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Design, characterization and testing of $tRNA_3^{Lys}$-based hammerhead ribozymes

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ABSTRACT

A hammerhead ribozyme targeted against the HIV-1 *env* coding region was expressed as part of the anticodon loop of human $tRNA_3^{Lys}$ without sacrificing tRNA stability or ribozyme catalytic activity. These tRNA-ribozymes were isolated from a library which was designed to contain linkers (sequences connecting the ribozyme to the anticodon loop) of random sequence and variable length. The ribozyme target site was provided *in cis* during selection and *in trans* during subsequent characterization. tRNA-ribozymes that possessed ideal combinations of linkers were expected to recognize the *cis* target site more freely and undergo cleavage. The cleaved molecules were isolated, cloned and characterized. Active tRNA-ribozymes were identified and the structural features conducive to cleavage were defined. The selected tRNA-ribozymes were stable, possessed cleavage rates lower or similar to the linear hammerhead ribozyme, and could be transcribed by an extract containing RNA polymerase III. Retroviral vectors expressing tRNA-ribozymes were tested in a human CD4+ T cell line and were shown to inhibit HIV-1 replication. These $tRNA_3^{Lys}$-based hammerhead ribozymes should therefore prove to be valuable for both basic and applied research. Special application is sought in HIV-1 or HIV-2 gene therapy.

INTRODUCTION

Ribozymes are small catalytic RNAs that can specifically bind and cleave other RNA species. The minimal structural requirements for the design of *trans*-acting ribozymes have been elucidated such that it is now possible to target ribozymes against any given RNA (1). Hammerhead and hairpin ribozymes have been designed against a variety of target RNAs (reviewed in 2,3). To address issues related to expression, size, stability and compartmentalization of RNAs containing ribozymes, tRNA-based ribozymes were developed. tRNAs have the advantage of being relatively small and possessing long half-lives. Furthermore, they are expressed under the control of RNA polymerase (pol) III in excess over mRNAs. Elements required for their expression are usually built within the tRNA coding region followed by a run of at least six T residues which function as an RNA pol III terminator (4–6).

The advantage of using tRNA and other RNA pol III-driven genes is evident by the increasing number of research groups that utilize them for expressing ribozyme (7–35) and other therapeutic (36–47) molecules. tRNA-driven transcriptional units were designed to express various RNAs as part of the tRNA anticodon loop (26–31) or as part of the 3′ region of the tRNA (7–25). Expression within the tRNA anticodon loop was achieved by cloning sequences between the tRNA intragenic promoter (4). Utilizing this method, ribozymes have been expressed as part of the anticodon loop of $tRNA_i^{Met}$ (26–30) and $tRNA^{Tyr}$ (31). Expression as part of the tRNA 3′ region was achieved by cloning ribozymes before the RNA pol III terminator of $tRNA_i^{Met}$ (7,20–25), $tRNA^{Val}$ (8–17,19) or $tRNA_3^{Lys}$ (18,19). Upon tRNA processing, this strategy allowed the liberation of expressed molecules as a separate RNA (41). Stem–loop structures were introduced upstream (23) and downstream of the released RNA to protect it from exonuclease degradation (41). In order to abolish processing, the last 18 nt at the 3′ end of the tRNA gene were deleted (48). This strategy allowed the production of tRNA-ribozymes which did not undergo processing (7,20–25).

We hypothesized that a $tRNA_{AC}$-Rz (ribozyme cloned within the anticodon loop of a tRNA), as opposed to a tRNA-Rz (ribozyme cloned downstream of a tRNA), would provide a more compact and stable structure to the ribozyme due to its location within the tRNA. Since the tRNA itself possesses a compact and stable structure, it is likely to impart this phenotype to the ribozyme. For this reason, $tRNA_3^{Lys}{}_{AC}$-Rzs were developed in which a ribozyme targeted against human immunodeficiency virus (HIV)-1 *env* coding region was inserted within the anticodon loop of human $tRNA_3^{Lys}$. The ribozyme inserted in the anticodon loop of $tRNA_3^{Lys}$ should not be spliced out since this tRNA does not contain introns (49). $tRNA_3^{Lys}$ was used with the intent of developing ribozymes which will cleave HIV-1 RNA both within the cell and HIV-1 progeny. The anticodon of $tRNA_3^{Lys}$ is not essential for packaging since a mutant $tRNA_3^{Lys}$ possessing a modified anticodon could still be packaged within HIV-1 virions (50,51; reviewed in 52). Similarly, $tRNA_1^{Lys}$ and $tRNA_2^{Lys}$ which possess a different anticodon were also shown to be packaged with the same efficiency as $tRNA_3^{Lys}$ (50,53).

Increased expression and stability of $tRNA_{AC}$-Rzs are highly desirable provided there is no structural constraint resulting in a decreased catalytic activity compared to a linear ribozyme. In $tRNA_{AC}$-Rzs, structural constraint may arise from suboptimal length of nucleotides connecting the ribozyme to the tRNA. The nucleotide composition of these linkers may also be important since potential base-pairing of tRNA sequences with ribozyme sequences could prevent hybridization of ribozyme with its target sequence.

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The length and sequence of linkers connecting the ribozyme to the tRNA sequences upstream (5′ linker) and downstream (3′ linker) of the ribozyme may therefore be optimized. To identify optimal lengths of 5′ and 3′ linkers, a selection strategy was used to isolate $tRNA_3^{Lys}$-based hammerhead ribozymes that were able to cleave a target site *in cis* (Fig. 1). These $tRNA_{AC}$-Rzs were cloned and characterized to determine whether they (i) can cleave a target RNA *in trans*, (ii) maintain catalytic activity similar to that of a linear ribozyme, (iii) are as stable as wild type $tRNA_3^{Lys}$, (iv) retain promoter elements (appropriate distance between boxes A and B) that permit RNA pol III-driven transcription, and (v) inhibit HIV-1 replication in a CD4+ human T cell line compared to an inactive tRNA-ribozyme or the vector alone.

MATERIALS AND METHODS

Construction of template DNA library encoding $tRNA_{AC}$-Rzs

Plasmids pSW2060 and pSW201 contain the $tRNA_3^{Lys}$ coding region. pSW2060 also contains a T7 promoter upstream of $tRNA_3^{Lys}$. pSW2060 was used to amplify the T7 promoter and 5′ half of $tRNA_3^{Lys}$, and pSW201 was used to amplify the 3′ half of $tRNA_3^{Lys}$. pHEnv contains the HIV-1 *env* coding sequences. PCRs were performed for 30 cycles (1 min at 95°C, 1 min at 56°C, 1 min at 72°C each). All products (20–50 ng of each) were ethanol precipitated and electroeluted from 1–2% agarose gels before serving as templates in subsequent PCRs.

Various overlap PCRs involved in generating the template DNA library are depicted in Figure 2 and its legend. Nucleotide sequences of the different primers used are as follows: a^+, 5′-CGA-GGC-CCT-TTC-GTC-TC-3′; a^-, 5′-ACT-CAT-CAG-TTG-CGA-TT$[N]_{0–20}$-AAG-TCT-GAT-GCT-CTA-CC-3′; b^+, 5′-GAG-GAC-GAA-ACC-AGC-CG$[N]_{0–20}$-TAA-TCT-GAG-GGT-CCA-GG-3′; b^-, 5′-TCA-AAA-AAG-GTA-CCC-CGC-CGT-GGC-GCC-CGA-ACA-G-3′; c^+, 5′-CGG-GGT-ACC-TTT-TTT-GAA-TTC-GTA-GCG-GGA-GAA-TGA-TA-3′; c^-, 5′-GTC-CGT-GAA-ATT-GAC-AG-3′; and d^+, 5′-*AAT-CGC-AA***C-TGA-TGA-GTC-CGT-GAG-GAC-GAA**-*ACC-AGC-CG*-3′. Primers a^- and b^+ are each equimolar mixtures of 11 individually synthesized primers binding either upstream (a^-) or downstream (b^+) of the anticodon. $[N]_{0–20}$ denotes 0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 nt-long random (A, T, C or G) sequence linkers. Primer d^+ contains the ribozyme catalytic domain (in bold) and 8 nt flanking sequences complementary to either side of the cleavage site (in italics). The final PCR products containing the template DNA library were analyzed to confirm the differences in the lengths of the 5′ and 3′ linkers of the $tRNA_{AC}$-Rzs.

