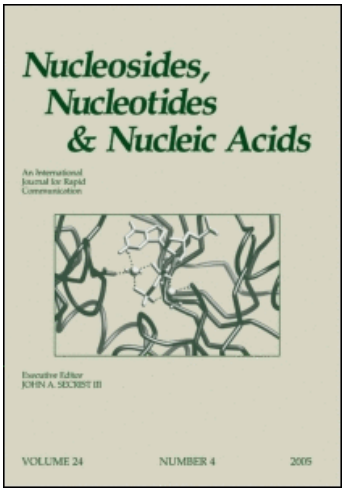

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SUPPRESSION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) REPLICATION BY AN HIV-1-DEPENDENT DOUBLE LOCKED VECTOR WITH THE CRE/LOXP SYSTEM

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□ *We previously demonstrated the function of an HIV-1-dependent ribozyme expression vector, with which the site-specific excision of loxP sequences can be achieved by using the Cre-loxP system (ON/OFF) as a molecular switch in an acute HIV-1 infection. However, this expression system also revealed the lower, non-specific expression of the anti-HIV-1 ribozyme in the absence of tat. To circumvent this problem, we used the more efficient HIV-1-dependent Cre recombinase gene expression vector, encoding the LTR-gag-p17 (extending from the 5′-LTR to the middle of the gag gene (pLTR-gag-p17-Cre)). Comparatively, the pLTR-gag-p17-Cre induces a higher Cre-protein expression level in an HIV-1 infection-dependent manner than the minimal pLTR-Cre. Furthermore, we constructed the ploxP-Rz-U5 and pLTR-gag-p17-Cre plasmids and also combined them into a single vector, pLTR-gag-p17-Cre/loxP-Rz-U5, for a comparison of their anti-HIV-1 activities. The resultant simultaneous expression of the Cre protein and the homologous recombination of the two loxP sequences induced a high level of HIV-1 replication inhibition (95%). Significantly, a high steady-state of ribozyme expression was observed in the RT-PCR analysis. These data imply that targeting the HIV-1 genes with the pLTR-gag-p17-Cre/loxP-Rz-U5 vector, which mediates HIV-1-dependent ribozyme expression, would be a useful tool for HIV-1 gene therapy applications.*

Keywords HIV-1; Gene therapy; Cre/loxP system; Ribozyme; Antiviral agent

In honor and celebration of the life and career of John A. Montgomery.

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INTRODUCTION

The limitations of gene therapy for human immunodeficiency virus type 1 (HIV-1) have led to the exploration of alternative approaches toward curative interventions.[1] There are various types of antiviral RNA expression vector systems for anti-HIV-1 gene therapy.[2–10] However, these vectors constitutively express the antiviral RNAs. Some small nuclear RNAs, such as tRNA and snRNA, have been used as ribozyme expression systems.[11] In general, the use of the $tRNA_i^{Met}$ promoter in expression vectors results in the constitutive expression of the desired anti-gene in transfected or infected cultured cells.[12] This expression could be considered non-specific in comparison with that of the HIV-1 LTR promoter, an inducible promoter dependent on the virus-encoded trans-activator (*tat*), with the potential for specific expression in HIV-1 infected cells.[13] Previously, we demonstrated the function of an HIV-1-dependent ribozyme expression vector, with which the site-specific excision of loxP sequences[14–16] can be achieved by using the HIV-1 long terminal repeat (minimal LTR)-Cre-loxP system (ON/OFF) as a molecular switch in an acute HIV-1 infection.[17,18] It is also demonstrated that the retroviral vector incorporated ribozyme inhibit HIV-induced apoptotic cell death.[19] However, our expression system also exhibited lower, non-specific expression of the anti-HIV-1 ribozyme in the absence of *tat*. Recently, Miyake et al. reported the use of the LTR promoter as an HIV-1-dependent LTR promoter, which includes the coding sequence from the 5′ U3 to the middle of *gag*.[20]

In this study, we constructed a double-locked vector by using the LTR-*gag-p17*/Cre-loxP-Rz-U5 (ON/OFF) system with the new LTR promoter, which was propagated in *E. coli* and converted into mammalian cells. We also investigated the functions of the vector in the presence and absence of HIV-1 (pNL4-3).[21] These results demonstrate the potential gains of using pLTR-*gag-p17*-Cre/loxP-Rz-U5 vectors in gene therapies for the treatment of HIV-1 infection.

