targeting the HIV-1 *env* gene can effectively and specifically inhibit HIV-1 gene expression by reducing viral mRNA expression (Park *et al.*, 2002). Furthermore, the siRNA duplexes are more potent inhibitors than the antisense RNAs. Our best siRNA candidate, E7490 (NL4-3, 7490-75089), targeting the CD4 binding site of the conserved regions on gp120, significantly inhibits HIV-1 gene expression (Park *et al.*, 2003).

In the present study, we designed a mammalian expression vector that directs the synthesis of siRNA-like transcripts and evaluated its application for siRNA-mediated gene interference targeting the *env* gene (E7490). We constructed siRNA expression vectors for two different strands (sense and antisense) and for the expression of the siRNA from hairpin RNA. These two vectors differentially inhibited the efficiency of HIV-1 replication. Furthermore, we examined the ability of lentiviral vectors expressing shRNA to suppress HIV-1 expression in infected SupT1 cells. The env-shRNA-E7490 almost completely suppressed HIV-1 expression in infected cells for up to 15 days.

Materials and methods

Construction of siRNA expression vector

For vectors constructed for the expression of siRNA from hairpin RNA, we used the pU6-ter plasmid, which includes the human U6 small nuclear RNA promoter and cytomegalovirus-green fluorescent protein (CMV-GFP). Hairpin siRNA sequences chemically synthesized as two complementary DNA oligonucleotides were mixed in an equimolar amount, heated for 5 min at 95°C and then gradually cooled down to room temperature in annealing buffer (10 mM Tris-HCl, 100 mM NaCl). The resultant duplex was ethanol precipitated and then ligated into the Kpn I (Acc65 I) and Xho I cloning sites downstream of the U6 promoter to produce the following: pU6-env-shRNA (5′-CCA TGT GGC AGG AAG TAG GAT TCA AGA GAT CCT ACT TCC TGC CAC ATG TTT TTA TCG ATC-3′ and 5′-TCG AGA TCG ATA AAA ACA TGT GGC AGG AAG TAG GAT CTC TTG AAT CCT ACT TCC TGC CAC ATG GGT AC-3′); pU6-m-env-shRNA (5′-CCA TGT GGC ACG AAC TAG GAT TCA AGA GAT CCT AGT TCG TGC CAC ATG TTT TTA TCG ATC-3′ and 5′-TCG AGA TCG ATA AAA ACA TGT GGC ACG AAC TAG GAT CTC TTG AAT CCT AGT TCG TGC CAC ATG GGT AC-3′); pU6-env-S (5′-GTA CAT GTG GCA GGA AGT AGG ATT TTT C-3′ and 5′-TCG AGA AAA ATC CTA CTT CCT GCC ACA TG-3′); pU6-env-A (5′-GTA CCT CCT ACT TCC TGC CAC ATG TTT TTC-3′, 5′-TCG AGA AAA ACA TGT GGC AGG AAG TAG GAG-3′); pU6-m-env-siRNA (m-sen[5′-GTA CCA TGT GGC ACG AAC TAG GAT TTT TC-3′ and 5′-TCG AGA AAA ATC CTA GTT CGT GCC ACA TG-3′) and m-as (5′-GTA CCT CCT AGT TCG TGC CAC ATG TTT TTC-3′, 5′-TCG AGA AAA ACA TGT GGC ACG AAC TAG GAG-3′]).

To construct the lentiviral vectors (CS-env-shRNA, CS-m-env-shRNA), the generated U6 hairpin vectors were digested with *Eco*R I and *Nhe* I and then cloned into the same site in the lentiviral transfer vector (CS-CDF-CG-PRE) (Naldini *et al.*, 1996; Manganini *et al.*, 2002; Rubinson *et al.*, 2003; Stewart *et al.*, 2003).

Cell culture

COS, 293T and SupT1 cells were grown in RPMI 1640 medium (Sigma-Aldrich Co., St. Louis, MO, USA) or Dulbecco's modified Eagle's medium (Sigma-Aldrich Co.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM). All cultures were maintained at 37°C under a 5% CO_2 atmosphere.

Northern blot analysis

Total RNA was extracted with Trizol reagent (GibcoBRL Life Technologies, Carlsbad, CA, USA) from vector-transfected cells, and samples (30 μg) were loaded onto a 20% polyacrylamide/8M urea gel. After electrophoresis, the RNA bands were transferred onto a Hybond-N™ nylon membrane (Amersham Co., Buckinghamshire, UK). The membrane was hybridized with synthetic oligonucleotides complementary to the antisense strand of the *env* shRNA. Hybridization was performed at 42°C, followed by washing with 2×SSC at 25°C prior to autoradiography.

Flow cytometry

Transduced SupT1 cells were washed twice in phosphate buffered saline (PBS), and fixed in PBS containing 1% formaldehyde. Direct fluorescence of GFP was analysed by FACS Calibur™ (Becton Dickinson, Franklin Lakes, NJ, USA). Data acquisition and analysis were performed with CellQuest software (Becton Dickinson). GFP detection parameters were established using mock-transduced cells as background.

Fluorescent microscopy

To evaluate the self-replicating function of the siRNA as an index for stable transgene expression in cells, transfected COS cells and SupT1 cells were trypsinized and seeded at a low cell density (3×10^5 cells). Direct fluorescence microscopy for GFP was performed at the mitotic stage of cell division after passages on days 3 and 15, and the data were obtained with a DP12 digital microscope camera (Olympus Co., Tokyo, Japan).

Inhibition of HIV-1 replication by expression vectors

The two different types of vectors (1 μg) were co-transfected with pNL4-3 (0.1 μg), using 3 μl of FuGENE™ 6 (Roche Diagnostics, Mannheim, Germany) in COS cells (3×10^5 cells). After 3 days of incubation, virus replication was monitored in cell-free culture supernatants with the HIV-1 p24 CLEIA assay (Lumipulse; Fujirebio, Inc., Tokyo, Japan). GFP expression was also observed and photographed with a fluorescent microscope.

Reverse transcription-PCR (RT-PCR) analysis

Total RNA from vector-transfected cells was extracted with the GenElute Mammalian Total RNA kit (Sigma-Aldrich Co.). Reverse transcription-PCR was then performed using an RNA PCR high-plus kit (Toyobo, Osaka, Japan) with *env* upstream (NL4-3 7070–7099) and downstream (NL4-3 7570–7600) primers; these are referred to as forward primer F1– (5′-ACA GCT GAA CAC ATC TGT AGA AAT TAA TTG-3′) and reverse primer R1– (5′-GTT GTT ATT ACC ACC ATC TCT TGT TAA TAG-3′). As an internal control, the mRNA of the human control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified simultaneously with the GAPDH-F (nucleotides 230–254) and downstream GAPDH-R (nucleotides 422–466) primers. These RT-PCR products were amplified using the following thermal cycle program: 1 cycle (60°C for 30 min and 94°C for 2 min), 25 cycles (94°C for 1 min and 51°C for 1.5 min) and 1 cycle (46°C for 7 min).

Virus preparation

A vector construct (15 μg) was co-transfected with the helper constructs encoding *gag/pol* (pMDLg/p.RRE) (15 μg), the Rev-expressing construct pRSV-rev (5 μg) and the VSV-G-expressing construct pMD.G (5 μg) into 293T cells using the calcium phosphate-precipitation method. The supernatants were harvested at 48 h post-transfection, filtered with a 0.45 μm filter disc and concentrated 100-fold by overnight centrifugation at 6,000×*g*. The resultant viral pellet was re-suspended in serum- and antibiotic-free RPMI medium (Sigma-Aldrich Co.) and stored at –80°C until use. To determine the viral titre, SupT1 cells were transduced with the prepared viral stock, and the numbers of GFP-positive cells were assessed after 72 h of culture by flow-cytometric analysis (Becton Dickinson).

HIV-1 challenge and culture assay

SupT1 cells were transduced by the CS-env-shRNA and control vectors at a multiplicity of infection (MOI) of 20 with 8 μg/ml polybrene. After incubation at 37°C for 8 h, the medium was removed before the HIV-1 challenge was initiated. SupT1 cells were infected with HIV-1_{NL4-3} at an MOI of 0.01. After the harvested culture was centrifuged, the cell-free medium was used for HIV-1 p24 CLEIA (Sakai *et al.*, 1999), while the pellet was used for GFP expression as an index of long-term expression of the transgenes, as monitored with a fluorescent microscope.

Results

Design and construction of the siRNA-driven expression system

The cellular machinery required for RNA interference (RNAi) exists in mammalian cells. To express siRNA targeting HIV-1 (NL4-3 7490–7508), we constructed vectors for two different strands (sense and antisense) and hairpin RNA, which were under the control of the RNA *pol* III promoters of the U6 small nuclear RNA gene (Figure 1) (Brummelkamp *et al.*, 2002). For the control vector, a G was mutated to a C in the siRNA sequence. We also constructed two different strand vectors, antisense (env-A) and sense (env-S), which were under the control of the U6 promoter (Figure 1). To construct the lentiviral vectors, the *Eco*R I site upstream from the U6 promoter and the *Eco*R I cloning site downstream the inserted fragments were digested and cloned into the *Eco*R I site of the lentiviral transfer vector (CS-CDF-CG-PRE) (Naldini *et al.*, 1996; Manganini *et al.*, 2002; Rubinson *et al.*, 2003; Stewart *et al.*, 2003), to generate the CS-env-shRNA and control transfer vectors (CS-m-env-shRNA). The sequences and orientations of the constructed vector inserts were confirmed by nucleotide sequencing.

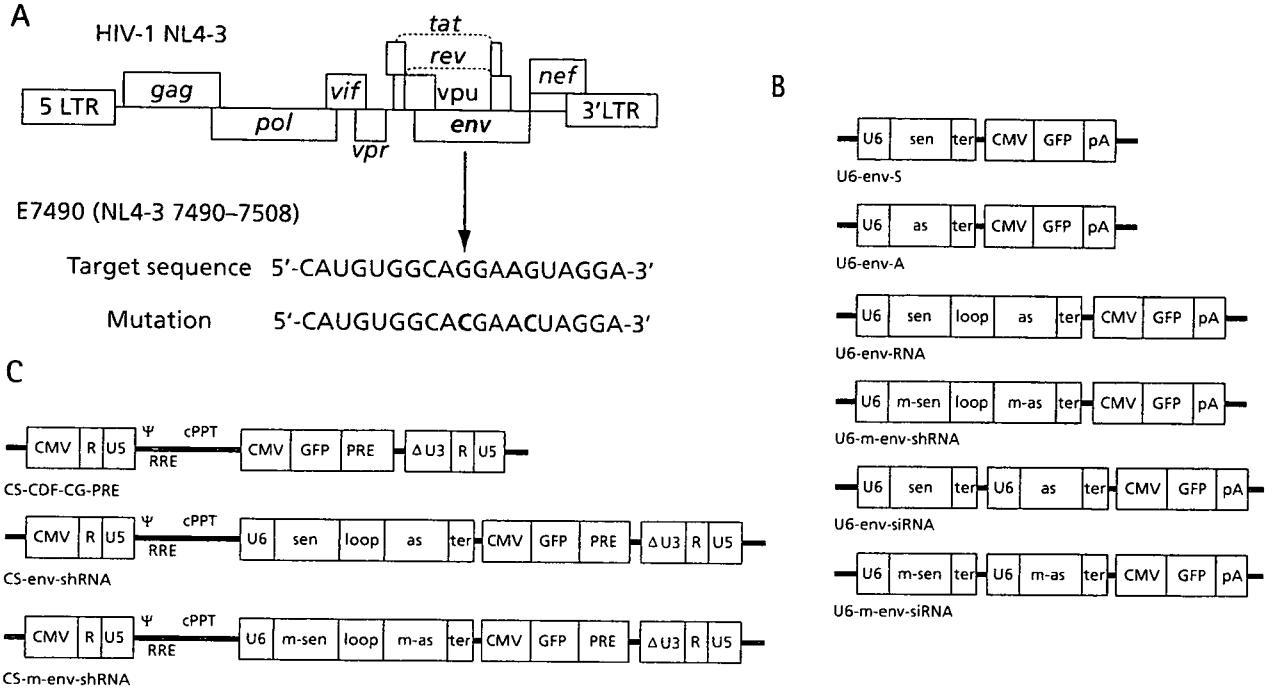
Expression of the shRNA in target cells

To characterize the effect of transgene expression, we monitored GFP expression in COS cells transfected with the six different plasmids. GFP expression in the transfected COS cells was observed for 3 days (Figure 2A). We also detected env-siRNA expression from pU6-env-shRNA in COS cells by Northern blot analysis. The env-siRNA was observed in pU6-env-shRNA-transfected COS cells (Figure 2B, lane 2).

