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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.

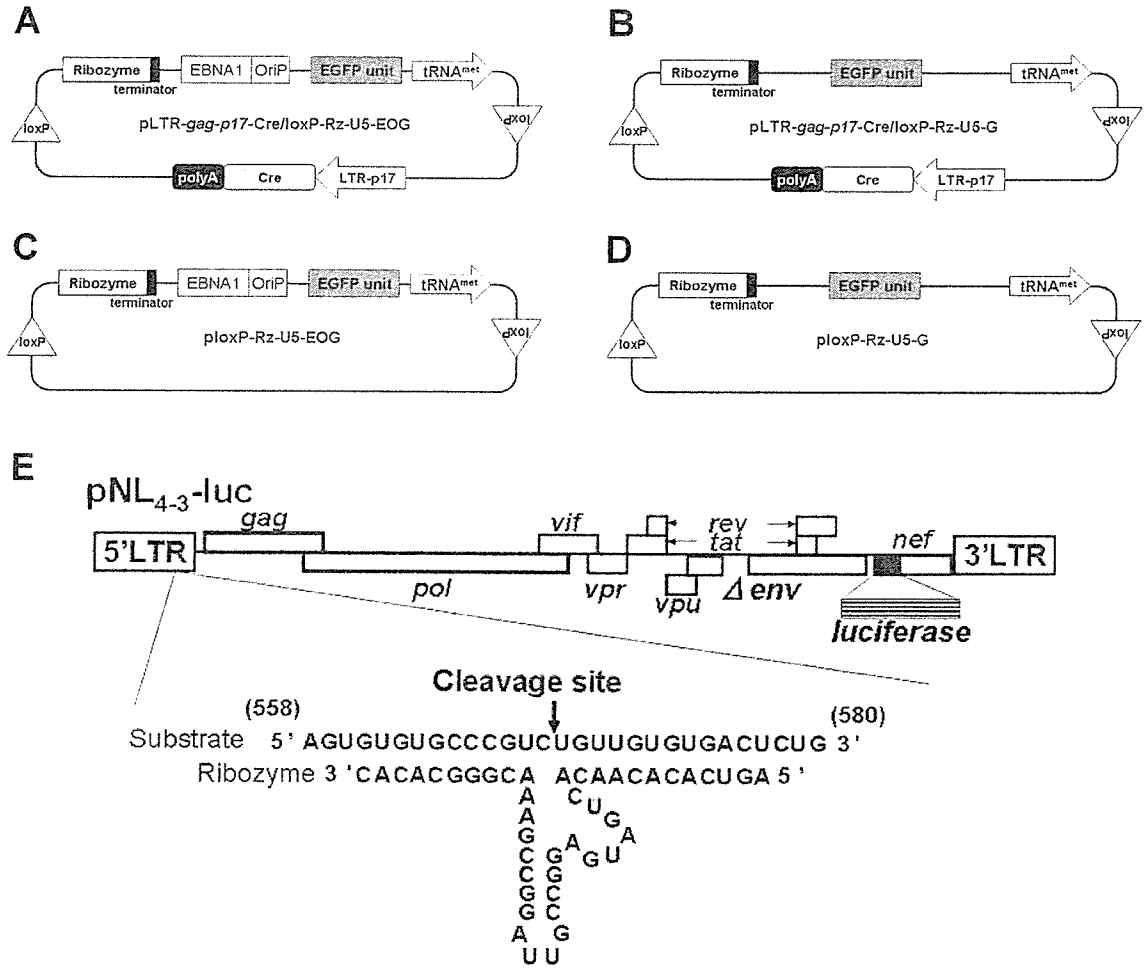

Figure 1. Schematic representation of HIV-1-dependent ribozyme-expression vectors. (**A**) The ribozyme expression (off switch) vector pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG, containing *EBNA-1* and *oriP* genes for long-term expression. (**B**) Control ribozyme-expression vector pLTR-*gag-p17*-Cre/loxP-Rz-U5-G, 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

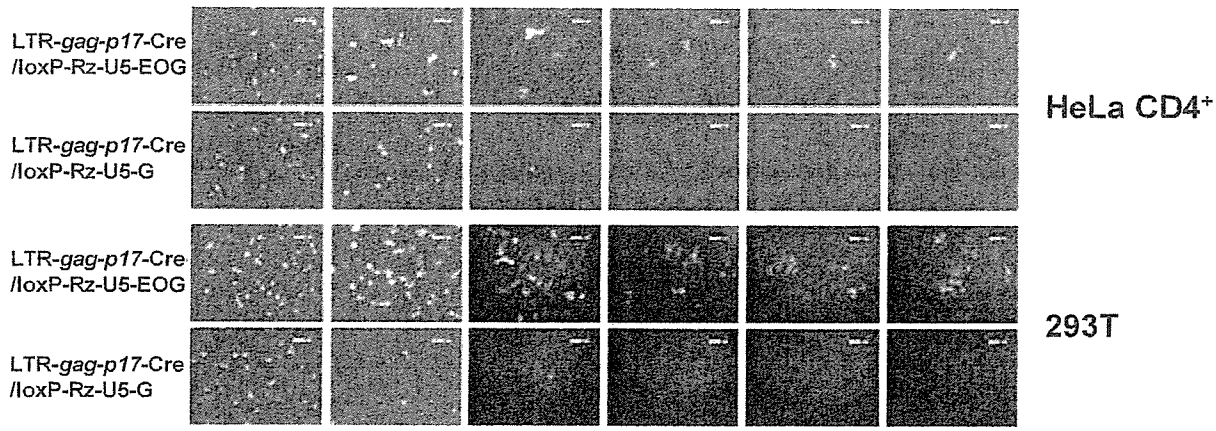

Figure 2. *EBNA-1* and *oriP* increase the duration of EGFP expression in transfected HeLa CD4$^+$ and 293T cells. EGFP expression was evaluated on days 1, 3, 7, 13, 19, and 25 post-transfection.

