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Effective inhibition of HIV-1 replication in cultured cells by external guide sequences and ribonuclease P

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Abstract—We examined the suppressive effect of HIV-1 RNA gene cleavge on HIV-1 expression, using the catalytic RNA subunit RNase P and the 3′-half $tRNA^{Try}$ [external guide sequence (EGS)] in cultured cells. HIV-1 expression was inhibited by the $tRNA^{met}$-EGS-U5 and U6-EGS-U5 from the $tRNA^{met}$ and U6 promoters, respectively. There was no difference in the inhibitory effects on HIV-1 expression between the $tRNA^{met}$ and U6 promoters.

1. Introduction

Small ribozymes require the presence of a specific nucleotide sequence in the target RNA but this requirement cannot always be fulfilled. This problem can be overcome, however, with the catalytic RNA subunit RNase P, which can be targeted to cleave any target RNA using external guide sequences (EGSs).[1] For example, the 5′-processing event in eukaryotic cells that generates the correct 5′-ends of mature tRNAs is performed by an endoribonuclease similar to the well-studied *Escherichia coli* RNase P.[2] Furthermore, the tRNA substrate can be deleted, and by joining the two pieces of the remaining precursor, a good substrate is formed. The resulting molecule resembles a T-stem stacked on the acceptor stem of a tRNA.[3] The structure recognized by RNase P can be approximated by a short RNA fragment, termed the EGS, which is complementary to the substrate.[4] RNase P will cleave a single-stranded 5′-leader sequence adjacent to any double-stranded RNA duplex, as long as the unpaired CCA nucleotides are present at the 3′-end of the EGS.[5] This method takes advantage of RNase P to cleave a targeted mRNA or viral RNA when it is in a complex with the appropriate EGS in mammalian cells.[6,7]

In this paper, we demonstrate the inhibition of HIV-1 products using a short EGS and RNase P to cleave an HIV–RNA target (substrate) in cultured cells. The EGS/substrate RNA complex forms a pre-tRNA-like structure with the 3′-half tRNA and T-stem–loop structures.

2. Results and discussion

2.1. Design and construction of the $tRNA^{met}$-EGS driven expression system

In the two half-tRNA mammalian system, pre-$tRNA^{Try}$ variants with the T-stem–loop region are cleaved by RNase P.[8] The utility of the methodology designed based on this system for human disease depends on the ability to deliver and express the guide sequence in target cells. We selected the U5 region (562–574) of LTR and tat (5931–5942) as the target sites, and tested

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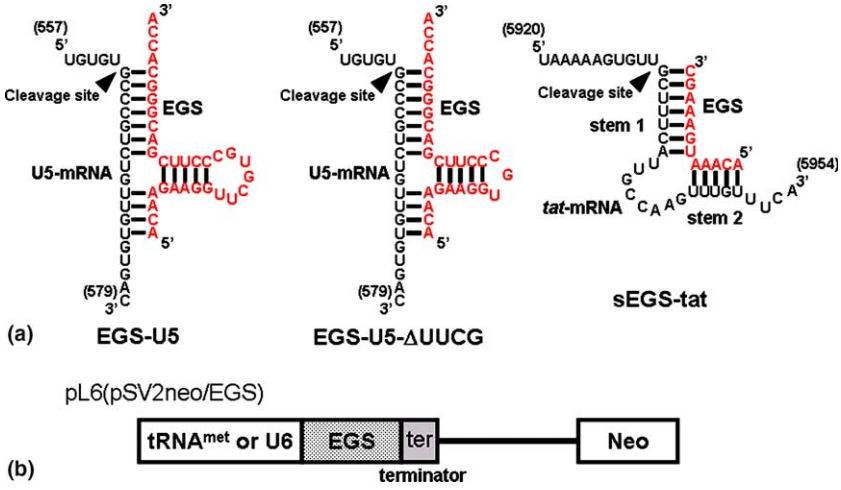

Figure 1. (a) Schematic representation of the HIV-1 genome, EGS target sequences, and complexes of target RNA (HIV–mRNA) and EGS that resemble the structure of a tRNA. The sEGS-tat binds to the target sequence with two helices. One of the stems forms adjacent to the UUCA sequence of the T-loop: this stem is named stem 1. Stem 1 is connected through a bulge to a second helix, which is named stem 2. (b) Schematic diagrams of EGS expression plasmid constructs. Construction of an EGS expression plasmid [pL6 (pSV2neo/EGS)].

