NEW METHODS AND TECHNOLOGIES

Long-term transgene expression and inhibition of HIV-1 replication by a Cre/loxP-EBNA-1/oriP HIV-1-dependent ribozyme vector: Applications for HIV-1 gene therapy

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ABSTRACT

The cleavage of target mRNA by ribozymes is being exploited as a means of gene silencing in nucleic-acid-based therapies. We previously established an HIV-1-dependent ribozyme-expression vector system, based on Cre-loxP technology with an LTR-*gag-p17* promoter as a molecular switch for use in acute HIV-1 infection. The simultaneous expression of the Cre protein and loxP homologous recombination induced a high level of HIV-1-replication inhibition, but ribozyme expression was transient. In the current study, we overcame this limitation by inserting *EBNA-1* and *oriP* genes from the Epstein-Barr virus (EBV) into the vector. When this plasmid was introduced into HeLa CD4^{+} cells, we observed long-term expression of both the *EGFP* reporter gene and the ribozyme. Moreover, HIV-1 replication was inhibited in the long-term in transfected cells. These data suggest that the HIV-1-dependent ribozyme-expression vector containing EBNA-1/oriP sequences would be a useful tool in HIV-1 gene therapy applications.

KEYWORDS: *Cre/loxP recombination, EBNA-1/oriP, gene therapy, HIV-1, ribozyme*

INTRODUCTION

In the application of therapeutics to HIV-1 infection, prevention is of greater importance than treatment. With this aim, many antiviral RNA-expression vector systems have been developed for use in anti-HIV-1 gene therapy (Banerjea et al, 2003; Barnor et al, 2004; Boden et al, 2004; Cordelier P et al, 2004; Habu et al, 2005; Mautino and Morgan, 2002; Takaku, 2004). Recently, small RNA molecules such as, siRNAs and snRNAs, have also been shown to affect gene silencing by RNA interference (Boden et al, 2003; Chang et al, 2002; Li et al, 2003).

Increasing the efficiency of transgene expression is of prime importance for human gene therapy (Davis and McNeilly, 2001). The Epstein-Barr Virus (EBV) is an autonomously replicating episomal vector that has been utilized to overcome the problem of rapid elimination of intracellularly delivered plasmid DNA in nonviral gene transfer. EBV is a gammaherpesvirus that is maintained as an episome of approximately 172 kb in size in a small number of resting B cells and epithelial cells in most of the human population. It latently infects human B cells with a high efficiency, after which its linear double-stranded genome circularizes and is sustained as a stable

episome (Hirai and Shirakata, 2001). The EBV replication system is present at a frequency of about 1-100 copies per cell, and maintains a non-covalent attachment to the host chromosome.

The latent origin of replication *oriP* and the viral transactivator protein EBNA-1 are essential components for EBV latent replication and maintenance of the viral genome (Daikoku et al, 2004; Lee et al, 1999). Both elements have been employed for long-term transgene expression in gene-therapy studies (Otomo et al, 2001; Tsujie et al, 2001).

Previously, we described an HIV-1-dependent ribozyme-expression vector capable of achieving site-specific excision of loxP sequences by using the HIV-1 minimal LTR-Cre-loxP system as a molecular switch in an acute HIV-1 infection (Habu et al, in press). However, we were unable to detect long-term expression of the anti-HIV-1 ribozyme. We hypothesized that the length of HIV-1-dependent transgene expression could be significantly increased in mammalian cells by introducing EBNA-1/oriP sequences to the vector.

In this study, we constructed an HIV-1-dependent long-term transgene (RNA ribozyme) expression vector (LTR-*gag-p17*/Cre-loxP-Rz-U5-*EBNA-1-oriP*-EGFP (EOG)) using the EBV replicon system, which was propagated in *Escherichia coli* and transfected into mammalian cells. We measured transgene-expression levels, including *EBNA-1* and *oriP*, in the presence and absence of HIV-1 infectious molecular clone (pNL4-3, Adachi et al, 1986). The potential anti-HIV-1 activity of the expression vector was evaluated with a view to establishing a highly effective therapeutic agent that could be further developed for HIV gene-therapy applications.