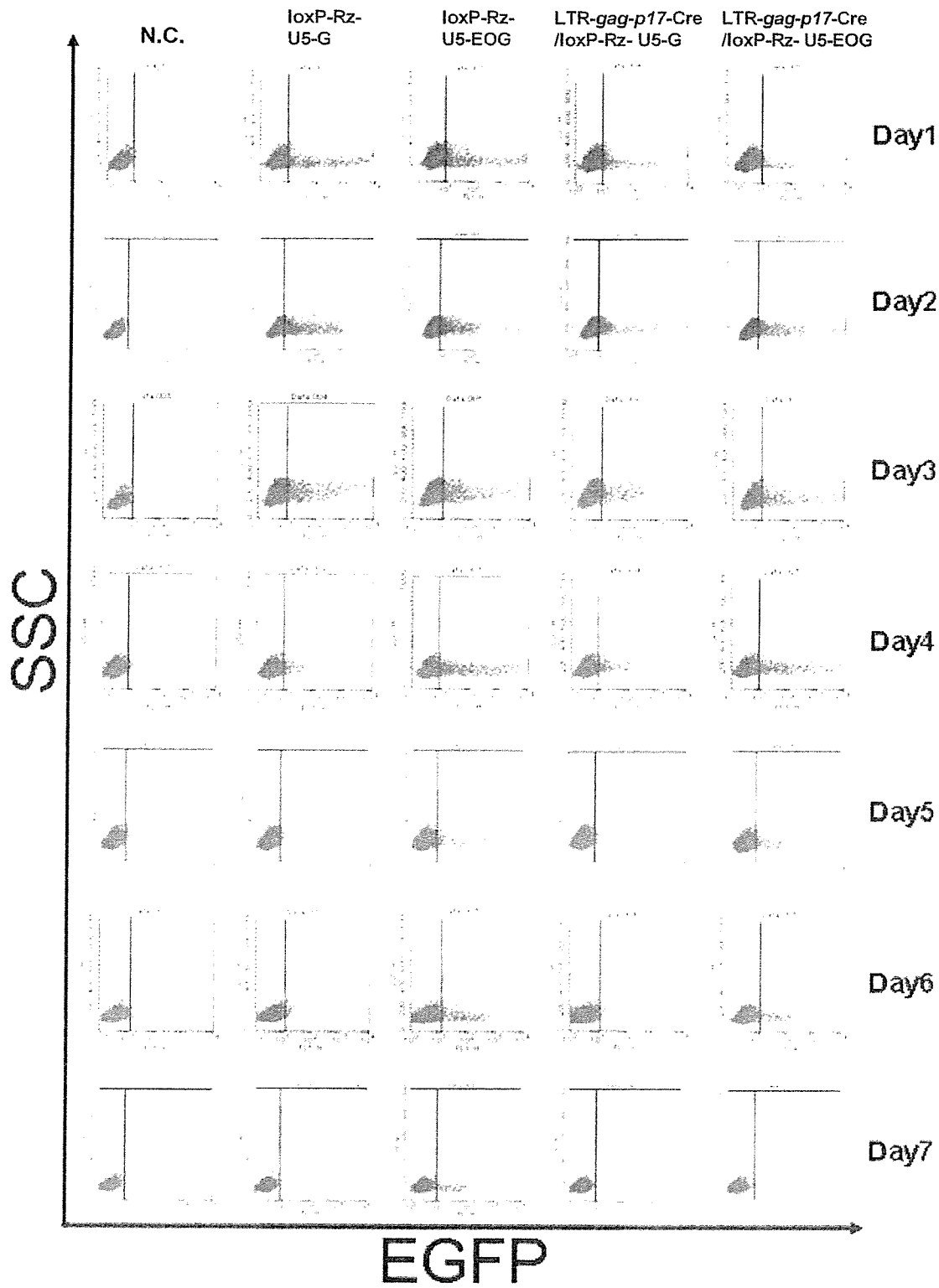

Figure 3. Enhancement of transgene expression by *EBNA-1* and *oriP* in HeLa $CD4^+$ cells. EGFP expression was evaluated on days 1 to 7 post-transfection by flow cytometry.