the two half-tRNA mammalian system for anti-HIV activity (Fig. 1). The HIV-1 tat protein is a *trans*-activator of transcription for all HIV-1 genes, acting primarily at the level of transcriptional elongation, rather than initiation.[9–11] Tat function depends on a bulging RNA stem–loop structure, TAR, at the 5′-end of all HIV-1 mRNAs. The highly conserved interaction between tat and TAR, and their combined effect on host cell transcription factors are essential for HIV-1 replication.[12] Therefore, blocking replication of the virus by inhibiting tat function is an attractive strategy for gene therapy of HIV-1 infection. On the other hand, the HIV-1 LTR is also a potential target, because of its high degree of conservation among the known HIV-1 isolates and because of its presence in both early and late viral gene products.[13] Also, several cellular factors interact with the LTR regulatory elements to regulate the expression of the HIV-1 genome.[14]

Various promoters have been used to express EGS in different cells, but there have been few direct comparisons to determine, which is most effective. Thus, two promoter designs (the human methionine tRNA ($tRNA^{met}$) promoter[15] and the human U6 snRNA promoter[16]) were constructed to express the EGS in tandem. We cloned the U5-EGS genes into mammalian expression vectors (pSV2neo) downstream of the human $tRNA^{met}$ or human U6 snRNA promoters and the pol III termination signal (Fig. 1). Furthermore, the control vector design was constructed to express the deletion of the T-loop analogue ($tRNA^{met}$-EGS-U5-ΔUUCG) of its 'tRNA' domain. Recently, Jarrous reported that human RNase P is located in the nucleolus.[17] The co-localization of the EGS with its target is an important determinant of the EGS efficiency, and thus it was essential to determine the intracellular localization of the $tRNA^{met}$-EGS target. Recent reports indicated that the $tRNA^{met}$–ribozyme, and the U6-EGS remain in the nucleus.[18,19] Therefore, binding of the EGS to its target might occur in the nucleus.

We designed a shorter oligonucleotide ($tRNA^{met}$-sEGS-tat) that acts as an EGS targeted to the tat mRNA (5931–5942) of HIV-1, and cloned the tat-EGS gene into mammalian expression vectors (pSV2neo) downstream of the human $tRNA^{met}$ promoter and the pol III termination signal (Fig. 1). Since, the minimal substrate for human RNase P consists of only the acceptor stem and the T-stem and loop, these two stems would comprise the binding helices of the EGS and target.[20,21] Recently, Werner et al. demonstrated that cleavage of the biomolecular construct by human RNase P does not require the presence of the remaining three nucleotides of the T-loop, implying that the interaction with the UUCR sequence in the T-loop is sufficient for recognition.[22]

2.2. Suppression of HIV-1 replication by the EGS

Expression of the EGS in COS cells transfected with these plasmids was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. The subgenomic RNA-driven by the $tRNA^{met}$ promoter was highly expressed in the transfected cells (Fig. 2).

To test the ability of the EGS-expressed by the mammalian expression vectors (pL6) to inhibit HIV-1 replication in a transient assay, a viral plasmid (pNL4-3-Luc) and the pL6-EGS vector ($tRNA^{met}$-EGS-U5 and U6-EGS-U5) or the pL6 control vector (pL6-$tRNA^{met}$-ter) with the $tRNA^{met}$ promoter and terminator (ter), were co-transfected into COS cells using the transfection reagent, FuGENE6. The virus production in the culture supernatant was monitored by the HIV-1 p24 antigen (gag gene product) assay. Low levels of the p24 product for both the $tRNA^{met}$-EGS-U5 and U6-EGS-U5 used in the challenge assays were detected, and there was no difference in the efficiency between the EGSs (Fig. 3). Recently, Kraus et al. reported that the inhibitory effects of RNase P-associated EGS are promising with regard to cross-clade protection and potency of the RNase P-mediated inhibition of HIV-1 infection.[23]