RESULTS AND DISCUSSION

Design and Construction of an HIV-1-Dependent Cre Expression Vector

Tissue-specific gene transfer and expression are crucial for the development of safe and effective gene therapy protocols. The HIV-1 LTR can serve as an efficient and inducible promoter dependent on the HIV-1 induced trans-activation factor, Tat.[13] In our previous study, we constructed the minimal pLTR (0.6 kb)-Cre and ploxP-luc for an HIV-1-dependent Cre/loxP vector system. However, the pLTR-Cre showed slight expression of the luciferase gene in HIV-1 uninfected HeLa $CD4^+$ cells.[18] Recently, Miyake

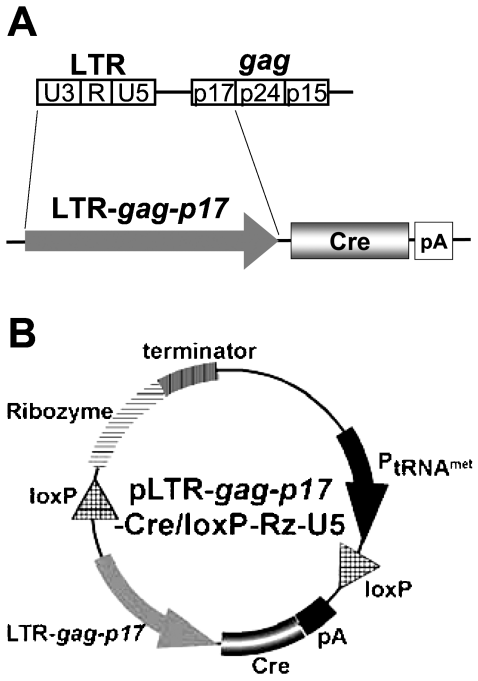

FIGURE 1 Schematic structures of the HIV-1-dependent Cre recombinase expression vector and the HIV-1-dependent Cre and ribozyme expression vector (pLTR-*gag-p17*-Cre/loxP-Rz-U5). A: The portion of the amplified LTR gene that has *tat*-dependent promoter activation. The HIV-1-dependent Cre expression vector (pLTR-*gag-p17*-Cre). B: The HIV-1-dependent Cre expression vector (pLTR-*gag-p17*-Cre) and the ribozyme gene expression vector, after loxP homologous recombination by the Cre protein (plox-Rz-U5) united them into one molecule.

et al. reported the use of the LTR promoter including the region from the 5′ U3 to the middle of *gag*.[20] Hence, we also constructed an HIV-1-specific Cre expression vector that includes the LTR-*gag-p17* promoter (from the 5′ LTR U3 to the end of *gag-p17*, 1.2 kb, Figure 1). Moreover, we constructed an intramolecular recombination double-locked vector system in HIV-1 infected cells (see Materials and Methods).

Intracellular Expression after loxP Recombination

In our previous work, we found that the pLTR-Cre and ploxP-luc system slightly expressed the luciferase gene in a non-specific manner.[18] First, to characterize the luciferase gene expression under the control of the Cre-loxP system, we compared the luciferase activities of an HIV-1 minimal LTR-driven luciferase expression plasmid (pLTR-luc) and a new HIV-1 LTR-*gag-p17* driven Cre expression plasmid, pLTR-*gag-p17*-Cre and ploxP-luc, in the absence or presence of pNL4-3 (Figure 2). When HeLa $CD4^+$ cells were transfected with the minimal pLTR-luc expression plasmid, the luciferase