Inhibition of HIV-1 replication by two different types of vectors, siRNA expression by a tandem promoter and an shRNA

To determine the potency and inhibitory efficacy of the two different types of vectors on HIV-1 replication in a transient assay, an HIV-1_{NL4-3} based-vector, pNL4-3 and the plasmid DNAs, pU6-env-shRNA and pU6-env-siRNA, were cotransfected into COS cells with the transfection reagent, FuGENE™ 6. After 72 h incubation, virus replication was monitored in the culture supernatants with the HIV-1 p24 CLEIA assay (Sakai *et al.*, 1999). The shRNA (pU6-env-shRNA) inhibited p24 expression more

Figure 1. Construction of the shRNA and siRNA expression vectors and the target sequence in siRNA design



(**A**) HIV-1 genomic sequence showing the target sequence in the *env* gene used to design the small interfering RNA (siRNA). (**B**) The siRNA expression vectors for two different strands (sense and antisense) and the expression of the siRNAs from the hairpin RNA included the promoter sequence from human U6 and a terminator sequence. The short hairpin RNA (shRNA) included the loop sequence (5'-UUCAAGAGA-3'). (**C**) The viral RNA genome is produced from the HIV-1 based lentiviral vector construct and contains the U6 promoter and transgene sequences (shRNA). In addition, the lentiviral vector contains the following *cis*-acting sequences: packaging signal (Ψ) comprising the 5'-untranslated region (5'-UTR) and the 5' sequences comprising the Rev-responsive element (REE), central polypurine tract (cPPT), and woodchuck hepatitis virus posttranscriptional regulatory element. The 3'-long terminal repeat contains a large deletion in the U3 region (ΔU3).

effectivcly than env-siRNA expression by the tandem promoter (pU6-env-siRNA) (Figure 3). Furthermore, we compared the inhibitory effects of these dsRNAs targeting the HIV-1 *env* gene with those of the corresponding sense (U6-env-S) and antisense RNAs (U6-env-A). In this experiment, in comparison with the control (U6-ter), p24 expression was suppressed 11-fold by cotransfection of the U6-env-shRNA and 2.5-fold by the antisense RNA (U6-env-A). In contrast, the control plasmids, sense RNA (U6-env-S), mutant shRNA (U6-m-env-shRNA) and mutant siRNA (U6-m-env-siRNA), allowed high levels of p24 expression (Figure 3).

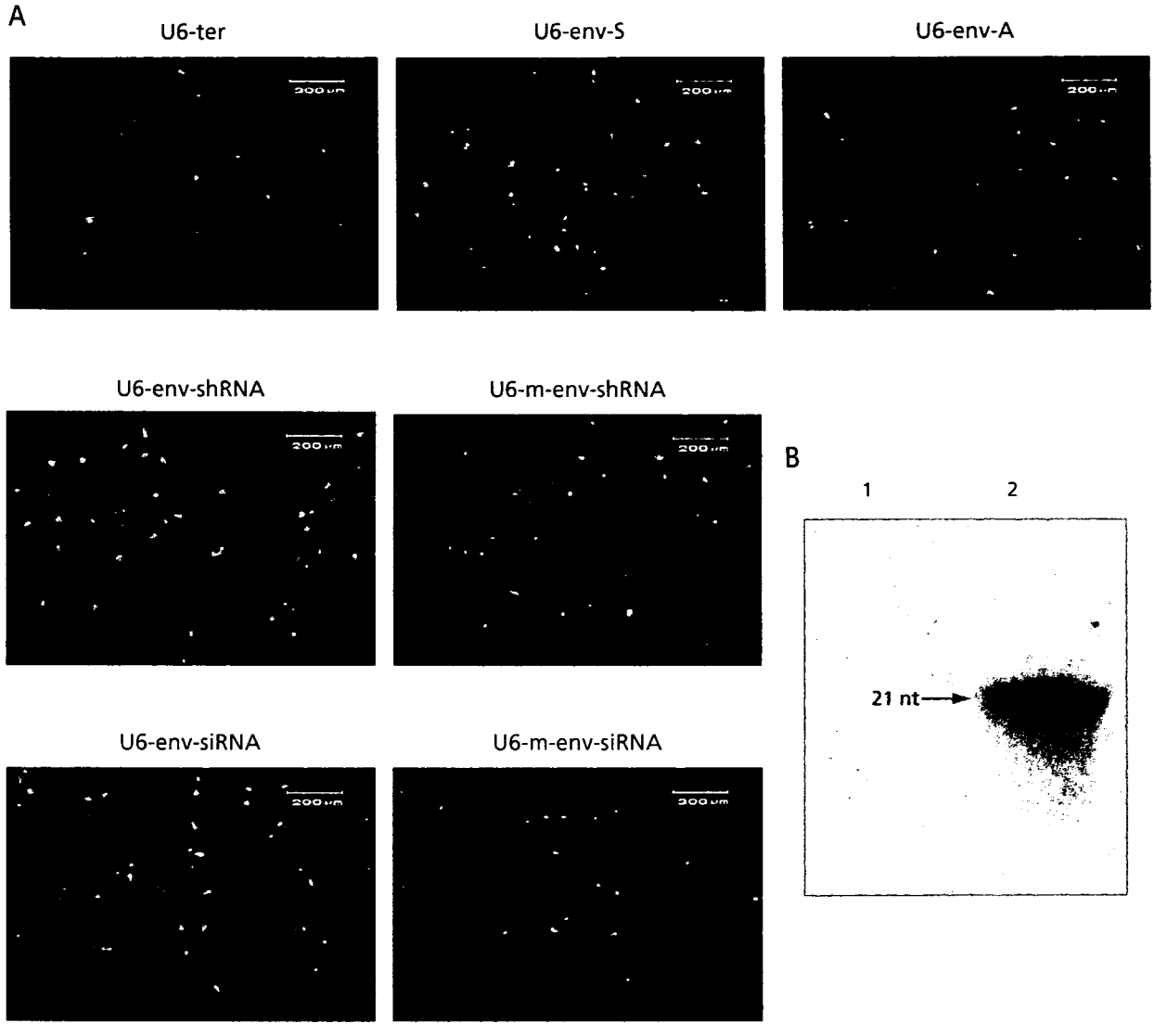
Effect of two different types of vectors on siRNA expression

We also examined the HIV-1 mRNA levels to identify the contribution of the RISC (siRNA–protein) complex-mediated RNA cleavage. RT-PCR reactions were used to establish the level of cleaved HIV-1 mRNA. An equal amount of total RNA of COS cells transfected with plasmid vectors and pNL4-3 was subjected to RT-PCR analysis. HIV-1 *env*-specific DNA primers (sense: 7072–7101; antisense: 7571–7600) were used to amplify a 529-bp (7072–7600) fragment in the transcripts (Figure 4A). The RNA obtained from the pU6-env-shRNA and pU6-env-siRNA transfected cells had drastically reduced HIV-1 transcript levels (Figure 4B). The mRNA from the cells transfected with other control plasmids, however, still contained abundant levels of uncleaved HIV-1 mRNA (Figure 4B). The reduction in the functional full-length HIV-1 mRNA is consistent with the siRNA-mediated cleavage effect at the post-transcriptional level.

Inhibition of HIV-1 gene expression by lentiviral vector-mediated shRNA in human T cells

Lentiviruses integrate into the chromosomal DNA so that the genome is stable in the host cells and is inherited by their progeny. Accordingly, long-term expression of a transduced gene can be achieved through lentivirus-mediated gene transfer. Other advantages of this vector include its

Figure 2. Expression of transgenes



(A) To monitor the continuous expression of the transgenes in the transduced cells throughout the culture period, the cells were transferred onto microscope slides and examined for green fluorescent protein expression during the sampling of the culture cells and supernatants. **(B)** The presence of small interfering (siRNA) was analysed by Northern blotting. Plasmids encoding U6-dsRNAs were introduced into COS cells. After 48 h, the cells were collected and total RNA was isolated and fractionated on a 15% polyacrylamide gel. Northern blotting was performed as described in the text (lane 1, control vector, U6-ter; lane 2, U6-env-siRNA). nt, nucleotide

broad host range and the availability of packaging cell lines for the large-scale production of high-titre vectors.

To investigate the sudden upsurge of viral replication in the cultures expressing the CS-env-shRNA, viral RNA from HIV-1_{NL4-3} was isolated from HIV-1-infected cells expressing the lentiviral vectors, CS-env-shRNA and CS-m-env-shRNA, on days 3, 6, 9, 12 and 15. The cells were infected with the wild-type HIV-1_{NL4-3}, and HIV-1 p24 antigen levels were measured in cell-free supernatants at 1-week intervals over a 15-day period. By day 15, HIV-1 replication was almost completely suppressed (~97%) in the cell cultures expressing env-shRNA (Figure 5A). In contrast, m-env-shRNA failed to inhibit viral replication under these experimental conditions, as did env-shRNA.

Furthermore, to characterize the effect of transgene expression, we observed the expression of env-siRNA and GFP in the CS-env-shRNA plasmid-transfected SupT1

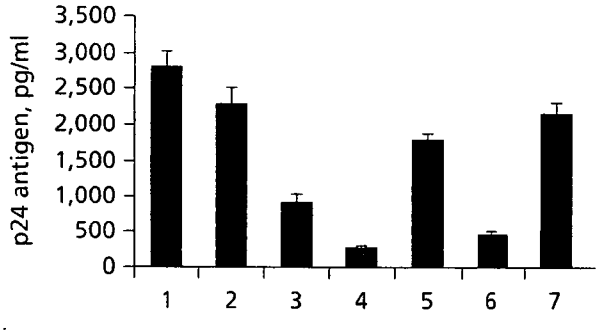
cells. Northern blot analysis was used to detect the production of siRNA in the SupT1 cells. siRNA expression was observed in the CS-env-shRNA plasmid-transfected SupT1 cells (Figure 5B). GFP expression in the CS-env-shRNA and CS-m-env-shRNA plasmid-transfected SupT1 cells was observed for 15 days (Figure 5C).

Discussion

To test whether siRNAs could effectively mediate the silencing of gene expression without inducing the interferon response, Elbashir *et al.* (2001a, 2001b, 2001c) introduced chemically synthesized siRNA into mammalian cells. They reported that siRNA transfection resulted in sequence-specific silencing of luciferase expression, as well as the endogenous nuclear envelope protein lamin A/C, in several mammalian cell lines without activating non-specific effects. However, chemically synthesized siRNA-directed silencing by transfection is limited in *Drosophila* and mammals due to its transient nature. To overcome some shortcomings of the transfection of chemically synthesized siRNA into cells, several groups have developed DNA-vector-mediated mechanisms to express substrates that can be converted into siRNAs (Brummelkamp *et al.*, 2002; Lee *et al.*, 2002; McManus *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002; Tavernarakis *et al.*, 2000; Yu *et al.*, 2002). Two approaches have been used for the expression of siRNA by the constructs driven by RNA *pol* III promoters.