MATERIALS AND METHODS

Construction of plasmids

The retroviral vector pLEGFP-C1 (Clontech, Mountain View, CA) was digested with *Nhe* I and *Xho* I to release the DNA fragment encoding enhanced green fluorescent protein (EGFP). This was inserted into the *Nhe* I/*Xho* I sites of pCEP4 (Invitrogen, Carlsbad, CA), which contains *EBNA-1* and *oriP*, to create pCEP4-EGFP. An *Ssp* I fragment containing *EGFP, EBNA-1*, and *oriP* was cloned into the *Stu* I sites of pLTR-*gag-p17*-Cre/loxP-Rz-U5 (Habu et al, in press) and ploxP-Rz-U5, which been previously described with a high cleavage affinity (Habu et al, 2002) to yield pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG (Figure 1A) and ploxP-Rz-U5-EOG (Figure 1C), respectively. The control plasmid vector ploxP-Rz-U5-EOG lacks the LTR-*gag-p17*-Cre gene and so does not trigger expression of the ribozyme. A *Pvu* II fragment containing the EGFP-expression unit was excised from pCMV-EGFP previously constructed (unpublished data) and cloned into the *Stu* I sites of ploxP-Rz-U5 (Habu et al, 2002) or pLTR-*gag-p17*-Cre/loxP-Rz-U5 to generate ploxP-Rz-U5-G (Figure 1D) and pLTR-*gag-p17*-Cre/loxP-Rz-U5-G (Figure 1B), which are the EBNA-1 and oriP negative-control plasmids, respectively.

Cell culture and transfections

HeLa CD4+ cells were grown in RPMI 1640 medium (Sigma, Saint Louis, MO) supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. HEK 293T cells were grown in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained at 37 °C in a 5% CO_2 atmosphere. HeLa CD4+ and 293T cell transfections were carried out using FuGENE™6 (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol.

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 (Akkina et al, 1996), lacking an *env* gene and with a firefly luciferase gene replacing the *nef* gene, was co-transfected into HeLa CD4+ cells with the pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG plasmid, which expresses the ribozyme following Cre/loxP homologous recombination. Transfected HeLa CD4+ cells were lysed in 200 μl PicaGene cell lysis buffer for 15 min and then harvested. Cell debris was removed by centrifugation. Centrifuged lysate (10 μl) was added to 100 μl luminous substrate, and the luminescent signal was immediately quantitated with a luminometer (Lumat LB 9507; Berthold, Bad Wildbad, Germany).

Flow cytometry

Transfected HeLa CD4+ cells were trypsinized, washed twice in PBS, and fixed in PBS containing 1% formaldehyde. Direct fluorescence of EGFP was analyzed by FACS Calibur (Clontech). Data acquisition and analysis were performed with CellQuest software (Clontech). Gates for detection of EGFP were established using mock-transfected cells as background.

Fluorescent microscopy

To evaluate the self-replicating function of the loxP ribozyme as an index for stable transgene expression in cells, transfected HeLa CD4+ cells were trypsinized and seeded at a low cell density. Direct fluorescence microscopy of EGFP was carried out at the mitotic stage of cell division, after each passage on days 1, 3, 7, 13, 19 and 25 and the data were acquired with a DP12 digital microscope camera (Olympus Company, Tokyo, Japan).

RNA isolation and RT-PCR

Total cellular RNA was isolated from transfected HeLa CD4+ cells with the GenElute Mammalian Total RNA kit (Sigma) using the manufacturer's protocol. RNA samples were treated with DNase I (Promega, Madison, WI) according to the manufacturer's instructions. RT-PCR assays were carried out using previously described primers (Habu et al, 2002) and the RT-PCR high-Plus-kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol.

Assay of HIV-1 replication

HIV-1 production was monitored by determining the HIV-1 p24 antigen concentration. The culture medium from HeLa CD4+ cells co-transfected with pNL4-3 and pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG was harvested on days 1,

3, 5, 7, 9, 11, 13 and 15 post-transfection. p24 Gag protein production was detected by the HIV-1 p24 CLEIA assay (Lumipulse, Fujirebio Inc., Tokyo, Japan), according to the manufacturer's protocol (Sakai et al, 1999).

