Improved accumulation and activity of ribozymes expressed from a tRNA-based RNA polymerase III promoter

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ABSTRACT

RNA polymerase III (pol III) transcripts are abundant in all cells. Therefore, pol III promoters may be ideal for expressing high levels of exogenous RNAs, such as antisense RNAs, decoy RNAs and ribozymes, in many different cell types. We have improved accumulation of recombinant RNAs expressed from a human met_t tRNA-derived pol III promoter >100-fold by modifying the 3' terminus of the transcripts to hybridize to the 5' terminus. This terminal duplex includes the 8 nt leader sequence present in the primary wild-type met_t tRNA transcript that is normally removed during processing to the mature tRNA. Expression of an anti-HIV ribozyme was analyzed in cells stably transduced with retroviral vectors encoding pol III transcription units containing this modification. High accumulation of recombinant pol III ribozyme transcripts was observed in all cell lines tested. Due to the enhanced transcript accumulation, ribozyme cleavage activity was readily detectable in total RNA extracted from stably transduced human T cell lines. One pol III transcription unit, termed 'TRZ', was optimized further for ribozyme cleavage activity. The improved pol III transcription units reported here may be useful for expressing a variety of functional and therapeutic RNAs.

INTRODUCTION

A number of RNA-based strategies have been developed to modulate gene expression and activity. Examples include antisense RNAs that hybridize to mRNAs and block translation (1,2), RNA decoys and aptamers that directly bind to proteins (3–8) and ribozymes that cleave other RNAs (9,10). The potential of these RNA gene modulators as therapeutics, particularly as antiviral agents, is currently being tested (11,12). One potential advantage of RNA therapeutics over conventional drugs is the ability to continually express functional RNAs in cells following stable gene transfer (11–13). The effectiveness of such gene transfer approaches will depend in part upon the level of RNA accumulation obtained in the treated cells. Pol III transcripts such as tRNAs, 5S RNA and small nuclear and cytoplasmic RNAs such

as U6 and 7SL are highly abundant in all cells. Therefore, pol III promoters may be ideal for obtaining high expression levels of functional and therapeutic RNAs in many different cell types.

Sullenger *et al.* used a pol III promoter to drive expression of antisense and decoy RNAs in cell lines stably transduced with a retroviral vector termed DCt5T' (3,14). The pol III promoter used, termed $\Delta 3'-5$, was derived from a human met_t tRNA gene (15). This met_t tRNA gene, like all tRNA genes, contains A and B box promoter elements encoded within the tRNA transcription unit (see Fig. 1) and an oligo(T) tract at the 3'-end of the gene where termination of pol III transcription occurs (16,17). The met_t tRNA gene from which the $\Delta 3'-5$ promoter was derived is normally transcribed as a precursor RNA containing 8 nt leader and trailer sequences that are removed during processing to the mature tRNA (see Fig. 1A). The $\Delta 3'-5$ gene was adapted to express exogenous RNAs by deleting sequences downstream of the B box promoter. Deletion of these sequences inactivates tRNA processing without affecting transcription, thus blocking the removal of exogenous sequences placed downstream of the B box promoter and co-transcribed with the tRNA sequences (15). Sullenger *et al.* incorporated the $\Delta 3'-5$ promoter into the 3' LTR of the N2 retroviral vector to generate the DCt5T' retroviral vector (14). The $\Delta 3'-5$ gene is duplicated within the 5' LTR upon transduction of cells with this vector (see Fig. 2A); therefore, this vector is termed a 'double copy' vector (14). Sullenger *et al.* expressed antisense and decoy RNAs from the $\Delta 3'-5$ gene of the DCt5T' vector (3,14). The chimeric pol III transcripts expressed from these constructs were comprised of 69 nt of met_t tRNA sequences followed by either the antisense or the decoy RNA sequences (3,14).

We explored the potential of expressing anti-HIV ribozymes in human T cell lines using the DCt5T' retroviral vector. Ribozymes were placed within the same location in the $\Delta 3'-5$ promoter as previously reported for antisense and decoy RNAs (3,14). However, we observed relatively low accumulation of the tRNA-ribozyme chimeric transcripts in human T cell lines. Recently it was shown that accumulation of pol III transcripts from the DCt5T' vector could be improved 3- to 10-fold by incorporating RNA structural elements into the recombinant transcripts to enhance intracellular stability (18). In an attempt to improve ribozyme accumulation we inserted a number of novel structural elements into one of our tRNA-ribozyme chimeras.

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Figure 1. (A) Sequences of the precursor transcript from the met₁ tRNA gene from which the $\Delta 3'-5$ gene was derived. (B) Predicted pol III tRNA-ribozyme chimeric transcript from the DC568 construct. The A and B box promoter elements and the anticodon of the tRNA portion of the transcripts are boxed. Arrows indicate sites cleaved in the precursor met₁ tRNA during processing. Bold lower case, met₁ tRNA sequences; bold upper case, ribozyme sequences; italic, non-tRNA sequences present in DC568 recombinant pol III transcript.

