Intracellular Traits of Chimeric tRNAlys3-ribozymes and their Inhibition of HIV

Zongli Chang

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INTRACELLULAR TRAITS OF CHIMERIC tRNA\textsuperscript{LYS3}-RIBOZYMES AND THEIR INHIBITION OF HIV

By

Zongli Chang

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in Microbiology and Molecular Genetics

March 2000
Each person whose signature appears below certifies that this dissertation, in their opinion, is adequate in scope and quality, as a dissertation for the degree Doctor of Philosophy.

Chairperson

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I would like to express my sincere thanks to my mentor, Dr. John J. Rossi for his expert guidance and suggestions throughout the study. I would also like to thank the Department of Microbiology and Molecular Genetics for support and guidance. I am especially grateful for the guidance and support by my committee members, Dr. William Langridge, Dr. Barry Taylor, Dr. John Zaia and Dr. Anthony Zuccarelli.

Dr. Barry Taylor has provided guidance throughout my academic studies. His experience and support has been a blessing in my academic pursuit. As a remote student, I deeply appreciate the care and assistance of Frieda Roos and the staff of the Department.

Special thanks also go to everybody in our laboratory. Dr. Shawn Westaway has provided numerous suggestions, technical assistance and advice. Haitang Li has been extremely supportive in techniques, suggestions as well as other aspects of my research. I also thank Dr. Nan Sook Lee for providing me with technical assistance for the in situ hybridization technique.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3'-tRNase</td>
<td>tRNA 3’ processing endoribonuclease</td>
</tr>
<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>ALV</td>
<td>avian leukemia virus</td>
</tr>
<tr>
<td>AMV</td>
<td>avian myeloblastosis virus</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescent in situ hybridization</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
</tr>
<tr>
<td>HDV</td>
<td>hepatitis delta virus</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>hr</td>
<td>inverted terminal repeats</td>
</tr>
<tr>
<td>ITR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>MDR-1</td>
<td>multidrug resistance gene</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>NC</td>
<td>nucleocapsid protein</td>
</tr>
<tr>
<td>NCP7</td>
<td>nucleocapsid protein of HIV</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PBS</td>
<td>primer binding site of HIV</td>
</tr>
<tr>
<td>Pol. II</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>Pol. III</td>
<td>RNA polymerase III</td>
</tr>
<tr>
<td>Pr160&lt;sup&gt;Gag-Pol&lt;/sup&gt;</td>
<td>Gag-Pol precursor (a polyprotein) part of which is the reverse transcriptase sequence</td>
</tr>
<tr>
<td>Pre-tRNA</td>
<td>precursor transcript RNA</td>
</tr>
<tr>
<td>RNase P</td>
<td>ribonuclease P</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>Rz</td>
<td>ribozyme</td>
</tr>
<tr>
<td>SELEX</td>
<td>Systemic Evolution of Ligands by Exponential Enrichment</td>
</tr>
<tr>
<td>snRNA</td>
<td>small nuclear RNA</td>
</tr>
<tr>
<td>tNtase</td>
<td>CCA-adding enzyme, tRNA nucleotidyl transferase</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>TRz</td>
<td>chimeric tRNA-ribozyme</td>
</tr>
<tr>
<td>U6 promoter</td>
<td>human U6 small nuclear RNA gene promoter</td>
</tr>
<tr>
<td>U6/tRNA expression cassette</td>
<td>an expression cassette with the U6 snRNA promoter adjoined upstream of the tRNA gene and its internal promoter.</td>
</tr>
<tr>
<td>VA1 promoter</td>
<td>adenovirus-associated RNA 1 (Ad VA1) gene promoter</td>
</tr>
</tbody>
</table>
ABSTRACT