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. To this end, the HIV-1 LTR can serve as an efficient and inducible promoter dependent on the HIV-1 trans-activation factor, Tat. In a previous study, we constructed the HIV-1-dependent RNA ribozyme expression Cre/loxP vector, pLTR-*gag-p17*-Cre/loxP-Rz-U5-G, which targets mRNAs encoded by the U5 region (548–578) of the LTR (Figure 1E). This vector showed HIV-1-dependent ribozyme expression in HeLa CD4$^+$ cells (Habu et al, 2002; Habu et al, in press), but expression was not long term. Hence, in the current study, we constructed an HIV-1-dependent expression vector containing Cre/loxP and EBNA-1/oriP sequences, with the aim of increasing the duration of transgene expression. Moreover, we inserted the reporter gene *EGFP* to enable visualization of transgene expression (Figure 1A). The plasmid vectors pLTR-*gag-p17*-Cre/loxP-Rz-U5-G (Figure 1B), ploxP-Rz-U5-EOG (Figure 1C), and ploxP-Rz-U5-G (Figure 1D) served as controls. The advantage of this vector system over previously reported ribozyme vector systems (Chang et al, 2002; Li et al, 2003) is that it is not constitutively expressed to trigger off non-specific inhibition, but specifically expressed only in the event of HIV infection.
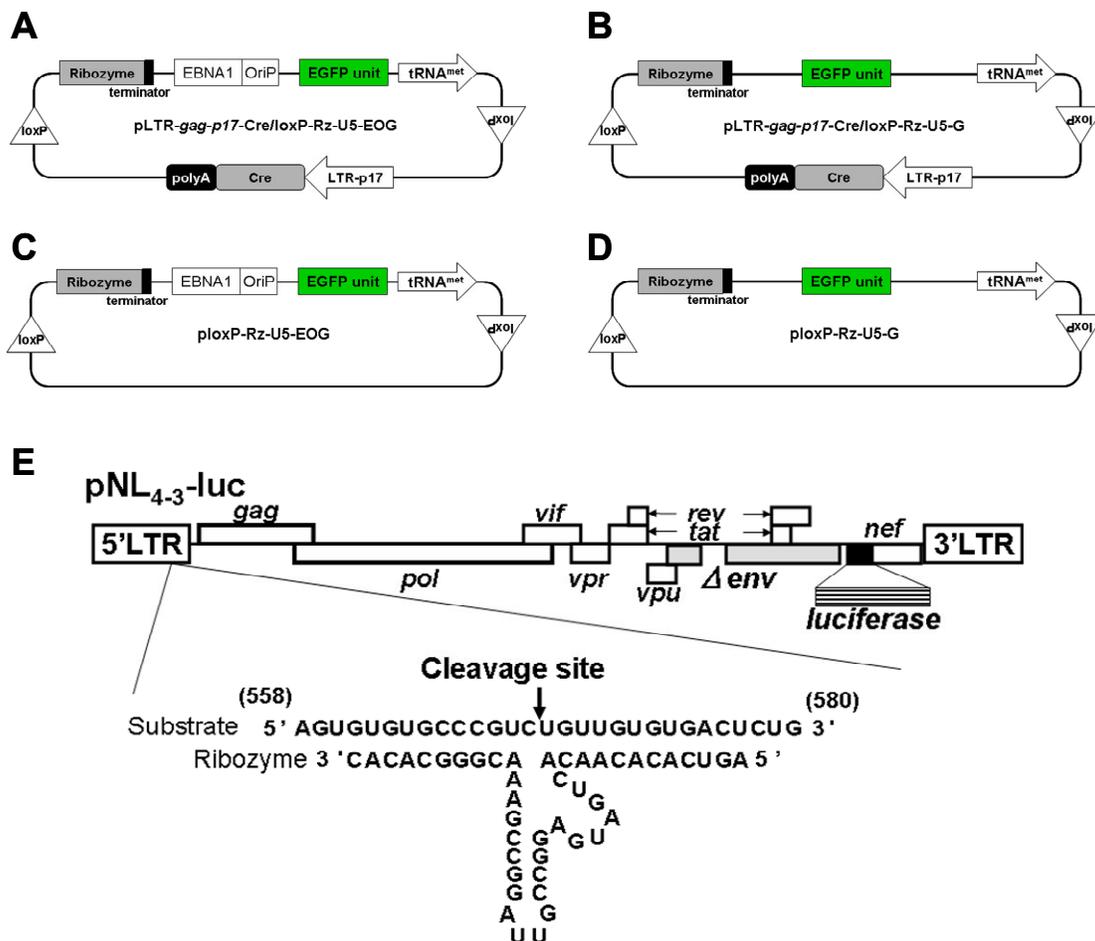
Figure 1. Schematic representation of HIV-1-dependent ribozyme-expression vectors. (**A**) The ribozyme expression (off switch) vector pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG, containing *EBNA-1* and *oriP* genes for long-term expression. (**B**) Control ribozyme-expression vector pLTR-*gag-p17*-Cre/loxP-Rz-U5-G, which lacks *EBNA-1* and *oriP* genes. (**C**) Control vector ploxP-Rz-U5-EOG, which lacks the *LTR-gag-p17-Cre* gene. (**D**) Control vector ploxP-Rz-U5-G, which lacks *EBNA-1/oriP* and *LTR-gag-p17-Cre* genes. (**E**) 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.