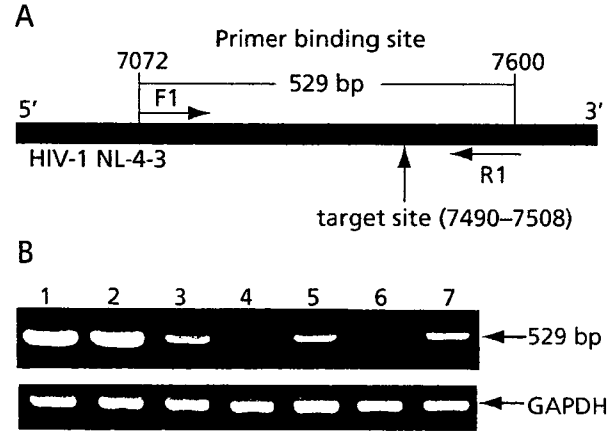
In this study, we demonstrated a potent inhibitory effect on HIV-1 replication for siRNA expressed from the short hairpin. We constructed siRNA expression vectors for two different strands (sense and antisense; tandem promoter) and for siRNA expressed from the short hairpin RNA, which were driven under the control of the RNA *pol* III promoters of the U6 small nuclear RNA gene (Figure 1). We also constructed shRNA-expressing lentiviral vectors, CS-env-shRNA and control transfer vectors (CS-m-env-shRNA). The level of siRNA expression from the two different types of vectors, env-siRNA expressed by the tandem promoter and the shRNA (env-shRNA) in COS cells will probably determine the extent of HIV-1 gene knockdown. These two vectors had stronger HIV-1 inhibitory effects than the antisense RNA (U6-env-A) (Figure 3). Furthermore, shRNA inhibited p24 expression more effectively than two different strands (sense and antisense; tandem promoter). The results in the anti-HIV-1 assay correlated with the HIV-1 mRNA levels to identify the contribution of the RISC (siRNA–protein) complex-mediated RNA cleavage (Figure 4B). These results suggest that the shRNA is more effective than the tandem promoter for expressing specifically targeted siRNAs.

Figure 3. Cotransfection of various RNA expression plasmids



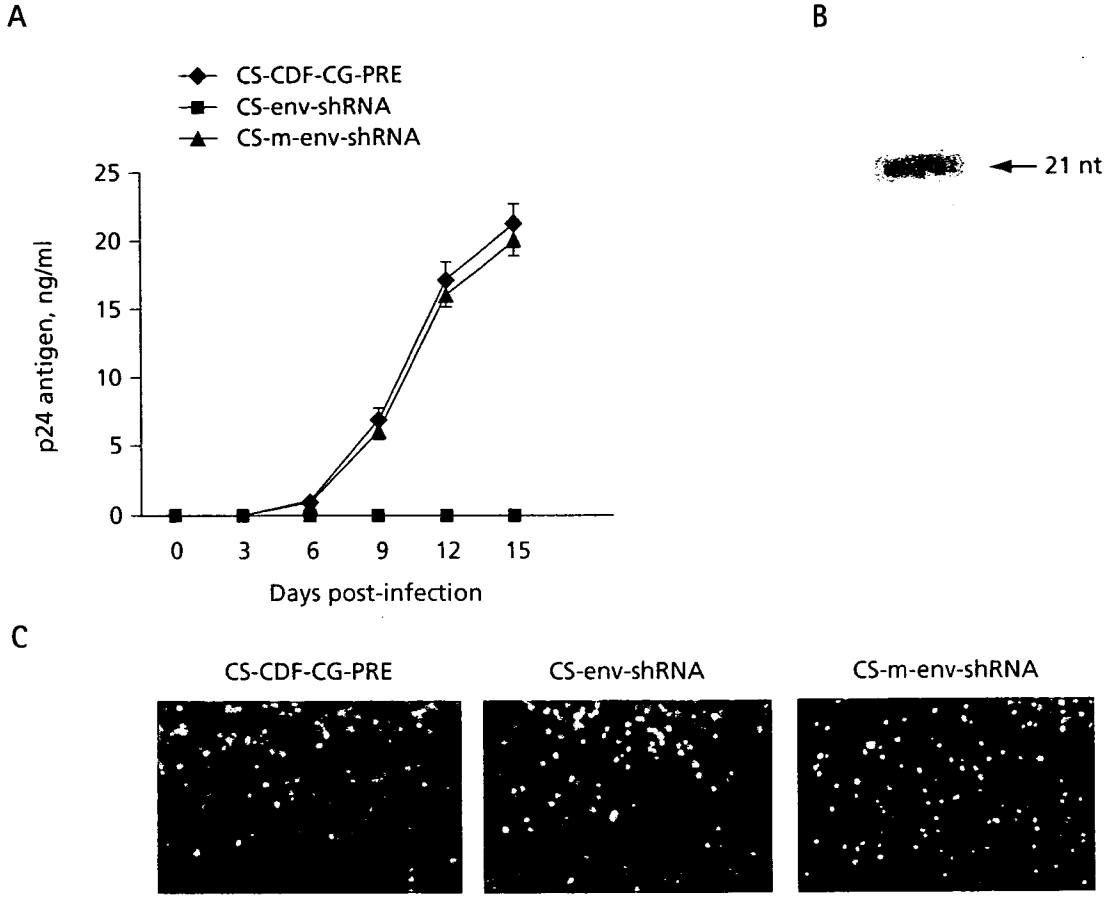
Co-transfection with *env*-specific sense, antisense, short hairpin RNA (shRNA), small interfering RNA (siRNA) and m-shRNA molecules with pNL4-3 in COS cells. The cell culture supernatants were determined for their p24 antigen levels on day 3 post-transfection, and a 91% reduction of p24 in cells transfected with env-shRNA was detected as compared with controls. Values represent the means with ranges of three independent experiments. Lane 1: U6-ter, lane 2: U6-env-S, lane 3: U6-env-A, lane 4: U6-env-shRNA, lane 5: U6-m-env-shRNA, lane 6: U6-env-siRNA, lane 7: U6-m-env-siRNA. All data are means ±standard deviations (error bar) from three independent experiments. A, antisense; m-shRNA, mutant shRNA; S, sense.

Figure 4. RT-PCR analyses of HIV-1 mRNA expression



RT-PCR analyses of uncleaved HIV-1 mRNA were performed using HIV-1 *env*-specific primers with concurrent amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. **(A)** Schematic representation of HIV-1-specific primer sites with respect to HIV-1 mRNA: F1 primer (7072–7101) and R1 primer (7571–7600). **(B)** RT-PCR amplification products fractionated by electrophoresis on a 2% agarose gel with ethidium bromide staining. Lane 1: U6-ter, lane 2: U6-env-S, lane 3: U6-env-A, lane 4: U6-env-shRNA, lane 5: U6-m-env- shRNA, lane 6: U6-env-siRNA, lane 7: U6-m-env-siRNA. A, antisense; S, sense; shRNA, small hairpin RNA; siRNA, small interfering RNA.

Figure 5. Inhibition of HIV-1 gene expression by lentiviral vector-mediated shRNA



(A) The short hairpin RNA (shRNA)-env stable expressing SupT1 cells were infected with HIV-1_{NL4-3} at an multiplicity of infection of 0.01, and p24 antigen levels were assessed over a 15-day period. Viral replication was suppressed by 98% until day 15 as compared with control cells. **(B)** Northern blot analysis of env-shRNA expression in SupT1 cells stably expressing env-shRNA. Env-shRNA expression was observed. **(C)** To monitor the continuous expression of the transgenes in the transduced cells throughout the culture period (day 15), the cells were transferred onto a microscope slide and examined for GFP expression during sampling of the culture cells and supernatants. All data are means ±standard deviations (error bar) from three independent experiments. GFP, green fluorescent protein.

When the sense and antisense strands are transcribed from separate promoters, the strands must anneal to form an siRNA. In contrast, the linked shRNA strands readily form a duplex, but the loop joining these strands must be processed with Dicer to generate siRNAs. The use of shRNAs allows for the insertion of multiple transcription units targeting various sites in the HIV-1 genome within a single vector.

Since gene therapy for HIV-1-infected patients is not likely to eliminate HIV-1 from their bodies, the persistence of a therapeutic anti-HIV-1 gene is important for long-term treatment. The lentivirus-based vectors can genetically modify non-dividing cells (Naldini *et al.*, 1996; Manganini *et al.*, 2002) and deliver genes into dividing cells at high efficiency (Tavernarakis *et al.*, 2000; Yu *et al.*, 2002). HIV-1-based vectors expressing various anti-HIV-1 genes have previously been reported to deliver them into lymphocytes, monocytes, stem cells and neuronal cells (Follenzi *et al.*, 2000; Li *et al.*, 2003; Mautino & Morgan, 2002; Mukhtar *et al.*, 2000; Pandya *et al.*, 2001; Qin *et al.*, 2003; Schroers *et al.*, 2002). We also demonstrated the inhibition of HIV-1 gene expression by lentiviral vector-mediated shRNA in SupT1 cells. The lentiviral vector-mediated shRNA strongly suppressed HIV-1 replication and exhibited long-term shRNA expression. By contrast, m-env-shRNA failed to inhibit viral replication under these experimental conditions, as did env-shRNA (Figure 5A). Furthermore, siRNA and GFP expression was observed in the CS-env-shRNA plasmid-transfected SupT1 cells for 15 days (Figures 5B,C).

In conclusion, shRNA was more effective than the tandem promoter for expressing specifically targeted siRNA because the sense and antisense strands are transcribed from separate promoters, and these strands must anneal to form an siRNA, whereas the linked shRNA strands readily form a duplex. We also demonstrated the long-term inhibition of HIV-1 infection in T-cells by lentiviral vector-mediated shRNA. This lentiviral vector-mediated shRNA anti-gene is a promising tool for practical RNAi-based HIV-1 gene therapy for the treatment of HIV-1 infection.

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Inhibition of HIV-1 replication by vesicular stomatitis virus envelope glycoprotein pseudotyped baculovirus vector-transduced ribozyme in mammalian cells ☆

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Abstract

The baculovirus has recently emerged as a promising vector for *in vivo* gene therapy. To investigate its potential as a delivery vector for an anti-virus ribozyme targeting HIV-1, we constructed recombinant baculovirus vectors bearing a ribozyme-synthesizing cassette driven by the $tRNA_i^{Met}$ promoter with enhanced transduction efficiency by displaying vesicular stomatitis virus glycoprotein (VSV-G) on the viral envelope. Transduction of HeLa $CD4^+$ cells with a recombinant baculovirus delivering the HIV-1 U5 gene-specific ribozyme dramatically suppressed HIV-1 expression in this cell line. The VSV-G pseudotyped baculovirus vector-transduced ribozyme potently inhibited HIV-1 replication compared to a recombinant baculovirus vector-transduced ribozyme lacking VSV-G. The use of a baculovirus vector might be beneficial for application in gene therapy.

Keywords: Baculovirus vector; Ribozyme; Gene silencing; Mammalian cells; Vesicular stomatitis virus glycoprotein; HIV-1

In recent years, the baculovirus (Autographa californica multiple nuclear polyhedrosis virus, AcMNPV) has emerged as a vector with great potential for gene transfer in mammalian cells [1]. Baculoviruses are a group of insect viruses possessing a rod-shaped capsid containing a condensed DNA genome, a double-stranded, covalently closed circular molecule ranging between 80 and 200 kbp in length [2]. The baculovirus (AcMNPV) has long been used as a biopesticide and as a tool for efficient recombinant protein production in insect cells [3]. Although its host specificity was originally thought to be restricted to cells derived from arthropods, the baculovirus infects a number of mammalian cells and animal models [4–16]. Gene transfer mediated by baculovirus vectors carrying a reporter gene under the control of a strong mammalian promoter, such as the immediate-early promoter of cytomegalovirus (CMV) or the chimeric CMV early enhancer (CAG) promoter, has been demonstrated in a number of human primary cell lines and liver tissue [5,8]. This vector is also capable of carrying large inserts and efficiently infects a variety of cell

☆ *Abbreviations*: AcMNPV, Autographa californica multiple nuclear polyhedrosis virus; VSV-G, vesicular stomatitis virus glycoprotein; MOI, multiplicity of infection; LTR, long terminal repeat; GFP, green fluorescent protein; HIV-1, human immunodeficiency virus type-1; Rz, ribozyme; U5, untranslated 5′ region.