Trans cleavage reaction using pooled $tRNA_{AC}$-Rzs

The template DNA library was PCR amplified to generate templates enabling T7 promoter driven transcription of $tRNA_{AC}$-Rzs using a primer which binds upstream of the T7 promoter in the template DNA library (5′-GTA-AAA-CGA-CGG-CCA-GT-3′) and a primer which binds at the 3′ end of $tRNA_{AC}$-Rzs (5′-TGG-CGC-CCG-AAC-AGG-GAC-3′). Target RNA was amplified using a primer which contains the T7 promoter sequence (5′-CGC-GGA-TCC-TAA-TAC-GAC-TCA-CTA-TAG-GGC-GAC-GGC-GGG-GTA-CCT-TTT-TTG-3′) and a primer which binds downstream of the ribozyme target site (c^- primer). PCR DNA (30 µl) was transcribed *in vitro* at 37°C in a reaction mixture (100 µl) containing 40 mM Tris–HCl, pH 8.0, 25 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 1 mM of each NTP and 200 U of T7 RNA polymerase (Life Technologies; Burlington, Canada). The reaction was stopped after 2 h by digesting the template DNA with 5 U of RQI RNase-Free DNase (Promega Corp.; Madison, WI) for 10 min. The mixture was extracted once with phenol and then ethanol precipitated. The resulting pool of $tRNA_{AC}$-Rzs and target RNA was used for *trans* cleavage reactions for 2 h as described previously (54). Essentially, $tRNA_{AC}$-Rzs and target RNA were combined in a reaction mixture containing 40 mM Tris–HCl, pH 8.0, and 10 mM NaCl. The sample was heated to 65°C for 5 min, cooled to 37°C, and the reaction initiated by adding 20 mM $MgCl_2$. After incubation for 2 h at 37°C, the reaction was stopped by adding 5 mM EDTA. Cleavage products were analyzed by 8 M urea–8% polyacrylamide gel electrophoresis (PAGE) followed by methylene blue staining (55).

Transcription, selection and cloning of $tRNA_{AC}$-Rzs

PCR-amplified template DNA library was transcribed *in vitro* for 2 h at 37°C in a reaction mixture containing 40 mM Tris–HCl, pH 8.0, 25 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 1 mM of each NTP and 200 U of T7 RNA polymerase. To visualize the product to be eluted, transcription was also performed in parallel using the same conditions in the presence of [α-^{32}P]UTP (3000 Ci/mmol; Amersham Canada Ltd; Oakville, Canada). *Cis* cleavage of the target sequence by the $tRNA_{AC}$-Rzs occurred under the condition used for transcription, without further incubation or addition of reagents. After phenol extraction and ethanol precipitation, the unlabeled reaction mixture was analyzed by 8 M urea–8% PAGE. The unlabeled $tRNA_{AC}$-Rzs (5′ cleavage products) were eluted (56) and reverse transcribed using the reverse primer which binds downstream of $tRNA_{AC}$-Rz sequences, contains the RNA pol III terminator and *Sst*I site (5′-ATA-TAT-ATA-ATC-GAT-GGA-GCT-CAA-AAA-AGG-TAC-CCC-GCC-3′), as described previously (54). The cDNA was PCR amplified using the forward primer which binds at the 5′ end of $tRNA_{AC}$-Rzs and contains the *Bam*HI site (5′-ATA-TAT-ATA-GGA-TCC-TAA-TAC-GAC-TCA-CTA-TAG-GGC-CCG-GAT-AGC-TCA-GTC-G-3′), and the reverse primer. PCR products were digested with *Bam*HI and *Sst*I and cloned at the same sites within pGEM4Z (Promega Corp.; Madison, WI). Twenty clones were identified by restriction enzyme and PCR analyses. Sequences were determined using the T7 Sequencing Kit (Pharmacia Biotech Inc.; Baie d'Urfé, Canada) following instructions provided by the supplier.

As a control, a linear ribozyme (*AAT-CGC-AA***C-TGA-TGA-GTC-CGT-GAG-GAC-GAA**-*ACC-AGC-CG*; ribozyme catalytic domain in bold and 8 nt flanking sequences complementary to either side of the cleavage site in italics) was designed to cleave the target site used by $tRNA_{AC}$-Rzs. pGEM-Rz_{Env} expressing the linear ribozyme was constructed as described (55) by cloning complementary oligonucleotides containing ribozyme sequences (5′-AGC-TTG-GAT-CC*A-ATC-GCA-A***CT-GAT-GAG-TCC-GTG-AGG-ACG-AA***A-CCA-GCC-G*TT-CGA-ATC-GGC-TGG-TTT-TGC-GAT-TCG-3′ and 5′-AAT-TCG-AAT-CGC-AAA-ACC-AGC-CGA-TTC-GAA-*CGG-CTG-GT***T-TCG-TCC-TCA-CGG-ACT-CAT-CAG**-*TTG-CGA-TT*G-GAT-CCA-3′) at the *Hin*dIII and *Eco*RI sites of pGEM4Z.

Trans cleavage activity of cloned pGEM-tRNA$_{AC}$-Rzs

Various tRNA$_{AC}$-Rzs were each PCR amplified from the respective pGEM4Z plasmids using a primer which binds at the 5′ end of tRNA$_{AC}$-Rzs and contains the T7 promoter sequence (5′-ATA-TAT-ATA-GGA-TCC-TAA-TAC-GAC-TCA-CTA-TAG-GGC-CCG-GAT-AGC-TCA-GTC-G-3′) and a primer which binds at the 3′ end of tRNA$_{AC}$-Rzs (5′-TGG-CGC-CCG-AAC-AGG-GAC-3′). The linear ribozyme was PCR amplified from pGEM-Rz$_{Env}$ using a primer which binds to the T7 promoter sequence (5′-CGA-AAT-TAA-TAC-GAC-TCA-CTA-TA-3′) and a primer which binds to the 3′ end of the ribozyme (5′-ATA-TAT-ATC-GAT-AAA-AAA-CGG-CTG-GTT-TCG-TCC-TC-3′).
The PCR products were transcribed *in vitro* as described above. Target RNA sequences were amplified from pHEnv using a forward primer which binds upstream of the ribozyme target site and contains the T7 promoter sequence (5′-ATA-TCA-TAT-GTA-ATA-CGA-CTC-ACT-ATA-GGG-CGA-GTG-CAG-AAA-GAA-TAT-GC-3′) and a primer which binds downstream of the ribozyme target site (c^{-} primer). Target RNA was internally labeled during *in vitro* transcription as described above.

For the characterization and to determine the kinetics of cleavage reactions, each tRNA$_{AC}$-Rz and linear ribozyme (2 µM) was incubated with [α-^{32}P]-labeled target RNA (0.2 µM) in a *trans* cleavage reaction as described above. The reaction was performed at 37°C, and aliquots were taken at various time intervals as indicated. The products were analyzed by 8 M urea–8% PAGE followed by exposure to a phosphor screen and scanning by Storm PhosphorImager (Molecular Dynamics; Sunnyvale, CA). The amount of target RNA cleaved was determined by measuring band intensities using ImageQuant software (Molecular Dynamics; Sunnyvale, CA).

