). CD4 is the main HIV-1 receptor for attachment; however, HIV-1 needs a coreceptor for entry into target cells. See text for details.

3. Genetic Polymorphisms Affecting HIV-1 Infection and Pathogenesis

Table 1 shows genetic polymorphisms that affect HIV-1 infection and pathogenesis [1-36].

3.1. CCR5 Δ32

The most potent anti–HIV-1 genetic variant, *CCR5 Δ32*, was discovered soon after the identification of CCR5 as an HIV-1 coreceptor. There have been reports of small numbers of individuals who do not become infected with HIV-1 despite repetitive sexual exposure to HIV-1 in extremely high-risk situations. $CD4^+$ cells obtained from 2 such individuals were found to be highly resistant in vitro to infection with R5 strains of HIV-1, but they were readily infected with X4 variants. A genetic analysis revealed that these 2 individuals carried a homozygous defect in the *CCR5* gene. The defective *CCR5* allele contained a 32-bp deletion corresponding to the second extracellular loop of CCR5. The encoded receptor was nonfunctional, explaining the resistance to infection with R5 strains of HIV-1 [1]. Several population studies have consistently revealed that approximately 1% of the Caucasian population of Western European ancestry are homozygous for the defect and that 10% to 20% are heterozygous for the defect. In contrast, DNA samples from western and central Africa and Asia did not reveal a single mutant allele, suggesting that the allele is either absent or extremely rare in Africa and Asia [37]. One study showed no homozygotes among 1400 HIV-1–infected Caucasian individuals, strongly supporting the concept that the homozygous defect confers protection against HIV-1 infection [2]. This finding is in a good agreement with the fact that transmitting viruses from individual to individual is strongly biased toward R5 strains of HIV-1. On the other hand, there was a higher frequency of heterozygotes among HIV-1–infected long-term nonprogressors than among rapid or standard progressors, suggesting that the heterozygous defect delays progression to AIDS. A few HIV-1–infected individuals who were in fact homozygous for the *CCR5 Δ32* allele have been identified. These individuals were found to have an X4 variant of HIV-1 [38].

Although *CCR5 Δ32* is present only in Caucasians, other defective *CCR5* alleles are distributed among different ethnic groups. The less frequent *CCR5 m303* allele, a nonsense mutation at codon 101, is present in both Caucasians and Africans [5]. A single nucleotide deletion at position 893 that impairs surface trafficking of CCR5 is observed only in eastern Asia countries such as China and Japan [8]. A point mutation at codon 106 that also impairs surface trafficking of CCR5 is observed only in southeastern Asia countries such as Thailand (unpublished data) and Vietnam [39].

3.2. CCR2 64I

The chemokine receptor CCR2B has been regarded as a minor HIV-1 coreceptor, because only a small number of HIV-1 strains have been shown to use CCR2B as an entry coreceptor. Nevertheless, a single-nucleotide polymorphism (SNP) in the *CCR2* gene, *CCR2 64I*, has been reported to be associated with delayed disease progression in HIV-1–infected individuals in a Caucasian cohort [9]. This polymorphism, a G-to-A transition at position 190, changes CCR2B codon 64 from valine to isoleucine, introducing a conservative amino acid change into the first transmembrane domain. Several independent cohort studies have shown that the effects of the *CCR2 64I* allele are more pronounced in earlier stages of disease than in later stages [6,40]. In a Dutch cohort, delay in HIV-1 disease progression was more pronounced in individuals with the *CCR2 64I* allele before the emergence of X4 variants and was not observed after the emergence of X4 variants [41]. These epidemiologic data suggest that the *CCR2 64I* allele affects replication of R5 HIV-1 in infected individuals. However, the initial biochemical studies failed to show any differences in HIV-1 coreceptor activity between variant CCR2B-64I and CCR2B without the 64I substitution. Furthermore, the results of these studies also disallowed the possibility that CCR2B-64I interferes with the expression and activity of CCR5. Recently, we showed that CCR2A, a splice variant of CCR2B that differs only in the carboxyl-terminal cytoplasmic tail, binds to CCR5 in the cytoplasm to down-modulate its surface expression. We also showed that the 64I substitution increased the half-life of CCR2A in cells and caused more severe CCR5 down-modulation than CCR2A without the 64I substitution. These results suggest that more severe CCR5 down-modulation by CCR2A with 64I is the major cause of the delay in HIV-1 disease progression in patients with this allele [42]. In contrast to *CCR5 Δ32*, this allele is more prevalent in Asian populations than in Caucasians, with the allele frequency approaching 30% in eastern Asia countries.

3.3. CCR5 P1

SNPs in the *CCR5* promoter region also affect levels of CCR5 expression and rates of HIV-1 disease progression. The disease of patients homozygous for *CCR5* promoter haplotype 1 (*P1*) and without *CCR5 Δ32* or *CCR2 64I* alleles progressed more rapidly than that of other patients [7]. The *P1* promoter fused with reporter genes showed higher pro-

Table 1.
Host Genetic Polymorphisms Affecting Human Immunodeficiency Virus (HIV) Infection*

Gene	Allele	Position	Molecular Mechanism	Effect on HIV	References
CCR5	*Δ 32*	ORF	Premature stop codon	Protection from viral infection (homozygotes)	[1,2]
				Decelerates disease progression	[3,4]
	m303	ORF	Premature stop codon	Protection from viral infection (homozygotes)	[5]
	927T	Intron	Linkage disequilibrium with *CCR2 64I*	Decelerates disease progression (homozygotes or heterozygotes)	[6]
	P1	Promoter	Increase in promoter activity	Accelerates disease progression (homozygotes)	[7]
	893(–)	ORF	Decrease in cell surface expression	Decelerates disease progression?	[8]
CCR2	*64I*	ORF	Single amino acid	Decelerates disease progression (homozygotes or heterozygotes)	[4,9]
			substitution	Protection from viral infection (homozygotes)	[10]
CX3CR1	*280M*	ORF	Single amino acid substitution	Accelerates disease progression (homozygotes)	[11,12]
SDF1 (CXCL12)	*3'A*	3' Noncoding	ND	Decelerates disease progression (homozygotes)	[4,13,14]
RANTES (CCL5)	*In1.1C*	Intron	Decrease in promoter activity	Accelerates disease progression (homozygotes or heterozygotes)	[15-18]
	–28G	Promoter	Increase in promoter activity	Decelerates disease progression (homozygotes or heterozygotes)	[18,19]
CCL3L1		Gene number	Lower mRNA copy numbers	Susceptible to viral infection	[20]
IL10	*5'A*	Promoter	Decrease in promoter activity	Accelerates disease progression (homozygotes or heterozygotes)	[21]
IL4	*–589T*	Promoter	Increase in promoter activity	Decelerates disease progression (homozygotes or heterozygotes)	[18,22]
CD45	*77G*	ORF	Alternative splicing	Susceptible to viral infection (heterozygotes)	[23]
KIRs (+HLA-B)	*KIR3DS1 (+HLA-B Bw4-80Ile)*	Wide (including ORF)	Active form of KIRs	Decelerates disease progression (homozygotes or heterozygotes)	[24]
APOBEC3G	*186R*	ORF	Single amino acid substitution	Accelerates disease progression (homozygotes or heterozygotes)	[25]
IL6	*–176G*	Promoter	Increase in promoter activity	Increased risk of Kaposi sarcoma (homozygotes)	[26]
SREBP1c/ADD1	*3'322C*	ORF	Change in mRNA structure?	Hyperlipoproteinemia (homozygotes or heterozygotes)	[27]
TNFα	*–238A*	Promoter	Decrease in promoter activity?	Increased risk of lipodystrophy (heterozygotes)	[28]
P450 (CYP)	*2D6*6 (172H and 262R)*	ORF	2 Amino acid substitutions	High plasma efavirenz concentration (homozygotes)	[29]
HLA	*B*27*			Decelerates disease progression	[30]
	*B*57*			Decelerates disease progression	[31]
	*B*35*			Accelerates disease progression	[32,33]
	*B*08*			Accelerates disease progression	[30]
	Homozygosity			Accelerates disease progression	[33]
	Rare HLA haplotype			Lower viral load	[34]
	*HLA-B*5701, HLA-DR7, HLA-DQ3*			Hypersensitivity to abacavir	[35,36]

*ORF indicates open reading frame; SDF1, stromal cell–derived factor 1; ND, not determined; RANTES, regulated on activation normal T-cell expressed and secreted; mRNA, messenger RNA; KIR, killer immunoglobulin-like receptor; APOBEC3G, apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G; SREBP1c/ADD1, strol-regulatory element–binding protein 1c/adipocyte determination and differentiation factor 1; TNFα, tumor necrosis factor α; HLA, human leukocyte antigen.

moter activity in transfected cells than the other haplotype [43]. Consistent with this finding were observations of higher levels of CCR5 expression in $CD14^+$ monocytes isolated from individuals homozygous for *P1* [44]. It is reasonable to assume that high levels of CCR5 expression would offer a more favorable environment for replication of the R5 strain of HIV-1 and hence accelerate disease progression. It is noteworthy that the protective *CCR5 Δ32* and *CCR2 64I* alleles are always associated with the *P1* haplotype. It is possible that a certain selective pressure during human and primate evolution has favored low levels of CCR5 expression and that such pressure may have facilitated expansion of these alleles in the human population.

3.4. SDF1 3'A

SDF-1α and SDF-1β are natural ligands of CXCR4 that inhibit replication of X4 variants of HIV-1. *SDF1 3'A*, a G-to-A SNP in the 3' noncoding region of SDF-1β, was first reported to be associated with a delay in HIV-1 disease progression [13]. However, subsequent studies did not show consistent results, and an international meta-analysis of large numbers of HIV-1–infected Europeans and Americans of European and African origins failed to confirm the protective effect of *SDF1 3'A* against HIV-1 disease progression [4]. So far, there is no evidence that this allele alters levels of SDF-1α or SDF-1β. Nevertheless, our recent study and those of others have shown a high allele frequency and apparent protective effects of *SDF1 3'A* against HIV-1 transmission and disease progression in Thais [45]. It is possible that *SDF1 3'A* exerts a stronger effect on HIV-1 infection in Thais than in Americans of European and African origins or that its high allele frequency in Thailand enables the detection of a very subtle difference.