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lines without any apparent viral replication or cytopathic effect, even at a high multiplicity of infection (MOI) [7,10]. Furthermore, enhanced gene transfer efficiency was observed in a variety of cell lines with recombinant baculovirus vectors expressing surface glycoprotein G of the vesicular stomatitis virus (VSV-G). VSV-G enhances the escape of baculovirus vectors from intracellular endosomes, thereby increasing the transduction efficiency of the virus [17].

The use of the baculovirus as a vector for vaccination was initially described by Aoki and co-workers, who demonstrated that injecting mice with a recombinant vector expressing pseudorabies virus glycoprotein B elicited a measurable humoral response directed against this viral glycoprotein [18]. More recently, we demonstrated that an immune response to the hemagglutinin glycoprotein of influenza virus was elicited upon vaccination with a baculovirus vector expressing the virus structural component. Additionally, the induction of a strong innate immune response was also detected upon injection of wild-type baculovirus [19]. The baculovirus is now recognized to induce a strong innate immune response, in addition to functioning as a gene delivery vector in mammalian cells. On the other hand, the effects of RNA interference by baculovirus vector mediated short-hairpin RNA have been reported by only a few groups [20–22]. Such advantages have resulted in baculovirus being increasingly explored as a possible alternative to more traditional types of viral vectors as a mammalian gene-delivery vehicle *in vitro* or *in vivo* [23]. Hammerhead ribozymes can be designed to cleave any RNA sequence. They are regarded as tools for *in vitro* and *in vivo* RNA intervention. HIV-1 proviral gene expression is tightly regulated by the binding of cellular host proteins to a variety of cis-acting DNA sequences located within the long terminal repeat (LTR) region of the viral genome [24]. The HIV-1 LTR is divided into three regions: U3, R, and U5. Recently, important motifs within the U5 region and gag leader sequences (GLS) have been described [25–28]. The long terminal repeat (LTR) of HIV-1 is a potential target of the ribozyme [29].

In this paper, we describe the inhibition of HIV-1 replication by a baculovirus vector-transduced ribozyme. In addition, a baculovirus vector encoding the U5-ribozyme downstream of the *Pol*III promoter ($tRNA_i^{met}$) and expressing the VSV-G in the viral envelope was generated by inserting the VSV-G coding sequence downstream of the polyhedrin promoter. The VSV-G-modified baculovirus (Ac/VSV-G) more efficiently transduced genes into mammalian cells than did wild-type baculovirus. This finding is also consistent with the inhibitory effect of baculovirus-mediated ribozyme on HIV-1 replication.

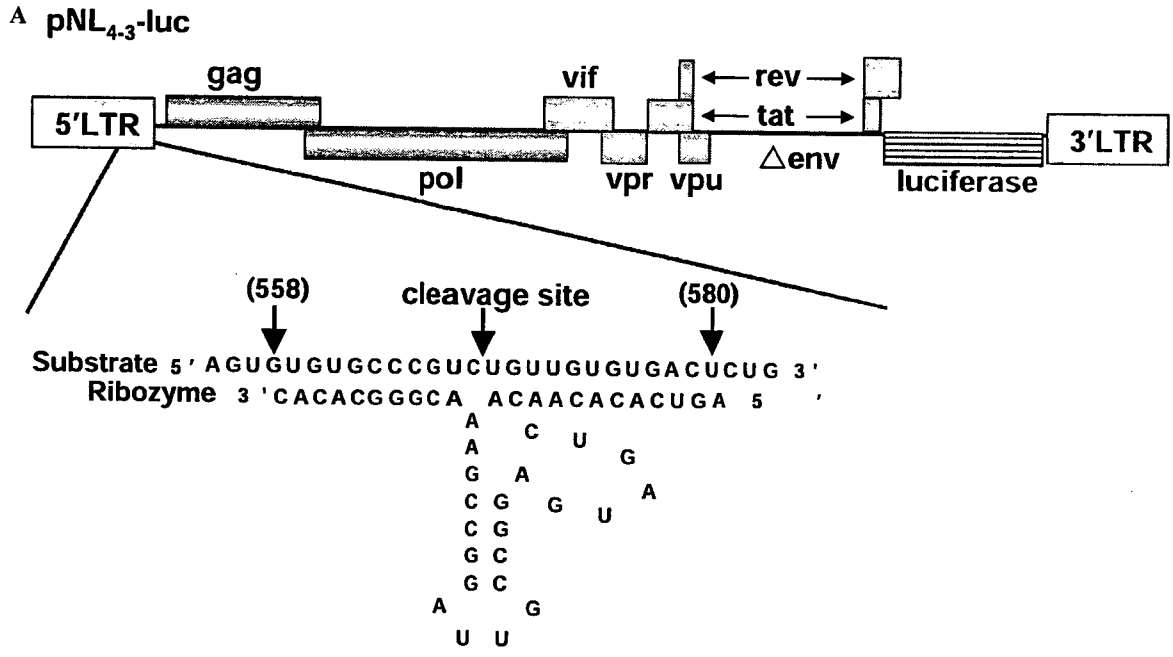

Fig. 1. Locations of the target sites and schematic representation of ribozyme-expression vectors. (A) HIV-1 NL4-3 molecular clone pNL_{4-3}-luc containing the luciferase reporter gene, showing the target site and structure of the constructed ribozyme. The arrow indicates the ribozyme cleavage point. (B) Baculovirus transfer plasmids were derived from pVL 1393 as described in Materials and methods. The U5-Rz and VSV-G coding sequences are indicated. The name of each vector is also indicated. Expression of U5-Rz is driven by the *Pol*III promoter, $tRNA_i^{Met}$. The baculovirus polyhedrin promoter drives the expression of VSV-G. (C) Western blot analysis of virion preparations of wild-type AcMNPV, Ac/$tRNA_i^{Met}$-U5-Rz, Ac/VSV-G/$tRNA_i^{Met}$-U5-Rz, Ac/$tRNA_i^{Met}$-ter, and Ac/VSV-G/$tRNA_i^{Met}$-ter. VSV-G, Gp65, and Vp39 proteins in wild-type AcMNPV, Ac/$tRNA_i^{Met}$-U5-Rz, Ac/VSV-G/$tRNA_i^{Met}$-U5-Rz, Ac/$tRNA_i^{Met}$-ter, and Ac/VSV-G/$tRNA_i^{Met}$-ter virion preparations processed for Western blot analysis as described in Materials and methods. MAb specificity is indicated at the side (VSV-G, Gp65, and Vp39), and virion preparations are indicated above each lane (wild-type AcMNPV, Ac/$tRNA_i^{Met}$-U5-Rz, Ac/VSV-G/$tRNA_i^{Met}$-ter, Ac/VSV-G/$tRNA_i^{Met}$-U5-Rz, and Ac/VSV-G/$tRNA_{iMet}$-ter). (D) HeLa CD4$^+$ cells were infected with Ac/CMV-GFP and Ac/VSV-G/CMV-GFP at MOI of 50. Direct fluorescence microscopy (Olympus Company, Tokyo, Japan) of the expressed reporter gene (GFP) was performed under mitotic stage of cell division, After each passage on days 1, 3, and 6, the data were acquired with a DP12 Digital microscope camera (Olympus Company).

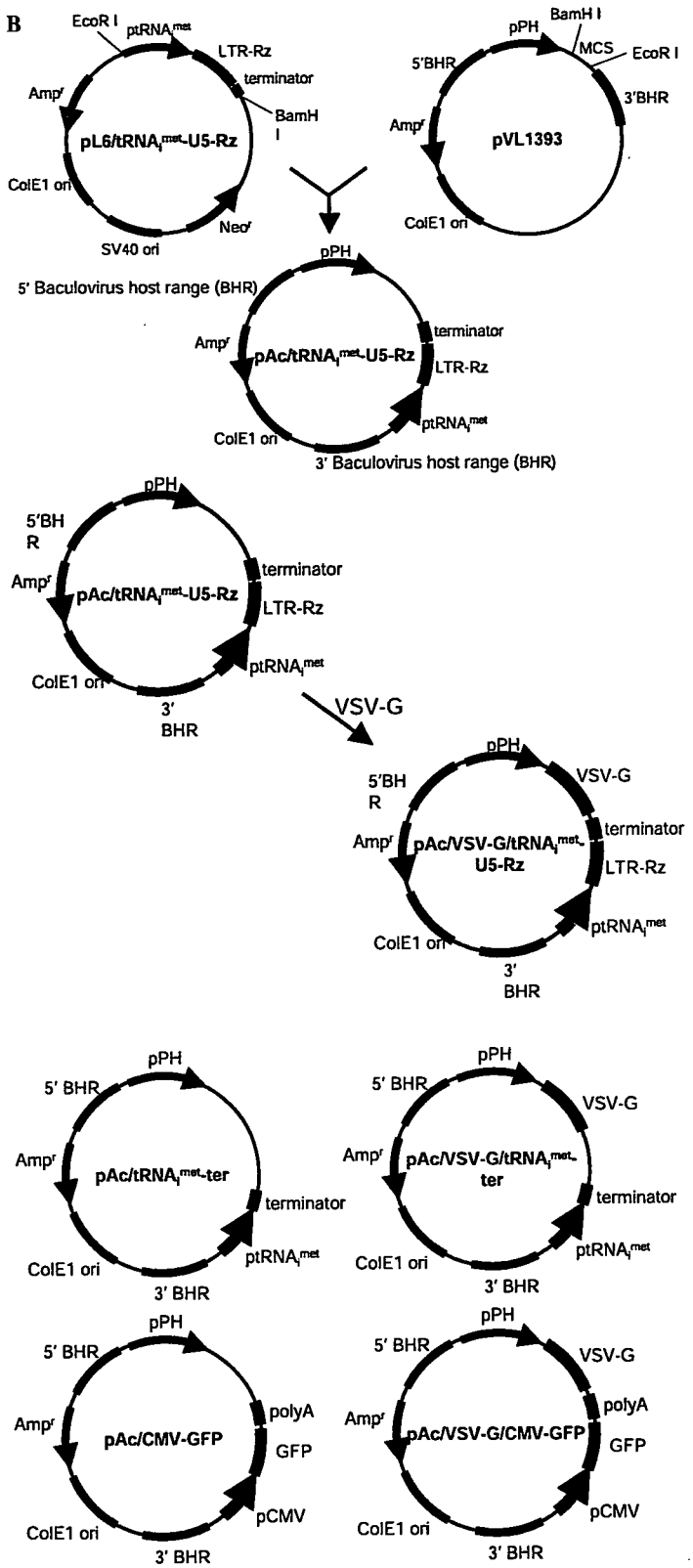

Fig 1. (*continued*)

Materials and methods

Cell culture and transfections. HeLa CD4$^+$ cells were grown in RPMI1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 atmosphere. The inset cell line Sf9 (ATCC CRL-1711) was grown in BD Baculogold™ medium (BD Biosciences, Pharmingen, San Diego, CA) supplemented with 100 ng/ml kanamycin.