One such modification was designed to form a duplex between the 5' and 3' termini of the transcripts. This duplex included the 8 nt leader sequence that is normally removed during processing of the wild-type met₁ tRNA. Incorporation of this structure resulted in a >100-fold increase in accumulation of the tRNA-ribozyme chimeric transcripts in stably transduced cell lines relative to transcripts expressed from the unmodified DCt5T' vector. One tRNA-based expression construct containing this modification, which we call 'TRZ', was optimized further for cleavage activity of ribozymes in the context of the flanking tRNA sequences. The improved pol III expression systems reported here may be used to achieve high expression levels of other functional RNAs besides ribozymes, such as antisense RNAs, decoy RNAs and RNA aptamers.

MATERIALS AND METHODS

Construction of retroviral vector plasmids

The DCt5T' double copy retroviral vector (14) was kindly provided by E. Gilboa. The DC568 plasmid was generated by cloning a fragment encoding the HH568 hammerhead ribozyme into the unique *Sac*II and *Bam*HI sites of the $\Delta 3'-5$ gene contained within the DCt5T' vector. The HH568 fragment was prepared from the following overlapping oligonucleotides: top oligonucleotide, 5'-ACG ACC GCG GCA CAA CAC TGA TGA GGA CCG AAA GGT CCG AAA CGG GCA-3'; bottom oligonucleotide, 5'-ACG AGG ATC CTG CCC GTT TCG GAC CTT-3'. The oligonucleotides were annealed, extended with T7 DNA polymerase (Sequenase version 2; US Biochemicals) and

digested with *Sac*II and *Bam*HI. The 568S3 plasmid, encoding a tRNA-ribozyme chimera containing a stem-loop at the 3'-end of the transcript, was constructed by cloning the following overlapping oligonucleotides into the unique *Bam*HI and *Mlu*I sites of the DCt5T' vector: top oligonucleotide, 5'-ACG ACC GCG GCA CAA CAC TGA TGA GGA CCG AAA GGT CCG AAA CGG GCA-3'; bottom oligonucleotide, 5'-ACG AAC GCG TGG ATC CAA AAA CGG CTT TCG CCG TTT TTT GCC CGT TTC GGA CCT T-3'. The 568S5 tRNA gene, encoding stem-loop structures at both the 5' and 3' termini of the tRNA-ribozyme chimeric transcripts, was generated by PCR amplification of the 568S3 plasmid using the following primers: top primer, 5'-ACG AAG ATC TTA GTA TAA AGT ACT GTG AGA CCG TGT GCT TGG CTG CGA AAG CAG CCT GGC GCA GCG GAA-3'; bottom primer, 5'-CAT GCC TTG CAA AAT GG-3'. The amplification product was digested with *Bgl*II and *Mlu*I and inserted into the *Bgl*II and *Mlu*I sites of the DCt5T' vector in place of the $\Delta 3'-5$ gene to generate the 568S5 plasmid. The DCS35 plasmid encodes an 'empty' recombinant tRNA transcript with termini designed to form an 18 bp duplex. This plasmid was generated by cloning the following overlapping oligonucleotides into the *Bam*HI and *Mlu*I sites of the DCt5T' vector: top oligonucleotide, 5'-GAT CCA CTC TGC TGT TCT GTT TTT GA-3'; bottom oligonucleotide, 5'-CGC GTC AAA AAC AGA ACA GCA GAG TG-3'. The 568S35 construct encodes a tRNA-ribozyme chimeric transcript with ends that are designed to hybridize to form a duplex. This plasmid was generated by cloning the HH568 ribozyme into the *Sac*II and *Bam*HI sites of the DCS35 vector using the HH568 oligonucleotides shown above. The 568STEM construct was similar to the 568S35

Figure 2. Accumulation of ribozyme transcripts in DC568-transduced MT-2 and CEM-SS cells. (A) Schematic representation of integrated version of the DC5T' retroviral vector. The Moloney LTR pol II transcript and the $\Delta 3'$ -5' pol III transcripts are indicated. The region of hybridization of the 218 nt DC568 antisense probe used in the LRPA is shown. (B) LRPA analysis of stably transduced MT-2 and CEM-SS cell lines. Cells were suspended to 10^7 cells/ml in lysis/hybridization buffer. DC568 antisense probe (A) was added directly to 45 μ l lysate (containing 450 000 cells) and hybridization was accomplished overnight at 37°C. A standard curve was generated by diluting known amounts of *in vitro* transcribed DC568 tRNA-ribozyme chimeric transcripts in non-transduced MT-2 lysate prior to addition of probe. Amounts of *in vitro* transcript added were corrected for copy number per cell. Lane 0 is non-transduced MT-2 lysate. Signals derived from the pol II (expected size 196 nt) and pol III (expected size 137 nt) transcripts are indicated.

construct, but encoded a tRNA-ribozyme chimeric transcript that contained more extensive base pairing within the transcript. The DC5STEM plasmid construct was produced by cloning the following oligonucleotides into the *Bam*HI and *Mlu*I sites of the DC5T' vector: top oligonucleotide, 5'-GAT CCT ACG ATC CGG GGT GTC GAT CCA TCA CTC TGC TGT TCT GTT TTT GA-3'; 3' oligonucleotide, 5'-CGC GTC AAA AAC AGA ACA GCA GAG TGA TGG ATC GAC ACC CCG GAT CGT AG-3'. The 568STEM plasmid then was produced by cloning the HH568 ribozyme into the *Sac*II and *Bam*HI sites of the DC5STEM plasmid using the HH568 oligonucleotides shown above.