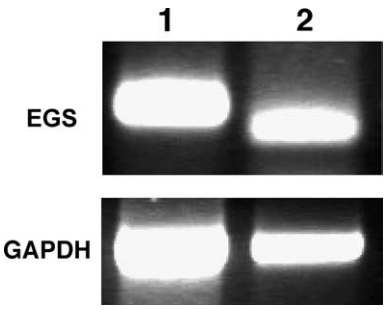

Figure 2. RT-PCR analysis of EGS expression in COS cells. The RT-PCR assay for EGS was performed using EGS-specific primers, with concurrent amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The RT-PCR amplification products were fractionated by electrophoresis on a 2% agarose gel with ethidium bromide staining. Lane 1, pL6-tRNA^{met}-EGS-U5 transfected COS cells; lane 2, pL6-tRNA^{met}-sEGS-tat transfected COS cells.

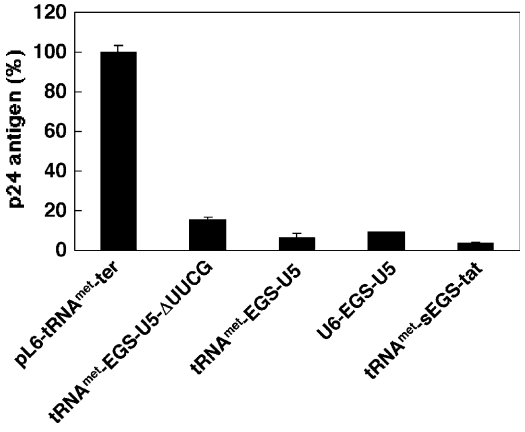

Figure 3. HIV-1-dependent EGS-mediated inhibition of HIV-1 expression. The target-expressing plasmid, the pL6 plasmid encoding EGS, and the pNL4-3-luc plasmid were co-transfected by the transfection reagent, FuGENE™6, into COS cells. The amount of p24 antigen production in the cultured cells was monitored by the HIV-1 p24 antigen assay.

The control EGS vector was designed for further comparisons of the RNase P cleavage efficiencies in vitro versus those in cultured cells. The latter EGS had a deletion of the T-loop analogue (tRNA^{met}-EGS-ΔUUCG) of its 'tRNA' domain; it was approximately twice as efficient in the inhibition of HIV-1 replication. This result suggests that the T-loop and the UUCG sequence of the T-loop are important for EGS catalytic efficiency. The same result was also observed for the biomolecular construct obtained though opening the T-loop between G57 and A58 (5′-UUCGAAU-3′), as described by Werner et al.[22] The UUCR sequence, adjacent to a 5 or 6-base pair helix, which in turn is connected through a bulge with a second short helix (seven–eight nucleotides), is sufficient for recognition and cleavage with human RNase P. This EGS is much shorter than the smallest reported EGSs for human RNase P.[20–22] This ability of the short oligonucleotide of function as an EGS provides promising new perspectives for developing oligonucleotide-based antiviral therapeutics. In this study, we also designed short EGSs for the tat mRNA of HIV-1 as the target (Fig. 1). A GenBank database search was conducted to find the pattern UUCA in the tat protein sequences of the HIV-1 strain. The sequence, composed of 12 nucleotides, was designed to hybridize as an EGS to a region of the viral RNA with the expected cleavage site located 5′ to the double stranded region (Fig. 1). Therefore, we tested the abilities of a virus plasmid (pNL4-3-luc), and pL6-tRNA^{met}-sEGS-tat or pL6-tRNA^{met}-ter as the control plasmid to inhibit HIV-1 replication in COS cells. The greatest inhibitory effect on HIV-1 replication was detected with the short EGS (tRNA^{met}-sEGS-tat) vector as the target of the HIV-1 *tat* gene (Fig. 3). These results suggest that the level of inhibition observed with the deletion of the T-loop analogue (tRNA^{met}-EGS-U5-ΔUUCG) was a lower than that of tRNA^{met}-EGS-U5 or tRNA^{met}-sEGS-tat. The inhibitory effect of the EGSs might be due to the sequence UUCR adjacent to a five base pair helix, which in turn, is connected through a bulge with a second short helix (seven nucleotides). We were able to design a short EGS of a length of only 12 nucleotides. This is significantly smaller than any other oligonucleotide used in gene inactivation reported thus far. This short EGS had the highest inhibitory effects similar to those of the EGS containing the T-stem and loop of the tRNA precursor described by Kraus et al.[23]