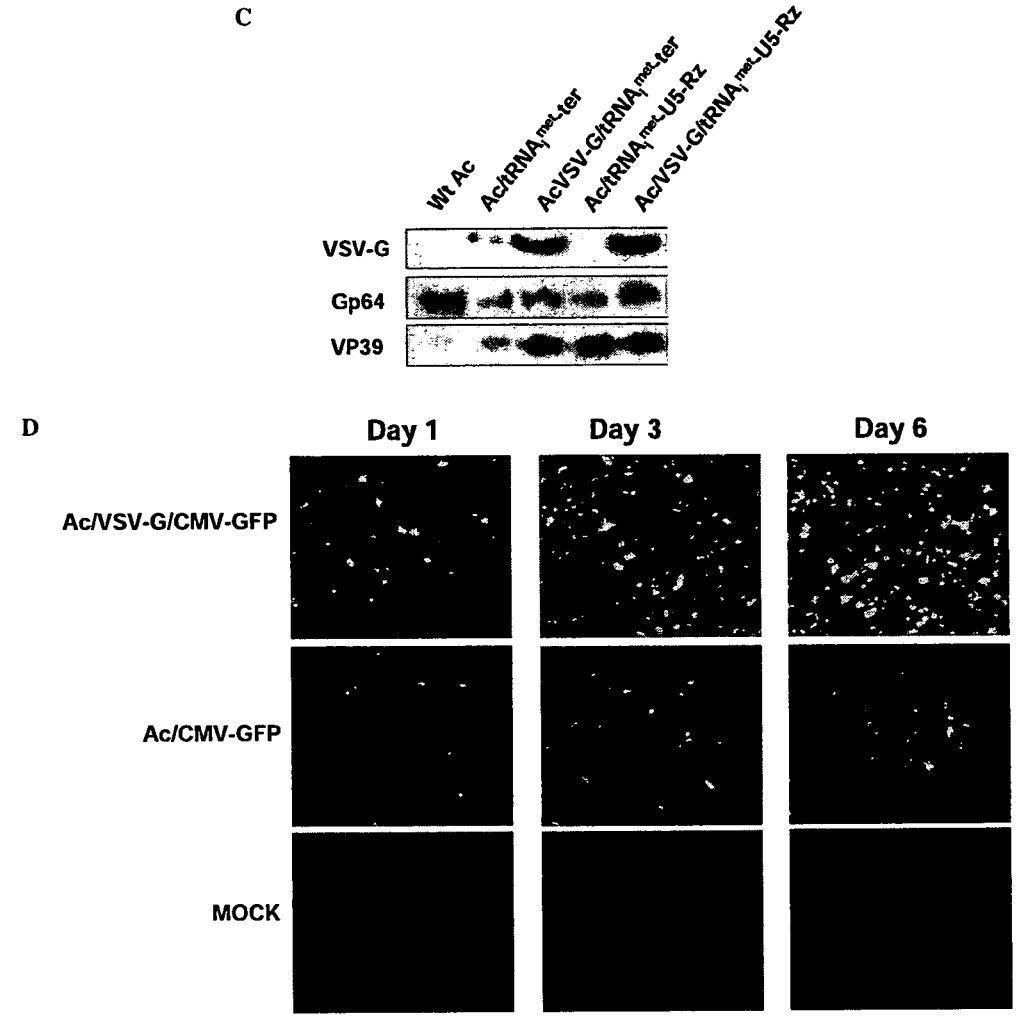

Fig 1. (*continued*)

Construction of recombinant baculoviruses. The ribozyme was designed to target the U5 region of the HIV-1 LTR RNA (Fig. 1A), and to be driven by the $tRNA_i^{Met}$ promoter. DNA fragments encoding the sense (5′-CTC ACA CAA CAC TGA TGA GGC CGT TAG GCC GAA ACG GGC ACA CCG TTT TTG GG-3′) and antisense (5′-GAT CCC CAA AAA CGG TGT GCC CGT TTC GGC CTA ACG GCC TCA TCA GTG TTG TGT GAG GTA C-3′) sequences of the ribozyme and terminator sequences were annealed and ligated into the *Kpn*I and *Bam*HI sites of pSV2neo (L6), with the $tRNA_i^{Met}$ promoter. The $tRNA_i^{Met}$-U5 ribozyme gene (Rz) was excised as an *Eco*RI/*Bam*HI fragment from the pL6-U5-Rz plasmid. This fragment was inserted into the *Eco*RI/*Bam*HI sites of the baculovirus transfer vector, pVL1393 MCS (BD Biosciences, San Jose, CA). Next, the VSV-G gene was excised as a *Bam*HI fragment from pMD.G (kindly provided by Dr. Miyoshi), and was inserted into the *Bam*HI sites of pAc/$tRNA_i^{Met}$-U5-Rz in a direct orientation with respect to the polyhedrin promoter to create pAcVSV-G/$tRNA_i^{Met}$-U5-Rz. The control vectors, pAc/$tRNA_i^{Met}$-ter and pAcVSV-G/$tRNA_i^{Met}$-ter, were constructed as described above.

Baculovirus production. Sf9 insect cells (American Type Culture Collection, Rockville, MD) were transfected with the linearized baculovirus DNA (BD Biosciences). with Cellfectin (Life Technologies), and the recombinant baculovirus vectors were amplified by repeated passages. To purify the virus, cell debris were first removed by centrifugation for 10 min at 2000 rpm. Infected cell supernatant was then layered over 27% sucrose and centrifuged at 24,000 rpm for 75 min in a Beckman SW28 ultracentrifuge tubes (Beckman Coulter Inc., Fullerton, CA). The virus pellet was resuspended in phosphate-buffered saline (PBS, pH 7.5) and centrifuged in SW28 tubes at 27,000 rpm for 150 min. The final pellet was resuspended in PBS. Purified virus was titrated in Sf9 cells with the BD BacPAK baculovirus rapid titer kit (BD Biosciences) and stored at 4 °C in the dark.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis. Samples were prepared for Western blot analysis in the following manner. Cell extracts from infected or uninfected cells were lysed in 1× Laemmli buffer (125 mM Tris, 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue, pH 6.8) and heated to 100 °C for 5 min prior to electrophoresis. Virions of wild-type AcMNPV or pseudotyped Ac/VSV-G were prepared from tissue culture supernatants by centrifugation at 80,000*g* for 75 min at 4 °C through a 25% sucrose cushion in PBS and subsequent resuspension of the pellet in 1× Laemmli buffer. Samples were heated to 100 °C for 5 min and subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. Approximately 2.6×10^4 cells or 8×10^6 virions were electrophoresed in each lane. Gels were blotted onto Immobilon-P filters (Millipore, Billerica, MA) and incubated with the following primary monoclonal antibodies (MAbs): an anti-GP64 MAb (AcV5) (a gift from Dr. Matsuura); an anti-VP39 MAb (a gift from Dr. Matsuura); or an anti-VSV-G MAb (P5D4) (Sigma Chemical Co.). The MAbs above were diluted 1:2000, 1:10000, and 1:1000, respectively, in TBST (10 mM Tris [pH 8], 150 mM NaCl, and 0.05% Tween 20) with 0.02% sodium azide. After washing, blots were incubated with a secondary antibody consisting of a goat anti-mouse immunoglobulin G (IgG)-alkaline phosphatase conjugate

(Promega, Madison, WI) at a dilution of 1:1000. Western blots were processed as described earlier [30].

Fluorescent microscopy. To investigate the efficiency of gene expression of the GFP as an index for the HeLa CD4$^+$ cells infected with pAc/CMV-GFP and Ac/VSV/CMV-GFP at an MOI of 50, HeLa CD4$^+$ cells were trypsinized and seeded at a low cell density. Direct fluorescence microscopy (Olympus Company, Tokyo, Japan) of the expressed reporter gene (GFP) was performed under the mitotic stage of cell division, after each passage on days 1, 3, and 6 of cell confluency and the data were acquired with a DP12 digital microscope camera (Olympus Company, Tokyo, Japan).

Luciferase assay. Luciferase activity was measured with the PicaGene kit (Toyo-inki, Tokyo, Japan) according to the manufacturer's protocol. The target gene-expressing plasmid pNL4-3-luc, with a deleted *env* gene and a firefly luciferase gene replacing the *nef* gene [31], was used to transfect COS cells together with the plasmids expressing U5-ribozyme and pNL4-3-luc (Fig. 1A). HeLa CD4$^+$ cells were infected with pAc/VSV-G/tRNA$_i^{Met}$-U5-Rz at a viral MOI of 1–100. After 90 min, the cells were washed with three times with PBS and then transfected with pNL4-3-luc using the FuGENE™6 reagent (Roche Diagnostics) according to the manufacturer's protocol. The HeLa CD4$^+$ cells were lysed in 200 μl of PicaGene cell lysis buffer (Toyo-inki) for 15 min and scraped off the plate. The cell debris were removed by centrifugation. After the addition of 10 μl of centrifuged lysate to 100 μl of luminous substrate, the luminescent signal was immediately quantified with a luminometer (Lumat LB 9507; Berthold, Bad Wildbad, Germany).

The amount of firefly luciferase activity was normalized with reference to the protein concentration in the lysate. The protein was quantified with the BCA Protein Assay Reagent Kit (Pierce Chemical, Rockford, IL), which is based on bicinchoninic acid (BCA).

RT-PCR analysis of U5-ribozyme expression. Total RNA from vector-transfected cells was extracted with the GenElute Mammalian Total RNA kit (Sigma Chemical Co.). RT-PCR was then performed using an RNA PCR high-plus kit (Toyobo, Osaka, Japan) with *U5-Rz* upstream (forward primer F:5′-ATC AAG CTT AGG ATC CGG AA-3′) and downstream (reverse primer R:5′-GTG CCC GTT TCG GCC TAA CG-3′) primers. As an internal control, the mRNA of the human control gene (G3PDH) was amplified simultaneously with the G3PDH-F (nucleotides 230–254) and downstream G3PDH-R (nucleotides 422–466) primers. These RT-PCR products were amplified using the following thermal cycle program: 1 cycle (60 °C for 30 min and 94 °C for 2 min), 25 cycles (94 °C for 1 min and 51 °C for 1.5 min), and 1 cycle (46 °C for 7 min). The use of the Rz-F and Rz-R primers generated a cDNA product from the expressed ribozyme RNA (152 bp). The G3PDH-F and R primers also generated the G3PDH gene (0.5 kb) as the internal control.

RT-PCR analysis of HIV-1 mRNA. Total RNA from vector-transfected cells was extracted with the GenElute Mammalian Total RNA kit (Sigma Chemical Co.). RT-PCR was then performed using an RNA PCR high-plus kit (Toyobo, Osaka, Japan) with *LTR* upstream (NL4-3, 506–530) and downstream (NL4-3 1171–1195) primers; these are referred to as the forward primer F-(5′-GGA ACC CAC TGC TTA AGC CTC AAT A-3) and reverse primer R-(5′-TTC AGC AAG CCG AGT CCT GC-3′). As an internal control, the mRNA of the human control gene (G3PDH) was amplified simultaneously with the G3PDH-F (nucleotides 230–254) and downstream G3PDH-R (nucleotides 422–466) primers. These RT-PCR products were amplified using the following thermal cycle program: 1 cycle (60 °C for 30 min and 94 °C for 2 min), 25 cycles (94 °C for 1 min and 51 °C for 1.5 min), and 1 cycle (46 °C for 7 min).

Results

Construction of recombinant expressing the ribozyme

To characterize the efficiency of baculovirus as a vehicle for gene therapy, we selected the U5 region (548–578) of the LTR as a target site [29] and constructed a recombinant baculovirus vector expressing the U5-ribozyme. Fig. 1B shows the vectors used in this study. Vectors pAcU5-Rz carry HIV-1 U5 under the control of the *Pol*III, tRNA$_i^{met}$ [32]. Furthermore, the latter virus also carries the VSV-G coding sequence inserted downstream of the poly-hedrin promoter, and vectors expressing the VSV-G glycoprotein infect cells more efficiently and transduce mammalian cells [17,33–35]. Lastly, pAc/CMV-green fluorescent protein (GFP) and pAcVSV/CMV-GFP carrying the CMV-GFP expression cassette were also constructed.

Viruses were produced at high titers, ranging from 1.5×10^8 to 1.2×10^9 pfu/ml, and the structure of the baculovirus genomic DNA was confirmed by Southern blot analysis (data not shown). Expression of VSV-G, Gp64, and Vp39 due to infection with recombinant baculoviruses was examined by Western blot analysis with VSV-G, Gp64, and Vp39-specific antibodies (Fig. 1C) [34]. A protein of approximately the same size as VSV-G was detected in Ac/VSV-G/tRNA$_i^{Met}$-ter and Ac/VSV-G/tRNA$_i^{Met}$-U5-Rz-infected HeLa CD4$^+$ cell lysates, whereas Gp64 and Vp39 were detected in the mock-infected samples (Ac/tRNA$_i^{Met}$-ter, Ac/tRNA$_i^{Met}$-U5-Rz, Ac/VSV-G/tRNA$_i^{Met}$-ter, and Ac/VSV-G/tRNA$_i^{Met}$-U5-Rz). To examine foreign gene expression in HeLa CD4$^+$ cells, we examined GFP expression in HeLa CD4$^+$ cells infected with Ac/CMV-GFP and Ac/VSV-G/CMV-GFP. The efficiencies of GFP expression in HeLa CD4$^+$ cells with VSV-G pseudotyped baculovirus vector, Ac/VSV-G/CMV-GFP, were greater than with the wild-type vector, Ac/CMV-GFP (Fig. 1D).