To determine the kinetic constants of tRNA$_{AC}$-Rz (10/0) and linear ribozyme, each ribozyme (0.25 µM) was mixed with varying concentrations of target RNA (0.5, 1, 2, 4 µM) and the *trans* cleavage reactions were performed as above for 5 min at 37°C. V_0 and V_0/S were determined as described in (57) and the Eadie–Hofstee graph was plotted.

Stability of tRNA$_{AC}$-Rzs

PCR-amplified templates containing the T7 promoter driving tRNA$_{AC}$-Rz (10/0), tRNA$_{AC}$-Rz (0/0) and tRNA$_3^{Lys}$ expression were each transcribed in the presence of [α-^{32}P]UTP as described above. Transcripts were ethanol-precipitated and purified by 8 M urea–8% PAGE. The relative stability of these RNAs (16 400 c.p.m. each) was then examined for up to 3 h at 30°C in the presence of MT4 cell (58,59) lysates (500 µg/ml) obtained as described in (60). Aliquots were taken at 0 min, 10 min, 20 min, 1 h, 2 h and 3 h, and the reactions were stopped by adding an equal volume of buffer containing 80% formamide, 10 mM EDTA, 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue and 1 mg/ml yeast tRNA. Samples were kept on dry ice until the last incubation and then analyzed by 8 M urea–8% PAGE. Bands were visualized using Storm PhosphorImager, and the intensities were measured using ImageQuant software.

RNA pol III-driven transcription of tRNA$_{AC}$-Rzs

pGEM4Z-based plasmids containing tRNA$_{AC}$-Rzs and pM13-Lys3 containing tRNA$_3^{Lys}$ (44) were each transcribed *in vitro* in the presence of [α-^{32}P]UTP using HeLa nuclear extract (Promega Corp.; Madison, WI) following instructions provided by the supplier. Transcripts were analyzed by 8 M urea–8% PAGE and the gel exposed to a phosphor screen. Band intensities were measured using ImageQuant software.

Construction of retroviral vectors

Selected tRNA$_{AC}$-Rzs were cloned in the retroviral vector pUCMoTiN (61). An inactive ribozyme (tRNA$_{AC}$-InRz) was also cloned in pUCMoTiN to serve as a control. tRNA$_{AC}$-InRz was isolated from the pool of tRNA$_{AC}$-Rzs and was shown to lack ribozyme activity. The nature of the mutation was determined by sequencing (55).

Various tRNA$_{AC}$-Rzs and tRNA$_{AC}$-InRz were PCR amplified from the respective pGEM4Z plasmids using 5′-ATA-TAT-ATA-GGA-TCC-GCC-CGG-ATA-GCT-CAG-TC-3′ and 5′-ATA-TAT-ATA-ATC-GAT-GGA-GCT-CAA-AAA-AGG-TAC-CCC-GCC-3′ primers containing *Bam*HI and *Cla*I sites and cloned at the same sites downstream of the neomycin phosphotransferase (*neo*) gene within pUCMoTiN. Ampicillin- and kanamycin-resistant colonies were isolated and correct clones were confirmed by restriction enzyme analysis. Clones expressing tRNA$_{AC}$-Rzs were then sequenced (55).

In vitro cleavage activity of cloned pUCMoTiN-tRNA$_{AC}$-Rzs

DNA templates containing various tRNA$_{AC}$-Rzs and tRNA$_{AC}$-InRz were PCR amplified from the respective pUCMoTiN-based plasmids using a primer which binds at the 5′ end of tRNA$_{AC}$-Rzs and contains the T7 promoter sequence (5′-ATA-TAT-ATA-GGA-TCC-TAA-TAC-GAC-TCA-CTA-TAG-GGC-CCG-GAT-AGC-TCA-GTC-G-3′) and a primer which binds at the 3′ end of tRNA$_{AC}$-Rzs (5′-TGG-CGC-CCG-AAC-AGG-GAC-3′ primers). The PCR products were transcribed *in vitro* to yield tRNA$_{AC}$-Rzs and tRNA$_{AC}$-InRz which were used in *trans* cleavage reactions as described above.

Transduction and selection of stable MT4 transductants

The ecotropic ψ-2 packaging cell line (62) was transfected with 25–50 µg of pUCMoTiN, pUCMoTiN-tRNA$_{AC}$-Rzs and pUCMoTiN-tRNA$_{AC}$-InRz (63–65). The vector particles released from pools of ψ-2 transductants were used to transduce the PA317 packaging cell line (66), and the amphotropic MoTiN, MoTiN-tRNA$_{AC}$-Rzs and MoTiN-tRNA$_{AC}$-InRz vector particles were then used to transduce MT4 cells as described previously (64,65). Pools of G418-resistant stable MT4 transductants lacking or expressing various tRNA$_{AC}$-Rzs or tRNA$_{AC}$-InRz were then selected and analyzed without cloning.

RT–PCR analysis of total RNA from stable MT4 transductants

Total RNA from stable MT4 transductants expressing various tRNA$_{AC}$-Rzs or tRNA$_{AC}$-InRz was extracted using acid guanidium thiocyanate–phenol–chloroform extraction (67) and incubated with RQI RNase-Free DNase (Promega Corp.; Madison, WI) for 15 min at 37°C to degrade any residual DNA. RT–PCRs were performed as described previously (54) using forward (5′-GAT-GGC-CGC-TTT-GGT-CC-3′) and reverse (5′-ATA-TAT-ATA-TAA-TAC-GAC-TCA-CTA-TAG-GCT-CGT-ACT-CTA-TAG-GC-3′) primers which bind to sequences flanking the tRNA$_{AC}$-Rzs. The RT–PCR products were then analyzed by electrophoresis on a 2% agarose gel.