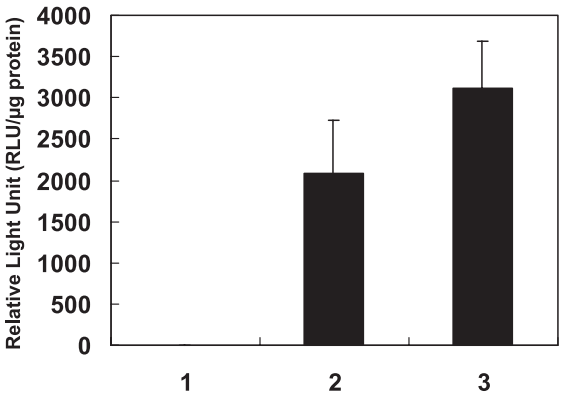

FIGURE 2 Luciferase activities after Cre/loxP homologous recombination in HeLa CD4$^+$ cells. Comparative luciferase background activities of pLTR-luc and pLTR-*gag-p17*-Cre, ploxP-luc and pNL4-3 in HeLa CD4$^+$ cells. Lane 1, pLTR-*gag-p17*-Cre and ploxP-luc transfected HeLa CD4$^+$ cells; lane 2, pLTR-*gag-p17*-Cre and ploxP-luc transfected HIV-1 (pNL4-3) infected HeLa CD4$^+$ cells; lane 3, pLTR-luc transfected HeLa CD4$^+$ cells. The LTR-luc plasmid exhibited a high luciferase background activity in the absence of HIV-1 infection.

activity was observed in the absence of pNL4-3 (lane 3). On the other hand, in the case of the LTR-Cre (pLTR-*gag-p17*-Cre) and ploxP-luc system, the luciferase activity was not observed in HeLa CD4$^+$ cells (lane 1). However, this system exhibited the luciferase activity in presence of pNL4-3 (lane 2). These results indicate that the new promoter, HIV-1 LTR-*gag-p17*, under the control of the pLTR-*gag-p17*-Cre and ploxP-luc system, can serve as an efficient and inducible promoter.

Ribozyme Expression in the HIV-1-Dependent Double-locking Vector-transfected HeLa CD4$^+$ Cells

Since high ribozyme-U5 (Rz-U5) expression is an important determinant of its efficiency, it was essential to determine the intracellular expression of the $tRNA_i^{Met}$-loxP-Rz-U5 target. Subconfluent cultures of HeLa CD4$^+$ cells were transfected with the pLTR-*gag-p17*-Cre/loxP-Rz-U5 plasmid. The LTR-*gag-p17* promoter can be induced by a variety of stimuli. The LTR-*gag-p17*-promoter of the pLTR-*gag-p17*-Cre expression plasmid in HeLa CD4$^+$ cells was stimulated with pNL4-3-luc. Two days after transfection, total cellular RNA was isolated from these cells and was analyzed by RT-PCR. We evaluated the expression of the U5 ribozyme from the ploxP-Rz-U5 vector after homologous recombination with the Cre protein. As compared to the human GAPDH-controlled expression, the Rz-U5 ribozyme, driven by the $tRNA_i^{Met}$ promoter, in pLTR-*gag-p17*-Cre/loxP-Rz-U5 transfected HeLa CD4$^+$ cells treated with pNL4-3-luc, was expressed well (Figure 3, lane 3). On the other hand, when pLTR-*gag-p17*-Cre, ploxP-Rz-U5, and pNL4-3-luc