3.5. RANTES SNPs

Two SNPs that have been found in the *RANTES* gene correlate with either increased or decreased expression of RANTES. The *RANTES –28G* allele is in the promoter region of the *RANTES* gene and up-regulates *RANTES* gene transcription and secretion of this chemokine. As described above, RANTES is an inhibitor of R5 strains of HIV-1. As expected, *RANTES –28G* is associated with delayed progression to AIDS [19]. The frequency of this allele is approximately 15% in eastern Asia, 5% to 8% in southeastern Asia, and 2% in Europeans and European Americans; it is absent in Africans and African Americans [46]. The *RANTES In1.1C* SNP is in the intron of the *RANTES* gene and negatively regulates *RANTES* gene transcription. Therefore, this SNP correlates strongly with rapid progression to AIDS [17]. It is interesting that the protective *RANTES –28G* allele is in strong linkage disequilibrium with the deleterious *RANTES In1.1C* allele, and *RANTES –28G* has been shown to exert a dominant effect over *RANTES In1.1C* in Thailand [18].

3.6. IL4 –589T

Interleukin (IL)-4 is known to down-regulate expression of CCR5. The SNP *IL4 –589T* is in the promoter region of the *IL4* gene and increases IL-4 transcription. This allele is associated with a slower progression to AIDS, presumably through the down-regulation of CCR5 by higher levels of IL-4. The frequency of this allele is 15% in Europeans but is as high as 75% in Asian countries [18,22].

3.7. IL10 5'A

IL-10 suppresses HIV-1 replication. Individuals who carry *IL10 5'A* in the *IL10* promoter are at increased risk of HIV-1 infection and progress more rapidly to AIDS following infection than those without this allele. This rapid progression is most likely because *IL10 5'A* decreases IL-10 transcription, resulting in the facilitation of HIV-1 replication [21].

3.8. Human Leukocyte Antigen

Heterozygosity for human leukocyte antigen genes (*HLA* genes) for class I loci (A, B, and C) has clearly been demonstrated to be associated with a delayed disease progression to AIDS among HIV-infected individuals, whereas homozygosity for these loci has been associated with a more rapid progression to AIDS and death [33]. This heterozygote advantage is most likely because individuals who are heterozygous for *HLA* loci are able to present higher numbers of antigenic epitopes to cytotoxic T-lymphocytes than homozygotes, resulting in a more effective immune response against HIV-1. It is noteworthy that *HLA* class I alleles *B*35* and *Cw*04* have consistently been associated with a more rapid progression to AIDS [33].

HIV-1 strains in infected individuals have been shown to escape from cytotoxic T-cells. In many cases, HIV-1 strains that enter a new host at the time of primary infection have already escaped from the cytotoxic T-cells of the infecting partner. Therefore, certain rare *HLA* class I and class II alleles facilitate a more effective immune response upon primary infection, leading to slower disease progression to AIDS. Conversely, common *HLA* alleles could lead to less effective immune responses, resulting in more rapid progression to AIDS [34].

A type of killer immunoglobulin-like receptor (*KIR*) gene, *KIR3 DS1*, was shown to be strongly associated with rapid progression to AIDS. However, HIV-1–infected individuals carrying the *KIR3 DS1* and *HLA-B Bw4-80I* alleles showed a slower progression to AIDS, even though this *HLA-B* allele alone has no effect on HIV-1 disease progression. These individuals showed a significantly reduced viral load, suggesting a potential role of natural killer cells in the control of viral load. These results also suggest that HLA-B Bw4-80I serves as a ligand for activating this KIR receptor to kill target cells [24].

4. Future Perspective

As described above, *CCR5 Δ32* and *CCR2 64I* were first identified as human genetic variants that modulate the susceptibility to HIV-1 infection and the rate of disease progression to AIDS. These polymorphisms are now under evaluation for their effects on other human diseases. *CCR5 Δ32* has been reported to reduce the risk of myocardial infarction [47]. *CCR2 64I* is underrepresented in renal transplant recip-

ients who experience acute rejection [48]. It is reasonable to assume that genetic polymorphisms that affect levels of CCR5 expression also have effects on other human diseases in which CCR5 or its natural ligands play major roles.

Efavirenz, a nonnucleoside reverse transcriptase inhibitor, is metabolized by cytochrome P450 2B6. An investigation of plasma efavirenz concentration and genetic polymorphisms in the *CYP2B6* gene of HIV-1–infected individuals who received this drug in Japan revealed that patients homozygous for the glutamine-to-histidine and lysine-to-arginine substitutions at positions 172 and 262, respectively, were able to maintain a significantly higher plasma concentration of efavirenz [29]. The frequency of this allele is approximately 20% in Japan, and 1 of 25 persons are homozygous for this allele. In these patients, the efavirenz dose could be decreased to reduce the adverse effects of this drug. Further studies on human genetic polymorphisms and the efficacy and/or adverse effects of antiretroviral therapy are now being undertaken to establish "tailor-made" therapy for HIV-1 infection.

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A three-base-deletion polymorphism in the upstream non-coding region of human interleukin 7 (IL-7) gene could enhance levels of IL-7 expression

H. Song,* E. E. Nakayama,* S. Likanonsakul,† C. Wasi,‡ A. Iwamoto§ & T. Shioda*

Summary

Interleukin 7 (IL-7) is a key factor in the survival, development and proliferation of B and T lymphocytes. Elevation of plasma IL-7 has been reported in several lymphopenia cases such as HIV-1 patients. After patients started to receive antiretroviral drugs and their $CD4^+$ cell counts had recovered, IL-7 in plasma decreased to normal levels. There are considerable variations in the levels of plasma IL-7 as well as the rate of $CD4^+$ T-cell restoration. Although pre-treatment plasma IL-7 levels have been shown to be prognostic for the rate of post-treatment $CD4^+$ T-cell restoration, the mechanisms responsible for the variations in plasma IL-7 and rate of $CD4^+$ T-cell restoration are still completely unknown. In the study here, we searched for genetic polymorphisms that might affect levels of IL-7 gene expression. For this purpose, we used 1658-bp PCR-amplified fragments of the IL-7 gene containing 1470 bp of the upstream non-coding region obtained from 151 Japanese and 234 Thai subjects. We found two novel human genetic polymorphisms in the upstream non-coding region of the IL-7 gene. The luciferase reporter assay demonstrated that one of those polymorphisms could increase the gene expression of IL-7. We speculate that this polymorphism, a three base ATC deletion just upstream of an out-of-frame ATG codon in the upstream non-coding region of the IL-7 gene, reduces the efficiency of translation from the upstream, out-of-frame ATG, resulting in increased translation efficiency from the authentic ATG of IL-7. Although the frequency of this allele is very low, it would be interesting to analyse this polymorphism in HIV-1-infected individuals with different rates of immune reconstitution after treatment with a highly active antiretroviral therapy.

* Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, † Bamrasnaradura Institute, Nonthaburi, Thailand, ‡ Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand and § Division of Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo, Japan

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Correspondence: Tatsuo Shioda, Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, 3–1 Yamada-Oka, Suita, Osaka 565-0871, Japan. Tel: +81 6 6879 8346; Fax: +81 6 6879 8347; E-mail: shioda@biken.osaka-u.ac.jp

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Haihan Song, E-mail: hhsong@biken.osaka-u.ac.jp
Emi E. Nakayama, E-mail: emien@biken.osaka-u.ac.jp
Sirirat Likanonsakul, E-mail: siratlik@health.moph.go.th
Chantapong Wasi, E-mail: sicws@mahidol.ac.th
Aikichi Iwamoto, E-mail: aikichi@ims.u-tokyo.ac.jp

Introduction

Human interleukin 7 (IL-7) is a cytokine produced by stromal cells of the thymus and bone marrow (Wolf & Cohen, 1992; Heufler *et al.*, 1993; Sudo *et al.*, 1993) and has the capacity to induce growth of immature B lymphocytes (Namen *et al.*, 1988). Similarly, IL-7 contributes to the development, proliferation and homeostatic maintenance of T cells (Grabstein *et al.*, 1990; Plum *et al.*, 1996; Schluns *et al.*, 2000; Fry *et al.*, 2001). Human IL-7 gene located on chromosome 8q12–13, has six exons that distributed to more than 33-Kb of genomic DNA (Lupton *et al.*, 1990; Fry & Mackall, 2002). It is known that the IL-7 gene has no canonical core promoter sequence in the 5′ upstream region (Lupton *et al.*, 1990; Oshima *et al.*, 2004). Recently, it has been reported, however, that transcription start sites of the IL-7 gene are clustered within two distinct regions that are approximately 515 bp to 600 bp and 130 bp to 217 bp upstream from the translation initiation ATG codon (Oshima *et al.*, 2004). Moreover, the region −282 to −251 upstream from the initiation ATG codon contains an interferon regulatory factor element (IRF-E) and could thus up-regulate the transcription of the IL-7 gene upon stimulation with gamma interferon (IFN-γ) in human intestinal epithelial cells (Oshima *et al.*, 2004). This study also revealed the presence of several out-of-frame ATG codons with unknown function in the upstream non-coding region of the IL-7 gene (Oshima *et al.*, 2004).

With respect to HIV-1 infection, there is a reverse correlation between $CD4^+$ T-cell numbers and IL-7 plasma levels in HIV-1-infected patients (Llano *et al.*, 2001; Beq *et al.*, 2004; Kopka *et al.*, 2005). After these patients started to receive antiretroviral drugs and their $CD4^+$ T-cell counts had recovered, the elevated IL-7 in the plasma decreased to normal levels (Llano *et al.*, 2001). Furthermore, it is well known that there are considerable variations in the levels of plasma IL-7 as well as the rate of CD4 T-cell restoration after HIV-1 patients started to

receive antiretroviral drugs, and pre-treatment plasma IL-7 levels have been shown to be prognostic for the rate of post-treatment CD4 T-cell restoration (Beq *et al.*, 2004). However, knowledge of the molecular mechanisms controlling IL-7 gene expression remains very limited, and the mechanisms responsible for the variations in plasma IL-7 levels and rate of CD4 T-cell restoration among individuals are still completely unknown.