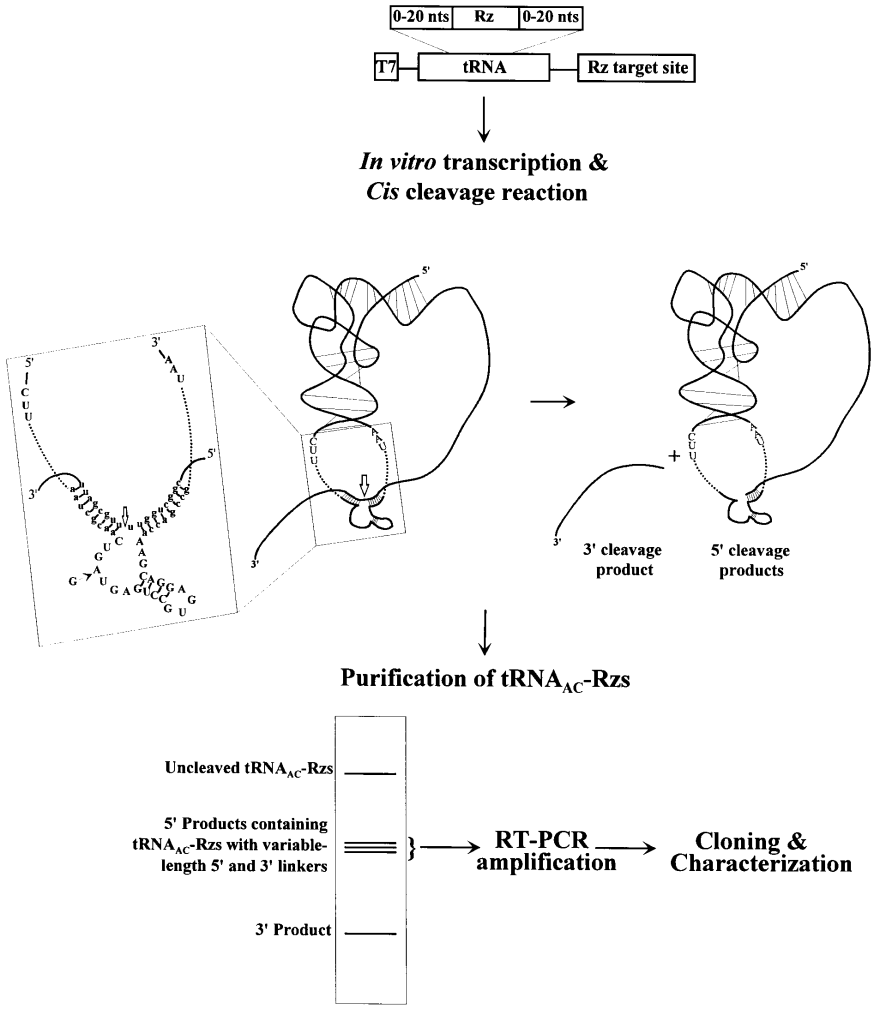

Figure 1. Overview of selection strategy for tRNA$_{AC}$-Rzs. A template DNA library containing the T7 promoter, the different tRNA$_{AC}$-Rzs with 5′ and 3′ linkers of variable length and sequences, and the ribozyme target site was transcribed *in vitro*. Active tRNA$_{AC}$-Rzs are expected to cleave the ribozyme target site provided *in cis*. The 5′ cleavage products (containing active tRNA$_{AC}$-Rzs) were separated from the 3′ products and uncleaved transcripts (containing inactive and less active tRNA$_{AC}$-Rzs) by denaturing PAGE. The 5′ cleavage products were eluted from the gel and used for RT–PCR followed by cloning and characterization. Enlarged view depicts the modified tRNA anticodon loop containing the ribozyme hybridized to its target sequence. Nucleotides 5′-CUU-3′ and 5′-UAA-3′ are part of the original tRNA$_3^{Lys}$ anticodon. The dotted lines correspond to the 5′ and 3′ linkers, which varied in length and sequence. Ribozyme flanking sequences and the target sequences to which they bind are shown in lower case. The open arrow denotes cleavage site. Ribozyme catalytic domain is shown in upper case. → corresponds to the A to G mutation in the tRNA$_{AC}$-InRz.

HIV-1 susceptibility of stable MT4 transductants

The pools of stable MT4 transductants lacking or expressing various tRNA$_{AC}$-Rzs or tRNA$_{AC}$-InRz were each infected with HIV-1 strain NL4-3 (68) as described previously (64,65) at a multiplicity of infection (m.o.i.) of 0.1 and 1. The amount of HIV-1 p24 antigen present in the cell culture supernatants was determined by a p24 ELISA kit (Abbott; Chicago, IL).

RESULTS

Selection strategy

A population of tRNA$_{AC}$-Rzs that contained 5′ and 3′ linkers connecting the ribozyme to the anticodon loop was generated. The 5′ and 3′ linkers ranged in size from 0 to 20 nt and possessed random sequences. These linkers varied in length with an increment of two, providing 11 different lengths of 5′ linkers that could have combined with any of the 11 3′ linkers. The 3 nt on either side of the anticodon loop (5′-CUU-3′ and 5′-UAA-3′) were retained and not counted as part of the 5′ or 3′ linker (Fig. 1). The ribozyme target site was located *in cis* with enough nucleotides between the tRNA$_{AC}$-Rz and the target site to allow hybridization. It was hypothesized that this design would allow tRNA$_{AC}$-Rzs which contain the ideal combinations of linkers to freely recognize and cleave the target site provided *in cis*. As the frequency of *cis* cleavage is expected to be much greater than that of *trans* cleavage, the 5′ cleavage products should mainly consist of active tRNA$_{AC}$-Rzs. These molecules could be easily separated from the less active tRNA$_{AC}$-Rzs present within the uncleaved RNA, RT–PCR amplified, cloned and characterized (Fig. 1).

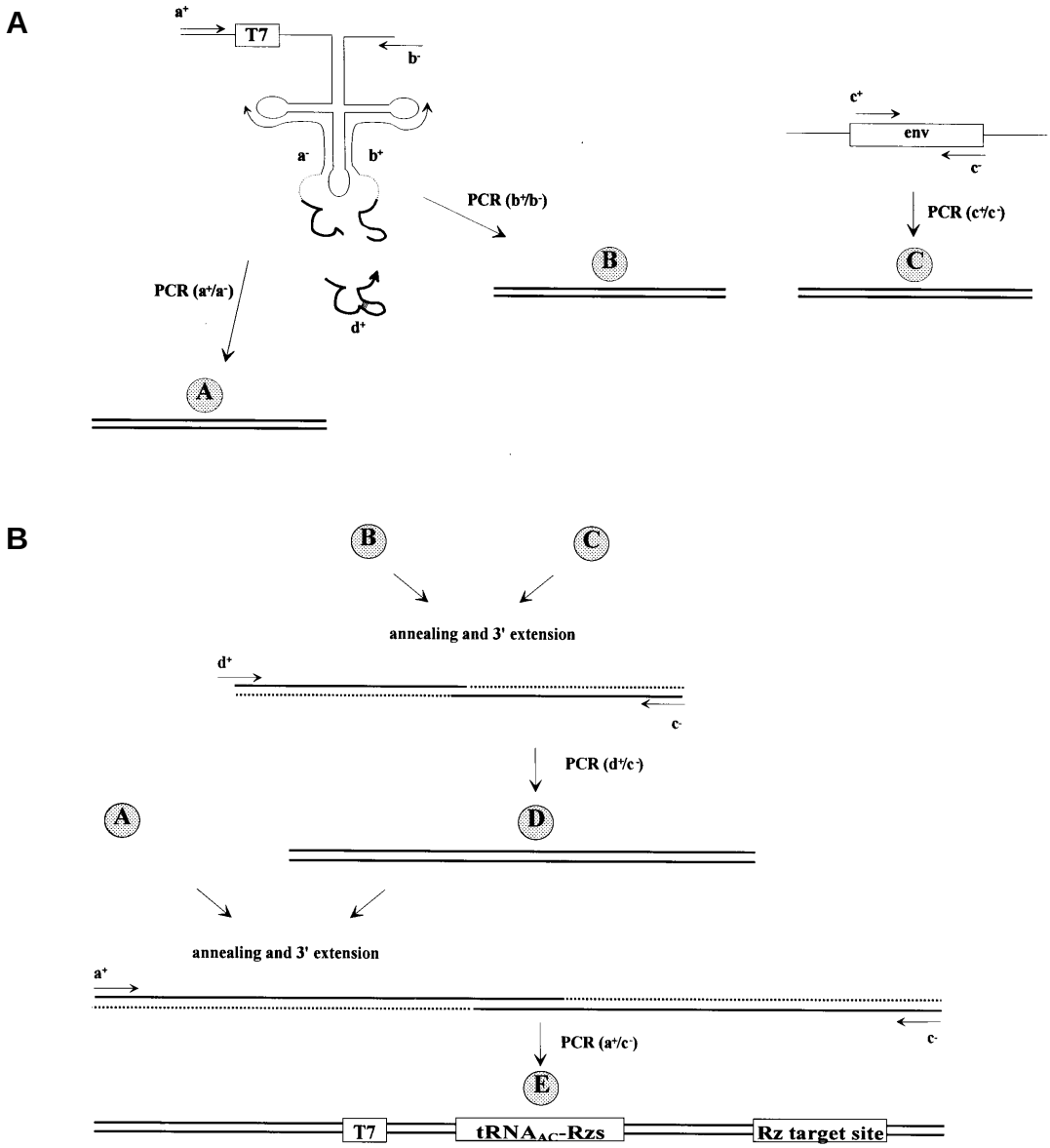

Figure 2. Overlap PCRs to generate the template DNA library containing the T7 promoter, the different $tRNA_{AC}$-Rzs and the ribozyme target sequence. (**A**) The secondary structures of a^-, b^+ and d^+ primers are shown. The dotted lines in a^- and b^+ primers correspond to the 5′ and 3′ linkers, respectively, which are of random sequence and range in length from 0 to 20 nt. A, B and C are products of regular PCR cycles. The templates used for products A (pSW2060) and B (pSW201), both containing $tRNA_3^{Lys}$, were amplified using a^+/a^- and b^+/b^- primer pairs, respectively. The template for product C (pHEnv) was amplified using c^+/c^- primers. This product contains the $tRNA_{AC}$-Rz target site. (**B**) PCR products B and C were used as overlapping templates which were PCR amplified using d^+/c^- primers to generate the product D. Products A and D were then used as overlapping templates with a^+/c^- primers to generate E, the $tRNA_{AC}$-Rz template DNA library. This library consists of a 988–1028 bp DNA population which contains the T7 promoter, the $tRNA_{AC}$-Rzs ($tRNA_3^{Lys}$ with random-sequence, variable-length 5′ and 3′ linkers connecting the ribozyme to the anticodon loop) and the ribozyme target site. The dotted lines correspond to the nucleotides incorporated by *Taq* polymerase after annealing of the overlapping templates.