The initial tRNA gene for constructing TRZ vectors was produced through two rounds of PCR amplification using the DC5T' retroviral vector as a starting template. The primers DC5PRIME (5'-GCA ACC TGT GGT AGC CAA-3') and TRZA

(5'-GTT ATG GGC CCA GCA CGC TTC CGC TGC GCC ACT CGA CTG TTC TGC CTA TAG TGA GTC GTA TTA GAA GCT TGG ACT ACT CAC AGG AGA TG-3') were used in the first round of amplification. DC5PRIME and TRZB (5'-TCG TAC GCG TTC GTG GAT CCT CGT CCG CGG CTC ATC AGG AGT GGC GCA GGT TTC GAT CCA TCG ACC TCT GGG TTA TGG GCC CAG CAC GCT-3') primers were used in the second round. The resulting amplification product was digested with *Bgl*II and *Mlu*I and cloned into the same sites in the DC5T' vector, replacing the $\Delta 3'$ -5' tRNA gene, to generate the TRZV vector. The HH568 ribozyme was then cloned into the *Sac*II and *Bam*HI sites of TRZV using the overlapping oligonucleotides TRZ568A (5'-ACA CAA CAC TGA TGA GCC GAA AGG CGA AAC GGG CAC ACA GGC GAT ACT GTT CTG TTT TTG-3') and TRZ568B (5'-GAT CCA AAA ACA GAA CAG TAT CGC CTG TGT GCC CGT TTC GCC TTT CGG CTC ATC AGT GTT GTG TGC-3') to generate the retroviral vector plasmid TRZ568.

Retroviral transduction of cell lines

Stable retroviral vector producer lines were generated by transfecting the retroviral vector plasmids described above into the AM12 amphotropic packaging cell line (19) as previously described (14) and pooling the G418-resistant colonies. Retroviral vector transduction of MT-2 (20), CEM-SS (21) and U937 (ATCC CRL 1593) cells was accomplished by co-culturing 2×10^5 cells on 10^6 stably transfected AM12 retrovirus producer cells for 3 days in 2 ml R3 medium consisting of RPMI-1640 medium (BioWhittaker) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine and 10% fetal calf serum (Hyclone). Stably transduced cells were selected by culturing in R3 medium containing G418 (0.4 mg/ml for MT-2 cells, 0.6 mg/ml for CEM-SS and U937 cells; Gibco BRL). Retroviral vector transduction of NIH 3T3 (ATCC CRL 1658) and 293 (ATCC CRL 1573) cells was accomplished by incubating cells with supernatant from producer cells in the presence of 8 μ g/ml polybrene (Sigma) for 16 h. Stably transduced cells were selectively expanded in DMEM-10, consisting of DMEM (BioWhittaker) supplemented with antibiotics, L-glutamine and 10% fetal calf serum and containing 0.7 mg/ml G418.

RNA analysis

Reagents for the lysate ribonuclease protection assay (LRPA) were purchased in kit form from US Biochemicals and the assay was performed as described by the manufacturer. Ribonuclease protection assays using purified RNA were performed using the LRPA kit, except that purified RNA diluted into the lysis/hybridization buffer was used in place of cell lysate. RNA was purified by the acid phenol method (22). Templates for the synthesis of antisense RNA probes were amplified by PCR from retroviral vector plasmid DNA using the following primers: top primer, 5'-ATT AAC CCT CAC TAA TAA ACT GTG AGA CCG TGT GCT-3'; bottom primer, 5'-CCA AGC TTC TAA TAC GAC TCA CTA TAG GGA CAT GCC TTG CAA AAT GG-3'. PCR reagents were purchased from Perkin-Elmer-Cetus Corp. The bottom primer encodes a T7 promoter for the synthesis of antisense RNA probes (23). The probe templates included the entire pol III transcription units, 18 bp upstream of the initiation site of tRNA transcription and 40 bp of adjacent MoMLV LTR sequences downstream of tRNA transcriptional termination.

For Northern blot analysis purified RNA (5 µg) was separated on 6% polyacrylamide–7 M urea denaturing gels, then transferred to Genescreen Plus membranes (NEN) with a BioRad electroblotter. The RNA was UV cross-linked to the membrane with a Strategene UV Stratalinker apparatus. Membranes were pre-hybridized at 65°C in 0.25 M sodium phosphate, pH 7.4, 0.5% SDS, 0.5% casein (Sigma) and hybridized with ³²P-labeled antisense RNA probes in the same buffer overnight at 65°C. Membranes were washed three times in 2× SSC (23) at 65°C, then once in 0.1× SSC at 65°C.

In vitro cleavage assays

The substrate RNA used in the *in vitro* cleavage assays corresponded to the first 613 nt of the primary HIV-1 transcript. The HH568 ribozyme cleaves after nucleotide 115 of this transcript. The template used for *in vitro* transcription of this target was amplified by PCR from the pNL4-3 HIV-1 proviral clone using the primers LTRA (5'-CCA AGC TTC TAA TAC GAC TCA CTA TAG GGA GGG TCT CTC TGG TAA GA-3'), which includes a T7 RNA polymerase promoter, and LTRB (5'-GAT GCA CAC AAT AGA GG-3'). Substrate RNA was transcribed *in vitro* in the presence of ³²P-labeled CTP (New England Nuclear) using the GeneScribe-Z transcription kit (US Biochemicals) and purified by G50 chromatography (23).