Human genetic polymorphisms have recently been shown to affect expression of the corresponding genes and to consequently modify the clinical course of several human diseases such as HIV-1 infection (Dean *et al.*, 1996; Michael *et al.*, 1997; Kostrikis *et al.*, 1998; Liu *et al.*, 1999; Nakayama *et al.*, 2000). We aimed to know the molecular mechanisms controlling variations in IL-7 gene expression among individuals. For this purpose, we searched for genetic polymorphisms that might affect levels of IL-7 gene expression in 1658-bp PCR-amplified fragments of the IL-7 gene containing 1470 bp of the upstream non-coding region, 9 bp of the first coding exon and 179 bp of the downstream intron, although there was no previous report on human genetic polymorphisms that alter the levels of IL-7 gene expression. We found two novel human genetic polymorphisms in the upstream non-coding region of the IL-7 gene, one of which could enhance IL-7 expression probably by reducing the efficiency of translation from an upstream, out-of-frame ATG that would result in diminished efficiency of translation from the downstream initiation ATG.

Materials and methods

Genotyping of IL-7 gene

Human genomic DNA was obtained from peripheral blood mononuclear cells of 52 unrelated non-HIV-1-infected and 99 HIV-1-infected Japanese, as well as 122 non-HIV-1-infected and 112 HIV-infected Thais, who provided written informed consent. Genomic regions of 1658 nucleotides containing 1470 nucleotides of the upstream non-coding region and the first exon and part of the intron of IL-7 were amplified by using the primer pair P1: 5′-TCCCTCCTCTTCCTTGTTC-3′ and P2: 5′-GGTTCAAGTGGCTATGTGC-3′. Polymerase chain reaction (PCR) was run for 40 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 2 min. Fluorescence-based automated cycle sequencing of the PCR products was then carried out by an ABI 3100 using P1, P2 (mentioned previously), P3: 5′-TGCTGCATTTGGGCTGTAGA-3′, P4: 5′-TGGTTTTTCCTGCGGTGAT-3′ and P5: 5′-GGTCTGCAGGTTCAATCT-3′ as sequencing primers.

Luciferase reporter gene assays

NheI and NcoI-tagged DNA fragments, corresponding to the sequences spanning positions −632 to +3, −632 to −67 and −297 to +3 from the initiation ATG of the IL-7 gene, were inserted into the corresponding restriction enzyme cleavage sites of the pGL3-Basic Vector in order to fuse ATGs in the IL-7 gene directly to the firefly luciferase open reading frame (Promega, Madison, WI). Constructs carrying an ATC deletion at position −29 to −27 from the initiation ATG of IL-7 were generated by PCR-based in vitro mutagenesis using P6: 5′-GGCTAGCAGACGACTTGGCATCGTCC-3′ and P8: 5′-TGGACCATGGTCTGCGGGAGGCGGGCGTAGTCATGACCGC-3′ or P7: 5′-GGCTAGCAGATTGAACCTGCAGACCA-3′ and P8 (mentioned previously) as the respective primer pairs for the −632 to +3 or −297 to +3 upstream region of the IL-7 gene with ATC deletion. All constructs were verified for sequence authenticity. Four micrograms of the resultant constructs was transfected with DMRIE-C (Gibco/BRL, Gaithersburg, MD) into Jurkat ($CD4^+$ T-lymphocyte cell line) and U937 cells (monocytic cell line). Transfection efficiency was normalized by cotransfection with 0.2 μg of pRL-CMV vector, which expresses *Renilla* luciferase under the control of the cytomegalovirus immediate early promoter. When necessary, INF-γ (Peprotech, Rocky Hill, NJ) was added to the transfected cell culture at a final concentration of 50 ng mL^{-1} 5 h after transfection. The cells were harvested 40 h after transfection, and firefly and *Renilla* luciferase activities were determined according to the manufacturer's instructions (Dual-Luciferase Reporter Assay System, Promega) with a Luminometer Centro LB960 (Berthold, Bad Wildbad, Germany). Relative luciferase expression (fold increase) was calculated with the following equation: fold increase = (firefly luciferase activity of upstream region of IL-7 gene construct/*Renilla* luciferase activity) / (firefly luciferase activity of promoterless vector pGL3-Basic/*Renilla* luiferase activity).

Statistical analysis

The unpaired *t*-test was used.

Results

Polymorphisms in the upstream non-coding region of the IL-7 gene

We sequenced a 1658-bp PCR-amplified fragment of the IL-7 gene containing 1470 bp of the upstream non-coding region, 9 bp of the first coding exon and 179 bp of the downstream intron. Samples were obtained from 52 unrelated non-HIV-1-infected and 99 HIV-1-infected Japanese, as well as from 122 non-HIV-1-infected and 112 HIV-infected Thais. Polymorphisms were identified at two positions: an A to G substitution at position −485 and an ATC deletion at a position from −29 to −27 upstream from the open frame ATG codon of the IL-7 gene (Fig. 1). Frequencies of these two polymorphisms are summarized in Table 1. As for the A to G mutation at position −485, there was no difference in frequency of the G allele between HIV-1-infected and non-HIV-1-infected individuals. For the allele of the ATC deletion, two of the 99 HIV-1-infected Japanese carried this allele, but none of the Thais. There was no linkage disequilibrium between these two mutations.

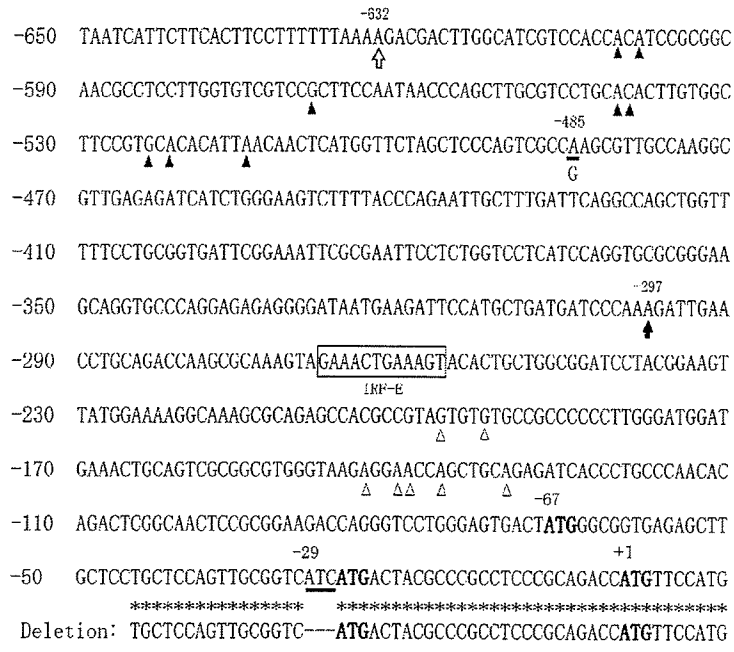

Figure 1. Fragment containing 650-bp of the upstream non-coding region and a part of the coding region of the IL-7 gene. Two polymorphisms, A to G at −485 and ATC deletion at −29 to −27 are underlined. The sequence with the ATC deletion is shown below the sequence without the ATC deletion. Asterisks denote sequence identity. Numbers denote positions from the initiation ATG of IL-7. An open arrow at −632 and a closed arrow at −297 denote the 5′ ends of the IL-7 upstream non-coding region inserted into reporter plasmids (see Figs 3 and 4). Triangles denote multiple transcription start sites that are clustered within the two distinct regions reported by Oshima *et al.* (2004). Open triangles denote transcription start sites specifically activated by IFN-γ. An open square denotes IRF-E (Oshima *et al.*, 2004).

In addition, calculation of nucleotide diversity in the 1470-bp fragment of the upstream non-coding region of IL-7 genes in all Japanese and Thai subjects showed 1.4×10^{-5} in Japanese and 0.9×10^{-5} in Thais. These results suggested that the human IL-7 gene has a highly conserved upstream non-coding region.

Roles of ATGs in the upstream non-coding region of IL-7 gene

In the upstream non-coding region, there are several out-of-frame ATGs (Fig. 1), with even the shortest transcript starting from position −130 containing two out-of-frame ATGs in the upstream non-coding region (Fig. 1). Because one of those out-of-frame ATGs occurred just downstream of the ATC deletion described previously, we then investigated roles of these upstream ATGs in IL-7 gene expression. For this purpose, we constructed a reporter plasmid in which the luciferase open reading frame was fused with the upstream ATG under the control of the upstream region of the IL-7 gene. As shown in Fig. 2, when a reporter plasmid carrying the region from position −632 to the authentic translation initiation ATG codon was transiently transfected into Jurkat or U937 cells, a significant increase in luciferase activity was observed, compared with the pGL3-basic vector employed as a control reporter plasmid in either cell, confirming a previous observation (Oshima *et al.*, 2004). When ATG at position −69 to −67 was fused with the luciferase open reading frame, luiferase activity became greatly enhanced (Fig. 2). These results indicated that the upstream AUG in IL-7 mRNA was more efficiently used for expression than the authentic AUG, and suggested that presence of upstream AUGs in IL-7 mRNA can be expected to reduce IL-7 translation levels.