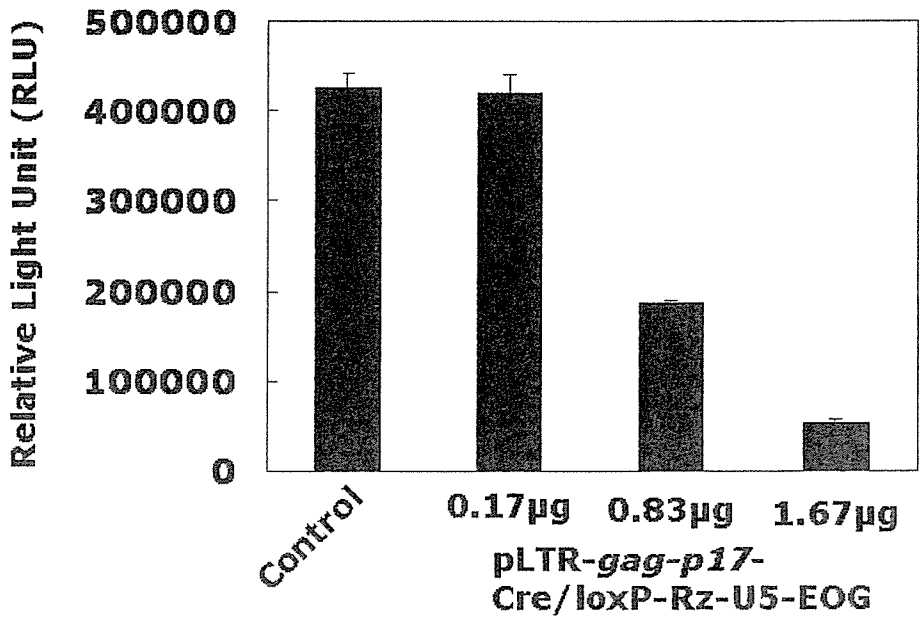

Figure 4. Dose-dependent inhibition of HIV-1 replication by pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG in HIV-1-transfected cells. Different amounts of pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG (0.17, 0.83 or 1.67 μg) were co-transfected with HIV-1 (pNL4-3-luc) into HeLa $CD4^+$ cells. At 72 h post-transfection, luciferase activity of the cell lysate was measured as an indirect marker for viral replication.

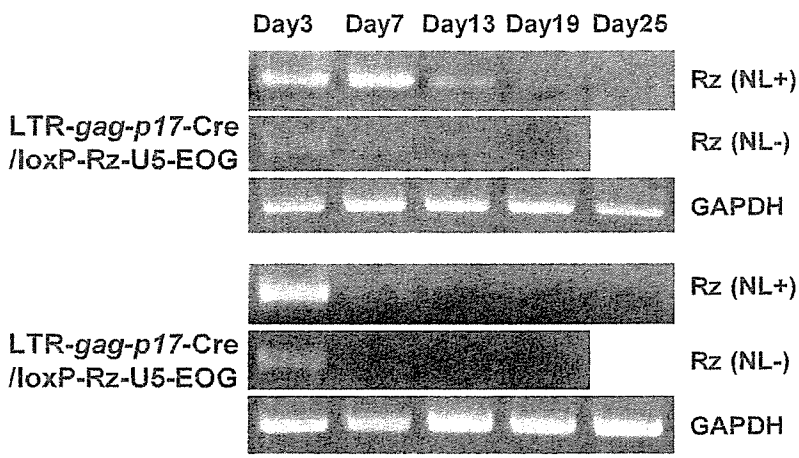

Figure 5. RT-PCR analysis shows persistent ribozyme expression of HeLa $CD4^+$ cells transfected with *EBNA-1/oriP*-containing vector. Total RNA was isolated from confluent pNL4-3 infected cell cultures transfected with pLTR-*gag-p17*-Cre/loxP-Rz-U5-G or pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG. RT-PCR using 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.

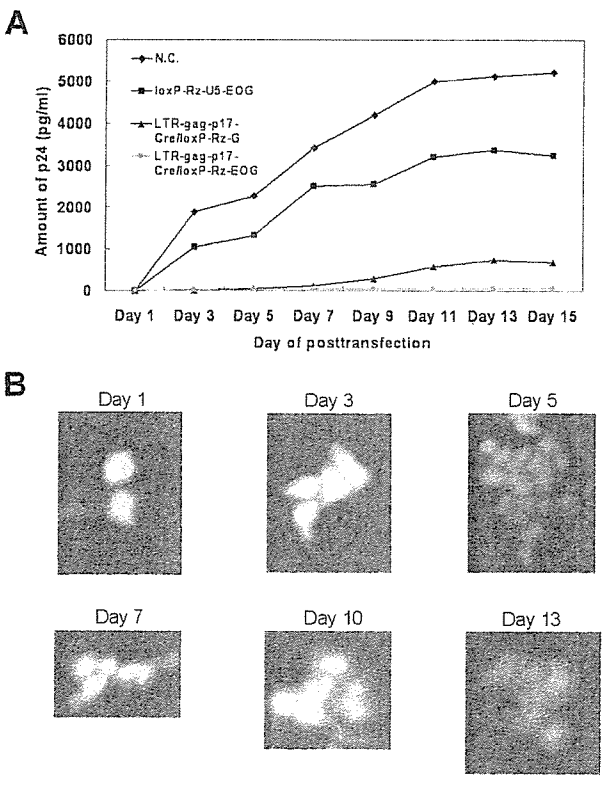

Figure 6. Inhibitory effects on HIV-1 p24 antigen production by pLTR-*gag-p17*-Cre/loxP-Rz-U5-EOG. **(A)** HeLa CD4$^+$ cells were co-transfected with HIV-1 pNL4-3 and either control vector (diamonds), ploxP-Rz-U5-EOG (squares), pLTR-*gag-p17*-Cre/loxP-Rz-U5-G (triangles), or pLTR-*gag-p17*-Cre/loxP-Rz-U5- 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.

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.

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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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SUPPRESSION OF HIV-1 REPLICATION BY A COMBINATION OF ENDONUCLEOLYTIC RIBOZYMES (RNase P AND tRNase ZL)

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□ *We examined the combinatorial action of RNase P and tRNase ZL-mediated specific inhibition of HIV-1 in cultured cells. We designed two short extra guide sequences (sEGS) that specifically recognize the tat and vif regions of HIV-1 mRNA and mediate the subsequent cleavage of hybridized mRNA by the RNase P and tRNase ZL components. We constructed an RNase P and tRNase ZL-associated vif and tat sEGS expression vector, which used the RNA-polymerase III dependent U6 promoter, as an expression cassette for EGS. Together, the RNase P and tRNase ZL-associated sEGS molecules allow more efficient suppression of HIV-1 mRNA production when separately applied. The possibilities offered by the vector to encode sEGS will provide a powerful tool for gene therapy.*

Keywords RNase P; tRNase ZL; External guide sequence; HIV-1; Gene therapy

INTRODUCTION

Small inhibitory RNAs (siRNAs) are small RNAs of 21-nucleotide length that trigger the destruction of target mRNA with which they share complementarity.[1] RNAi has emerged as a powerful tool to probe the function of genes of known sequence *in vitro* and *in vivo*. Advances in vector design

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This article is dedicated to Professor Eiko Ohtsuka on the occasion of her 70th birthday.
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permit the effective expression of siRNA in human cells by transfer of short hairpin RNA (shRNA) expression cassettes.[2] Recent investigations have described the ability of RNAi to decrease the replication of human immunodeficiency virus type 1 (HIV-1) in lymphocytic cells using siRNA targeting viral (e.g., tat, gag, rev, nef)[3–7] and host (e.g., CCR5, CXCR4)[8,9] proteins. RNAi can be used as a form of genetic therapy for HIV-1 and associated infections.