Suppression of HIV-1 replication by the VSV-G pseudotyped baculovirus vector

Because ribozyme-U5 (Rz-U5) expression is an important determinant of its efficiency, it was essential to determine the intracellular expression of the Ac/tRNA$_i^{Met}$-U5-Rz and Ac/VSV-G/tRNA$_i^{Met}$-U5-Rz targets. HeLa CD4$^+$ cells were infected with the Ac/tRNA$_i^{Met}$-U5-Rz and Ac/VSV-G/tRNA$_i^{Met}$-U5-Rz. Two days after infection, total cellular RNA was isolated from these cells and analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Rz-U5 ribozyme expression, driven by the tRNA$_i^{Met}$ promoter, was observed in the Ac/tRNA$_i^{Met}$-U5-Rz and Ac/VSV-G/tRNA$_i^{Met}$-U5-Rz-infected HeLa CD4$^+$ cells (Fig. 2B, lanes 3 and 5). The control baculovirus vectors, Ac/tRNA$_i^{Met}$-ter and Ac/VSV-G/tRNA$_i^{Met}$-ter, did not express the Rz-U5 ribozyme (Fig. 2B, lanes 2 and 4). These results suggest that the Rz-U5 ribozyme was expressed by the VSV-G pseudotyped baculovirus vector Ac/VSV-G/tRNA$_i^{Met}$-U5-Rz-infected cells, and its expression was more effective than that of wild-type vector Ac/tRNA$_i^{Met}$-U5-Rz.

To test the ability of the U5-Rz-expressed by baculovirus vectors (Ac/tRNA$_i^{Met}$-U5-Rz and Ac/VSV-G/tRNA$_i^{Met}$-U5-Rz) to inhibit HIV-1 replication in HeLa-CD4$^+$, HeLa-CD4$^+$ cells infected with an MOI (1–100) of baculo-

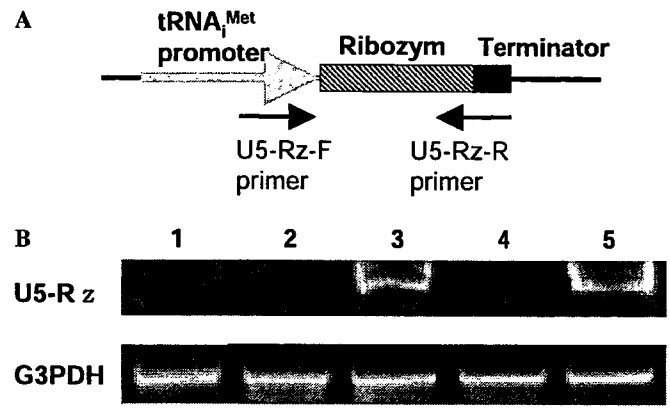

Fig. 2. RT-PCR assay for ribozyme RNA expression. The RT-PCR assay for ribozyme RNA was carried out using Rz-U5-specific primers with concurrent amplification of G3PDH mRNA. (A) Schematic representation of the Rz-U5-specific primer locations (F and R primers) with respect to the ribozyme expression pattern after baculovirus vectors infected HeLa CD4$^+$ cells. (B) RT-PCR amplification products, fractionated by electrophoresis on a 2% agarose gel with ethidium bromide staining. Lane 1, mock-infected HeLa CD4$^+$ cells; lane 2, Ac/tRNA$_i^{Met}$-ter-infected HeLa CD4$^+$ cells; lane 3, Ac/tRNA$_i^{Met}$-U5-Rz-infected HeLa CD4$^+$ cells; lane 4, Ac/VSV-G/tRNA$_i^{Met}$-ter-infected HeLa CD4$^+$ cells, and lane 5, Ac/VSV-G/tRNA$_i^{Met}$-U5-Rz-infected HeLa CD4$^+$.

virus vectors (Ac/tRNA$_i^{Met}$-U5-Rz and Ac/VSV-G/tRNA$_i^{Met}$-U5-Rz) were transfected with the pNL4-3-luc using the transfection reagent FuGENE™6. At 72 h post-transfection, the luciferase activity of the cell lysate was measured as an indirect marker of viral replication. The baculovirus vectors (Ac/tRNA$_i^{Met}$-ter and Ac/VSV-G/tRNA$_i^{Met}$-ter) (Fig. 1B), which do not trigger ribozyme expression as it lacks the ribozyme sequence, were used as controls. Our analysis revealed a dose-dependent inhibition of HIV-1 replication by Ac/VSV-G/tRNA$_i^{Met}$-U5-Rz with a maximum inhibitory efficacy of greater than 95% at an MOI of 100 of the VSV-G pseudotyped baculovirus vector (Fig. 3). On the other hand, the wild-type vector

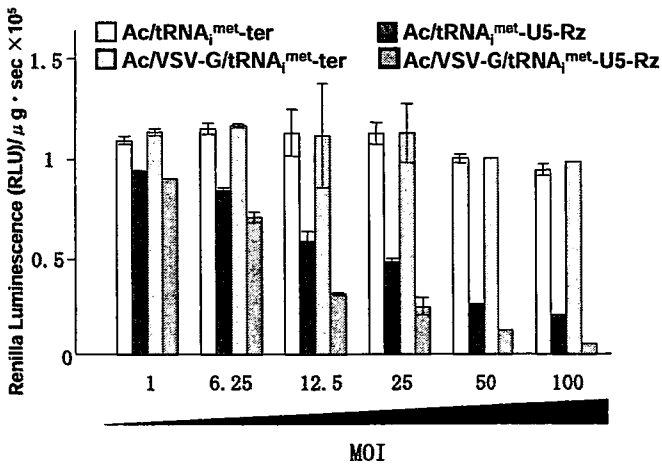

Fig. 3. Dose-dependent inhibition of HIV-1 expression in baculovirus-mediated U5-Rz-infected HeLa CD4$^+$ cells. HeLa CD4$^+$ cells were infected with Ac/VSV-G/tRNA$_i^{Met}$-U5-Rz at a viral MOI of 1–100. After 90 min, the cells were washed three times with PBS and then transfected with pNL4-3-luc using the FuGENE™6 reagent.

Ac/tRNA$_i^{Met}$-U5-Rz inhibited 85% of HIV-1 replication at an MOI of 100. On the other hand, the control baculovirus vectors (Ac/tRNA$_i^{Met}$-ter and Ac/VSV-G/tRNA$_i^{Met}$-ter) had no inhibitory effect on HIV-1 replication. This result suggests that the VSV-G pseudotyped baculovirus vector significantly inhibited HIV-1 replication as compared with the wild-type baculovirus vector (Fig. 3).

Effect of the HIV-1 mRNA levels of the VSV-G pseudotyped baculovirus vector-transduced ribozyme

We also examined HIV-1 mRNA levels to identify the contribution of HIV-1 mRNA cleavage to the ribozyme-mediated anti-HIV-1 effect [29,36]. RT-PCRs were used to establish the level of uncleaved HIV-1 mRNA. The uncleaved HIV-1 mRNA was amplified by the U5-Rz-F and U5-Rz-R primers (Fig. 4A). The RNA level was expected to decrease after cleavage of the HIV-1 mRNA. The U5-Rz expression system reduced the amount of full-length HIV-1 mRNA, whereas Ac/tRNA$_i^{Met}$-ter-infected HeLa CD4$^+$ cells did not have a significantly altered level of intact HIV-1 mRNA (Fig. 4B). The reduction in functional full-length HIV-1 mRNA was consistent with the cleavage effects of U5-Rz. These results indicate that binding of U5-Rz to its target HIV-1 mRNA and cleavage of HIV-1 mRNA might occur. The reduction in functional full-length HIV-1 mRNA was consistent with the Rz cleavage effect at the post-transcriptional level.

Discussion

Replicating forms of a number of different virus species, including adenovirus, herpes virus, vaccinia virus, reovirus, poliovirus, and vesicular stomatitis virus, are highly adapted to their natural hosts, providing the means for efficient gene delivery into cultured cells, and possibly patients [36,37]. The retrovirus system has the advantage of mediating stable gene transfer with a low potential for immunogenicity [38], but the vector delivery system has limitations for *in vivo* usage [39–41]. Baculovirus vectors are used for a multitude of applications, including the production of virus-like particles and viral display systems. Gene delivery into mammalian cells by baculoviruses has been enhanced by the incorporation of a foreign envelope protein into virions [33].

In this study, we demonstrated the inhibition of HIV-1 replication by VSV-G pseudotyped or wild-type baculovirus vector-transduced ribozyme in mammalian cells. The expression of VSV-G was controlled by the polyhedrin promoter, and is therefore expressed at high levels in the infected insect cells, but not in transduced mammalian cells. This vector also contains the U5 ribozyme expression sequence to target the mRNAs encoded by the U5 region (548–578) of the LTR (Fig. 1A). Rz-U5 ribozyme expression, driven by the tRNA$_i^{Met}$ promoter in the Ac/tRNA$_i^{Met}$-U5-Rz and Ac/VSV-G/tRNA$_i^{Met}$-U5-Rz-infected HeLa CD4$^+$ cells, was observed (Fig. 2B, lanes 3 and 5).

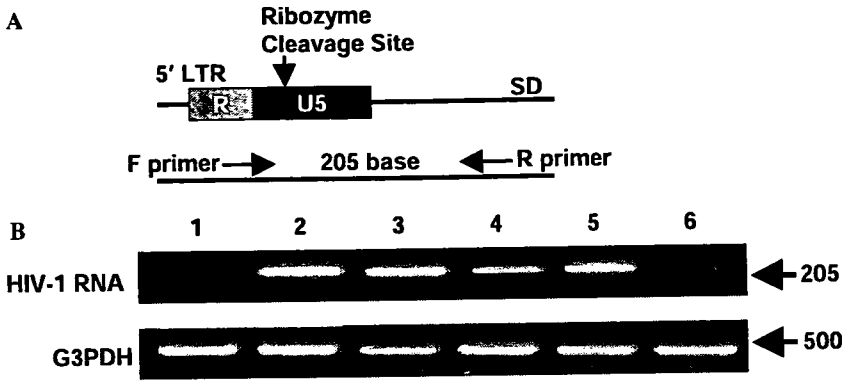

Fig. 4. RT-PCR analyses of HIV-1 mRNA expression. RT-PCR analyses of HIV-1 mRNA were performed using HIV-1 LTR-specific primers with concurrent amplification of G3PDH mRNA. (A) Schematic representation of HIV-1 LTR-U5-specific primer sites (F and R primers) with respect to HIV-1 LTR mRNA. (B) RT-PCR amplification products, fractionated by electrophoresis on a 2% agarose gel with ethidium bromide staining. Lane 1, untreated HeLa CD4$^+$ cells; lane 2, HIV-1 (pNL4-3-luc) transfected HeLa CD4$^+$ cells; lane 3, pNL4-3-luc transfected Ac/tRNA$_i^{Met}$-ter-infected HeLa CD4$^+$ cells; lane 4, pNL4-3-luc transfected Ac/tRNA$_i^{Met}$-U5-Rz transfected infected HeLa CD4$^+$ cells; lane 5, pNL4-3-luc transfected Ac/VSV-G/tRNA$_i^{Met}$-ter-infected HeLa CD4$^+$ cells; and lane 6, pNL4-3-luc transfected Ac/VSV-G/tRNA$_i^{Met}$-U5-Rz-infected HeLa CD4$^+$.