Long-term transgene expression of pLTR-gag-p17-Cre/loxP-Rz-U5-EOG in 293T and HeLa CD4$^+$ cells

To characterize the ability of the EBV replication system to effect long-term transgene expression we compared EGFP expression of control vector (pLTR-*gag-p17*-Cre/loxP-Rz-U5-G) with that of the EBNA-1/oriP plasmid pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG. EGFP expression in pLTR-*gag-p17*-Cre/loxP-Rz-U5-G-transfected 293T cells was observed for a maximum of 3 days, while EGFP expression persisted for more than 25 days in pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG-transfected 293T cells (Figure 2). As it has been reported that the EBNA-1 protein potentiates gene transcription (Mackey and Sugden, 1999), we measured the enhancement of gene expression by determining EGFP fluorescence intensity. FACS analysis showed that HeLa CD4$^+$ cells transfected with the EBNA-1-containing plasmids demonstrated longer-term EGFP expression (until day 7; Figure 3) than HeLa CD4$^+$ cells transfected with plasmids lacking EBNA-1. This is of therapeutic importance, because EBNA retains the therapeutic molecule to sensor for infective HIV-1 to release the catalytic ribozyme for cleavage.

Measurement of the mean fluorescence intensity (MFI) revealed a twofold increase in the EGFP expression level of HeLa CD4$^+$ cells transfected with pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG compared with pLTR-*gag-p17*-Cre/loxP-Rz-U5-G-transfected cells (data not shown). These results indicate that EBNA-1/oriP sequences mediate efficient and stable replication of transgene expression by enhancing nuclear localization of EBNA-1 (Mackey and Sugden, 1999; Marechal et al, 1999). The nuclear localization of this vector system is of cardinal importance since its function is induced by HIV-1 tat which is nuclear-based.

Dose-dependent inhibition of HIV-1 replication by pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG

The effect of pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG on HIV-1 (pNL$_{4-3}$-luc) replication was measured in a transient assay following its co-transfection with pNL$_{4-3}$-luc into HeLa CD4$^+$ cells. At 72 h post-transfection, the luciferase activity of cell lysate was measured as an indirect marker of viral replication. The plasmid vector ploxP-Rz-U5-EOG (Figure 1C), which does not trigger ribozyme expression as it lacks the LTR-*gag-p17*-Cre gene, was used as a control. Our analysis showed a dose-dependent inhibition of HIV-1 replication by pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG with a maximum inhibitory efficacy of >90% at a vector DNA concentration of 1.67 µg (Figure 4). Control vector alone had no inhibitory effect. This result suggests that the Rz-U5 ribozyme was expressed using Cre/loxP recombination and EBNA/oriP systems in HIV-1 infected cells, and successfully cleaved its target HIV-1 mRNA (Figure 1E).

EBNA-1 mediates long-term ribozyme expression in HIV-1-dependent vector-transfected HeLa CD4$^+$ cells