When profound inhibition of virus replication is obtained by means of RNAi technology, one has to consider the possibility of viral escape. This potential problem is particularly true for viruses that exhibit significant genetic variation due to an error-prone replication machinery. Thus, this problem may be more severe for RNA viruses and retroviruses than DNA viruses. Indeed, RNAi-resistant poliovirus and HIV-1 variants have already been reported.[10–12]

This problem can be overcome with the catalytic RNA subunit RNase P which can in principle be targeted to cleave any target RNA using the external guide sequences (EGS).[13] This strategy is unique in that cleavage of a specific target mRNA occurs after hybridization of the EGS to form a structure resembling a tRNA substrate.[14,15] RNA-based EGSs have been expressed endogenously as transgenes in both bacteria and mammalian cells,[14,16] and have been effective in inhibiting gene expression by HIV-1.[17,18] Mammalian cells contain the essential enzyme, tRNA 3′-processing endoribonuclease (tRNase Z or 3′-tRNase; EC 3.1.26.11), which removes 3′trailers from pre-tRNAs.[19] The long-form enzyme (tRNase ZL) is unique in that it can cleave any RNA at any site when directed by a small-guide RNA (sgRNA) in vitro.[20–22] Recently, we demonstrated the efficacy of this method in specifically targeting RNA in HIV-1 infected T-cells by introducing sgRNAs encoded by expression retroviral vector. Mo-MLV-based sgRNA-SL4 targeting the HIV-1 gag gene could suppress sgRNA-dependent HIV-1 expression in human T cells.[23]

In this article, we demonstrate the combinatorial action of RNase P and tRNase ZL-mediated specific inhibition of HIV-1 in cultured cells. We designed two truncated short extra guide sequences (sEGS) specifically recognize the tat and vif regions of HIV-1 mRNA and mediate subsequent cleavage of hybridized mRNA by the RNase P and tRNase ZL components. Combination of RNase P and tRNase ZL-associated sEGS molecules allows more efficient suppression of HIV-1 mRNA than separate application.

RESULTS AND DISCUSSION

Design and Construction of the U6–EGS Driven Expression System

In a previous paper, we demonstrated the inhibition of HIV-1 products using sEGS and RNase P to cleave an HIV-RNA target (substrate) in cultured

cells.[18] The short EGS, 12 nucleotides long, was designed to hybridize as an sEGS to a region of the viral RNA with the expected cleavage site located 5′ to the double stranded region (Figure 1B). The greatest inhibitory effect on HIV-1 replication was detected with the sEGS (sEGS-tat) vector as the target of the HIV-1 tat gene (Figures 1A and B). Furthermore, we demonstrated the inhibition of HIV-1 gene products in cultured cells by inducing HIV-1 mRNA cleavage using a modified 5′-half-tRNA^{Arg} (sgRNA) and mammalian tRNase ZL.[23] The greatest inhibitory effect on HIV-1 expression was achieved using sgRNA targeting the HIV-1 gag gene.

In this article, we selected the vif (5521-5533) and tat (5921-5940) as the target sites and tested the RNase P and tRNase ZL-associated sEGS (RNase P-tat-sEGS and tRNase-ZL-vif-sEGS) in one molecule for anti-HIV activity (Figures 1B–D). The complete T loop of RNase P-*tat*-sEGS was replaced by single-stranded sequence UUCA, whereas the T-stem loop of tRNase ZL-vi*f*-sEGS included the T-stem loop (5′-CCAGGUUCGACUCCUGG-3′) of wild-type tRNA^{Arg}.

Alternatively, small RNA molecules might also be expressed in the cell following the cloning of siRNA templates into RNA polymerase III (pol III) transcription units, which are based on the sequences of the natural transcription units of the small nuclear RNA U6.[24,25] We cloned the vif-sEGS, tat-sEGS, and vif-tat-sEGS lined with linker (5′-CGCUCGAGU-3′) between either the vif-sEGS-5′ end and tat-sEGS-3′ end genes into Kpn I and BamH I sites downstream of the human U6 snRNA promoter[24] of mammalian expression vector (pSV2neo-U6) (Figure 2). These vectors have been previously described with a high cleavage affinity.[18,23] Furthermore, the control vector designs were constructed to express pSV2neo-U6-mutant-EGS expression vectors (U6-mvif-sEGS, U6-mtat-sEGS, and U6-mvif-mtat-sEGS) (Figure 2).

Expression of the sEGS in Target Cells

To characterize the effect of transge expression, we monitored green-fluorescent protein (GFP) expression in the COS cells transfected with three different plasmids (U6-vif-sEGS, U6-tat-sEGS, and U6-vif-tat-sEGS). GFP expression in these transfected COS cells were observed for 3 days (Figure 3A). To ensure that the therapeutic gene expression by a plasmid vector, pU6-vif-tat-sEGS in target cells, we carried out Northern blot analysis on total RNAs extracted from transduced COS cells. As shown in Figure 3B, a combination of U6-vif-tat-sEGS was expressed at readily detectable levels.