2.3. Effects of EGSs on HIV-1 mRNA levels

We examined the HIV-1 mRNA levels to identify the contribution of HIV-1 LTR cleavage in the tRNA^{met}-EGS-U5 and tRNA^{met}-EGS-U5-ΔUUCG-mediated anti-HIV-1 effects. Two sets of RT-PCR reactions were used to establish the level of uncleaved HIV-1 mRNA (product 1) and the total amount of (uncleaved and cleaved) HIV-1 mRNA (product 2).[24] The uncleaved HIV-1 mRNA was amplified by the LTR-F1 and LTR-R primers (Fig. 4a). The level of product 1 was expected to decrease upon cleavage of the HIV-1 mRNA. Since total HIV-1 mRNA is amplified by the LTR-F2 and LTR-R primers (Fig. 4a), product 2 reflects the total amount (tRNA^{met}-EGS-U5 and tRNA^{met}-EGS-U5-ΔUUCG-cleaved and uncleaved) of HIV-1 LTR mRNA, and the 3′ fragment of tRNA^{met}-EGS-U5 and tRNA^{met}-EGS-U5-ΔUUCG-cleaved HIV-1 mRNA serves as a viable template for product 2 amplification. The results indicate that tRNA^{met}-EGS-U5 and tRNA^{met}-EGS-U5-ΔUUCG reduced the amount of full length HIV-1 LTR mRNA (Fig. 4b, product 1, lanes 3 and 4), whereas COS cells transfected with pL6-tRNA^{met}-ter and tRNA^{met}-sEGS-tat did not exhibit a significantly altered level of intact HIV-1 LTR mRNA (Fig. 4b, lanes 2 and 5). The reduction in the amount of functional full length HIV-1 mRNA is consistent with the EGS cleavage effect at the post-transcriptional level.

We believe that RNase P, in conjunction with short EGSs in its mammalian form, is a promising tool for inactivating genes in mammalian cells. Inhibition of

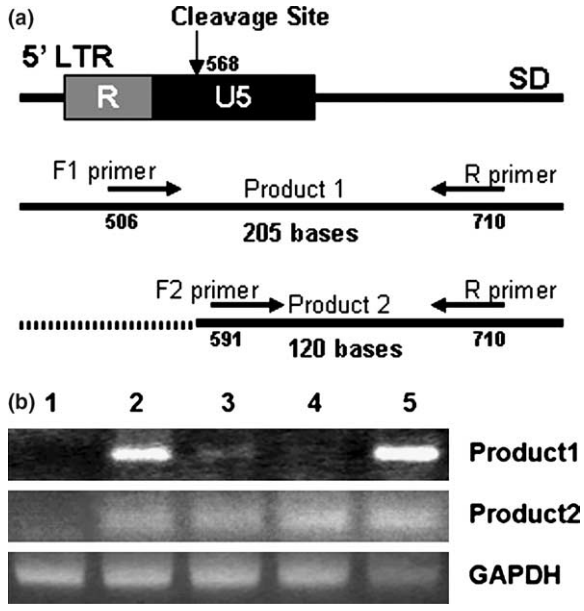

Figure 4. RT-PCR analyses of HIV-1 mRNA expression. RT-PCR analyses of uncleaved (product 1) and total (cleaved + uncleaved; product 2) HIV-1 mRNA were performed using HIV-1 LTR-specific primers with concurrent amplification of GAPDH mRNA. (a) Schematic representation of HIV-1 LTR-U5-specific primer sites (F1, F2, 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 COS cells; lane 2, pL6-tRNA^{met}-ter transfected HIV-1 (pNL4-3-luc) infected COS cells; lane 3, pL6-tRNA^{met}-EGS-U5-ΔUUCG transfected HIV-1 (pNL4-3-luc) infected COS cells, and lane 4, pL6-tRNA^{met}-EGS-U5 transfected HIV-1 (pNL4-3-luc) infected COS cells, and lane 5, tRNA^{met}-sEGS-tat transfected HIV-1 (pNL4-3-luc) infected COS cells.

HIV-1 by this methodology has important therapeutic potential.

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