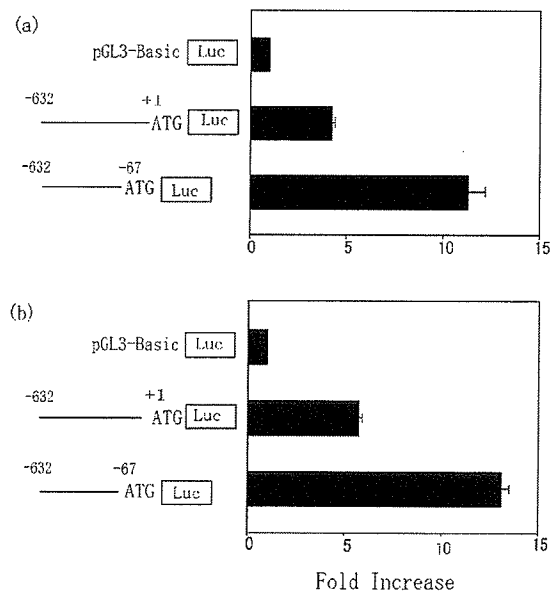

Figure 2. Luciferase activity mediated by the upstream non-coding region of the IL-7 gene. Jurkat (a) or U937 cells (b) were transfected with the plasmids indicated. The fold increase of each construct is represented by a bar. Data represent three independent experiments with similar results. Error bars show actual fluctuations among measurements of fold increase in four clones of each construct.

ATC deletion could affect the gene expression of IL-7

As mentioned previously, the ATC deletion occurred just upstream of the ATG located at position −26 to −24 (Fig. 1). Kozak previously reported that ACCATGG is

Table 1. Allele and genotype frequencies of A to G mutation at −485 and deletion mutation at −29 to −27 in HIV-1-infected and non-HIV-1-infected Japanese and Thai people

	Japan				Thailand			
	HIV-1-Infected *n* (%)	Non-HIV-1-Infected *n* (%)	HIV-1-Infected *n* (%)	Non-HIV-1-Infected *n* (%)	HIV-1-Infected *n* (%)	Non-HIV-1-Infected *n* (%)	HIV-1-Infected *n* (%)	Non-HIV-1-Infected *n* (%)
Allele			Genotype		Allele		Genotype	
A	196 (99.0)	102 (98.1)	AA	97 (98.0)	50 (96.2)	A	221 (98.7)	241 (98.8)	AA	109 (97.3)	119 (97.5)
G	2 (1.0)	2 (1.9)	AG	2 (2.0)	2 (3.8)	G	3 (1.3)	3 (1.2)	AG	3 (2.7)	3 (2.5)
			GG	0 (0)	0 (0)				GG	0 (0)	0 (0)
Total	198	104		99	52	Total	224	244		112	122
Allele			Genotype			Allele			Genotype		
W[a]	196 (99.0)	104 (100)	WW	97 (98.0)	52 (100)	W[a]	224 (100)	244 (100)	WW	112 (100)	122 (100)
D[b]	2 (1.0)	0 (0)	WD	2 (2.0)	0 (0)	D[b]	0 (0)	0 (0)	WD	0 (0)	0 (0)
			DD	0 (0)	0 (0)				DD	0 (0)	0 (0)
Total	198	104		99	52	Total	224	244		112	122

[a] W denotes the wild type at −29 to −27.

[b] D denotes deletion at −29 to −27.

the optimal sequence for translation initiation of pre-proinsuline by eukaryotic ribosomes and that substitution of G for A at position −3 (3-bp upstream from the ATG codon) reduced translation efficiency (Kozak, 1986). The A to G substitution at position −3 of an upstream, out-of-frame ATG condon also reportedly diminished translation from the corresponding upstream ATG and consequently increased translation from the authentic downstream ATG (Kozak, 1986). In the case of the human IL-7 gene, the sequence surrounding the ATG at position −26 to −24 is ATCATG but the ATC deletion observed in our study converted it into GTCATG (Fig. 1). These data indicate that the ATC deletion altered the A at position −3 into G (Fig. 1), thus hypothetically reducing translation efficiency from the upstream ATG at position −26 to −24 and increasing translation from the authentic IL-7 ATG. We therefore decided to test experimentally whether the ATC deletion polymorphism actually affected levels of expression from the authentic IL-7 ATG.

We constructed a reporter plasmid containing the upstream non-coding region from −632 to +3 with the ATC deletion and compared its luciferase activity with that of the wild-type version. As shown in Fig. 3(a), the reporter activity of the deletion mutant was approximately 30% higher than that of the wild-type plasmid. We also generated shorter versions of the wild type as well as mutant constructs carrying the upstream non-coding region from −297 to +3, which spans the minimal promoter region containing IRF-E (−268 to −257) (Oshima *et al.*, 2004). Again, the reporter activity of the deletion mutant was approximately 30% higher than that of the wild-type plasmid (Fig. 3b). We repeated the same experiments by using monocytic U937 cells. Here too, luciferase activity in the deletion mutant was approximately 25% higher than that in the corresponding wild-type plasmid when the upstream non-coding region of −632 to +3 was used (Fig. 4a). An approximately 20% increase in luciferase activity was observed in the deletion mutant when the upstream non-coding region −297 to +3 was used (Fig. 4c). It is known that INF-γ is capable of up-regulating the gene expression of IL-7 in intestinal epithelial cells through the IRF-E in the region −268 to −257 from the initiation ATG codon (Oshima *et al.*, 2004). As shown in Fig. 4(b,d), the addition of INF-γ to the transfected cells in fact did augment luciferase activity in U937 cells. Moreover, the deletion mutant exhibited significantly higher luciferase activity than the wild-type constructs (Fig. 4b,d). These results clearly indicate that ATC deletion in the upstream non-coding region resulted in higher expression from the authentic IL-7 ATG.

Discussion

In the study reported here, we demonstrated that an out-of-frame ATG in the upstream non-coding exon of IL-7 gene was more efficiently used for expression than the authentic ATG of IL-7 gene. We also found a naturally occurring ATC deletion polymorphism at position −29 to

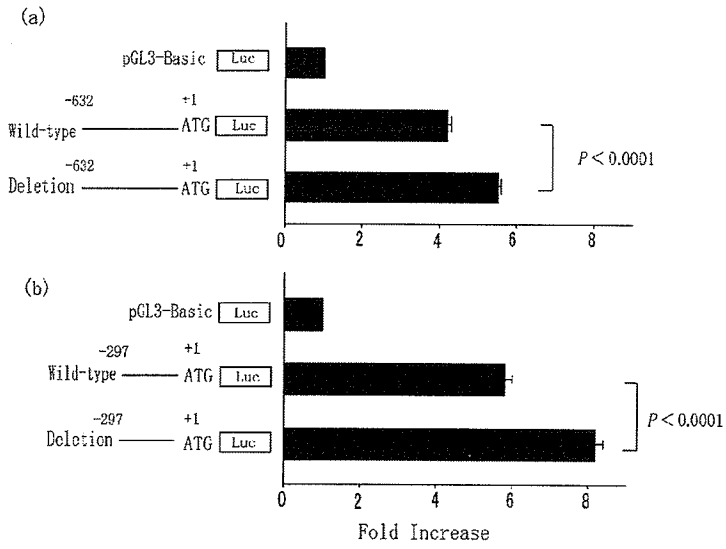

Figure 3. Luciferase activity mediated by the upstream non-coding region of the wild-type and ATC deletion in Jurkat cells. (a) A region from position −632 to +3 of the upstream non-coding region of the wild-type and ATC deletion. (b) A region from position −297 to +3 of the upstream non-coding region of the wild-type and ATC deletion. Data represent three independent experiments with similar results. Error bars show actual fluctuations among measurements of fold increase in four clones of each construct. *P* values for differences in fold increase are shown.

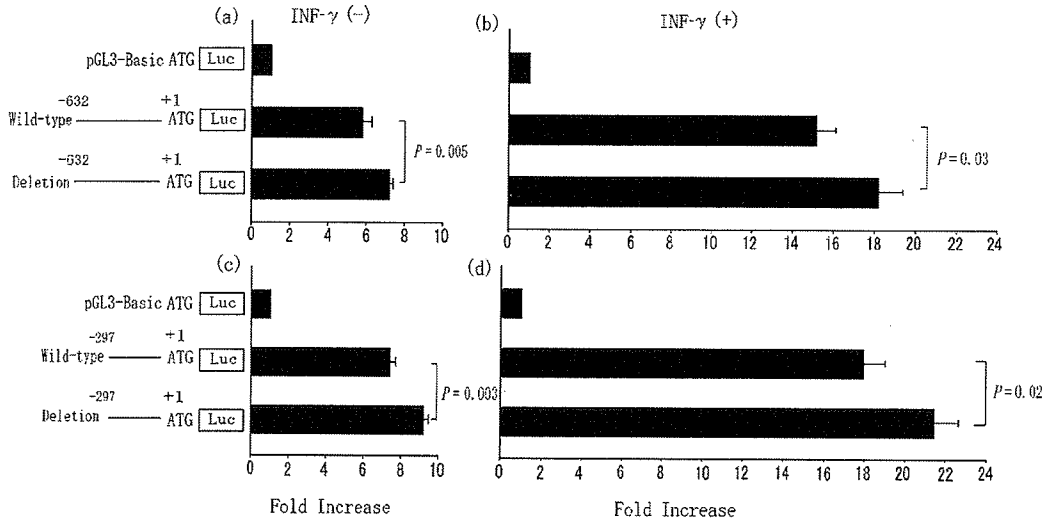

Figure 4. Luciferase activity mediated by the upstream non-coding region of the wild-type and ATC deletion in U937 cells. (a) A region from position −632 to +3 of the upstream non-coding region of the wild-type and ATC deletion without INF-γ. (b) A region from position −632 to +3 of the upstream non-coding region of the wild-type and ATC deletion with INF-γ. (c) A region from position −297 to +3 of the upstream non-coding region of the wild-type and ATC deletion without INF-γ. (d) A region from position −297 to +3 of the upstream non-coding region of the wild-type and ATC deletion with INF-γ. Data represent three independent experiments with similar results. Error bars show actual fluctuations among measurements of fold increase in four clones of each construct. *P* values for differences in fold increase are shown.