Templates for the *in vitro* synthesis of tRNA-ribozyme chimeric transcripts were amplified by PCR from the appropriate retroviral plasmid templates using a 5' primer containing the T7 promoter sequence 5'-CCA AGC TTC TAA TAC GAC TCA CTA TA-3' and sequences corresponding to the first 18 nt of the tRNA-ribozyme transcripts. Primers corresponding to the 3' termini of the predicted pol III transcripts were used in conjunction with the specific 5' primers, except for the 568S35 and 568STEM constructs, where a MoMLV LTR-specific 3' primer 5'-CAT GCC TTG CAA AAT GG-3' was used. The resulting PCR amplification products for the 568S35 and 568STEM templates were cleaved with *Bam*HI prior to *in vitro* transcription and contained an additional 8 nt downstream of the predicted pol III termination site. T7 transcripts were generated using the Megascript kit (Ambion) and were subjected to electrophoresis in denaturing polyacrylamide gels. Transcripts corresponding to the full-length tRNA-ribozyme chimeric transcripts were excised from the gel, eluted overnight at 4°C in TNE (23) and precipitated with ethanol.

Cleavage assays were accomplished in ribozyme cleavage buffer (75 mM Tris, pH 7.4 at 37°C, 10 mM MgCl₂) in the presence of 10 mM dithiothreitol and 1 U/µl pancreatic ribonuclease inhibitor. tRNA-ribozyme transcripts and substrate were heated in separate tubes in ribozyme cleavage buffer to 65°C for 5 min, equilibrated to 37°C and combined to initiate the cleavage reactions (final concentrations of tRNA-ribozyme transcripts and substrate were 1 µM and 1 nM respectively). Samples were removed at the indicated times, diluted 1:5 in sequencing loading buffer (US Biochemicals), heated to 95°C for 2 min, cooled on ice and subjected to electrophoresis in 6% denaturing polyacrylamide gels. Bands in the gels corresponding to uncleaved and cleaved substrate were quantified using a phosphorimager.

Assay for cleavage activity of expressed ribozymes

The sequence of the RNA oligonucleotide used as substrate in the cleavage assays was UGC CCG UCU GUU GUG. This

oligonucleotide was 5'-end-labeled with ³²P using polynucleotide kinase (23). Cleavage assays in RNA extracted from stably transduced cells were performed as follows. RNA (5 µg) purified from either non-transduced or stably transduced cells was dissolved in 5 µl 75 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM dithiothreitol, 1 U/µl pancreatic ribonuclease inhibitor (US Biochemicals) and ≤1 nM end-labeled RNA substrate. The reactions were incubated either at 37°C (Fig. 3C) or at 45°C (Fig. 6B) for 16 h, diluted to 10 µl with sequencing loading buffer (US Biochemicals) and cleavage products were separated from non-cleaved substrate by electrophoresis in 20% polyacrylamide–7 M urea denaturing gels.

RESULTS

Ribozyme accumulation using the DCt5T' vector

To examine the feasibility of expressing ribozymes from the Δ3'-5 promoter we cloned a hammerhead ribozyme, termed HH568, into the DCt5T' retroviral vector to generate the DC568 plasmid. The HH568 ribozyme was designed to cleave the sequence 5'-UGC-CCGUC↓UGUUGUG-3' (↓ site of cleavage) found in the leader sequence of most HIV-1 isolates (24). We and others have found this region to be particularly susceptible to ribozyme cleavage (25–27). The sequence of the predicted pol III-derived tRNA-HH568 chimeric transcript is shown in Figure 1B. The DC568 plasmid was used to establish stably transfected retroviral vector producer lines. The retroviral particles produced from this line were used to transduce two human T cell lines, MT-2 (20) and CEM-SS (21). Ribozyme accumulation in the stably transduced, pooled cultures was quantified using a lysate ribonuclease protection assay (LRPA) (Fig. 2). The DCt5T' is a double copy retroviral vector in which the Δ3'-5 gene is duplicated in the U3 region of the LTRs of the integrated proviral vector (Fig. 2A). Because of this configuration, two types of ribozyme-containing transcripts were expressed in the transduced T cells: pol III transcripts expressed from the duplicated Δ3'-5 genes and pol II transcripts derived from the Moloney retroviral LTR (Fig. 2A). The LTR-driven pol II transcript extends through the U3 region of the 3' LTR of the vector and encodes the *neo* selectable marker (Fig. 2A). The probe used in the LRPA was designed to distinguish Moloney LTR pol II transcripts from Δ3'-5 pol III transcripts. In both the MT-2 and CEM transduced cells pol III-derived tRNA-HH568 chimeric transcripts accumulated to low levels (<100 copies/cell; Fig. 2B). In fact, the Moloney LTR-derived pol II transcripts were more abundant than the Δ3'-5 pol III transcripts in the transduced CEM-SS cells (Fig. 2B). Interestingly, pol III transcripts were more prevalent in MT-2 cells relative to CEM-SS cells, while the pol II transcripts were more abundant in the CEM-SS cells. These results indicate that factors that regulate the overall expression and accumulation of pol II and pol III transcripts expressed from the DCt5T' vector can vary significantly among different cell lines.