Synthesis of template DNA library encoding different $tRNA_{AC}$-Rzs

Template DNA library encoding $tRNA_{AC}$-Rzs containing various linkers was constructed by overlap PCRs (Fig. 2A and B). Each template contained a T7 promoter, a $tRNA_{AC}$-Rz with random-sequence, variable-length 5′ and 3′ linkers, and the ribozyme target site. Upon transcription of this DNA library, a pool of RNAs each containing a $tRNA_{AC}$-Rz and the ribozyme target site was generated.

Variability within the 5′ and 3′ linker regions of the $tRNA_{AC}$-Rz pool

5′ and 3′ primers containing 0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 nt-long linkers were used to amplify the respective linker regions of $tRNA_{AC}$-Rzs. A total of 121 combinations of linkers should exist since any of the 11 5′ linkers could have combined with any of the 11 3′ linkers. The variability in the lengths of the 5′ and 3′ linkers present in the pool was confirmed by PCR (data not shown).

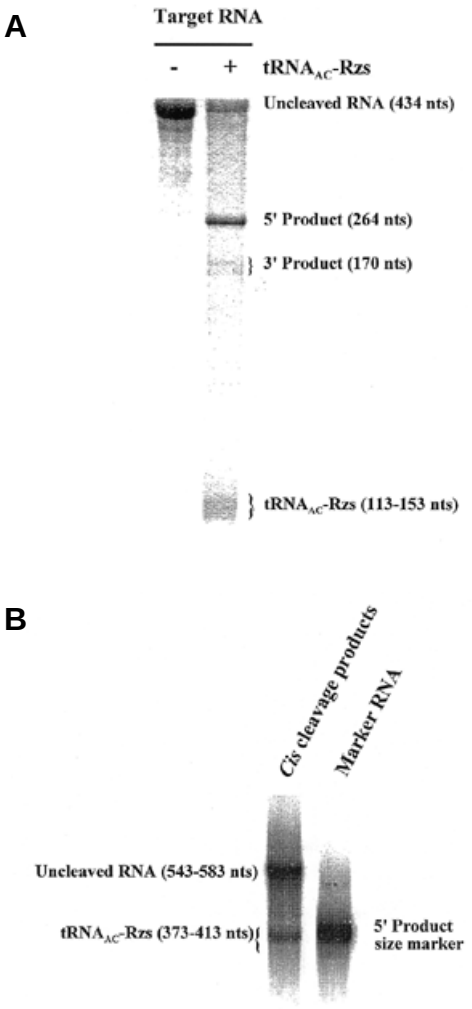

Figure 3. *Trans* and *cis* cleavage by the pool of tRNA$_{AC}$-Rzs transcribed from the template DNA library. (**A**) Ability of the pool of tRNA$_{AC}$-Rzs to cleave the target RNA *in trans*. The two bands corresponding to the 3′ cleavage product may have resulted from cleavage of premature transcription products. (**B**) *In vitro cis* cleavage by the pool of tRNA$_{AC}$-Rzs. A 5′ product marker (396–436 bp) corresponding to the size of the expected product was analyzed in parallel. The 3′ cleavage products ran out of the gel and are therefore not visible.

Trans cleavage ability of the tRNA$_{AC}$-Rz pool

Although selection was based on the *cis* cleavage ability of active tRNA$_{AC}$-Rzs, the selected ribozymes would be eventually used to cleave target RNAs *in trans*. Thus, before proceeding to selection, the pool of tRNA$_{AC}$-Rzs and the ribozyme target RNA were separately transcribed and used in a *trans* cleavage reaction. The cleavage products were analyzed by gel electrophoresis. 5′ and 3′ products of expected sizes were observed (Fig. 3A). This result confirms that the tRNA$_{AC}$-Rzs transcribed from the library can cleave the target RNA *in trans*.

Selection and cloning of tRNA$_{AC}$-Rzs

The tRNA$_{AC}$-Rz template DNA library was transcribed *in vitro*. Active tRNA$_{AC}$-Rzs capable of cleaving the target site *in cis* were able to do so during *in vitro* transcription (Fig. 3B). The 5′ cleavage products were separated from the uncleaved RNA by gel electrophoresis. These products were then eluted, RT–PCR amplified and cloned in pGEM4Z. Twenty independent clones containing different tRNA$_{AC}$-Rzs with variable linkers were isolated.

Identification of active tRNA$_{AC}$-Rzs allowing *trans* cleavage

Plasmids containing tRNA$_{AC}$-Rzs, linear ribozyme and ribozyme target site were each PCR amplified and the PCR products transcribed *in vitro*. The tRNA$_{AC}$-Rzs and linear ribozyme were then incubated with the target RNA to allow *trans* cleavage. All of the cloned tRNA$_{AC}$-Rzs were active except for two which failed to cleave (Table 1).

Table 1. Length, sequence and relative cleavage activity of various tRNA$_{AC}$-Rzs

Type	Number of clones	Linker length and sequence 5′	3′	Relative cleavage activity**
tRNA$_{AC}$-Rz (0/0)	6	0*	0	0.48
tRNA$_{AC}$-Rz (2/0)	2	2 (CU)*	0	0.86
		2 (GA)	0	0.48
tRNA$_{AC}$-Rz (4/0)	5	4 (CUCA)*	0	1.34
		4 (AGGC)	0	0.98
		4 (CGAC)	0	0.92
		4 (GGCA)	0	0.92
		4 (CCCC)	0	0.34
tRNA$_{AC}$-Rz (8/0)	1	8 (AGAACCAC)*	0	1.11
tRNA$_{AC}$-Rz (10/0)	1	10 (ACCACCCGAU)*	0	1.08
tRNA$_{AC}$-Rz (16/0)	1	16 (CCACCAACAAAAUCCA)	0	1.19
tRNA$_{AC}$-Rz (8/2)	1	8 (GCCAAUUU)*	2 (AU)	0.33
tRNA$_{AC}$-Rz (6/6)	1	6 (AACAAU)*	6 (UAUUUU)	0.45
Linear ribozyme	1	NA	NA	1.00

*Clones selected for further characterization. For tRNA$_{AC}$-Rz (0/0), one among the six clones was used for further experiments.
***Trans* cleavage reactions were performed for 10 min at 37°C as described in the Materials and Methods. Relative cleavage activity was then determined by normalizing these values to the % target RNA cleaved by the linear ribozyme, which was 38%.
NA, not applicable.

Structure of selected tRNA$_{AC}$-Rzs

All of the plasmids encoding tRNA$_{AC}$-Rzs were sequenced (Table 1). The two inactive tRNA$_{AC}$-Rzs that failed to cleave contained a mutation in one of the conserved residues of the ribozyme catalytic domain. Sixteen tRNA$_{AC}$-Rzs did not possess 3′ linkers. These were classified based on the length of the 5′ linkers (Table 1): tRNA$_{AC}$-Rz (0/0), tRNA$_{AC}$-Rz (2/0), tRNA$_{AC}$-Rz (4/0), tRNA$_{AC}$-Rz (8/0), tRNA$_{AC}$-Rz (10/0) and tRNA$_{AC}$-Rz (16/0). tRNA$_{AC}$-Rzs with 5′ linkers of a given length were not identical as these linkers varied in their sequence composition. The two remaining tRNA$_{AC}$-Rzs possessed 8/2 and 6/6 nt-long 5′ and 3′ linkers.