−27 in the upstream non-coding exon next to one of the upstream ATGs. This polymorphism was found to be capable of increasing the expression from the authentic IL-7 ATG in Jurkat T cell and U937 monocytic cell lines. This is the first time human genetic polymorphism has been identified that is supposed to affect expression of a protein by changing the translation efficiency from the out-of-frame AUG in the upstream non-coding region of mRNA.

There are a few precedents for a human genetic polymorphism near the initiation ATG codon affecting translation efficiency. A single nucleotide polymorphism (SNP) that switches C to T at position −1 upstream from the open frame ATG codon in the human annexin V gene has been found to increases translation efficiency and plasma levels of annexin V, and to decreases the risk of early myocardial infarction (Gonzalez-Conejero *et al.*, 2002). Also, an SNP that switches G to T at position −3 upstream from the open frame ATG codon of the BRCA1 gene in sporadic breast cancer causes down-modulation of translation efficiency (Signori *et al.*, 2001). Moreover, a mutation of G into A at +4 downstream from the open frame ATG codon of the human androgen receptor gene observed in

a family with partial androgen insensitivity syndrome can reduce the efficiency of protein translation (Choong *et al.*, 1996). Finally, it has been reported recently that a Graves'-disease–associated SNP that substitutes T for C at position −1 upstream from the open frame ATG codon of the CD40 gene enhances translation and could predispose to disease (Jacobson *et al.*, 2005). However, all these SNPs are located near the authentic translation initiation ATG codon of the proteins and directly affect the translation efficiency from the open frame ATG codon. In the case of the IL-7 gene, however, the mutation is located at −29 to −27 upstream from the open frame ATG codon, rather than near the authentic translation initiation codon. Nevertheless, it could up-regulate the IL-7 gene expression probably by changing the translation efficiency from the upstream, out-of-frame ATG codon. Our data showed that the consensus sequence for translation initiation is important, not only for the open-frame initiation ATG codon, but also for the upstream, out-of-frame ATG that is thought to reduce translation efficiency from the downstream initiation ATG. Similar to mutations within the consensus sequence of the open-frame initiation ATG codon, nucleotide substitution within the consensus sequence of the upstream, out-of-frame ATG can also modulate translation efficiency.

Our data also showed that the 1470-bp upstream non-coding region of the IL-7 gene exhibited extremely low levels of diversity in both Japanese and Thai populations. Also, no non-synonymous polymorphism has yet been identified in the IL-7 coding region. The reason for the low levels of diversity of the IL-7 gene is not clear at present, but is probably the result of its importance for the survival, development and proliferation of B and T cells. Experiments with IL-7 deficient mice proved that IL-7 is a non-redundant cytokine (von Freeden-Jeffry *et al.*, 1995). It is therefore reasonable to assume that low levels of diversity of the upstream non-coding region of the IL-7 gene that regulates the transcription of this gene are needed to provide a stable condition for IL-7 production.

In conclusion, we have identified a polymorphism in the upstream non-coding region of the IL-7 gene that could up-regulate gene expression. Although the frequency of this allele is very low in Japan and Thailand, it would be interesting to analyse this polymorphism in HIV-1-infected individuals with different rates of immune reconstitution after treatment with a highly active antiretroviral therapy. It would be important to analyse this polymorphism in other ethnic groups. On the other hand, an elevation of plasma IL-7 has also been reported in lymphopenia cases, including patients undergoing bone marrow transplantation or chemotherapy for cancer, or patients with idiopathic $CD4^+$ lymphopenia (Fry & Mackall, 2005; Bolotin *et al.*, 1999). It would also be of interest to analyse this polymorphism in those patients.

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Retro-transduction by virus pseudotyped with glycoprotein of vesicular stomatitis virus

Masahisa Ohishi, Tatsuo Shioda, Jun-ichi Sakuragi *

Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita City, Osaka 565-0871, Japan

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Abstract

A virus pseudotyped with glycoprotein of vesicular stomatitis virus (VSV-G) can enter various cell types at a relatively high titer. We observed that the amount of viral antigen from VSV-G pseudotyped human immunodeficiency virus type 1 (HIV-1) producing cells was much higher than that from their non-pseudotyped counterparts. This enhanced viral antigen production was not observed when we used HIV-1 *pol* mutant, viral enzyme inhibitors, HIV Env protein, or VSV-G fusion defective mutants. The transfection experiment using GFP-expressing virus showed time-dependent expansion of GFP-positive cells and viral DNA integration. These results suggested that the increase in viral antigen yield was caused by the release of a progeny virus following retro-transduction by the pseudotyped virus of the cells within the transfected cell culture. The infectivity as well as the amount of VSV-G on virus particles per unit of viral antigen was significantly different before and after the onset of the yield enhancement. This suggests that results of infection assays of the virus pseudotyped with VSV-G may be affected by the occurrence of such enhancement. This means that, while pseudotyping with VSV-G is a simple and effective method, this procedure should be carefully considered when the virus is produced for infectivity assays.

Keywords: VSV-G; HIV-1; Pseudotyping; Viral vector; Retro-transduction

Introduction

Gene delivery using retroviral vectors has become a very popular and indispensable method. To ensure safety of the vectors and to increase their efficiency, envelope pseudotyping of viral vectors lacking their own envelope gene is used in almost all cases. Glycoprotein of vesicular stomatitis virus (VSV-G) in particular is one of the most frequently used envelope proteins for pseudotyping since it is very stable and promises highly effective and broad-spectrum gene delivery. However, there seems to be very little awareness of its adverse effect, that is, re-infection/retro-transduction. VSV-G interacts with a phospholipid component of the cell surface membrane and mediates viral entry by membrane fusion (Burns et al., 1993; Mastromarino et al., 1987). As viral entry does not seem to require specific protein receptors, VSV-G pseudotyped vectors could infect virtually all kinds of cells. Thus, the VSV-G pseudotyped viral vector has the potential to infect its producer cells to re-generate progenies, which may contain unexpected characteristics. In the study presented here, we examined retro-transduction of VSV-G pseudotyped HIV-1 vectors. Our findings suggest that this problem could confound the interpretation of the experimental results under certain conditions and must therefore be taken into careful consideration.

Results

Enhancement of virion production by VSV-G pseudotyping

We previously noticed that the viral antigen yield of VSV-G pseudotyped HIV-1 by transfection was always much higher than that of the non-pseudotyped virus. We first reconfirmed this phenomenon by using a simplified experimental design. For this purpose, 2.5 μg of HIV-1 proviral plasmid pNLNh, which lacks Env expression, was transfected into 3×10^6 293T cells

* Corresponding author. Fax: +81 6 6879 8347.
E-mail address: sakuragi@biken.osaka-u.ac.jp (J. Sakuragi).

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along with 2.5 μg of either pCG-VSVG, which expresses VSV-G under the CAG hybrid promoter (Niwa et al., 1991), or pGEM-5Zf(+) (Promega, Madison, WI) as a control. The quantity of virus production was monitored by measuring CA-p24 antigen in the culture supernatant every 12 h. The amounts of CA-p24 detected in VSV-G pseudotyped virus and in control were almost equal up to 24 h post-transfection. However, CA-p24 production of the former became 10 times higher than that of control at 36 h and finally 20 times higher at 48 h (Fig. 1A). The actual amount of CA-p24 of virus from 3×10^6 of transfected cells with pseudotyping was typically 20–40 μg in total. This enhancement may have been caused by some transactivation effects of VSV-G, or by retro-transduction of the cells within the transfected cell culture, which then leads to production of the progeny virus. The absence of the enhancement at 12 or 24 h post-transfection suggests that transactivation was unlikely since the transactivation effect should become apparent from the onset of protein expression. In subsequent experiments, we therefore examined the possibility of retro-transduction of virus thus produced.

Retro-transduction of pseudotyped virus during transfection

The transfected cells were harvested 48 h post-transfection and viral antigens within the cells were detected by western blotting (Fig. 1B). The viral antigens within cells producing the *env*-lacking virus were mostly large, seemingly uncleaved, and with the precursor protein Gag-Pr55 dominant. On the other hand, the protein profile within the cells producing the VSV-G pseudotyped virus was strikingly different. The majority of viral antigens were mature proteins, such as CA-p24 and MA-p17, while the number of immature proteins was very limited. This observation was in agreement with a hypothesis that the viral antigen within cells producing the pseudotyped virus mainly came from mature virions, which would support the concept of retro-transduction of the released virus.

To examine this possibility, a *pol* mutant pNLN-RI was constructed from pNLNh. The mutant had a large deletion at the RT and IN genes in addition to an Env frameshift, while the expression of functional Env, RT, and IN was eliminated. The VSV-G pseudotyped NLN-RI was therefore not able to complete retro-transduction to produce its progeny. Next, 293T cells were co-transfected with pNLN-RI along with either pCG-VSVG or pGEM-5Zf(+), and the quantity of virus production was monitored by measuring CA-p24. No difference in virus production was observed between VSV-G pseudotyped and non-pseudotyped viruses during 48 h post-transfection (Fig. 1C). This indicates that the enhancement of virus production was impaired by the elimination of RT expression and subsequent inhibition of retro-transduction.