Improved pol III transcript accumulation

In an attempt to improve the intracellular accumulation of the pol III transcripts we incorporated structural elements into the tRNA-ribozyme transcripts to protect the transcripts from nucleolytic degradation. Two strategies to protect the termini of the chimeric transcripts from exonucleases were tested. One strategy involved the incorporation of stem-loop structures into the termini of the transcripts (constructs 568S3 and 568S5 in

Figure 3. Accumulation of tRNA–HH568 ribozyme chimeric transcripts in stably transduced MT-2 cells. (A) Schematic representation of RNA structural motifs inserted into the DC568 pol III transcription unit. Boxed regions indicate the HH568 hammerhead ribozyme. (B) Northern blot analysis of stably transduced MT-2 lines. Total RNA (5 µg) from each cell line was separated on an 8% polyacrylamide–7 M urea denaturing gel and electroblotted onto a nylon membrane. The membrane was hybridized with antisense RNA probes specific for each construct tested (10^6 c.p.m./ml each probe) as described in Materials and Methods. The probes contained met_i tRNA sequences and therefore hybridized to the cellular precursor and mature met_i tRNA transcripts indicated. Expected sizes of the recombinant pol III transcripts are as follows: DC568, 133 nt; 568S3, 144 nt; 568S35, 146 nt; 568S5, 145 nt; 568STEM, 170 nt. (C) Ribozyme activity in total RNA extracted from stably transduced MT-2 lines. Total RNA from each cell line (5 µg) was incubated overnight with a 5′-end-labeled 15 nt RNA oligonucleotide containing the cleavage site for the HH568 ribozyme in 5 µl 10 mM Mg²⁺ (see Materials and Methods for details). Cleavage products were separated from the non-cleaved substrate by electrophoresis on a 20% polyacrylamide–7 M urea denaturing gel and visualized by autoradiography. S, substrate; P, cleavage product.

Fig. 3A). The second strategy involved making the 3′-ends of the transcripts homologous to the 5′-ends to favor the formation of an intramolecular duplex between the 5′ and 3′ termini of the transcripts (568S35 and 568STEM in Fig. 3A). 568STEM was similar to the 568S35 construct, but included more extensive internal structure in an attempt to also protect the transcript from endonuclease degradation (Fig. 3A). These constructs were cloned into the DCt5T′ vector, retroviral vector producer cell lines were generated and stably transduced human T cell lines (MT-2 and CEM-SS) were established.

Figure 3B shows the results of a comparative Northern blot analysis of ribozyme transcript accumulation in stably transduced MT-2 cells. Incorporation of stem–loop structures into the ends of the DC568 pol III transcript had a negative effect on accumulation of the tRNA–ribozyme chimeric transcripts (568S3 and 568S5 constructs; Fig. 3A and B). Additional experiments showed that stem–loop structures at the 3′ termini of Δ3′–5 transcripts lowered the efficiency of pol III transcriptional termination (not shown). This reduced efficiency of termination probably contributed to the decreased accumulation observed with these two constructs.

A dramatic improvement in accumulation was observed, however, when the termini of the transcripts were designed to hybridize to each other to form a duplex (568S35 and 568STEM constructs; Fig. 3A and B). tRNA–ribozyme chimeric transcripts were >100-fold more abundant in the 568S35-transduced MT-2 cells relative to the original DC568-transduced cells, while transcripts in the 568STEM-transduced cells were ≥20-fold more abundant (Fig. 3B). An additional B box pol III promoter element was duplicated in the reverse orientation within the 568STEM construct, which may have caused the reduced expression of this transcript relative to the 568S35 transcript.

To determine if active ribozymes were expressed in the transduced cells we incubated a labeled RNA substrate containing the HH568 cleavage site with total RNA purified from the stably transduced MT-2 cell lines (Fig. 3C). Ribozyme levels within cells transduced with the DC568, 568S3, 568S5 and 568STEM constructs were too low to detect cleavage of the RNA substrate. Ribozyme transcripts were sufficiently abundant in the 568S35-transduced T cell RNA to readily detect ribozyme cleavage activity (Fig. 3C). These results

Figure 4. Ribonuclease protection analysis of transcript accumulation in cells stably transduced with 568S35 or DCS35 constructs. (A) Accumulation of 568S35 transcripts in various cell lines. Ribonuclease protection was performed as in Figure 2B, except 5 μ g total RNA purified from the cell lines indicated was used instead of crude cell lysate. A standard curve was generated by diluting known amounts of *in vitro* transcribed 568S35 tRNA-ribozyme chimeric transcripts in non-transduced MT-2 RNA prior to addition of the 229 nt 568S35 antisense probe. The amounts of *in vitro* transcript added were corrected for copy number per cell, assuming that 1 cell contains 10 pg RNA (23). Accurate copy numbers of 568S35 transcripts in the stably transduced cells could not be determined, because the signals exceeded the linear range of the standard curve. The 568S35 *in vitro* transcript used to generate the standard curve contained an additional 8 nt on the 3'-end relative to the predicted 568S35 transcript, resulting in a larger protected fragment (predicted to be 154 nt). Predicted sizes of protected 568S35 fragments are: pol II, 207 nt; pol III, 146 nt. Refer to Figure 2B for marker sizes. (B) Accumulation of DCS35 transcripts in stably transduced MT-2 and CEM-SS cells. Ribonuclease protection was accomplished as described in (A), except using a 189 nt DCS35 antisense probe. The DCS35 samples were assayed in parallel with the 568S35 samples, run on the same gel and the photographs shown in (A) and (B) were taken from the same autoradiograph. Predicted sizes of protected DCS35 fragments are: pol II, 167; pol III, 109 nt.

demonstrate that active ribozymes were synthesized in the 568S35-transduced MT-2 T cells.