Kinetics of *trans* cleavage of selected tRNA$_{AC}$-Rzs

tRNA$_{AC}$-Rz (0/0), tRNA$_{AC}$-Rz (2/0), tRNA$_{AC}$-Rz (4/0), tRNA$_{AC}$-Rz (8/0) and tRNA$_{AC}$-Rz (10/0) with 0, 2, 4, 8 and 10 nt-long 5′ linkers (Table 1) were each used in a *trans* cleavage reaction. A linear ribozyme and tRNA$_3^{Lys}$ served as positive and negative controls, respectively. Compared to the linear ribozyme, each tRNA$_{AC}$-Rz cleaved a similar amount of total target RNA by 5 h (Fig. 4A). tRNA$_{AC}$-Rz (0/0) without any linkers demonstrated

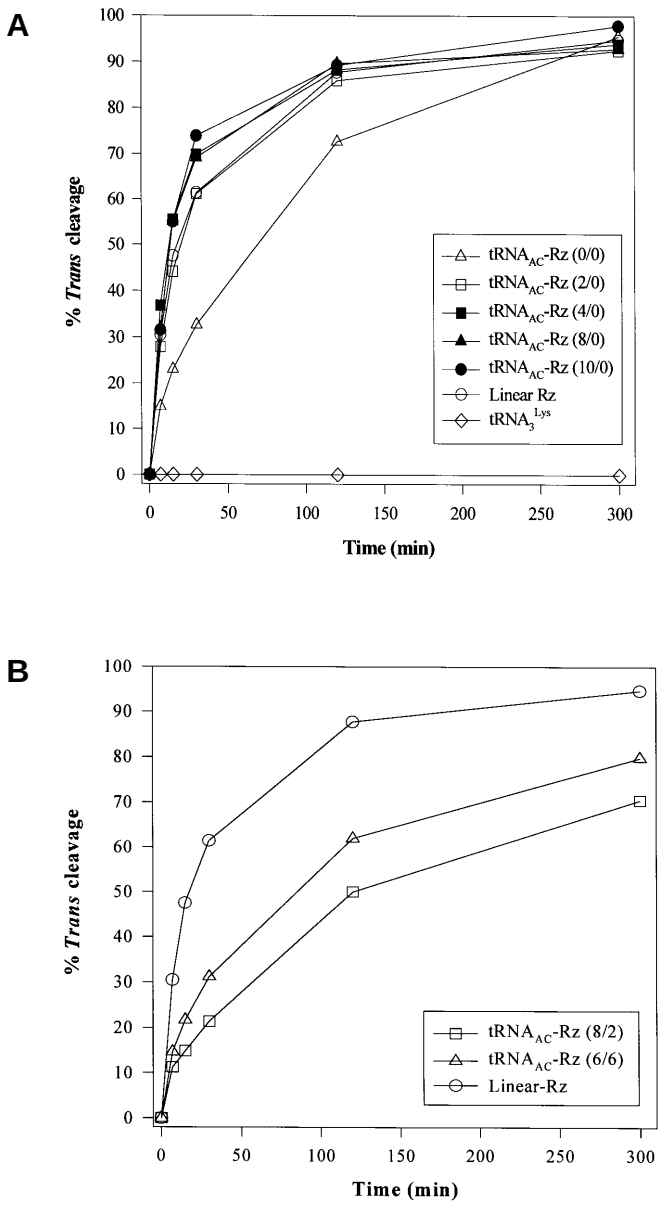

Figure 4. Kinetics of *trans* cleavage reactions for tRNA$_{AC}$-Rzs. *Trans* cleavage kinetics for tRNA$_{AC}$-Rzs with 5′ linkers (**A**) or with 3′ linkers (**B**).

the slowest rate of cleavage. All the other tRNA$_{AC}$-Rzs possessed similar initial rates of cleavage.

The tRNA$_{AC}$-Rzs with 3′ linkers, tRNA$_{AC}$-Rz (8/2) and tRNA$_{AC}$-Rz (6/6), were also used in *trans* cleavage reactions. Both of these tRNA$_{AC}$-Rzs demonstrated cleavage rates lower than that observed for the linear ribozyme (Fig. 4B). In addition, tRNA$_{AC}$-Rz (8/2) with a 2 nt-long 3′ linker possessed a cleavage rate lower than that of tRNA$_{AC}$-Rz (8/0) which does not contain a 3′ linker (Fig. 4A and B).

To further compare the catalytic activities of the linear ribozyme and tRNA$_{AC}$-Rzs, *trans* cleavage reactions were performed using the same amount of either linear ribozyme or tRNA$_{AC}$-Rz (10/0) but with varying concentrations of target RNA. The kinetic constants for both of these RNAs were very similar (Fig. 5).

Stability of selected tRNA$_{AC}$-Rzs

To compare the stability of tRNA$_{AC}$-Rzs with tRNA$_3^{Lys}$, tRNA$_{AC}$-Rz (0/0), tRNA$_{AC}$-Rz (10/0) and tRNA$_3^{Lys}$ were each incubated with a lysate obtained from MT4 cells, a human CD4+ T cell line. Both tRNA$_{AC}$-Rz (0/0) and tRNA$_{AC}$-Rz (10/0) appeared to be very stable (Fig. 6). The half-lives of tRNA$_{AC}$-Rz (0/0), tRNA$_{AC}$-Rz (10/0) and tRNA$_3^{Lys}$ in the presence of MT4 cell lysate were 50, 80 and 25 min, respectively. No RNA degradation was observed in the absence of cell lysate for up to 2 h incubation (data not shown). Thus, insertion of ribozyme sequences in the anticodon loop does not decrease the inherent stability of tRNA$_3^{Lys}$.

RNA pol III-driven transcription of tRNA$_{AC}$-Rzs from the tRNA promoter

Plasmids containing tRNA$_{AC}$-Rzs and tRNA$_3^{Lys}$ were individually transcribed using HeLa nuclear extract. Transcripts corresponding to tRNA$_{AC}$-Rz (0/0), tRNA$_{AC}$-Rz (10/0) and tRNA$_3^{Lys}$ were detected (Fig. 7). tRNA$_{AC}$-Rz (2/0), tRNA$_{AC}$-Rz (4/0) and tRNA$_{AC}$-Rz (8/0) could also be transcribed (data not shown). A negative control consisting of HeLa nuclear extract alone without the addition of template DNA did not yield any transcript (Fig. 7). This result shows that the intragenic tRNA promoters are intact and that the insertion of the ribozyme in the anticodon loop does not disrupt transcription by RNA pol III.

Retroviral vectors expressing tRNA$_{AC}$-Rzs and tRNA$_{AC}$-InRz

The ribozyme in all tRNA$_{AC}$-Rzs is designed to cleave a highly conserved sequence within the *env* coding region of HIV-1 B subtype. In order to assess the ability of various tRNA$_{AC}$-Rzs to inhibit HIV-1 replication in a human CD4+ T cell line, a Moloney murine leukemia virus-based pUCMoTiN (61) vector was used to construct retroviral vectors (Fig. 8) expressing various tRNA$_{AC}$-Rzs. A retroviral vector expressing tRNA$_{AC}$-InRz which lacks ribozyme activity was constructed to serve as a control. tRNA$_{AC}$-InRz contains an A to G mutation in one of the conserved residues of the hammerhead catalytic domain (Fig. 1).