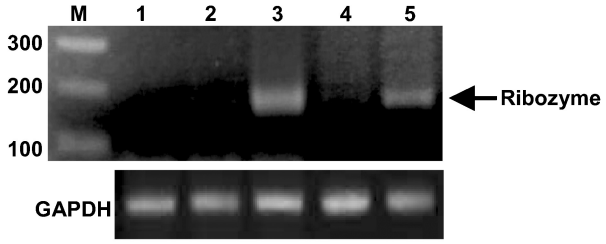

FIGURE 3 RT-PCR analysis of the ribozyme expression in HeLa $CD4^+$ cells. The RT-PCR assay for ribozyme RNA was carried out using Rz-specific primers, with concurrent amplification of GAPDH mRNA. The RT-PCR amplification products, fractionated by electrophoresis on a 2% agarose gel with ethidium bromide staining. Lane 1, pLTR-*gag-p17* and HIV-1 (pNL4-3) transfected HeLa $CD4^+$ cells; lane 2, pLTR-*gag-p17*-Cre and ploxP-Rz-U5 transfected HeLa $CD4^+$ cells; lane 3, pLTR-*gag-p17*-Cre, ploxP-Rz-U5, and HIV-1 (pNL4-3) transfected HeLa $CD4^+$ cells; lane 4, pLTR-*gag-p17*-Cre/loxP-Rz-U5 transfected HeLa $CD4^+$ cells; lane 5, pLTR-*gag-p17*-Cre /loxP-Rz-U5 and HIV-1 (pNL4-3) transfected HeLa $CD4^+$ cells.

were triple-transfected into HeLa $CD4^+$ cells, the Rz-U5 ribozyme, driven by the $tRNA_i^{Met}$ promoter, was also highly expressed (Figure 3, lane 5). However, the Rz-U5 ribozyme expression was not observed when the pLTR-*gag-p17*-Cre and ploxP-Rz-U5 plasmid or pLTR-*gag-p17*-Cre/loxP-Rz-U5 plasmid was present within HIV-1-uninfected HeLa $CD4^+$ cells (Figure 3, lanes 2 and 4). These results suggest that the Rz-U5 ribozyme was expressed by the LTR-*gag-p17*-Cre/loxP-Rz-U5 (ON/OFF) system in HIV-1 infected cells.

Effect of the LTR-*gag-p17*-Cre/loxP-Rz-U5 in HIV-1 Infected Cells

HIV-1-dependent ribozyme-mediated inhibition of HIV-1 expression with the LTR-*gag-p17*-Cre/loxP-Rz-U5 system was designed and tested in vivo, with promising results. In this study model, acute HIV-1 infected cells initiated *tat* protein expression. This triggered Cre protein expression from the pLTR-*gag-p17*-Cre in the presence of the ploxP-Rz-U5 vector. The expressed Cre protein subsequentially initiated the homologous recombination of the two loxP sequences from the ploxP-Rz-UP5 vector. This resulted in the expression and release of the ribozyme, which finally suppressed the HIV-1 gene expression (Figure 4). The inhibition of HIV-1 replication by the combined plox-Rz-U5 and pLTR-*gag-p17*-Cre (pLTR-*gag-p17*-Cre/loxP-Rz-U5) was compared with that by the triple-transfected plox-Rz-U5, pLTR-*gag-p17*-Cre, and pNL-4-3-luc vectors in HeLa $CD4^+$ cells. The pLTR-*gag-p17*-Cre/loxP-Rz-U5 (95%) showed a very high inhibitory effect as compared to the triple-transfected vector (60%). This result demonstrates the potential anti-HIV-1 effect by the expressed ribozyme from the Cre/loxP system. Thus, the LTR-*gag-p17*-Cre/loxP-Rz-U5 system is a potential gene therapy tool for controlling HIV-1 infection. In contrast, neither the ploxP-Rz-U5 transfected HeLa