Suppression of HIV-1 Replication by the EGS

To test the ability of the sEGS-expressed by the mammalian expression vectors (pSV2-neo-U6-sEGS) to inhibit HIV-1 replication in a transient assay,

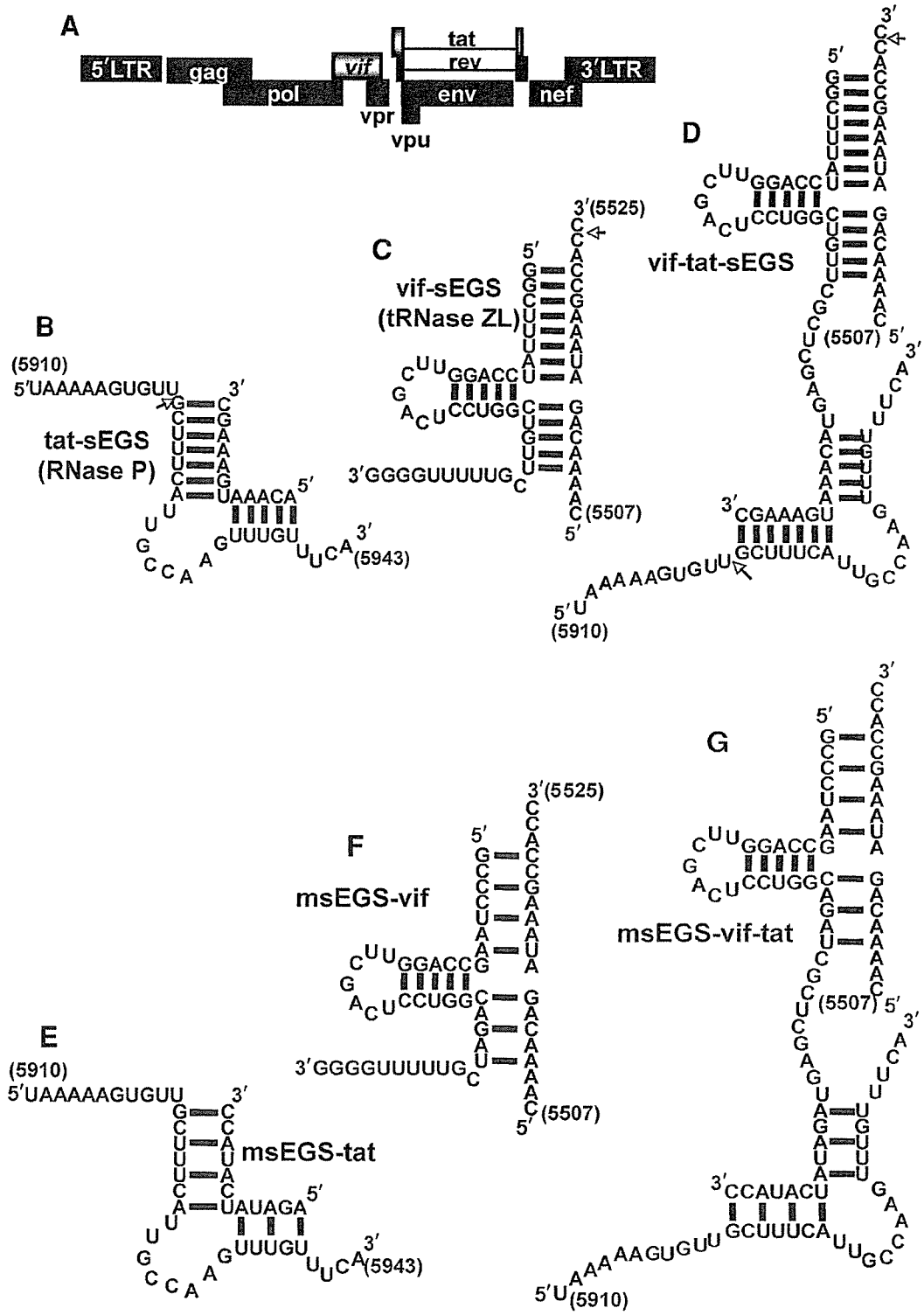

FIGURE 1 Locations of the target sites and secondary structures in the HIV-1 gene of sgRNAs. (A) Locations of the target sites in the HIV-1 gene of sEGS. (B-G) Plausible secondary structures of complexes of the two target sites within the HIV-1 genome (the vif and tat within the HIV-1 gene) with the modified 5′-half-tRNA^{Arg} (tat-sEGS) containing 9 and 5 nt sequences and the T-stem and loop as well as the acceptor stem to the complementary to the target HIV-1 vif site and the tat gene. The arrow indicates the RNase P and tRNase ZL cleavage point.

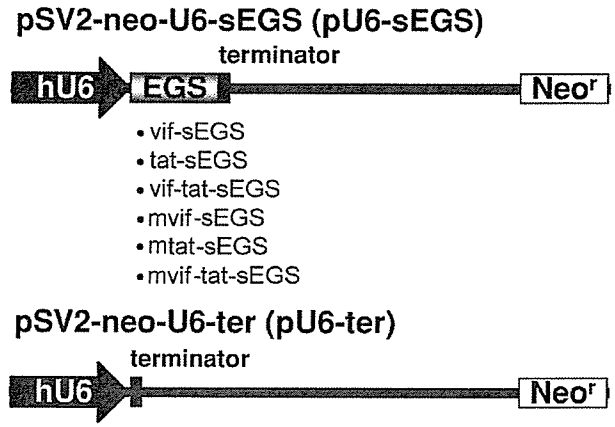

FIGURE 2 Schematic representation of sEGS-expression vectors. The pSVneo-U6-sEGS vectors, pU6-vif-sEGS, pU6-tat-sEGS, pU6-vif-ta-sEGS, pU6-mvif-sEGS, pU6-mtat-sEGS, and pU6-mvif-tat-sEGS. Control vector, pSVneo-U6-ter, lacked sEGS in pSVneo-U6-sEGS.