Accumulation of 568S35 transcripts in other cell lines

To test whether the improved accumulation observed above was specific to human T cell lines we transduced other cell lines with the 568S35 construct. The cell lines tested were the U937 human monocytic cell line, the 293 adenovirus-transformed human embryonic kidney cell line and the NIH 3T3 murine fibroblast cell line. Ribozyme accumulation in stably transduced, pooled cultures was examined by ribonuclease protection analysis (Fig. 4A). High levels of pol III transcripts accumulated in all three cell lines and the levels were comparable with those observed in MT-2 and CEM-SS human T cell lines. In fact, the pol III transcripts were significantly more abundant than the pol II transcripts derived from the Moloney LTRs of the DC5T' vector. In contrast, the Moloney LTR pol II transcripts were more abundant than the $\Delta 3'-5$ pol III transcripts in CEM-SS cells transduced with the original DC568 vector (Fig. 2B).

Enhanced accumulation of $\Delta 3'-5$ transcripts

The ends of the 568S35 and the 568STEM transcripts were designed to hybridize to form a duplex (Fig. 5). To test whether this modification was sufficient in the absence of ribozyme sequences to enhance accumulation of pol III transcripts derived from the $\Delta 3'-5$ gene we removed the ribozyme from the 568S35 construct to produce the DCS35 retroviral vector. Pol III transcripts derived from the DCS35 vector are also predicted to form a duplex between the ends of the transcripts, but lacked any ribozyme sequences (Fig. 5). Transcript accumulation in MT-2 and CEM-SS T cells stably transduced with the DCS35 retroviral vector was analyzed by ribonuclease protection (Fig. 4B). High levels of DCS35-derived pol III transcripts lacking ribozyme sequences were observed (Fig. 4B). These levels were comparable with the levels observed in 568S35-transduced lines (compare Fig. 4A and B). Pol III transcript levels in DCS35-transduced cells were >100-fold greater than levels of pol III transcripts expressed from the original DC5T' vector, which lacked the duplex structure (data not shown). These results indicate that the enhanced accumulation of pol III transcripts observed with the 568S35 and 568STEM constructs was not due to the presence of the ribozyme sequences in these constructs. Instead, these results suggest that the improved accumulation was due to the potential of the 5' and 3' termini of the 568S35, 568STEM and DCS35 pol III transcripts to form a duplex.

Variation and persistence of expression from the 568S35 construct

We further characterized expression from the 568S35 construct by examining how expression varied within the pooled CEM-SS-transduced culture. To accomplish this we generated 14 clonal lines by limiting dilution of the pooled culture. Ribozyme accumulation in the clonal lines was analyzed by Northern analysis (Fig. 6A). In addition, ribozyme cleavage activity was assayed in purified total RNA from all of the clones (Fig. 6B). All 14 clonal lines were found to express active pol III ribozyme transcripts (Fig. 6). Ribozyme accumulation in the clonal lines ranged from 20 to 210% of the pooled culture. No obvious difference in growth rate was observed between the low and high expressers. Thus high accumulation of tRNA-ribozyme chimeric transcripts did not adversely affect cell proliferation. Ribozyme cleavage activity was detectable in total RNA from all clonal lines and was directly proportional to the expression levels measured by Northern analysis (compare Fig. 6A and B). In contrast, the cleavage activity observed did not correlate with pol II transcript levels measured in these clonal cell lines by RNase protection analysis (data not shown).

To examine the persistence of expression from the 568S35 construct we propagated the pooled 568S35-transduced CEM-SS culture for 3 months in the absence of G418 selection. During this period the cell line was passaged every 3–4 days. We estimate that ≥ 90 cell doublings occurred during this period. Ribozyme accumulation in the culture propagated for 3 months in the absence of selection was comparable (70%) with that of the original culture, as determined by Northern analysis (Fig. 6A).

Figure 5. Sequences and predicted secondary structures of DCS35, 568S35 and 568STEM pol III transcripts. The outputs from the program Mulfold (28–30) were displayed with Loopviewer and represent the lowest free energy fold of each sequence. Bold lower case, met_t tRNA sequences; bold upper case, ribozyme sequences; italic, non-tRNA sequences present in the recombinant transcripts. Also shown are unique *Sac*II and *Bam*HI sites that can be used to insert exogenous sequences into the DCS35 pol III transcription unit.