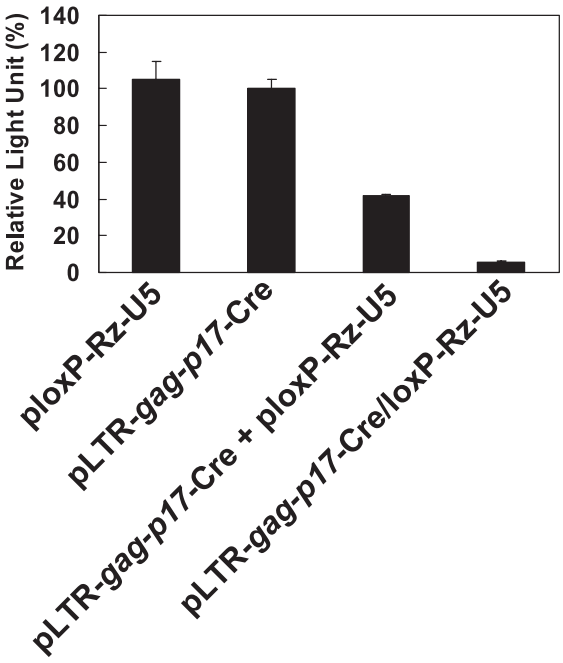

FIGURE 4 Inhibition of pNL4-3-luciferase activities by pLTR-*gag-p17*-Cre/loxP-Rz-U5. Inhibition of pNL4-3-luciferase fusion gene production in transiently transfected HeLa CD4$^+$ cells. The target-expressing plasmid (pNL4-3-luc), the plox-Rz-U5 plasmid, encoding the ribozyme, and the pLTR-*gag-p17*-Cre plasmid, encoding an HIV-1-dependent Cre, were triple-transfected or the pNL4-3-luc and the pLTR-*gag-p17-Cre*/loxP-Rz-U5 were co-transfected, by the FuGENE^{TM}6 transfection reagent, into HeLa CD4$^+$ cells.

CD4$^+$ cells without the Cre expression vector (pLTR-*gag-p17*-Cre) nor the pLTR-*gag-p17*-Cre transfected HeLa CD4$^+$ cells showed any inhibition of HIV-1 replication.

In addition, to clarify the ability of the Rz-U5 ribozyme to inhibit viral replication in HIV-1 infected cells, we used the HeLa CD4$^+$ cell line and pLTR-*gag-p17*-Cre/loxP-Rz-U5. The HIV-1 pNL4-3 infected HeLa CD4$^+$ cells were treated with either 0.1 μg, 1.0 μg, or 3.0 μg of pLTR-*gag-p17*-Cre/loxP-Rz-U5 for 2 days. As expected, the LTR-*gag-p17*-Cre/loxP-Rz-U5 system induced the HIV-1-dependent ribozyme-mediated inhibition in a dose-responsive manner compare with pLTR-*gag-p17*-Cre/loxP-luc as a control (Figure 5A).

Moreover, we examined the HIV-1 mRNA levels to identify the contribution of the Rz-U5 ribozyme-mediated specific RNA cleavage. RT-PCR reactions were used to establish the level of uncleaved HIV-1 mRNA. Equal amounts of total RNA from HeLa CD4$^+$ cells, transfected with pLTR-*gag-p17*-Cre/loxP-Rz-U5 and pNL4-3, were subjected to an RT-PCR analysis. HIV-1 *env*-specific DNA primers (sense, 6610-6634; antisense, 7783-7807) were used to amplify a 1198 bp (6610-7807) fragment in the transcripts. The RNA