In vitro cleavage activities of tRNA$_{AC}$-Rzs and tRNA$_{AC}$-InRz cloned within the retroviral vector

tRNA$_{AC}$-Rzs, tRNA$_{AC}$-InRz and Env target RNA containing the ribozyme cleavage site were each transcribed from DNA templates containing the T7 promoter, which were PCR amplified from pUCMoTiN-tRNA$_{AC}$-Rzs, pUCMoTiN-tRNA$_{AC}$-InRz and pHEnv plasmids, respectively. The target RNA was internally labeled and subjected to *in vitro* cleavage by each tRNA$_{AC}$-Rz and tRNA$_{AC}$-InRz. As expected, all tRNA-Rzs were capable of cleaving the target RNA while tRNA-InRz failed to cleave (Fig. 9). This result shows that tRNA$_{AC}$-Rzs cloned in the retroviral vector are capable of cleaving their target RNA in *trans*.

Development of pools of stable MT4 transductants expressing tRNA$_{AC}$-Rzs and tRNA$_{AC}$-InRz

Amphotropic MoTiN, various MoTiN-tRNA$_{AC}$-Rzs and MoTiN-tRNA$_{AC}$-InRz vector particles were each used to transduce MT4 cells, a human CD4+ T cell line. Pools of stable MT4

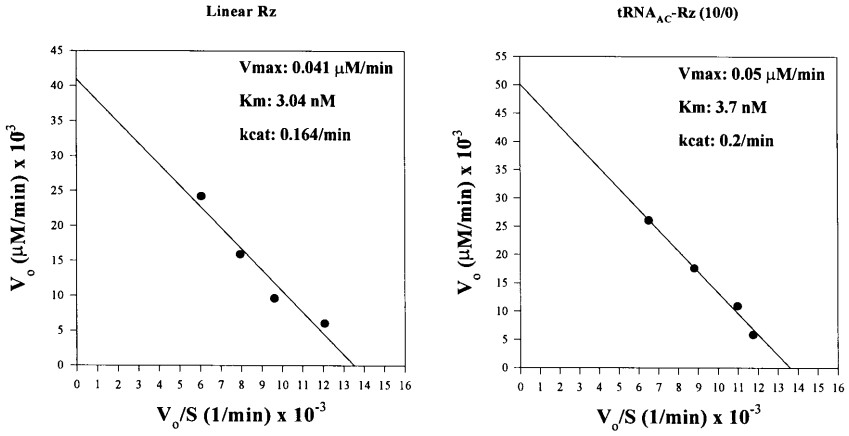

Figure 5. Eadie–Hofstee plot for tRNA$_{AC}$-Rz (10/0) and Linear Rz. *Trans* cleavage reactions using each ribozyme and increasing concentrations of [α-^{32}P]-labeled target RNA were performed. V_o and V_o/S were calculated and plotted.

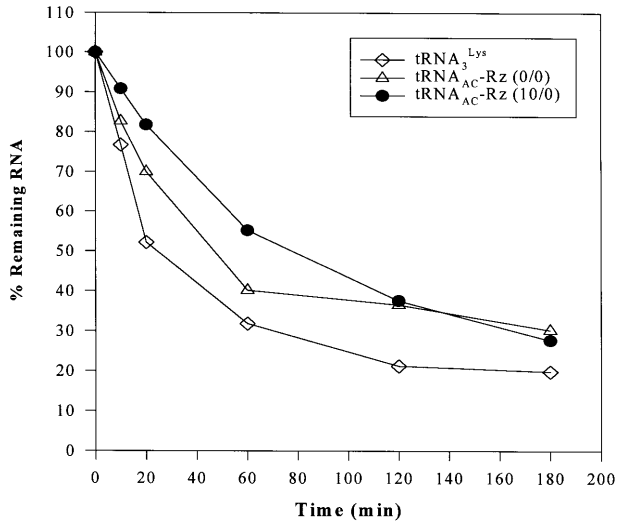

Figure 6. Stability of tRNA$_{AC}$-Rzs. [α-^{32}P]-labeled tRNA$_{AC}$-Rzs and tRNA$_3^{Lys}$ were each incubated with MT4 cell lysate at 30°C and aliquots were taken at various time intervals. Values indicate percent intact RNA remaining compared to time 0.

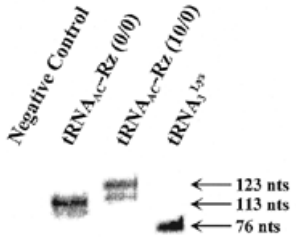

Figure 7. RNA pol III-driven expression of tRNA$_{AC}$-Rzs. tRNA$_{AC}$-Rzs were transcribed *in vitro* using a HeLa nuclear extract and analyzed by 8 M urea–8% PAGE.

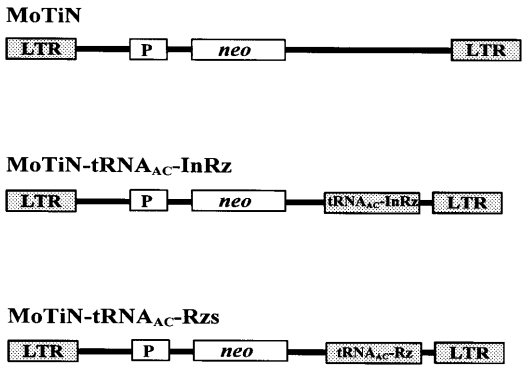

Figure 8. Retroviral vectors expressing tRNA$_{AC}$-Rzs and tRNA$_{AC}$-InRz. Genes encoding tRNA$_{AC}$-Rz (0/0), tRNA$_{AC}$-Rz (2/0), tRNA$_{AC}$-Rz (4/0), tRNA$_{AC}$-Rz (8/0), tRNA$_{AC}$-Rz (10/0) or tRNA$_{AC}$-InRz were each cloned in a retroviral vector pUCMoTiN. This vector contains the herpes simplex virus type-1 thymidine kinase-HIV-1 TAR fusion promoter (P) that allows *neo* gene expression. LTR, long terminal repeat.

transductants lacking or expressing various tRNA$_{AC}$-Rzs or tRNA$_{AC}$-InRz were selected and tested without cloning.

tRNA$_{AC}$-Rzs and tRNA$_{AC}$-InRz expression was confirmed by RT–PCR analysis of total cellular RNA extracted from stable MT4 transductants expressing tRNA$_{AC}$-Rzs or tRNA$_{AC}$-InRz. The expected products were detected in each case (Fig. 10).

HIV-1 susceptibility of pools of stable MT4 transductants expressing tRNA$_{AC}$-Rzs and tRNA$_{AC}$-InRz

The pools of stable MT4 transductants lacking or expressing tRNA$_{AC}$-Rzs or tRNA$_{AC}$-InRz were each infected with HIV-1 strain NL4-3 at an m.o.i. of 0.1 and 1. Virus production was measured by determining the amount of HIV-1 p24 antigen in the infected cell culture supernatants (Fig. 11A and B). Cells expressing retroviral vector sequences alone (MoTiN) produced high amounts of virus. In contrast, HIV-1 production was greatly reduced in tRNA$_{AC}$-Rz-expressing cells. The inhibition conferred by the tRNA$_{AC}$-Rzs could not have been due to an antisense effect by the ribozyme flanking sequences, since cells expressing an

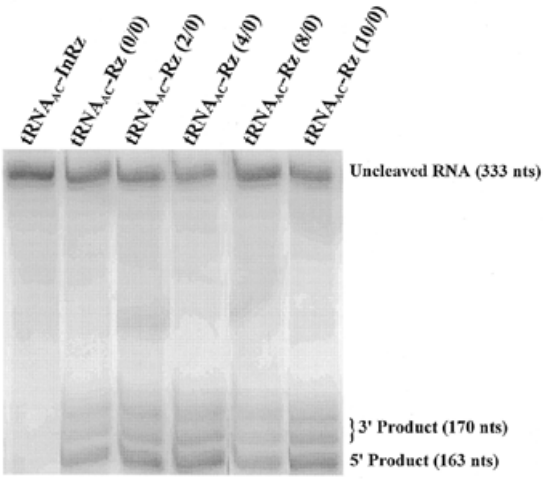

Figure 9. *In vitro* cleavage activity of cloned pUCMoTiN tRNA$_{AC}$-Rzs. tRNA$_{AC}$-Rz sequences were amplified from the respective pUCMoTiN clones and transcribed *in vitro*. Transcripts were used in *trans* cleavage reactions with [α-^{32}P]-labeled target RNA and the products analyzed by 8 M urea–8% PAGE. The two bands corresponding to the 3′ cleavage product may have resulted from cleavage of premature transcription products.