As long-term ribozyme-U5 (Rz-U5) expression is an important determinant of its efficiency, we compared the level of expression in HeLa CD4$^+$ cells transfected with pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG or pLTR-*gag-p17*-Cre/loxP-Rz-U5-G in the presence or absence of pNL4-3 (Figure 5). RT-PCR analysis showed that Rz-U5 expression persisted for more than 19 days following pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG transfection in the absence of pNL4-3 (Figure 5). By contrast, pLTR-*gag-p17*-Cre/loxP-Rz-U5-G-transfected cells demonstrated Rz-U5 expression for only 3 days. Ribozyme expression was not observed following transfection of either plasmid DNA in the absence of pNL4-3. These results confirm that
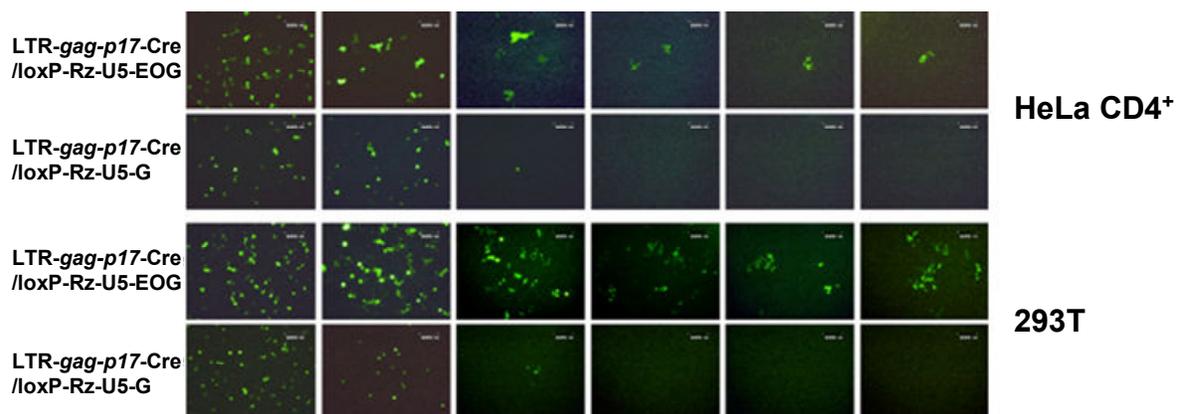
Figure 2. *EBNA-1* and *oriP* increase the duration of EGFP expression in transfected HeLa CD4$^+$ and 293T cells**.** EGFP expression was evaluated on days 1, 3, 7, 13, 19, and 25 post-transfection.
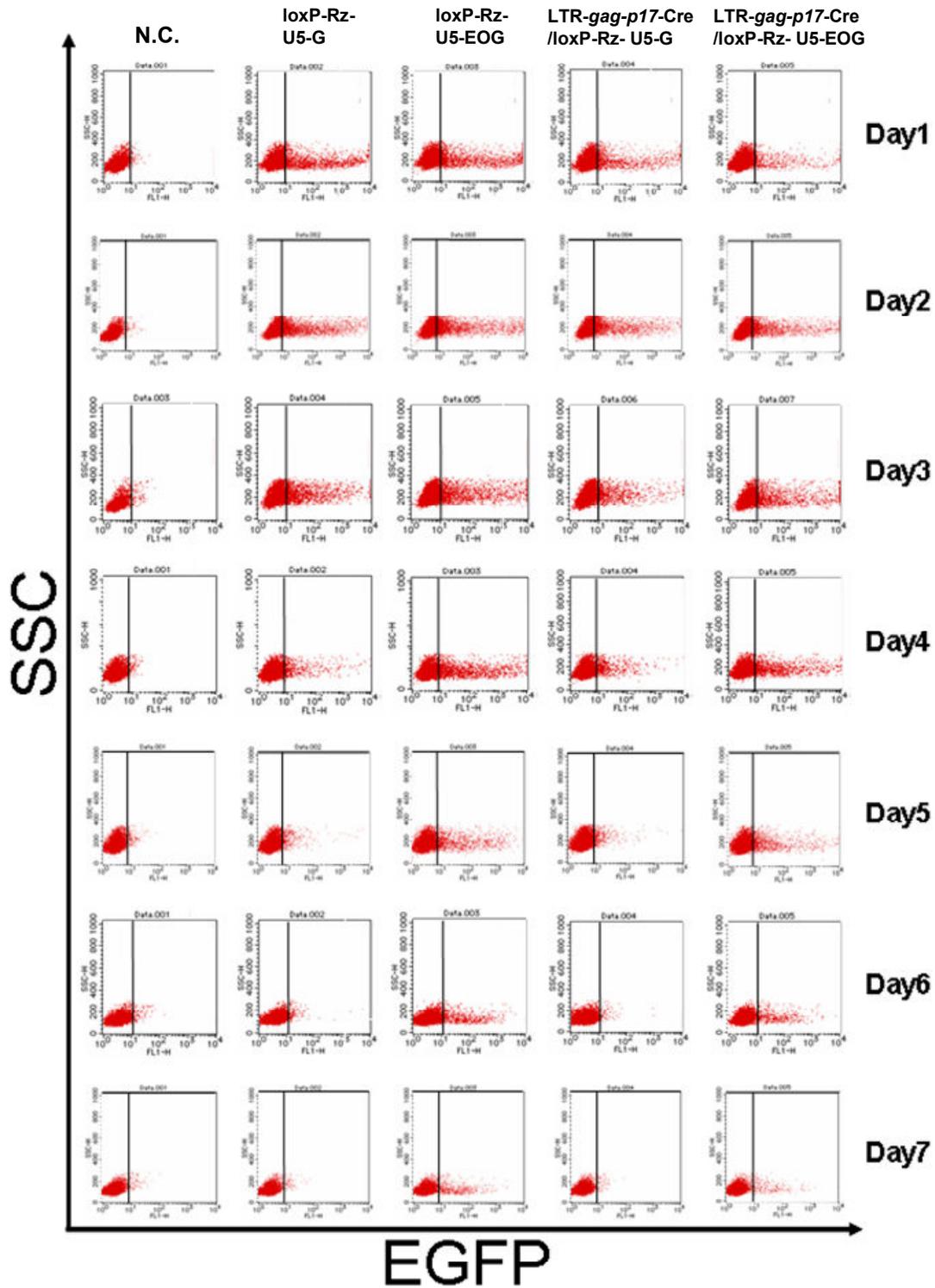