a viral plasmid (pNL4-3-luc)[26] and the pSV2-neo-U6-sEGSs (U6-vif-sEGS, U6-tat-sEGS, and U6-vif-tat-sEGS) or pSV2-neo-U6-ter (U6-ter) with the U6 promoter and terminator as the control plasmid, were cotransfected into COS cells by the transfection reagent, FuGENE6. The virus production in the culture supernatant was monitored by the HIV-1 p24 antigen (gag gene

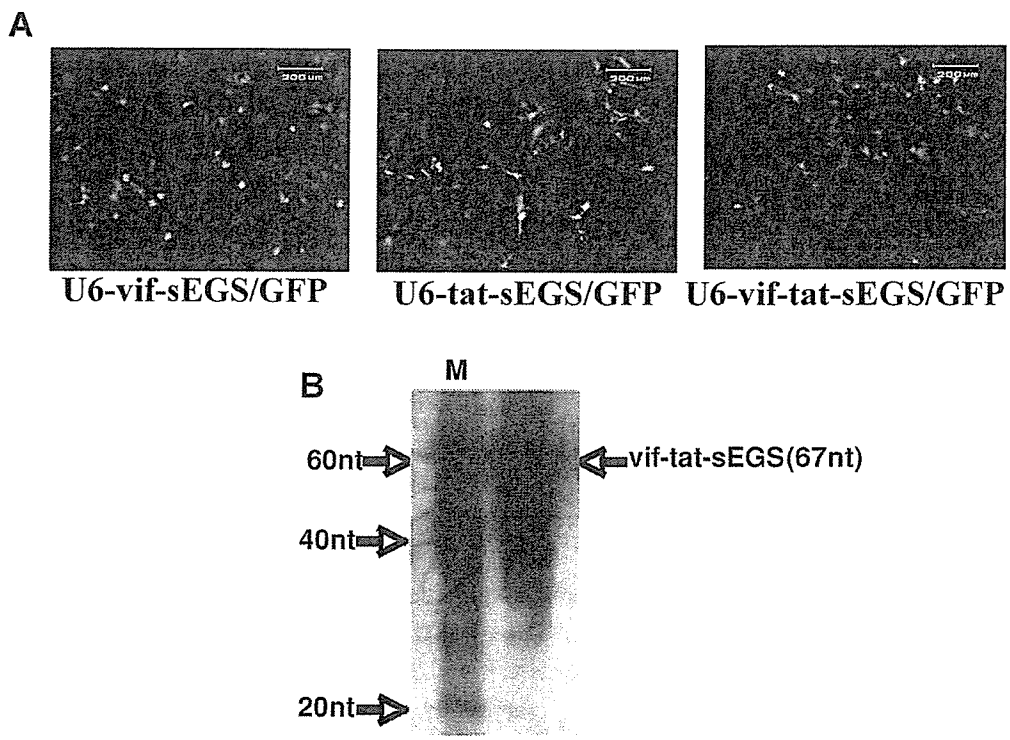

FIGURE 3 Expression of transgenes. (A) To monitor the continuous expression of the transgenes in the transduced cells throughout the culture period, the cells were transferred onto microscope slides and examined for GFP expression during the sampling of the culture cells and supernatants. (B) The presence of RNA was analyzed by northern blotting. Plasmids encoding vif-tat-sEGS were introduced into COS cells. After 72 h, the cells were collected, total RNA was isolated and fractionated on a 15% polyacrylamide gel.

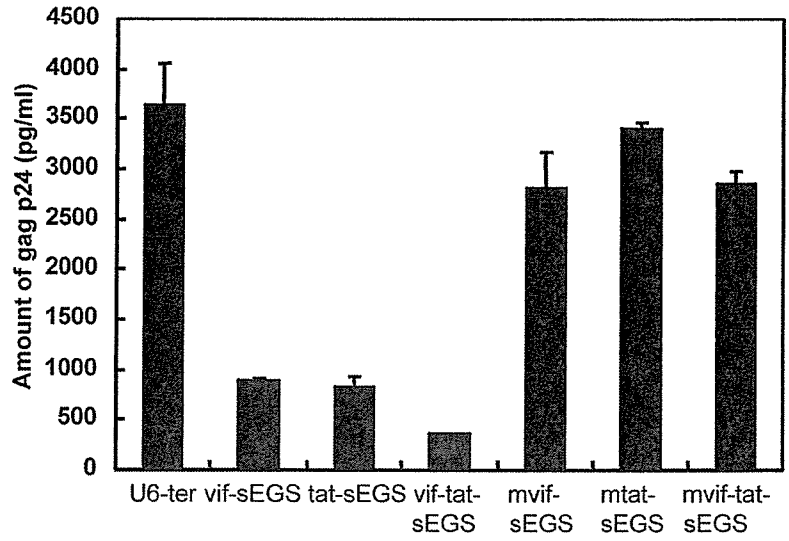

FIGURE 4 Inhibition of production of HIV-1 gag p24 antigen in COS cells transduced with pU6-sEGS. COS cells were transduced with pU6-sEGS and pNL4-3-luc. The pU6-vif-tat-sEGS showed significant suppression of the HIV-1 p24 antigen expression in COS cells. Data are representative of three independent experiments.

product) assay.[27] Low levels of p24 product for both U6-vif-sEGS and U6-tat-sEGS used in the challenge assays were detected, and no differences between the EGS efficiencies were observed (Figure 4). In contract, combination of RNase P and tRNase ZL associated sEGSs molecules significantly reduced the level of HIV-1 p24 antigen as compared with that of either U6-vif-sEGS and U6-tat-sEGS (Figure 4). On the other hand, the control sEGSs (U6-mvif-sEGS, U6-mtat-sEGS, and U6-mvif-tat-sEGS) had no inhibitory effect on HIV-1 p24 antigen. These results suggest that the combination of two different antiviral RNAs provides more than separate application.