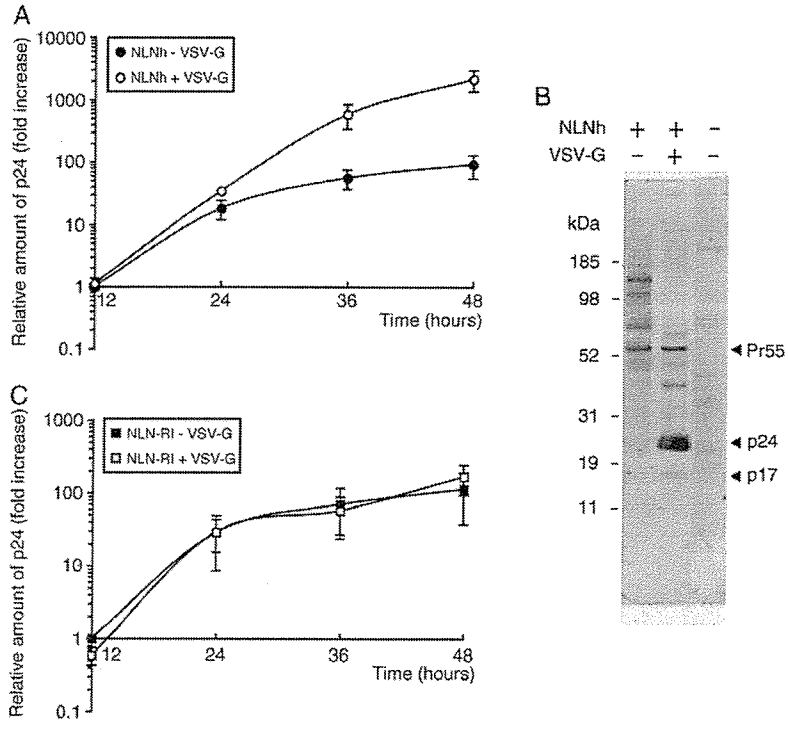

Fig. 1. Enhancement of virus production by pseudotyping. (A) Time course of HIV-1 production during transfection. The plasmid pNLNh was transfected into 293T cells along with either pCG-VSVG or pGEM-5Zf(+), and the quantity of virus production in the culture supernatant was monitored at 12, 24, 36, and 48 h post-transfection by measuring CA-p24 antigen. The value for NLNh without pseudotyping at 12 h was set at 1. Results show the averages of three separate experiments. Error bars represent the mean standard error for the difference between experiments. (B) Profiles of viral antigen in transfected cells. Cells were harvested at 48 h post-transfection and lysed. Viral antigens within cell lysates were detected by western blotting using serum from HIV-1 infected patients. (C) Similar experiment as in A except for the use of viral *pol* mutant pNLN-RI instead of the wild-type pNLNh.

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Effect of viral enzyme inhibitors and fusion defective mutation to retro-transduction

To further test our hypothesis, two more experiments were performed. First, 293T cells were co-transfected with pNLNh and pCG-VSVG in the presence or absence of viral enzyme inhibitors. We used an effective RT inhibitor AZT (Mitsuya et al., 1985) and a protease inhibitor Ritonavir (Markowitz et al., 1995) (Fig. 2). For efficient functioning of AZT, the amount of plasmid DNAs used in AZT experiment was reduced to 0.5 μg in total, and the day after transfection, the medium was replaced with fresh medium with or without the inhibitors. The amounts of virus production were measured 48 h post-transfection. When 0.5 μg of DNA was transfected into 3×10^6 cells, actual amount of CA-p24 of pseudotyped virus was typically 1–2 μg. Virion production was reduced to the same level as that of control in the presence of 5 μM AZT, and the enhancement was recovered as the concentration of the drug was reduced (Fig. 2A). The effect of Ritonavir was similar to that of AZT since 0.417 μM or more Ritonavir severely blocked the enhancement of virion production measured by pr55-Gag amounts (Fig. 2B). For the second experiment, two VSV-G fusion defective mutants, pCGVG-G124E and pCGVG-P127D, were constructed. Both mutants contain one amino acid substitution at the fusion domain of the protein and reportedly lose their membrane fusion activity although their expression level at cell surface remains similar to that of the wild-type (Fredericksen and Whitt, 1995). The mutants were compared to the wild-type VSV-G (WT-G) in terms of viral production enhancement by pseudotyping (Fig. 3A). Both of the mutants showed very similar effects. Unexpectedly, the amount of virus production by the mutants was reduced throughout by approximately half compared to that by the non-pseudotyped virus. Nonetheless, the time course of virus production kinetics was almost identical for the mutants-pseudotyped and non-pseudotyped virus and drastically different from that of WT-G. Some of the data may

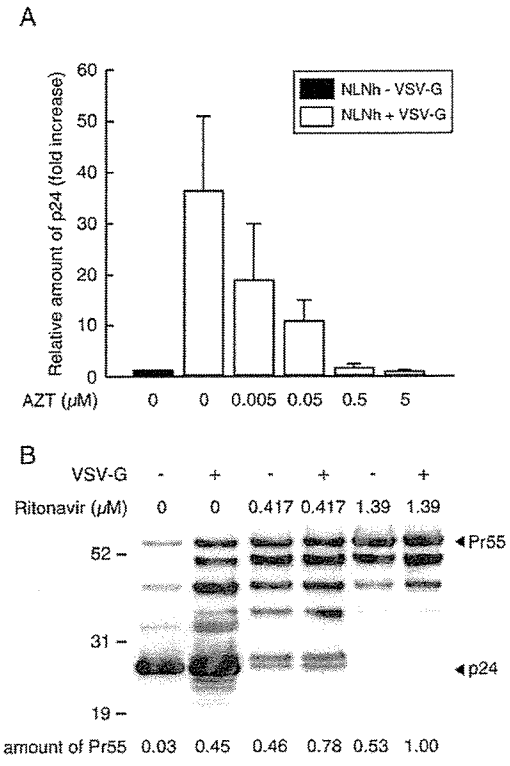

Fig. 2. Effect of viral enzyme inhibitor on enhancement. (A) 293T cells were co-transfected with 0.25 μg of pNLNh and 0.25 μg of either pCG-VSVG or pGEM-5Zf(+) in the absence or presence of 0.005, 0.05, 0.5, and 5 μM AZT. The virus particles derived from the cells were collected at 48 h post-transfection, and CA-p24 antigen was quantified. The value for NLNh without pseudotyping and AZT was set at 1. Results show the averages of three separate experiments. Error bars represent the mean standard error for the difference between experiments. (B) 293T cells were co-transfected with 2.5 μg of pNLNh and 2.5 μg of either pCG-VSVG or pGEM-5Zf(+) in the absence or presence of 0.417 or 1.37 μM Ritonavir. Western blotting of released virion was performed and viral antigen was detected by anti-HIV-1 p24-CA monoclonal antibody. A series of dilution controls was generated (not shown), and the amount of Pr55-Gag were measured. The value for a sample with pseudotyping with 1.37 μM Ritonavir was set at 1. The amount of CA-p24 in the samples was efficiently reduced by the effect of Ritonavir. Results show the representative data of two independent experiments with similar results.

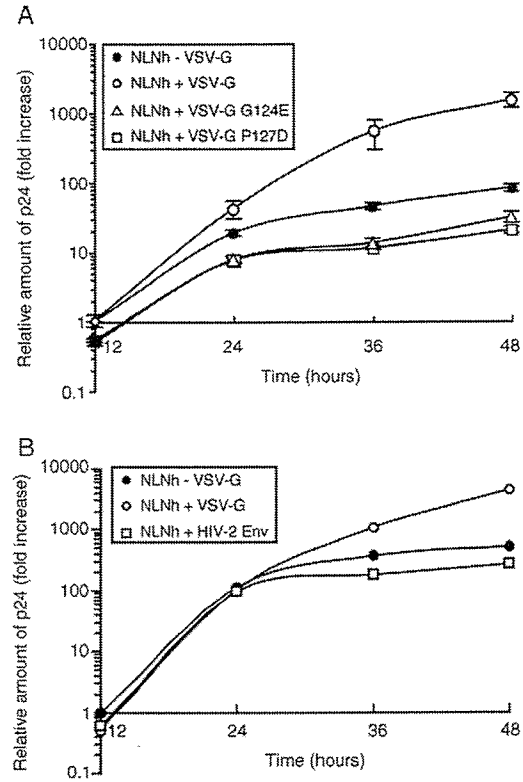

Fig. 3. Effects of fusion-defective VSV-G or HIV-2 Env on enhancement. pNLNh was transfected into 293T cells along with either pCG-VSVG, pCGVG-G124E, pCGVG-P127D, pCGH2Env or pGEM-5Zf(+), and the virus in the culture supernatant was monitored at 12, 24, 36, and 48 h post-transfection by measuring CA-p24 antigen. The values for NLNh without pseudotyping at 12 h was set at 1. (A) Effect of mutation lacking VSV-G fusion. Results show the averages of three separate experiments. Error bars represent the mean standard error of the difference between experiments. (B) Effect of HIV-2 Env pseudotyping. Results show the representative data of two independent experiments with similar results.

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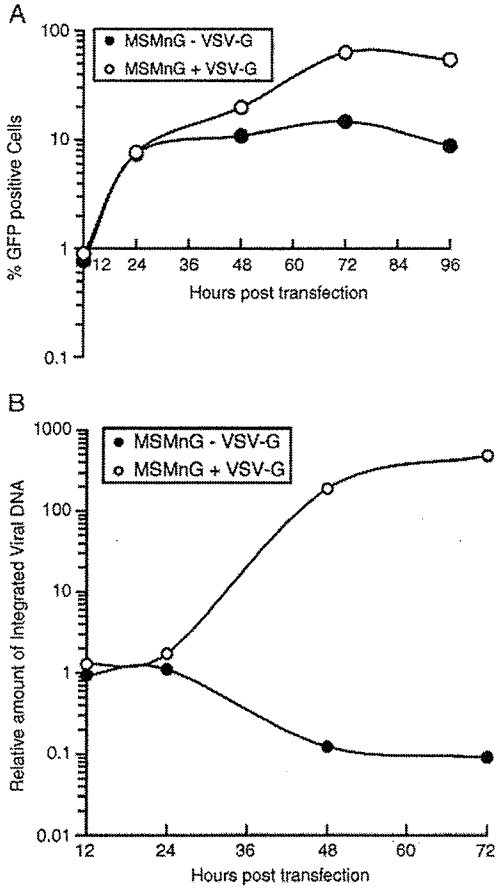

Fig. 4. GFP-virus transfection and viral DNA integration. 293T cells were co-transfected with 0.25 μg of pMSMnG and 0.25 μg of either pCG-VSVG or pGEM-5Zf(+) and were harvested at 12, 24, 48, 72, and 96 h post-transfection. Half of each sample was fixed and subjected for FACS analysis, and total DNA of latter half was extracted for Alu-PCR analysis. (A) GFP expression of transfected cells. Percentages of GFP-positive cells per total cells were indicated. (B) Alu-PCR analysis for integrated viral DNA quantification. The value for the VSV-G(−) sample at 12 h was set at 1. Results show the representative data of two independent experiments with similar results.

be due to reduced virion production in the absence of a suitable envelope. To verify this possibility, we tried pseudotyping of envelope protein (Env) of HIV-2, which is a closely related virus of HIV-1. As the CD4 antigen, the cell surface receptor for HIV-1 and 2 (McClure et al., 1987), is not expressed on the surface of non-lymphocyte cells such as 293T, virion pseudotyped with HIV-2 Env is noninfectious to 293T cells. The data of HIV-2 Env pseudotyping were very similar to that of the fusion defective mutants of VSV-G (Fig. 3B), suggesting that envelope suitability does not affect virion production. HIV-2 Env pseudotyping was confirmed by infectivity assay. The infectivity of it per viral antigen was comparable to that of VSV-G pseudotyped one, suggesting that HIV-2 Env was efficiently incorporated into HIV-1 virion (data not shown). These results are a clear indication that the viral production enhancement by VSV-G pseudotyping was completely eliminated by the blocking of viral infectivity to producer cells and suggest that the enhancement was caused by retro-transduction and subsequent replication of the virus.