The Rz-U5 ribozyme expression from the VSV-G pseudotyped baculovirus vector-infected cells was higher than that from cells infected by the wild-type vector, Ac/tRNA$_i^{Met}$-U5-Rz (Fig. 2B). This finding was supported experimentally by GFP expression of the VSV-G pseudotyped baculovirus vector (Fig. 1C). Barsoum et al. [33] reported that baculoviruses displaying heterologous envelope proteins, such as VSV-G, transduce human hepatoma and rat neuronal cells at efficiencies 1- to 100-fold greater than those of baculoviruses lacking VSV-G. Based on the Rz-U5 ribozyme expression data, we evaluated the effectiveness of ribozyme-mediated inhibition of HIV-1 expression by VSV-G pseudotyped or wild-type baculovirus vector-transduced ribozyme in HeLa CD4$^+$ cells. The results indicated a dose-dependent inhibition of HIV-1 replication by both pseudotyped baculovirus vector (Ac/VSV-G/tRNA$_i^{Met}$-U5-Rz) and wild-type baculovirus vector (Fig. 3). HIV-1 replication was more significantly inhibited by the VSV-G pseudotyped baculovirus vector than by the wild-type baculovirus vector (Fig. 4). The level of Rz-U5 ribozyme expression, driven by the tRNA$_i^{Met}$ promoter in the Ac/tRNA$_i^{Met}$-U5-Rz and Ac/VSV-G/tRNA$_i^{Met}$-U5-Rz-infected HeLa CD4$^+$ cells, correlated with the results of the HIV-1 anti-viral activity.

We also determined whether the reduced expression of HIV-1 proteins directly resulted from a lower amount of HIV-1 mRNA. Equal amounts of total RNA from Ac/tRNA$_i^{Met}$-U5-Rz or Ac/VSV-G/tRNA$_i^{Met}$-U5-Rz-infected HeLa CD4$^+$ cells transfected with the pNL4-3-luc plasmid were subjected to RT-PCR analysis. The results indicate that the Rz-U5 ribozyme expression from Ac/tRNA$_i^{Met}$-U5-Rz or Ac/VSV-G/tRNA$_i^{Met}$-U5-Rz reduced the amount of full-length HIV-1 LTR mRNA (Fig. 4, lanes 4 and 6), whereas there was no significant change in the level of intact HIV-1 LTR mRNA in HeLa CD4$^+$ cells transfected with the control vectors (Ac/tRNA$_i^{Met}$-ter or Ac/VSV-G/tRNA$_i^{Met}$-ter; Fig. 4, lanes 3 and 5). The reduction in the amount of functional full-length HIV-1 LTR mRNA is consistent with an Rz-U5 ribozyme-mediated cleavage effect at the post-transcriptional level. The results of these experiments indicate that the ribozyme causes a specific reduction in target HIV-1 mRNA levels [29,36].

In conclusion, we designed a ribozyme-delivery system mediated by a VSV G-displaying baculovirus vector. A reduction of HIV-1 replication in tissue culture was achieved in our primary study towards developing an alternative anti-virus approach based on our vector system. Now that a recombinant baculovirus vector-transduced RNA against has been extensively characterized, enough information exists on the design of more RNAs against suitable for specific applications.

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HIV gene therapy using RNA virus systems

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ABSTRACT

We designed a vector to produce single-stranded DNA (ssDNA). We used HIV-1 reverse transcription for the purpose of constructing a DNAzyme expression vector against the HIV-1 env V3 loop. Initiation of HIV-1 reverse transcription requires the formation of a complex containing the viral RNA, tRNALys and reverse transcriptase. The expression vector contains the HIV-1 primer binding site (PBS) and tRNALys at the 3' end of its RNA transcript, thus enabling to an ssDNA would be synthesized by HIV-1 reverse transcriptase. We have demonstrated that the DNAzyme expressed by the lentiviral vectors suppressed HIV-1 replication in SupT1 cells.

INTRODUCTION

A DNAzyme (also known as deoxyribozyme, DNA enzyme) is a specifically structured DNA sequence that possesses catalytic RNA-cleaving activity, by recognizing its target RNA in a highly sequence-specific manner and blocking the expression of the corresponding RNA. mRNAs from oncogenes and viral genomes are ideal targets for DNAzyme therapeutic agents. A number of investigators have reported the sequence-specific cleavage of a variety of target RNAs, including HIV-1 RNA. The inhibition of infection by an incoming HIV-1 was reported by Zhang et al., who used DNAzymes that were targeted against the V3 loop of the envelope region[1].

In our laboratory, Kusunoki et al. designed a vector for single-stranded DNA expression using HIV-1 RT[2]. The expressed DNAzyme had site-specific cleavage activity in vitro. In this study, we describe that a DNAzyme expression system transduced by a lentiviral vector, and assessed HIV-1 suppression in mammalian cells.

MATERIALS AND METHODS

Construction of lentiviral vectors.

In order to construct the lentiviral vectors, the generated DNAzyme expression vectors[2] were digested with EcoR I, and then were cloned into the same site in the CS-CDF-CG-PRE vector[3].

Cell culture.

293T and SupT1 cells were grown in RPMI 1640 medium or D-MEM (Sigma Aldrich) supplemented with 10%(v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). All cultures were maintained at 37°C under a 5% CO_2 atmosphere.

RT-PCR analysis.

Total RNA from vector-transfected cells was extracted with a GenElute Mammalian Total RNA kit (Sigma-Aldrich). RT-PCR was then performed using an RNA PCR high-plus kit (Toyobo) with env upstream (NL4-3 7070-7099), envneutral (NL4-3 7241-7271) and downstream (NL4-3 7570-7600) primers; these are referred to as forward primer F1- (5'-ACA GCT GAA CAC ATC TGT AGA AAT TAA TTG-3'), F2- (5'-AAA CAG ATA GCT AGC AAA TTA AGA GAA CAA-3') and reverse primer R1- (5'-GTT GTT ATT ACC ACC ATC TCT TGT TAA TAG-3'). These RT-PCR products were amplified using the following thermal cycle program: 1 cycle (60°C for 30 min and 94°C for 2 min), 25 cycles (94°C for 1 min and 51°C for 1.5 min), and 1 cycle (51°C for 7 min).

Lentiviral vector preparation.

A vector construct (15 μg) was co-transfected with the helper constructs encoding gag/pol (pMDLg/p.RRE) (15 μg), the rev expressing construct pRSV-rev (5 μg), and the VSV-G expressing construct pMD.G (5 μg), into 293T cells using the calcium phosphate-precipitation method. The supernatants were harvested 48h post-transfection, filtered through a 0.45 μm filter disc and concentrated 100-fold by centrifugation at 6,000g overnight. The resultant viral pellet was re-suspended in serum- and antibiotic-free RPMI medium and stored at -80°C until use. To determine the virus titer, SupT1 cells were transduced with the prepared viral stock, and the numbers of GFP-positive cells were assessed after 72h of culture by flow-cytometric analysis (Becton, Dickinson).

Flow cytometry.

Transduced SupT1 cells were washed twice in PBS, and then fixed in PBS containing 1% formaldehyde. Direct fluorescence of GFP was analyzed by a FACS Calibur system (Becton, Dickinson). Data acquisition and analysis were performed with the CellQuest software (Becton, Dickinson). Gates for GFP detection were established using mock-transduced cells as the background.

HIV-1 challenge and culture assay.

After transduction by the lentiviral vectors, the GFP-positive SupT1 cells were sorted by a FACS vantage system (Becton, Dickinson), and were infected with HIV-1NL4-3 at an MOI of 0.01. After the harvested culture was centrifuged, the cell-free medium was used for an HIV-1 p24 CLEIA.

RESULTS AND DISCUSSION

In order to express the DNAzyme targeted to HIV-1 (NL4-3 *env*: 7196-7210), we constructed three types of DNAzyme expression lentiviral vectors (CS-DZ-tRNA-0, CS-DZ-tRNA-1, CS-DZ-tRNA-2), which were under the control of the $tRNA^{met}$ promoter (Figure 1). As shown in Figure 1, we designed the $tRNA^{Lys}$ gene with three different lengths. Furthermore, we constructed an shRNA (NL4-3 *env*: 7193-7213) expression lentiviral vector (CS-env-shRNA).

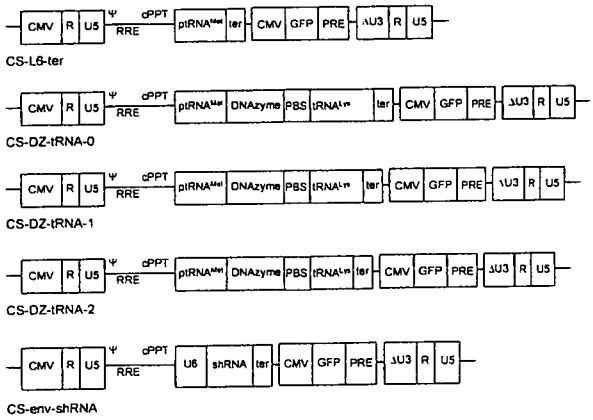

Figure 1. Construct of lentiviral vectors.

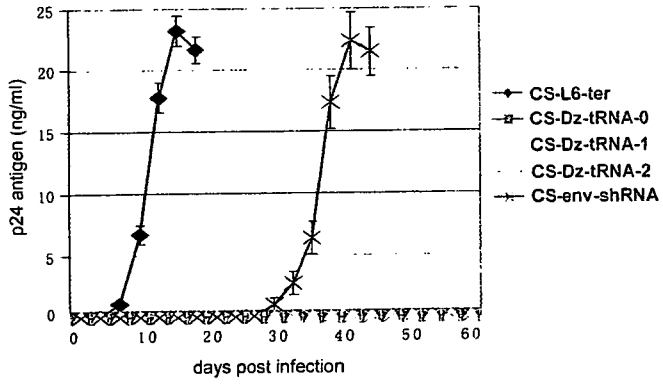

Figure 2. Inhibition of HIV-1 replication in SupT1 cells.

In other to investigate the sudden upsurge of viral replication, SupT1 cells were infected with the lentiviral vectors and wild-type $HIV\text{-}1_{NL4\text{-}3}$, and the HIV-1 p24 antigen levels were quantified in the cell-free supernatant (Figure 2). First, we observed the ssDNA expression under the above conditions (data not shown). Next, CS-DZ-tRNA-0, CS-DZ-tRNA-1 and CS-DZ-tRNA-2 were tested, and they almost completely suppressed HIV-1 replication. Although CS-env-shRNA had been almost completely suppress HIV-1 replication, after four weeks a mutation in the target site was observed. Furthermore, HIV-1 RNA cleavage by the DNAzyme and siRNA was examined by an RT-PCR analysis (Figure 3), which confirmed that HIV-1 RNA cleavage occurred in these transduced cells.

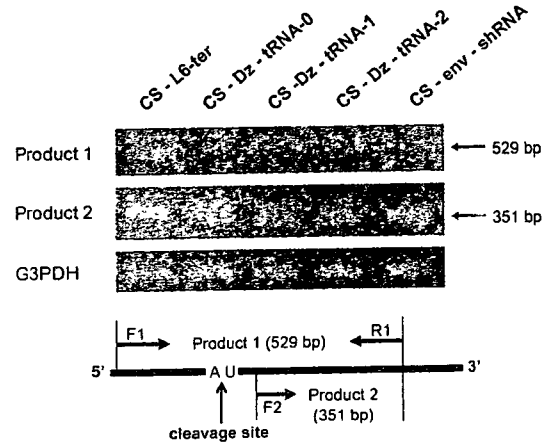

Figure 3. Confirmation of HIV-1 RNA cleavage.

CONCLUSION

Our DNAzyme expression system was able to suppress HIV-1 replication for a relatively long period and was more effective than RNAi. Hence, this system might be an effective strategy for gene therapy applications in HIV-1/AIDS treatment.