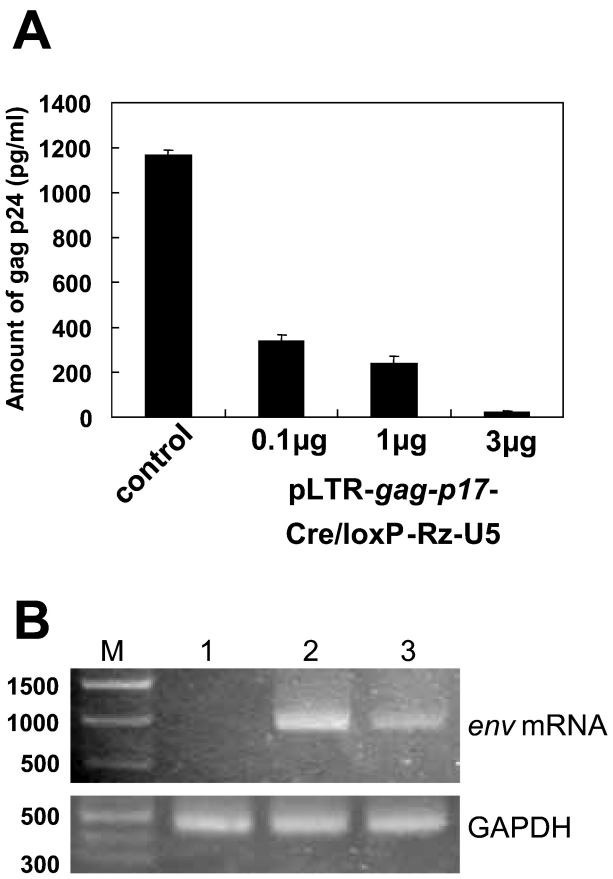

FIGURE 5 Inhibition of HIV-1 replication by pLTR-*gag-p17*-Cre/loxP-Rz-U5 in HeLa CD4$^+$ cells. A: Dose-dependent inhibition of HIV-1 replication by pLTR-*gag-p17*-Cre/loxP-Rz-U5 in HIV-1 infected cells. B: RT-PCR analysis of HIV-1 mRNA expression. Lane 1, negative control (untransfected cells); lane 2, positive control cells transfected with pLTR-*gag-p17*-Cre/loxP-luc and pNL4-3 proviral DNA; lane 3, cells transfected with pLTR-*gag-p17*-Cre/loxP-Rz-U5. The GAPDH RT-PCR was run in parallel to normalize the mRNA levels in the samples.

from pLTR-*gag-p17*-Cre/loxP-Rz-U5 transfected cells showed a high reduction in HIV-1 transcripts (Figure 5B, lane 3). However, the mRNA from the pLTR-*gag-p17*-Cre/loxP-luc transfected cells still contained an abundance of HIV-1 mRNA (Figure 5B, lane 2). The reduction in the functional full length HIV-1 mRNA is consistent with the Rz-U5 ribozyme-mediated cleavage effect at the post-transcriptional level. These results suggest that the HIV-1-dependent Rz-U5 ribozyme was expressed by using the LTR-*gag-p17*-Cre/loxP-Rz-U5 recombination system in HIV-1 infected cells, and cleaved its target HIV-1 mRNA.

In conclusion, the HIV-1-dependent ribozyme-mediated inhibition of HIV-1 expression using the Cre/loxP system with the new LTR promoter

(pLTR-*gag-p17*) was designed and tested in vivo, with promising results. In this study model, acute HIV-1 infected cells initiated *tat* protein expression. This triggered Cre protein expression from the pLTR-*gag-p17*-Cre in the HIV-1 infected HeLa $CD4^+$ cells. The expressed Cre protein subsequentially initiated the homologous recombination of the two loxP sequences from the plox-Rz-U5 vector. This resulted in the expression and release of the ribozyme, which finally suppressed the HIV-1 gene expression. The combination of plox-Rz-U5 and pLTR-*gag-p17*-Cre (pLTR-*gag-p17*-Cre/loxP-Rz-U5) was more potent in the suppression of the HIV-1 gene expression than the separately co-transfected ploxP-Rx-U5 and pLTR-*gag-p17*-Cre vectors in HeLa $CD4^+$ cells. Moreover, pLTR-*gag-p17*-Cre/loxP-Rz-U5 inhibited the production of both *gag* p24 and viral mRNA. These results demonstrate the potential anti-HIV-1 effect by the expressed HIV-1-dependent ribozyme from the Cre/loxP system with the new LTR (LTR-*gag-p17*) promoter. Thus, the Cre/loxP system with a new LTR promoter is a potential gene therapy tool for controlling HIV-1 infection.