Intracellular Traits of Chimeric tRNA\textsuperscript{Lys3}-Ribozymes and Their Inhibition of HIV-1

by

Zongli Chang

Host tRNA\textsuperscript{Lys3} is the primer recruited by HIV-1 for initiating reverse transcription. When ribozymes targeted upstream of the primer binding site of the HIV-1 genome are tethered to the 3' end of the tRNA\textsuperscript{Lys3}, it is expected that the tRNA will carry the ribozymes into the virions. We made constructs that express chimeric tRNA\textsuperscript{Lys3}-ribozymes from internal tRNA promoters. These chimeras were also adjoined downstream of a U6 promoter, resulting in a hybrid U6/tRNA expression cassette with extragenic U6 and intragenic tRNA promoters. Juxtaposition of the tRNA and U6 promoters significantly enhanced expression levels compared to the tRNA promoter alone. A U6/tRNA expression cassette with two mutations in B box of the tRNA moiety gave rise to the highest expression level, suggesting that the mutations reduce the steric interference of the tRNA gene with the U6 promoter. Qualitative changes in the initiation site of one minor species of transcripts were observed. These may have resulted from steric interference between the U6 and tRNA promoter elements. In an anti-HIV assay, it
was found that tRNA-ribozyme chimeras could be encapsulated into HIV virions leading to reduced infectivity.

The intracellular stability and processing of the chimeric tRNA\textsuperscript{\text{Lys3}}-ribozymes were also studied. Point mutations in B box were found to reduce the intracellular processing of the tRNA-ribozymes in a tc/t analysis of the transcripts by Northern hybridization analysis. A deletion of one guanidine residue immediately upstream of the B box led to increased stability in a processing assay using cell extract with tRNA processing activities. Nevertheless, the transcripts from these constructs can still be exported to the cytoplasm as demonstrated by fluorescence in situ hybridization and can be packaged into the virions. Constructs with these changes were also found to inhibit HIV infectivity more efficiently. Changes in the tRNA sequence that enhance transcript stability could be useful in the design of other tRNA expression cassettes for RNA-based therapeutics. The tc/t analysis proposed could be useful for screening other changes in tRNA expression cassettes for increased transcript stability.
I. INTRODUCTION

A. tRNA<sub>Lys</sub><sup>3</sup> as the primer for reverse transcription in HIV

Human immunodeficiency virus (HIV) is the causative agent of acquired immune deficiency syndrome (AIDS). During its replication cycle (Figure 1), the HIV single-stranded RNA genome is reverse transcribed into double stranded DNA (proviral DNA). The process is catalyzed by reverse transcriptase, a viral-encoded enzyme that is encapsidated into the virion (Figure 2). Proviral DNA is then transported into the nucleus and integrated into the host chromosome. The primer for HIV DNA synthesis is tRNA<sub>Lys</sub><sup>3</sup> (Figure 3), which is derived from the host cells and packaged into virions (Figure 1). The nucleotide sequence at the 3' end of the primer tRNA hybridizes to a complementary 18 nucleotide viral sequence, termed the primer binding site (PBS), near the 5' end of the RNA genome (Figure 4). The following introduction provides more details about this process; review articles are also available (Litvak et al., 1994; Marquet et al., 1995; Gotte et al., 1999).

1. HIV reverse transcriptase

Reverse transcriptase (RT) is the key enzyme for the replication of the retroviral RNA genome in the process of reverse transcription (Figure 2, see Goff, 1990; DeVico et al., 1992; Kupiec et al., 1996, for reviews). It is an RNA-dependent DNA polymerase with three enzymatic activities: 1) an RNA-dependent DNA polymerase activity involved in
Figure 1. Replication cycle of HIV-1 and the mechanism of packaging of tRNA<sub>Lys</sub> and chimeric tRNA<sub>Lys</sub>-ribozymes.

tRNA<sub>Lys</sub>-ribozymes: chimeric tRNA<sub>Lys</sub>-ribozymes. Adapted and modified from Mak and Kleiman (1997).
Figure 2. Reverse transcription of retroviral genomic RNA into double-stranded proviral DNA.