Effect of a Combination of RNase P and tRNase ZL-Associated sEGS

We also examined the HIV-1 mRNA levels to identify the contribution of HIV-1 mRNA cleavage to the sEGS-mediated anti-HIV-1 effect. The RT-PCR reactions were used to establish the level of uncleaved HIV-1 mRNA (product I and II).[28] The uncleaved HIV-1 mRNA was amplified by the vif-F1and tat-F1 primers, and the vif-R and tat-R primers (Figure 5A). The level of product I and II were expected to decrease after cleavage of the HIV-1 mRNA. The results, which are shown in Figure 5B, indicate that the vif-sEGS and tat-dependent expression system reduced the amount of full-length HIV-1 mRNA (product I and II), whereas COS cells transfected with pNL4-3-luc/mutant-sEGS did not exhibit a significantly altered level of intact HIV-1 mRNA (Figure 5B). The reduction in functional full-length HIV-1 mRNA was consistent with the cleavage effects of vif-sEGS and tat-sEGS at the

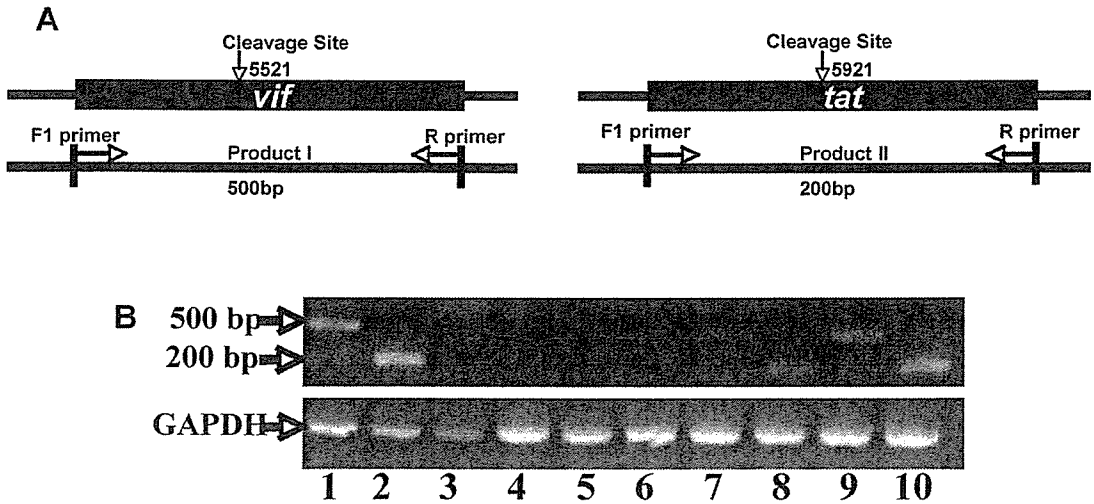

FIGURE 5 RT-PCR analyses of HIV-1 mRNA expression. RT-PCR analyses of uncleaved (product I and II), HIV-1 mRNA were performed using HIV-1 vif and tat-specific primers with concurrent amplification of GAPDH mRNA. (A) Schematic representation of HIV-1-specific primer sites with respect to HIV-1 mRNA: F1 primers, vif and tat; R-primers, vif and tat. (B) RT-PCR amplification products analysed by 2% agarose gel electrophoresis with ethidium bromide staining RT-PCR analysis of HIV-1 mRNA expression in COS cells. RT-PCR analysis of uncleaved HIV-1 mRNA was carried out using HIV-1 specific primers with concomitant amplification of GAPDH mRNA. Lane 1: control vif; lane 2: control tat; lane 3: vif-sEGS; lane 4: tat-sEGS; lane 5: vif-tat-sEGS (vif); lane 6: vif-tat-sEGS (tat); lane 7: mvif-sEGS; lane 8: mtat-sEGS; lane 9: mvif-tat-sEGS (vif); lane 10: mvif-tat-sEGS (tat).

post-transcriptional level. These results indicate that the binding of the sEGS to its target HIV-1 mRNA, and cleavage of pre-tRNA complexes with RNase P and tRNase ZL, might occur in the nucleus. The reduction in functional full-length HIV-1 mRNA was consistent with the RNase P and tRNase ZL cleavage effect at the post-transcriptional level.

This work explores the combination of two inhibitor RNAs (RNase P and tRNase ZL) in one molecule, generating a multifunctional RNA tool, which we have termed an "endonucleolytic ribozymes" (enR). The combination of an RNase P and a tRNase ZL-associated EGSs molecules would be advantageous than separate application. It possible to take advantage of the possibilities offered by the vector to encode sEGS and provides a powerful tool for HIV-1 gene therapy.

EXPERMENTAL PROCEDURES

Cell Cultures

COS and MT-4 cells were grown in complete culture medium consisting of either RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) or D-MEM (Sigma Chemical Co.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). All cultures were maintained at 37°C under a 5% CO_2 atmosphere.