Figure 3. Enhancement of transgene expression by *EBNA-1* and *oriP* in HeLa CD4$^+$ cells. EGFP expression was evaluated on days 1 to 7 post-transfection by flow cytometry.
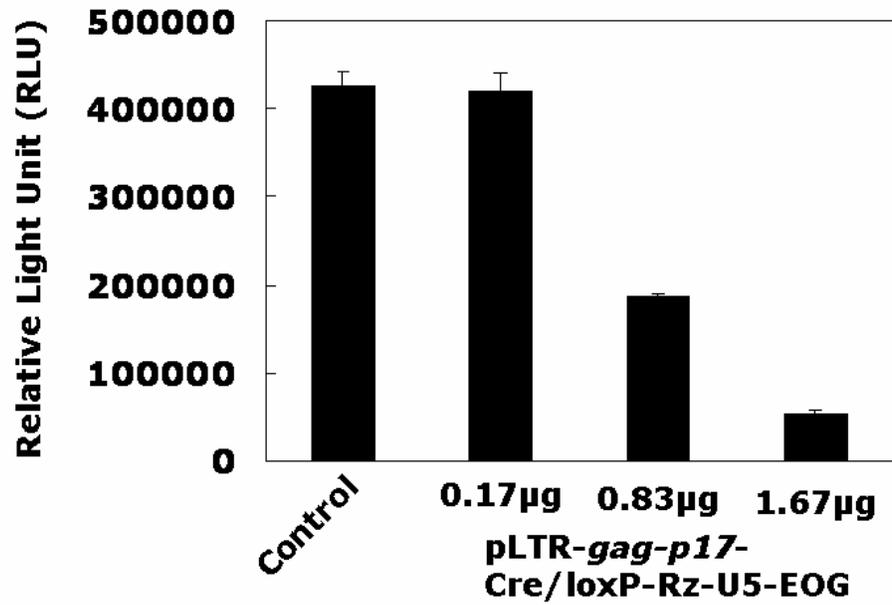
Figure 4. Dose-dependent inhibition of HIV-1 replication by pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG in HIV-1-transfected cells. Different amounts of pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG (0.17, 0.83 or 1.67 µg) were co-transfected with HIV-1 (pNL4-3-luc) into HeLa CD4+ cells. At 72 h post-transfection, luciferase activity of the cell lysate was measured as an indirect marker for viral replication.
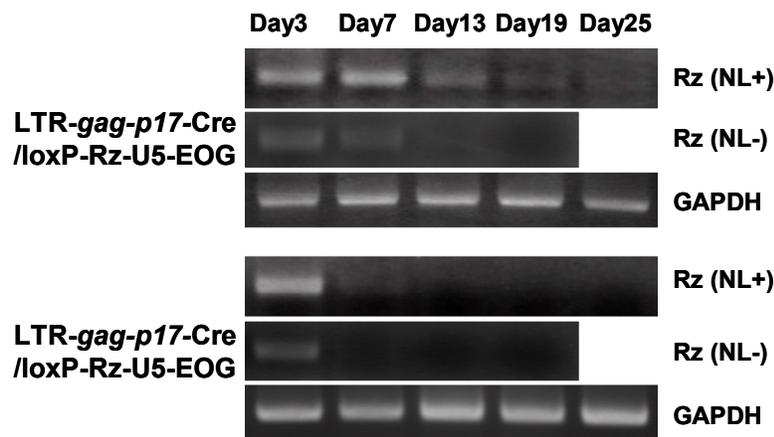
Figure 5. RT-PCR analysis shows persistent ribozyme expression of HeLa CD4+ cells transfected with *EBNA-1/oriP*-containing vector. Total RNA was isolated from confluent pNL4-3 infected cell cultures transfected with pLTR-*gag-p17*-Cre/loxP-Rz-U5-G or pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG. RT-PCR using ribozyme-specific primers was performed concurrently with amplification of GAPDH mRNA.