The GFP virus experiment and detection of provirus integration

To gain direct evidence of the retro-transduction, we constructed pMSMnG, a derivative of pNL4-3 expressing the green fluorescent protein (GFP) instead of viral nef protein. The 293T cells were transfected with 0.25 μg of pMSMnG along with or without 0.25 μg of pCG-VSVG, and GFP expression and viral DNA integration of the cells were analyzed from 12 h to 96 or 72 h after transfection (Fig. 4). The amount of GFP-positive cells was similar between with and without VSV-G expression until 24 h. After that, that of VSV-G positive sample was increased dramatically and reached nearly to 70% at 72 h whereas that of VSV-G negative ones was only slightly increased to 15% (Fig. 4A). Viral DNA integration was much drastic. It was virtually not observed in VSV-G negative sample throughout the experiment, whereas in VSV-G positive sample, very large amount of viral DNA integration was detected later than 24 h post-transfection (Fig. 4B). These data clearly

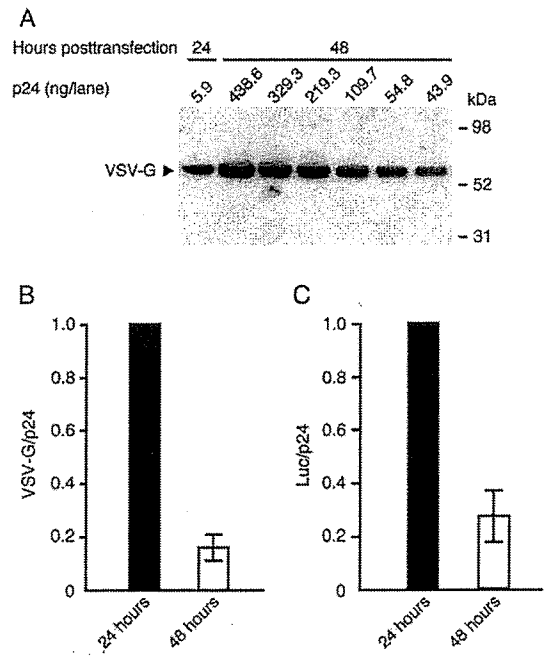

Fig. 5. VSV-G content and infectivity per viral antigen in early and late stages of transfection. Virions derived from 293T cells co-transfected with pNLNh and pCG-VSVG were harvested at 24 and 48 h post-transfection, purified, and lysed. CA-p24 antigen and VSV-G in the lysate were measured by ELISA and by western blot, respectively. (A) Western blot of VSV-G on the virion. The time the virion was harvested and the amount of CA-p24 antigen for each lane are indicated. (B) VSV-G content per viral antigen. Signal intensities of VSV-G on the membrane were quantified as described in Materials and methods. (C) Infectivity of 'early' and 'late' virus. M8166/H1luc cells (10^6) were infected with three serially diluted viruses. At 24 h post-infection, cells were lysed and luciferase activity in the cell lysate was measured. The values for the virus at 24 h were set at 1. Results show the averages of at least three separate experiments. Error bars represent the standard deviation between experiments.

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indicated the occurrence of massive retro-transduction and subsequent progenitor production.

Reduction of viral infectivity by retro-transduction

Finally, we examined differences between 'early' and 'late' virus particles. The virion production of the WT-G pseudotyped virus 48 h post-transfection was enhanced up to 20 times compared to that of non-pseudotyped viruses, whereas no such difference was observed at 24 h. This implies that most of the pseudotyped viruses at 48 h can be assumed to have been the result of replication, which might have resulted in some characteristics different from those of the virion produced solely by transfection. To verify this possibility, we harvested the total virus at 24 h ('early') and 48 h ('late') post-transfection and measured the amounts of virus production by CA-p24 ELISA and VSV-G on virus by western blotting (Fig. 5). When we calculated the ratio of VSV-G to CA-p24 at the two time points, we found that the VSV-G content of the 'early' virus was approximately 5 times higher than that of the 'late' virus. This indicates that, under certain conditions, the relative amount of VSV-G of the 'late' virus is drastically reduced compared to that of the 'early' virus particle. To determine the effect of this reduction on virus infectivity, a single-round infection assay using the M8166/H1luc reporter cell line (Nagao et al., 2004) was performed (Fig. 5C). The actual value of infectivity of 'early' virus was typically 1.3×10^5 RLU/s CA-p24 μg. The infectivity of the 'early' virus per CA-p24 was nearly 4 times higher than that of the 'late' virus. This shows that the infectivity of the 'early' and 'late' viruses mostly depends on the amount of VSV-G per virion and therefore could alter dramatically under certain conditions.

Discussion

Retrovirus pseudotyping is one of the most commonly used methods for not only gene delivery, but also virological studies. Since transfection of cells with envelope and *gag/pol* expression vectors makes it easy to generate infectious particles, this method rarely causes concern. However, notable incidents are sometimes observed even in the case of common experiments. In the study reported here, we focused on the enhancement of HIV-1 production by VSV-G protein pseudotyping. In the first set of experiments, we observed up to 20-fold enhancement of viral production by VSV-G pseudotyping, with no enhancement occurring on or before 24 h post-transfection (Fig. 1A). We therefore hypothesized the occurrence of retro-transduction of the virus produced and subsequent virion production. Altered viral antigen profiles in transfected cells with or without pseudotyping indicated the presence of mature viral proteins in the cells with pseudotyping, thus corroborating our hypothesis (Fig. 1B). The experiment using the HIV-1 *pol* mutant also suggested a close correlation between enhancement and viral infectivity (Fig. 1C). In the subsequent experiments, we eliminated the infectivity of the produced virions by viral enzyme inhibitors (Fig. 2), fusion defective mutation in VSV-G, or HIV-2 Env (Fig. 3). Both of the experiments resulted in loss of the enhancement. These data all strongly suggested that the retro-transduction of the produced virus by the transfectant resulted in the release of large quantities of progeny virions.

The virus production was reduced in the virus pseudotyped with VSV-G mutants and HIV-2 Env compared to that in control from 12 h to 48 h post-transfection (Fig. 3). This was possibly due to overexpression of the genes by the CAG promoter since CAG promoter activity is very strong (Niwa et al., 1991) and may take away the transcription/translation factors from other promoters. Reduction of the viral protein was not observed in case of WT-G pseudotyping, probably because the reduction of viral expression offset the enhancement of viral production by WT-G at 12 h, while the enhancement became dominant after that.

The experiment using GFP-virus gained direct evidence for retro-transduction (Fig. 4). From 24 h to 72 h post-transfection, the percentage of GFP-positive cells just doubled in non-pseudotyped sample, which might reflect the period required for maturation of GFP in cells. In contrast, the increase was more than eight times in VSV-G pseudotyped sample. The result of Alu-PCR experiment gave us more solid evidence. The amount of integrated viral DNA was rapidly increased after 24 h post-transfection and reached more than 200-fold higher than the background levels from 24 h to 72 h post-transfection (Fig. 4B). As the viral DNA integration was not detected in non-pseudotyped sample, this result directly demonstrated retro-transduction by pseudotyping during transfection.

The final experiment produced a striking result in terms of alteration of viral envelope incorporation and infectivity (Fig. 5). There was a more than 80% reduction in the quantity of VSV-G per virus at 48 h post-transfection compared to that at 24 h. Moreover, the infectivity of the 'late' virus was reduced in parallel to one-fourth that of the 'early' virus. Throughout the experiments, we used the calcium phosphate method for DNA transfection (Aldovini and Walker, 1990), which is very popular due to its low cost, ease of manipulation, and efficiency. Co-transfection of multiple vectors enables a cell to uptake multiple genes simultaneously, and it is known that, with this method, usually 10–50% of the cells take up the DNA and express the genes thus introduced (Ausubel et al., 1995). In our experiment, we usually noted a transfection efficiency of 20% at most (data not shown). In other words, far more than half the numbers of cells within a dish remained untransfected. The VSV-G pseudotyped virus could equally retro-transduce both transfected and untransfected cells during a transfection experiment. The genome of the pseudotyped virion lacked its original *env* gene and many of the cells were untransfected and thus did not express VSV-G on their surface. As a result, the majority of the cells probably produced progeny virions without envelope proteins. This implies that the reduction of VSV-G per virion observed represents a vast increase in noninfectious particles and a relative decrease in the ratio of pseudotyped virions. As this ratio can vary greatly depending on the time and conditions during transfection, infection experiments using such a mixture of infectious and noninfectious viruses may sometimes produce inconsistent results.