Ribozyme cleavage activity in the context of the various tRNA transcription units

An effective *trans*-cleaving ribozyme transcript should accumulate to high levels within a cell and should exhibit good cleavage activity. We therefore examined the cleavage activity of the tRNA–HH568 transcripts shown in Figure 3A. *In vitro* transcripts corresponding to these chimeric transcripts were generated and their cleavage activity was tested on a substrate RNA comprising the first 613 nt of the genomic HIV-1 transcript (Fig. 7A). The HH568 ribozyme incorporated into these tRNA chimeric transcripts cleaves after position 115 of the 613 nt HIV-1 RNA substrate used. The cleavage activity of the tRNA–HH568

transcripts shown in Figure 3A was significantly reduced relative to a chemically synthesized HH568 ribozyme which lacked any flanking tRNA sequences (Fig. 7A). In fact, the two tRNA–HH568 transcripts that exhibited the best accumulation in stably transduced cell lines, 568S35 and 568STEM, demonstrated the poorest cleavage activity.

Improved cleavage activity of tRNA–ribozyme chimeric transcripts

We therefore set out to improve ribozyme cleavage activity of tRNA–ribozyme transcripts containing the duplex structure, present in the 568S35, 568STEM and DCS35 constructs,

Figure 6. Ribozyme accumulation and activity in pooled and clonal CEM-SS cell lines stably transduced with the 568S35 construct. (A) Clonal cell lines were generated from the pooled 568S35-transduced CEM-SS culture by limiting dilution cloning. In addition, the pooled transduced population was cultured for 3 months without G418. Northern analysis was performed as in Figure 3B. (B) Ribozyme activity in total RNA extracted from pooled and clonal CEM lines. Ribozyme cleavage activity in 5 μ g RNA purified from either the pooled culture or clonal lines was analyzed as in Figure 3, except that the overnight incubation was performed at 45°C instead of 37°C to facilitate detection of ribozyme cleavage activity in the clones expressing lower levels of the 568S35 transcript. S, substrate; P, cleavage product.

responsible for the enhanced transcript accumulation. One construct, termed TRZ, contained a number of changes (Fig. 7B) relative to the 568S35 transcript. These changes included the incorporation of a base paired region preceding the site of ribozyme insertion, a bulge to separate the ribozyme from the flanking tRNA sequences and a shorter terminal duplex structure (compare Figs 5 and 7B). The HH568 ribozyme was inserted within the stem-loop structure indicated in Figure 7B to yield the TRZ568 construct. *In vitro* cleavage activity of the TRZ568 tRNA-ribozyme transcript was significantly improved relative to the 568S35 transcript (compare Fig. 7A and C) and was only slightly reduced relative to the HH568 synthetic ribozyme that lacked any flanking tRNA sequences (Fig. 7C). In addition, cleavage activity of another *trans*-cleaving ribozyme motif, the hairpin ribozyme, was tested in the context of the DCS35 and TRZ constructs. The hairpin ribozyme tested cleaves at the same GUC site in HIV-1 RNA as the HH568 hammerhead ribozyme (26). Cleavage activity of the hairpin ribozyme was markedly

reduced in the context of the DCS35 construct relative to a chemically synthesized hairpin ribozyme that lacked any tRNA flanking sequences (data not shown). However, the hairpin ribozyme, when tested in the TRZ transcript, exhibited cleavage activity that was identical to the chemically synthesized hairpin ribozyme (data not shown).

We next examined whether the TRZ568 transcript would accumulate to the high levels observed with the 568S35 transcript in stably transduced T cells. RNase protection analyses showed that TRZ568 transcripts accumulated to levels comparable with 568S35 transcripts in stably transduced MT-2 and CEM-SS T cells when incorporated into the DC retroviral vector in place of the original $\Delta 3'-5$ tRNA expression cassette (Fig. 7D). Therefore, although the TRZ- and DCS35-based constructs both accumulate to high levels in stably transduced cells, the TRZ vector is preferable for expressing ribozymes due to the improved cleavage activity of the TRZ tRNA-ribozyme chimeric transcripts.

DISCUSSION

Enhanced accumulation of recombinant tRNA chimeric transcripts containing an 'S35' motif

We report a significant improvement in accumulation of recombinant pol III tRNA transcripts by enabling the transcripts to form an intramolecular duplex between the 5' and 3' termini. For simplicity, we will refer to this structure as the 'S35' motif (stem formed between the 3' and 5' termini). tRNA-ribozyme chimeric transcripts containing the S35 motif showed a >100-fold increase in accumulation relative to three other different tRNA-ribozyme pol III transcripts lacking this motif (see Figs 3 and 7D). Accumulation of S35-containing ribozyme transcripts was sufficient to readily detect ribozyme cleavage activity in total RNA extracted from stably transduced human T cell lines (Figs 3C and 6B). Ribozyme expression in T cell lines was stable for at least 3 months in the absence of selection (Fig. 3B) and the enhanced accumulation was observed in all cell lines tested (Fig. 4A).