Construction of U6 Expression Plasmid Vectors

Expression plasmids were constructed using standard techniques. EGS-RNA sequences that were chemically synthesized as two complementary DNA oligonucleotides were mixed in equimolar amounts, heated for 5 min at 95°C, and then gradually cooled down to RT in annealing buffer (10 mM Tris-HCl/100 mM NaCl). The resultant duplex was ethanol precipitated and then ligated into the Kpn I and BamH I cloning sites downstream of the human U6 snRNA promoter of mammalian expression vectors (pSV2neo) (BD Biosciences Clontech, Mountain View, CA) in order to produce the following: the pSV2neo-U6-vif-tat-sEGS vector encoding both HIV-1 vif and tat RNA as dsRNA sense sequences containing KpnI and BamH I cloning sites (5′-GGCTTTATCCAGGTTCGACTCCTGGCTGTT-CGCTCGAGACAAATGAAAGCTTTTTG-3′) and antisense sequences (5′-GATCCCAAAAAGCTTTCATTTCTCTCGAGCGAACAGCCAGGAGTCGAA-CCTGGATAAAGCCGTA-3′); the pSV2neo-U6-vif-sEGS vector encoding HIV-1 vif sense fragment sequences (5′-CGCTTTATCCAGGTTCGACTC-CTGGCTGTTCGTTTTTGGGGTACG-3′) and antisense sequences (5′-GAT-CCGTACCCCAAAAACGAACAGCCAGGAGTCGAACC- TGGATAAAGCCG-TAC-3′); the pSV2neo-U6-tat RNA vector encoding HIV-1 tat sense sequences (5′-ACAAATGAAAGCTTTTTG-3′) and antisense sequences (5′-GATCCAA-AAAGCTTTCATTTGTGTAC-3′); the pSV2neo-U6-mvif-sEGS vector encoding mutated vif sense sequences (5′-CCCCTAAGCCAGGTTCGAC-TCCTGGCAGATCGTTTTTGGGGTACG-3′) and antisense sequences (5′-GATCCGTACCCCAAAAACGATCTGCCAGGAGTCGAACCTGGCTTA-GGCCGTAC-3′); the pSV2neo-U6-mtat-sEGS vector encoding mutated tat sense sequences (5′-AGATATCATACCTTTTTG-3′) and antisense sequences (5′-GATCCAAAAAGGTATGATATCTGTAC-3′); and, finally, the pSV2neo-U6-mvif- tat-sEGS vector encoding mutated vif and tat sense sequences (5′-GCCCTAAGCCAGGTTCGACTCCTGGCAGATCGCTCGAGAGATATC-ATACCTTTTTG-3′) and antisense sequences (5′-GATCCCAAAAAGGTA-TGAATCCTCTCGAGCGATCTGCCAGGAGTCGAACCTGGCTTAGGGCG-TAC-3′); as control expression vectors.

Fluorescent Microscopy

COS cells were grown to ~80% confluence (3×10^5 cells) and transfected with 1 μg each of the sEGS-RNA vectors using 3 μl of FuGENE 6 (Roche Applied Science, Indianapolis, IN). The transfected COS cells were trypsinized, washed twice in PBS, and fixed in PBS containing 1% formaldehyde. Direct fluorescence microscopy of GFP was carried out at the mitotic stage of cell division after each passage on day 3; the data were acquired with a DP12 digital microscope camera (Olympus Company, Tokyo, Japan).

Northern Blot Analysis

Total RNA was extracted with Trizol reagent (GibcoBRL Life Technologies, Carlsbad, CA) 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^{TM} nylon membrane (Amersham Co., Buckinghamshire, UK). The membrane was probed with synthetic oligonucleotides that were complementary to the antisense strand of the env shRNA. Hybridization was carried out at 42°C, and was followed by washing with 2 × SSC at 25°C prior to autoradiography.

Antiviral Assay

To each well of a six-well plate were added COS 3×10^5 cells in RPMI 1640 medium (Sigma Chemical Co.) supplemented with 10% fetal bovoine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Approximately 24 h after plating, when the cells had reached 80% confluence, sEGS-RNA vectors (1 μg) and pNL4-3-luc (0.1 μg) using 3 μl of FuGENE 6 (Roche Applied Science) were generated according to the manufacturer's optimized protocols. The envelope-defective HIV-1NL4-3-based retroviral vector containing a luciferase expression marker (pNL4-3-luc) was generated by substituting nef gene sequences of the HIV-1NL4-3 genome with the firefly luciferase gene and deleting the envelope gene sequences located between two Bgl II restriction endonuclease sites.[26] After 3 days of incubation, the virus replication was monitored in the cell-free culture supernatants with the HIV-1 p24 CLEIA assay (Lumipulse, Fujirebio Inc., Tokyo, Japan).[27]

Cleavage Activities of sEGS in COS Cells

COS cells were grown to ~80% confluence (3×10^5 cells) and transfected with 1 μg each of the sEGS-RNA vectors and the pNL4-3-luc plasmid. The cells were incubated for 3 days before the total cellular RNA was isolated. RNA samples were treated with DNase I according to the manufacturer's specifications. To quantify the level of HIV-1 RNA, amplifications of HIV-1 mRNA and the internal control message GAPDH were incorporated into the reaction mixtures for the RT and PCR steps. A sample of 1 μg of total RNA was used as the template with the vif, tat, and GAPDH primers (20 pmol each). Reverse transcription (final volume: 50 μl) was carried out at 60°C for 30 min. The cDNA products were amplified by PCR (94°C, 60 sec; 60°C, 90 sec; 40 cycles). The vif-F1 (5′-AGGAGAAAGAGACTGGCATTTGGG-3′) and tat-F1 (5′-ATGGAGCCAGTAGATCCTAGATCAGA-3′) primers and the vif-R (5′-CTCCTTCTGTCGAATAACGCCTATTCTG-3′) and tat-R (5′-CTATTCCTTCGGGCCTGTCGGGTC-3′) primers only generated a cDNA product from the uncleaved HIV-1 mRNA (RT-PCR product I, vif = 500 base

pairs, product II, tat = 200 base pairs), whereas the GAPDH-F and R primers generated the GAPDH gene (0.45 kb) as the internal control.

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