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The single-round replication assay involves another issue. To compare the infectivity of mutants to that of the wild-type virus, single-round replication assays of pseudotyped viruses are often performed. If the infectivity of the mutants is reduced, which is very likely, the quantity of noninfectious progeny virions from cells retro-transducted by the mutants pseudotyped with VSV-G would be reduced. As a result, the quantity of infectious virions pseudotyped with the VSV-G contained in the mutants could be much larger than that of the wild-type. In such a case, the infectivity of the mutant per viral antigen would be greatly overestimated and may produce a misleading interpretation of the results.

Construction of a helper-vector system with self-inactivating vector (Miyoshi et al., 1998) eliminates the possibility of reproducing noninfectious viruses, and thus can solve the problems concerned. Nonetheless, retro-transduction of the vector is bound to occur as a result of VSV-G pseudotyping, and retro-transduction of the vector by the vector producing cells might yield somewhat unexpected results such as increase of pseudo-transduction (Liu et al., 1996).

In conclusion, we have demonstrated that retro-transduction of HIV-1 pseudotyped with VSV-G into 293T cells resulted in the enhancement of virus production during transfection. The quantity of infectious viruses pseudotyped by VSV-G to that of the total quantity of viruses is significantly different depending on whether or not the enhancement occurs. In a simple experiment, such as making gene transfer vectors by VSV-G pseudotyping, this issue should not matter much. However, when the harvested virus is used for assays relating to infectivity, there are certain problems that possibly cause misleading of the results. Although VSV-G pseudotyping is very easy and convenient, the conditions under which this procedure is used should be carefully considered.

Materials and methods

Constructs

Replication-competent HIV-1 proviral clone pNL4-3 (Adachi et al., 1986) was digested with *Nhe*I (position 7250), blunted with KOD DNA polymerase, and self-ligated with T4 DNA ligase for the construction of pNLNh. pNLNh thus carries a 4-base insertion mutation within the *env* region, and Env protein expression is abrogated. To construct pNLN-RI, the *Bal*I fragment encoding a part of the *pol* gene (position 2619 to 4551) was removed from pNLNh. EGFP gene was amplified using pCMX-SAH/Y145F (Kallio et al., 1998) (a generous gift from Dr. Takao Masuda, Tokyo medical and dental university) as a template and primers (5′-GGATTTTGCTATAAGATGGT-GAGCAAGGGCG-3′/5′-CTCGAGTTACTTGTACAGCTC-3′), and a portion of the *env* gene of pNL4-3 was amplified using primers (5′-GTGCTGTTAACTTGCTCAATGCC-3′/5′-CGCCCTTGCTCACCATCTTATAGCAAAATCC-3′). Two fragments were purified, mixed, and amplified again with the second and third primers to generate a fragment including EGFP utilizing ATG codon of *nef* gene as its own start codon and obtaining *Xho*I site just downstream of its stop codon. The fragment was cloned into pGEM-T Easy (Promega) and verified its nucleotide sequence to make pGEMHnGX. The plasmid pMSMBA (McBride and Panganiban, 1996), a derivative of pNL4-3, was digested with *Hpa*I and *Xho*I, and the *Hpa*I–*Xho*I fragment of pGEMHnGX including EGFP gene was inserted in the corresponding position to construct pMSMnG.

pCGVG-G124E and pCGVG-P127D were generated by replacing the 1123-bp *Xba*I–*Acc*65I fragment in pCG-VSVG (a generous gift from Prof. Hideo Iba, Institute of Medical Science, University of Tokyo) with the corresponding fragment carrying the G124E and P127D mutation, respectively. The 5′ part of G124E and P127D fragments were amplified using primer pairs 5′-GCTCTAGAGCCTCTGCTAAC-3′/5′-AGG-GAACTCTGGATTCAGC-3′ and 5′-GCTCTAGAGCCTCT-GCTAAC-3′/5′-TTTGATCAGGGAAGCCTG, respectively. The 3′ part of the G124E and P127D fragments was amplified using primers 5′-GCTGAATCCAGAGTTCCC-3′/5′-TAGGG-TACCATTGATTATGGT-3′ and 5′-CCAGGCTTC-CCTGATCAAA-3′/5′-TAGGGTACCATTGATTATGGT-3′, respectively. The total G124E fragment was amplified with primers 5′-GCTCTAGAGCCTCTGCTAAC-3′/5′-TAGGG-TACCATTGATTATGGT-3′ (underlined nucleotides represent *Xba*I and *Acc*65I restriction sites) using the 5′ and 3′ parts of G124E as templates. The total P127D fragment was amplified in the same way as described above. The *env* coding region of pGH123 (Shibata et al., 1990), a molecular infectious clone of HIV-2, was amplified with primers (5′-GCCGCCATGTGTGG-TAAGAGTCTAC-3′/5′-CTACAAGTCGTAACCATCGTC-3′) to add kozak sequence at 5′ end. The amplified fragment was cloned into pGEM-T Easy to generate pGEMH2env, and the sequence was verified. The HIV-2 *env* expression vector pCGH2env was generated by replacing an *Eco*RI fragment of pCG-VSVG including whole VSV-G fragment with the *Eco*RI fragment of pGEMH2env including the *env* gene.

Cell culturing and transfection

293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. The cells were transfected with the calcium phosphate method (Aldovini and Walker, 1990) using either pNLNh or pNLN-RI and either pGEM-5Zf(+), pCGH2env, pCG-VSVG, or its derivatives.

Virus purification

At 24 or 48 h after transfection, the culture supernatants of the transfected cells were centrifuged at 1570×*g* for 20 min at 4 °C, and virus particles in the supernatants were pelleted by ultracentrifugation (151,000×*g*, 1 h) through a 20% sucrose cushion. The pelleted viruses were then resuspended in growth medium for infection experiment or lysed with lysis buffer (50 mM Tris–HCl—pH 7.5, 100 mM NaCl, 10 mM EDTA, 1% SDS) for western blotting or CA-p24 quantitation. CA-p24 antigen was quantified with an ELISA kit (ZeptoMetrix Corp., Buffalo, NY) according to the manufacturer's instructions.

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Virus infection

At 24 and 48 h post-transfection, the resultant virion was purified and used for infection into M8166/H1luc cells (Nagao et al., 2004), which contain integrated reporter DNA carrying HIV-1 long terminal repeat (LTR) and luciferase. Upon infection with HIV-1, HIV-1 LTR is activated along with the expression of viral transactivator Tat, and luciferase expression in cytoplasm is induced. For our study, the cells (1×10^6) were infected at 37 °C with 300 μl of three serially diluted viruses. After 90 min, the cells were washed with PBS, added to 1.5 ml of media and cultured for 40 h at 37 °C. The infected cells were then washed with PBS and lysed with 125 μl of Glo lysis buffer (Promega), and a 50 μl sample of each lysate was assayed for photon emission after the addition of 50 μl of Bright-Glo Reagent (Promega) with a microplate luminometer (Centro LB 960; Berthold Technologies, Bad Wildbad, Germany).

Western blotting analysis

The lysates from the pelleted virus particles and cell lysates were prepared as described previously (Willey et al., 1988), while the proteins were resolved on SDS–4–12% polyacrylamide gels and then electrophoretically transferred to polyvinylidene difluoride membranes. ECL western blotting detection reagents (Nakalai Tesque, Kyoto, Japan) were used to detect VSV-G or HIV-1 antigen on the membranes. Briefly, the membranes were incubated for 1 h at room temperature with anti-VSV-G polyclonal antibody (Rockland Immunochemicals, Gilbertsville, PA), the serum of HIV-1 infected patients, or anti-HIV-1 CA-p24 monoclonal antibody (Advanced Biotechnologies Inc., Columbia, MD) and washed. They were then incubated for another 1 h with horseradish peroxidase-conjugated anti-rabbit or anti-human IgG (Vector Laboratories, Burlingame, CA), washed, and visualized by chemiluminescence with LAS-1000 (Fujifilm, Tokyo, Japan) according to the manufacturer's manual. The relative intensity of each band was quantified by digital image analysis using ImageGauge software (Fujifilm).

Cell cytometry analysis

The 293T cells were harvested, washed twice with PBS (−), and fixed by suspending in 1% formaldehyde–PBS(−). The fixed cells were analyzed by FACScan (Bekton Dickinson, Franklin Lakes, NJ) to measure the expression of GFP.

Genomic DNA preparation

Transfected cells were harvested at 12, 24, 48, and 72 h post-transfection, washed twice with PBS(−), and pelleted. GenElute mammalian genomic DNA miniprep kit (Sigma, St. Louis, MO) was used to extract total DNA of the cells. DNA was digested overnight with *DpnI* restriction enzyme at 37 °C to eliminate contaminating plasmids which were methylated.

Alu PCR analysis

For integrated proviral DNA quantitation, a modified Alu-PCR method from a recent report (Brussel and Sonigo, 2003) was employed. In the first round of PCR, two outward-facing Alu primers that anneal within the conserved regions of the Alu repeat element were used together with an HIV-1 LTR specific primer (L-M667) to optimize the probability of amplifying an LTR sequence since Alu elements could be present in either orientation relative to the integrated provirus. L-M667 consisted of an HIV-1 LTR-specific sequence fused with a lambda phage-specific tag sequence at the 5′ end (Brussel and Sonigo, 2003). For the second round of PCR (real-time PCR), a lambda-specific primer (Lambda T) (Brussel and Sonigo, 2003) was used as a sense primer to detect only the amplified fragments in the first round of PCR, and a Taqman probe and an anti-sense primer were selected from the set for R/U5 DNA detection in the previous report (Julias et al., 2001). Cycling conditions of the first round of PCR were 94 °C for 3 min followed by 22 cycles of 94 °C for 30 s, 66 °C for 30 s, 70 °C for 10 min, and then 72 °C for 10 min (Ikeda et al., 2004). Equal volume of DNA (0.15 μg) was applied for amplification. Ten-fold serially diluted DNA samples of 72 h post-transfection with VSV-G pseudotyping were employed as standards. Total amount of DNA in each standard was adjusted by adding total DNA of mock-transfected cells. The resultant PCR products were diluted 100-fold and subjected to real-time PCR using ABI7500 (Applied Biosystems, Foster City, CA).

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