EXPERIMENTAL

Construction of Plasmids

The DNA fragment encoding the LTR to the middle of the *gag* gene sequence was amplified by PCR using pHXNeo/L(s)[20] as the template, with the following upper primer, including an *Spe*I site (5′-ACTAGTGTGCCAGA CGTGCTCGAGAC-3′), and lower primer, including an *Eco*RI site (5′-GAATT CCTTCCTTGGGTCTTTTATCTCTA-3′). The DNA fragment encoding the Cre gene was amplified by PCR from pBS185 (Invitrogen). The PCR product of the partial Cre gene was inserted into the pGEM-T TA cloning vector system (Promega), to yield pGEM-Cre. After digestions of the PCR amplified LTR product with *Spe*I and *Eco*RI, and the pGEM-Cre vector with *Mfe*I and *Bam*HI, each fragment was ligated into the *Spe*I/*Bam*HI sites of the plasmid containing the Cre gene (pBS185), to yield pLTR-*gag-p17*-Cre. In order to add the *Xho*I cloning site to the plox-Rz-U5[18] fragment, plox-Rz-U5 was first digested by *Not*I and *Spe*I. This fragment was then cloned into the *Not*I and *Spe*I sites in pVAX-1 (Invitrogen) to yield pVAX-LRU, which contained the needed *Xho*I site. Similarly, pLTR-*gag-p17*-Cre was digested with *Spe*I and *Mlu*I and was cloned into the *Xba*I and *Bss*HII sites of pDNR-3 (BD Biosciences Clontech) to yield pDNR-LTR-*gag-p17*. Thus, the *Sal*I site in pDNR-3 was incorporated. To finally construct the HIV-1-dependent ribozyme expression vector (pLTR-*gag-p17*-Cre/loxP-Rz-U5), pVAX-LRU was digested with *Spe*I and *Xho*I, and pDNR-LTR was digested with *Spe*I and *Sal*I. The resultant digested products were then ligated to generate pLTR-*gag-p17*-Cre/loxP-Rz-U5.

Cell Culture and Transfections

HeLa $CD4^+$ cells were grown in RPMI1640 medium (SIGMA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 50 units/mL penicillin, and 50 units/mL streptomycin at 37°C in a 5% CO_2 atmosphere. Transfection was carried out using the FuGENE^{TM}6 reagent (Roche Diagnostics) according to the manufacturer's protocol.

RNA Purification and RT-PCR

Total cellular RNA was isolated from transfected HeLa $CD4^+$ cells with the GenElute Mammalian Total RNA Kit (SIGMA) according to the manufacturer's protocol. After isolation, RNA samples were treated with DNase I (Promega) according to the manufacturer's specifications. Reverse transcription (RT)-PCR assays were carried out using RT-PCR high-plus- (TOYOBO) according to the manufacturer's protocol. The sequences of the primers used in the RT-PCR analysis were described in our previous study.[18]

Luciferase Assay

Luciferase activity was measured with the PicaGene kit (Toyo-inki) according to the manufacturer's protocol. The target gene-expressing plasmid (pNL4-3-luc), with a deleted *env* gene and a firefly luciferase gene instead of the *nef* gene, was used to transfect HeLa $CD4^+$ cells together with the pLTR-*gag-p17*-Cre plasmid and the plox-Rz-U5 plasmid, which express the ribozyme after loxP homologous recombination. The HeLa $CD4^+$ cells were lysed in 200 μl of PicaGene cell lysis buffer (Toyo-inki) for 15 min and were scraped off the plate. The cell debris was removed by centrifugation. After the addition of 10 μl of the centrifuged lysate to 100 μl of luminous substrate, the luminescent signal was immediately quantitated with a luminometer (Lumat LB 9507; Berthold).

Furthermore, the amount of firefly luciferase activity was normalized with reference to the protein concentration in the lysate. The protein concentrations were determined with the BCA Protein Assay Reagent Kit (PIERCE), which is based on bicinchoninic acid (BCA).

Assay of HIV-1 Production

HIV-1 production was monitored by determming of the HIV-1 p24 antigen concentration. The target gene-expressing plasmid (pNL4-3) was used to transfect HeLa $CD4^+$ cells, together with the pLTR-*gag-p17*-Cre/loxP-Rz-U5 plasmid, which expresses the ribozyme after loxP homologous recom-

bination. The HeLa $CD4^+$ cells were lysed in 200 μl of PicaGene cell lysis buffer for 15 min and were scraped off the plate. The cell debris was removed by centrifugation. The amount of cellular p24 was quantitated with a LUMIPULSE®*f* (forte), a fully automated chemiluminescent enzyme immunoassay (CLEIA) system (Fujirebio).[22]

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