Mechanism of enhanced accumulation of S35 constructs

Transcript accumulation is a function of the rate of transcription and the stability of the transcript. It is unlikely that the rate of transcription was altered in the constructs containing an S35 motif, since any modifications that were made occurred downstream of the B box promoter of the $\Delta 3'-5$ gene and therefore outside the regions critical for pol III transcription (15). In previous studies the rate of transcription from the $\Delta 3'-5$ gene was not affected by the insertion of sequences downstream of the B box promoter (15). These data suggest that the observed increase in accumulation was related to improved stability of the transcripts as a consequence of the potential intramolecular interaction between the 5' and 3' termini of the transcripts. We are studying whether similar improvements in transcript accumulation will occur in other pol III promoter systems, such as U6 (31) and adenovirus VA promoters (32), by the incorporation of an S35 motif.

Cleavage activity of tRNA-ribozyme chimeric transcripts

Ribozyme cleavage activity was markedly reduced in all of the tRNA-ribozyme chimeras shown in Figure 3A (Fig. 7A). There are several possible explanations for the reduced cleavage

Figure 7. Optimization of ribozyme cleavage activity in the context of tRNA transcription units. (A) Cleavage activity of the tRNA-ribozyme chimeric transcripts shown in Figure 3A. *In vitro* T7 transcripts corresponding to the predicted tRNA-ribozyme transcripts shown schematically in Figure 3A were synthesized and their cleavage activities measured on a 613 nt HIV-1 RNA substrate as described in Materials and Methods. Shown for comparison is the cleavage activity of a chemically synthesized HH568 ribozyme lacking any flanking tRNA sequences. (B) Predicted secondary structure of the optimized TRZ transcription unit. The site where *trans*-cleaving ribozymes were inserted is indicated. (C) HH568 ribozyme cleavage activity in the context of the optimized TRZ transcription unit. The HH568 ribozyme incorporated into the TRZ construct had 9 and 11 nt binding arms and a 3 bp stem II relative to the HH568 ribozyme incorporated into the tRNA-ribozyme chimeras shown in (A), which had 7 nt binding arms and a 5 bp stem II (see Fig. 1B). These changes slightly reduced the cleavage activity of a chemically synthesized ribozyme on the 613 nt HIV-1 substrate (compare HH568 activity in A and C), but did not alter the cleavage activity on a 15 nt substrate (data not shown). (D) Comparison of 568S35 and TRZ568 transcript accumulation in stably transduced MT-2 and CEM-SS T cells. Ribonuclease protection was performed as in Figure 4. Lanes 1-4 are 568S35 samples and controls and lanes 5-8 are TRZ568 samples. Lanes 1 and 5, antisense probes; lanes 2 and 4, non-transduced CEM-SS cellular RNA; lanes 3 and 5, RNA from stably transduced MT-2 cells; lanes 4 and 8, RNA from stably transduced CEM-SS cells. Signals derived from pol II and pol III transcripts are indicated. Predicted sizes of the TRZ568 protected fragments are: pol II, 216 nt; pol III, 148 nt.

activity. Ribozymes, like protein enzymes, must fold into proper secondary and tertiary structures for activity. Computer folding predictions of the tRNA-ribozyme chimeras shown in Figure 3A revealed potential base pairing interactions between the HH568 ribozyme and the non-ribozyme flanking sequences (e.g. the fold

of 568S35 in Fig. 5). These interactions could interfere with optimal folding of the HH568 ribozyme (33-35). In addition, the ribozyme should hybridize efficiently to its intended RNA target for maximum cleavage activity. Formation of the S35 motif in 568S35 and 568STEM causes these transcripts to adopt a circular

conformation, thus reducing the freedom of movement of the ends of these transcripts (see Fig. 5). This constraint could restrict the ability of the ribozyme to hybridize to its target RNA. In support of this, cleavage activity of several hammerhead ribozymes was significantly reduced when incorporated into small (<65 nt) covalently closed circular RNA molecules (P. Pavco, unpublished observation).

The TRZ transcription unit was designed to overcome these difficulties. Cleavage activity of a hammerhead and a hairpin ribozyme placed within the TRZ transcription unit was comparable with that of the corresponding synthetic ribozymes that lacked any flanking sequences (Fig. 7C and unpublished observations). The TRZ transcript differed from the 568S35 transcript in that the non-ribozyme sequences were designed to be sequestered in secondary structure, thus making them unavailable to interact with the ribozymes (Fig. 7B). The ribozymes were also placed within a bulge, which may have provided sufficient flexibility to allow the ribozymes to fold optimally. In addition, the TRZ transcript contained a shorter S35 motif (compare Figs 5 and 7B). The shorter S35 motif in the TRZ construct may be capable of unwinding during hybridization of the ribozymes with their target RNAs, thereby improving the hybridization efficiency of the ribozymes.

Potential uses of DCS35 and TRZ vectors

The DCS35 and TRZ vectors may be useful for expressing other functional RNAs besides ribozymes, such as decoy RNAs, RNA aptamers and antisense RNAs. In fact, accumulation of anti-HIV RRE and TAR decoy RNAs cloned between the *SacII* and *BamHI* sites of the DCS35 construct (see Fig. 5) was as high as levels obtained with the 568S35 construct in stably transduced human T cell lines (B. Sullenger and J. Thompson, unpublished observation). The TRZ vector is preferable for expressing ribozymes, since ribozyme activity in the context of TRZ transcripts was superior to the DCS35-based construct 568S35. The TRZ construct may also be more suitable for expressing antisense RNAs that, like hammerhead and hairpin ribozymes, must hybridize to their respective target RNAs for activity.

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