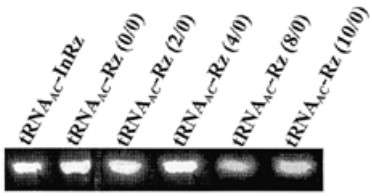

Figure 10. tRNA$_{AC}$-Rz expression in transduced MT4 cells. Total RNA was extracted from MT4 cells expressing tRNA$_{AC}$-Rzs, treated with DNase, and used for RT–PCR analysis.

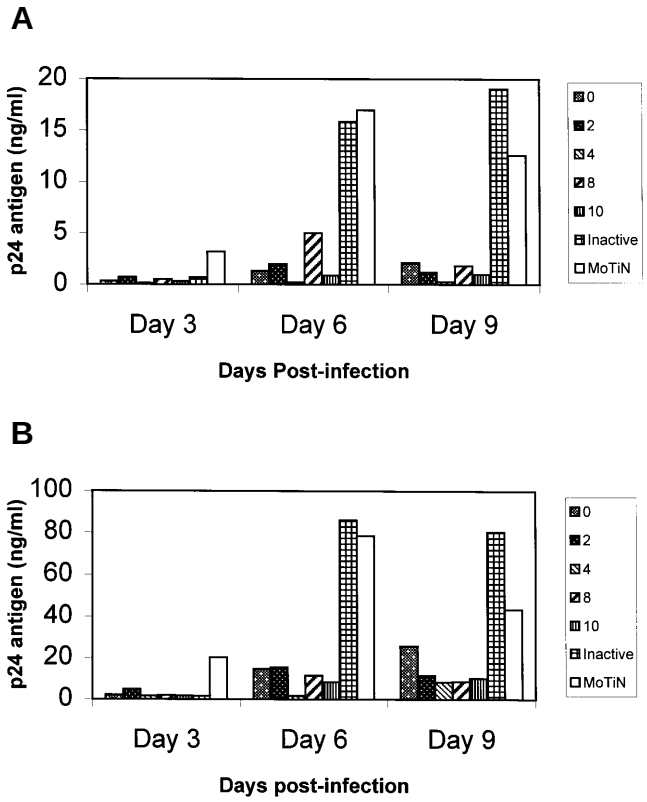

Figure 11. HIV-1 susceptibility of transduced MT4 cells. Transduced MT4 cells were infected with HIV-1 at an m.o.i. of 0.1 (**A**) or 1 (**B**) and the amount of HIV-1 p24 antigen released in the culture supernatants was measured at various time intervals.

inactive ribozyme (tRNA$_{AC}$-InRz) also produced high amounts of virus. Similar results were obtained in challenge experiments performed at the two (0.1 and 1) m.o.i.'s. Comparisons could not be made beyond day 9 as extensive cell death was observed in some of the cultures. Nevertheless, these preliminary results demonstrate that all tRNA$_{AC}$-Rzs are capable of inhibiting HIV-1 replication up to day 9 post-infection. Long term HIV-1 challenge experiments are underway.

DISCUSSION

tRNAs are produced in very high concentrations and possess stable secondary structures in cells. Intragenic sequences constitute the promoter elements used for tRNA expression under the control of RNA pol III. tRNA genes are therefore ideal for expression of small RNA molecules. Sequences encoding these RNAs may be cloned either within the tRNA gene between the promoter sequences, or downstream of the tRNA gene. In both cases, tRNA processing sites may be preserved such that the transcripts produced will be processed to yield two separate RNAs. Alternatively, these sites may be modified to prevent tRNA processing. In this case, the RNA of interest will remain as part of the tRNA. To develop tRNA$_3^{Lys}$-based ribozymes, cloning between the promoter elements seems the most attractive as the ribozyme is expressed within a region of highly ordered secondary structure which should confer protection against nucleases within the cell. The only drawback of this strategy is that the catalytic activity of the ribozyme may be decreased. Care should, therefore, be taken to design or select tRNA$_{AC}$-Rzs that retain full ribozyme activity.

We describe in this paper the design and characterization of tRNA$_3^{Lys}$-based tRNA$_{AC}$-Rzs. While cloning within the anticodon loop may allow ribozymes to be expressed in high concentrations and possess stable secondary structures, structural constraint may force ribozymes to adopt a conformation detrimental to their activity. Based on the results we obtained, it is clear that sequences immediately surrounding the ribozyme influence the catalytic activity. Our strategy allowed selection of tRNA$_{AC}$-Rzs that contained 5′ and 3′ linkers optimal for ribozyme cleavage (Fig. 1). tRNA$_{AC}$-Rzs with different linkers were isolated (Table 1). These tRNA$_{AC}$-Rzs could be transcribed by RNA pol III (Fig. 7) and were as stable as tRNA$_3^{Lys}$ (Fig. 6). *Trans* cleavage rates of these ribozymes varied (Fig. 4A and B). tRNA$_{AC}$-Rz (0/0) without a 5′ linker demonstrated the slowest rate, while tRNA$_{AC}$-Rz (4/0), tRNA$_{AC}$-Rz (8/0) and tRNA$_{AC}$-Rz (10/0) possessed rates similar to that of the linear ribozyme (Figs 4A and 5). As the selected tRNA$_{AC}$-Rzs possessed 100% activity compared to the linear ribozyme, subsequent rounds of selection were not required. Since

only the linker sequences were optimized, an increase in ribozyme catalytic activity was not anticipated.

Out of 20 cloned $tRNA_{AC}$-Rzs, two were found to be inactive. These may have been selected due to *trans* cleavage by the active $tRNA_{AC}$-Rzs prior to their purification. Two clones that possessed 3′ linkers had a decreased activity compared to the linear control ribozyme (Fig. 4B). The absence of 3′ linkers in the rest of the clones must be due to the selection procedure, as varying lengths of 3′ linkers were detected upon PCR analysis of the $tRNA_{AC}$-Rz template DNA library. The selection procedure resulted in more $tRNA_{AC}$-Rz clones that contained shorter linkers (Table 1). However, further analysis revealed that $tRNA_{AC}$-Rzs with longer linkers are better at performing *trans* cleavage (Fig. 4A). As selection relied on *cis* cleavage, it is possible that $tRNA_{AC}$-Rzs with shorter linkers are better at performing *cis* cleavage. Another possibility is that the position of the downstream target favoured the selection of $tRNA_{AC}$-Rzs with shorter linkers.

Retroviral vectors encoding $tRNA_{AC}$-Rzs were constructed (Fig. 8). Stable MT4 transductants were shown to express active $tRNA_{AC}$-Rzs (Figs 9 and 10) and inhibit HIV-1 replication (Fig. 11A and B). Virus production from the infected MT4 cells expressing $tRNA_{AC}$-Rzs was consistently lower compared to control cells.

The ribozyme used in this study was designed to target a highly conserved sequence within the HIV-1 *env* coding region. However, this does not limit the application of the active $tRNA_{AC}$-Rzs to this target sequence alone. Now that the structures conducive to ribozyme cleavage have been determined, the antisense sequences flanking the ribozyme catalytic domain may be easily modified to alter target RNA specificity. The strategy described in this paper may also be adapted to identify suitable tRNA cassettes for expression of other therapeutic molecules such as decoy and antisense RNAs.

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