the Cre/loxP vector containing EBNA-1 has the potential to confer long-term transgene expression in HIV-1 infected cells. This property therefore puts our vector system in the class of prophylactics, since the vector will function to prevent infection of the target cells.

Long-term inhibition of HIV-1 replication by pLTR-*gag-p17*-Cre/loxP-Rz-U5- EOG in pNL4-3 infected HeLa CD4^{+} cells

To evaluate further the persistency of the EBNA-1/oriP-dependent ribozyme effect on HIV-1 replication we co-transfected 0.3 μg pNL4-3 with 5.0 μg pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG, pLTR- *gag-p17*-Cre/loxP-Rz-U5-G or ploxP-Rz-U5-EOG into HeLa CD4^{+} cells. As shown in Figure 6A, pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG inhibited HIV-1 replication for 15 days. By contrast, ploxP-Rz-U5-EOG (lacking ribozyme expression) showed little inhibitory activity towards HIV-1 replication compared with the negative control (pNL4-3 only).

EGFP reporter gene expression was monitored as an indicator of transgene expression (Figure 6B). Its persistence throughout the experiment (up to 13 days) confirms our earlier findings, and suggests that the HIV-1-dependent ribozyme-expression vector containing EBNA-1/oriP sequences mediates long-term ribozyme expression, inhibits HIV-1 replication and would be a useful tool for HIV-1 gene-therapy applications.

CONCLUSIONS

- Ribozyme expression is HIV-1 Tat dependent, as we were only able to detect it in the presence of pNL4-3.

- Transgene expression is long-term and is extended by the presence of oriP and EBNA-1. Hence, inhibition is also shown to be long-term.

- The Cre/loxP system induces ribozyme-mediated inhibition of HIV-1 replication in a dose-dependent manner.

- These results demonstrate the anti-HIV-1 effect of the expressed ribozyme from the Cre/loxP system, clearly illustrating the potential of the Cre/loxP-oriP/EBNA-1 system as a gene-therapy tool for controlling HIV-1 infection.

STATEMENT OF COMPETING INTERESTS

The authors declared no competing interests.

LIST OF ABBREVIATIONS

EBV; Epstein-Barr virus
EBNA-1; EBV nuclear antigen 1
EOG; EBNA-1/OriP/EGFP
MFI; Mean fluorescence intensity
OriP; EBV latent origin of replication

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Figure 6. Inhibitory effects on HIV-1 p24 antigen production by pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG. **(A)** HeLa CD4^{+} cells were co-transfected with HIV-1 pNL4-3 and either control vector (diamonds), ploxP-Rz-U5-EOG (squares), pLTR-*gag-p17*-Cre/loxP-Rz-U5-G (triangles), or pLTR-*gag-p17*-Cre/loxP-Rz-U5- EOG (circles). HIV-1-virus production was measured by p24 ELISA at the indicated times post-transfection. **(B)** Transgene replication in dividing cells. Cells were passaged every 2 days, and monitored for transgene replication by way of EGFP expression in singly dividing cells.

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