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Impaired GATA3-Dependent Chromatin Remodeling and Th2 Cell Differentiation Leading to Attenuated Allergic Airway Inflammation in Aging Mice[1]

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Age-related changes in lymphocytes are most prominent in the T cell compartment. There have been substantial numbers of reports on T cell function in aged mice and humans, such as on the production of Th1 and Th2 cytokines, but the results show considerable variation and contradictions. In the present study, we used 8- to 12-mo-old aging mice and a well-established in vitro Th1/Th2 cell differentiation culture system to identify molecular defects in Th1/Th2 cell differentiation that can be detected in the relatively early stages of aging. The capability to differentiate into Th2 cells is reduced in aging mouse $CD4^+$ T cells. Decreased activation of the ERK MAPK cascade upon TCR stimulation, but normal intracellular-free calcium ion concentration mobilization and normal IL-4-induced STAT6 activation were observed in aging mouse $CD4^+$ T cells. In addition, reduced expression of GATA3 was detected in developing Th2 cells. Chromatin remodeling of the Th2 cytokine gene locus was found to be impaired. Th2-dependent allergic airway inflammation was milder in aging mice compared with in young adult mice. These results suggest that the levels of Th2 cell differentiation and resulting Th2-dependent immune responses, including allergic airway inflammation, decline during aging through defects in the activation of the ERK MAPK cascade, expression of GATA3 protein and GATA3-dependent chromatin remodeling of the Th2 cytokine gene locus. In the present study, we provide the first evidence indicating that a chromatin-remodeling event in T cells is impaired by aging. *The Journal of Immunology,* **2006, 176: 2546–2554.**

The $CD4^+$ T cells consist of two distinct Th cell subpopulations, Th1 and Th2 cells (1). Th1 cells produce IFN-γ and are involved in cell-mediated immunity against intracellular pathogens. Th2 cells produce IL-4, IL-5, and IL-13 and control humoral immunity and allergic reactions. Naive $CD4^+$ T cells differentiate into Th1 cells following recognition of Ags in the presence of IL-12, whereas IL-4 drives differentiation into Th2 cells (2–4). In addition to the cytokines mentioned above, TCR stimulation by Ags is also indispensable for both Th1and Th2 cell differentiation. We reported that the efficient TCR-mediated activation of $p56^{lck}$, calcineurin, and the Ras-ERK MAPK signaling cascade is required for Th2 cell differentiation (5–7). Several transcription factors that control Th1/Th2 cell differentiation have been identified (8, 9). Among them, GATA3 appears to be a master transcription factor for Th2 cell differentiation (10–13) and Th2 cell maintenance (14, 15). Recently, we reported that the activation of the ERK MAPK cascade inhibits the ubiquitin-dependent degradation of GATA3 in developing Th2 cells and facilitates GATA3-dependent chromatin remodeling of the Th2 cytokine gene locus (16).

In the elderly, there is an increase in the frequency and severity of infectious diseases (17–19). Age-related changes in the immune system occur mainly in the T cell compartment (20–22). There may be related to a decrease in the ability of T cells to proliferate, and are associated with a reduction in IL-2 production (23) and reduced IL-2R expression (24–26). Various alterations in signaling have been described in comparison with young T cells. $CD4^+$ T cells from old mice show defects in TCR signal transduction that include diminished TCR-ζ phosphorylation, decreased elevation of intracellular Ca^+, and diminished activation of the MEK/ERK pathway (20, 27, 28). In contrast, aging does not affect Zap70-TCR-ζ association (29).

T cells in the elderly are often characterized by a shift from naive to memory phenotypes (30, 31). The production of the type 1 cytokine IFN-γ has been reported to be increased (32–34) or decreased (35–37) in aged mice and humans. Also, the production of type 2 cytokines such as IL-4 and IL-5 has been reported to be increased (38, 39) or decreased (33, 35) in vitro. These controversial observations on cytokine production may be a result of variations among the species or strains used in experiments, housing conditions or experimental culture systems. At present, age-related molecular defects in developing Th1/Th2 cells that control Th1/Th2 cytokine gene chromatin remodeling have not been formally investigated. In addition, it is not well clarified whether the severity of Th2-dependent allergic responses, such as allergic asthma, is modulated by aging.

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In the present study, we demonstrate that Th2 cell differentiation and Th2-dependent immune responses in vivo, including OVA-induced airway inflammation, are attenuated in aging mice. We detected several molecular defects in aging mouse $CD4^+$ T cells that may account for the attenuated Th2 responses, i.e., 1) reduced activation of the ERK MAPK cascade upon TCR stimulation, 2) decreased GATA3 expression in developing Th2 cells, and 3) impaired chromatin remodeling of the Th2 cytokine gene locus.

Materials and Methods

Mice

C57BL/6 and BALB/c were purchased from Charles River Laboratories. All young adult (5–6 wk) and aging (8–12 mo) mice including OVA-specific TCR$\alpha\beta$ transgenic (DO.11.10 Tg) mice (40) used in this study were maintained under specific-pathogen-free conditions. Animal care was in accordance with the guidelines of Chiba University.

Immunofluorescent staining and flow cytometric analysis

In general, 1 million cells were incubated on ice for 30 min with the appropriate staining reagents, according to a standard method (41). The reagents used in this study, anti-CD4-PE (RM4-1-PE), anti-CD4-FITC (RM4-1-FITC), anti-CD44-PE, anti-IL-4Rα Ab, anti-CD25-FITC, and anti-CD69-FITC, were purchased from BD Pharmingen. Anti-rat Ig-FITC was purchased from CAPPEL. Anti-TCRβ-FITC (H57-FITC), anti-TCRβ-biotin, and anti-CD3-FITC (2C11-FITC) were prepared in our laboratory. Flow cytometric analysis was performed on a FACSCalibur (BD Biosciences), and the results were analyzed with CellQuest software (BD Biosciences). Intracellular staining of IL-4 and IFN-γ was performed as described previously (6). FITC- or allophycocyanin-conjugated anti-IFN-γ Ab (XMG1.2; BD Pharmingen) and PE- or allophycocyanin-conjugated anti-IL-4 Ab (11B11; BD Pharmingen) were used for detection.

Cell purification

Splenic $CD4^+$ T cells were stained with anti-CD4-FITC and then purified using anti-FITC magnetic beads (Miltenyi Biotec) and an AutoMACS sorter (Miltenyi Biotec), yielding a purity of >98%. In some experiments, spleen cells were stained with anti-CD4 and anti-CD44, and naive $CD4^+CD44^{low}$ T cells were sorted by a FACSVantage (BD Biosciences) and used as responder T cells as described previously (42).

Proliferation assay

Splenic $CD4^+$ T cells (2×10^5) prepared by the AutoMACS sorter were stimulated in 200-μl cultures for 40 h with immobilized anti-TCRβ mAb (H57-597). [^{3}H]Thymidine (37 kBq/well) was added to the stimulation culture for the last 16 h, and the incorporated radioactivity was measured on a beta plate (6).

Analysis of the efficiency of cell division

Splenic $CD4^+$ T cells purified by the AutoMACS sorter were labeled with CFSE (Molecular Probes) as described previously (42).

Measurement of intracellular-free calcium ion concentration ($[Ca^{2+}]_i$)[3]

Splenic $CD4^+$ T cells purified by the AutoMACS sorter were loaded with Indo-1 (Indo-1 AM; Molecular Probes) in the presence of F127 (41). After washing, the cells were incubated with anti-CD4-FITC and anti-TCRβ-biotin on ice. The stained cells were washed and subjected to Ca analysis on a FACSVantage (BD Biosciences). TCR was cross-linked with avidin, the $[Ca^{2+}]_i$ was monitored for 512 s, and the results were analyzed with CellQuest software (BD Biosciences).

In vitro Th1/Th2 cell differentiation cultures

DO11.10 Tg $CD44^{low}CD4^+$ T cells (1.5×10^4) purified by cell sorting were stimulated with antigenic OVA peptide (OVA; 323–339, 10 μM) and irradiated (3000 rad) BALB/c APCs (1×10^5) in the presence of exogenous IL-4 or IL-12 as described previously (6).

ELISA for the measurement of cytokine concentration

The productions of IL-2, IL-4, IL-5, IL-13, and IFN-γ were measured by ELISA as described previously (43).

RT-PCR analysis

The quantitative RT-PCR analysis of GATA3 expression was performed as described previously (44).

Retroviral vectors and infection

cDNA for human GATA3 was inserted into a multicloning site of pMX-IRES-GFP (16). The methods for the generation of the virus supernatant and infection were described previously (16).

OVA immunization and ELISA for the measurement of serum Ig concentration

Young (6 wk old) and old (10 mo old) BALB/c mice were immunized i.p. with 100 μg of OVA emulsified in CFA (Difco) on days 0 and 7. Blood was collected from the tail vain on day 14. The concentrations of IgE in the serum were measured with a mouse IgE ELISA kit (BD Biosciences). The concentrations of OVA-specific Igs (IgG1 and IgG2a) in the serum were determined by ELISA as described previously (5).

Immunoblotting

Immunoblotting was performed as described previously (6). For ERK1 and ERK2 phosphorylation, naive $CD4^+$ T cells from C57BL/6 mice were purified with anti-CD4 mAb (RM4-5) and magnetic beads sorting (MACS sorting), and then the cells were incubated with anti-TCR mAb (H57-597) on ice. After incubation, the cells were stimulated with anti-hamster Igs (which cross-reacts with both H57-597) for 3, 10, or 30 min at 37°C, and then total cell lysates were subjected to phospho-ERK immunoblotting (Cell Signaling Technology). For STAT6 phosphorylation, naive $CD4^+$ T cells from C57BL/6 mice were activated with immobilized anti-TCR mAb and IL-4 (100 U/ml) for 2 days (induction culture). To assess IL-4-induced tyrosine phosphorylation, stimulated cells were washed, cultured for 8 h without cytokines, and stimulated with IL-4 (100 U/ml) for 3, 10, 30, or 60 min at 37°C. For IL-4 titration, 10–100 U/ml IL-4 was used. Anti-phosphotyrosine (RC20; BD Transduction Laboratories) or antiserum reactive with STAT6 (R&D Systems) was used for detection. For the detection of GATA3 or JunB, nuclear extracts were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce) according to the manufacturer's protocol. Immunoblotting was performed with anti-GATA3 mAb or anti-JunB mAb (Santa Cruz Biotechnology). HRP-conjugated anti-mouse Ig Ab (Amersham Biosciences) was used for GATA3 or JunB visualization (45).

Chromatin immunoprecipitation (ChIP) assay

Acetylation status of histone H3-K9/K4 was assessed using histone H3 (K9/14) ChIP assay kits (no. 17-245; Upstate Biotechnology) as described previously (46). The ChIP assay for di-methylated histone H3-K4 was performed using anti-histone H3 dimethyl K4 antiserum (no. 07-030; Upstate Biotechnology) (47). Semiquantitative PCR was performed with DNA samples from 3×10^4 or 1×10^4 cells at 28 cycles. PCR products were resolved in an agarose gel and visualized and quantified using an ATTO L&S analyzer (ATTO). The primers used were described previously (46).

Sensitization and inhalation with OVA

Young (6 wk old) and old (12 mo old) BALB/c mice were immunized i.p. with 250 μg of OVA (chicken egg albumin purchased from Sigma-Aldrich) in 4 mg of aluminum hydroxide gel (alum) on days 0 and 7. Mice were made to inhale aerosolized OVA in saline (10 mg/ml) for 30 min. using a supersonic nebulizer (model NE-U07; Omron) on days 14 and 16 to assess eosinophilic inflammation as described previously (48).

Collection of bronchioalveolar lavage (BAL) fluid and lung histology

Two days after the last OVA inhalation on day 16, BAL was performed as described previously (49). Total BAL fluid was collected and the cells in 100-μl aliquots were counted. One hundred thousand viable BAL cells were cytocentrifuged onto slides using a Cytospin3 (Thermo Shandon) and stained with May-Grünewald-Giemsa solution (Merck) as described previously (50). Two hundred leukocytes were counted on each slide. Cell types were identified using morphological criteria. The percentages of each cell type were calculated.

[3] Abbreviations used in this paper: $[Ca^{2+}]_i$, free calcium ion concentration; ChIP, chromatin immunoprecipitation; BAL, bronchioalveolar lavage.