Step 1: Annealing of primer tRNA to the PBS, and synthesis of minus-strand strong-stop cDNA, with the resulting degradation of R and U5 RNA by the RNase H activity of the reverse transcriptase. Step 2: The first strand transfer, in which minus-strand strong-stop cDNA is annealed to the 5' terminus of the genomic RNA via R-R' hybridization. Steps 3 and 4: Further synthesis of minus-strand cDNA, during which the genomic RNA is further degraded by RNase H. A small piece of RNA, the polyuridine tract (PPT), remains undegraded and serves as the primer for plus-strand strong-stop cDNA (step 5). Step 5: Termination of plus-strand strong-stop cDNA synthesis 18 nucleotides into the primer tRNA, thereby generating a new PBS sequence; the tRNA is released from the minus-strand cDNA. Step 6: The second strand transfer, in which plus-strand strong-stop cDNA is annealed to the 3' terminus of minus-strand cDNA via PBS-PBS' hybridization. Step 7: Completion of synthesis of double-stranded proviral DNA. Figure adapted from Mak and Kleiman (1997).
Figure 3. Nucleotide sequence of tRNA$_{\text{Lys}}$ presented in cloverleaf form.

Modified nucleotides are: D, dihydrouridine; Ψ, pseudouridine; S, 5-(methoxycarbonylmethyl)-2-thiouridine; R, N-[N-(9-β-D-ribofuranosyl-2 methylthiopurine-6-yl)carbamoyl] threonine; Tm, 2'-O-methyl ribosylthymine; at other position, m indicates a methylated G or A, with the position of methylation indicated. The solid line indicates 18 nucleotides at the 3' end of the tRNA complementary to the HIV-1 primer binding site. (Adapted from Wilson and Abbotts, 1992).
Figure 4. Schematic representation of nucleic acid interactions leading to the formation of a complex between primer tRNA and the complementary region on the retroviral genome.

PBS, primer-binding site.

(Adapted from Litvak et al., 1994).
the synthesis of the minus strand of the proviral DNA; 2) a DNA dependent DNA polymerase activity that catalyzes the synthesis of the plus DNA strand; and 3) an RNase H activity, a nuclease activity that degrades the RNA portion of the RNA-DNA hybrid, generating the RNA primer used for the synthesis of the plus DNA strand.

RT is encoded downstream from the gag gene in a large coding region. This coding region is translated to yield a 160 kD Gag-Pol fusion protein Pr160Gag-Pol (Figure 1). This polyprotein is subsequently cleaved by a viral protease to generate the mature RT present in the infectious virion. The initial product is a 66-kD polypeptide that has both a polymerase domain and an RNase H domain. Limited proteolysis of p66 by a retrovirus-encoded protease generates a p51 polypeptide. The reverse transcriptase isolated from HIV particles is a heterodimer of p66 and p51. The p66 subunit has the DNA polymerase and RNase H activities, and is considered to be the catalytic core for the enzyme. The precise role of p51 in the virion active polymerase remains to be determined.

2. tRNA^{Lys3} as the initiator of DNA synthesis: complementarity of tRNA^{Lys3} and PBS

Reverse transcriptase requires a primer with a free 3'-hydroxyl to initiate DNA synthesis. All retroviruses are known to use host cellular tRNAs as primers to initiate reverse transcription during their replication cycles. Different retroviruses use different tRNAs as primers (Table 1).
<table>
<thead>
<tr>
<th>Retroviruses</th>
<th>Primer tRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human immunodeficiency virus (HIV)</td>
<td>tRNA$_{\text{Lys}^3}$</td>
</tr>
<tr>
<td>Mouse mammary tumor virus (MMTV)</td>
<td>tRNA$_{\text{Lys}^3}$</td>
</tr>
<tr>
<td>Equine infectious anemia virus (EIAV)</td>
<td>tRNA$_{\text{Lys}^3}$</td>
</tr>
<tr>
<td>Feline immunodeficiency virus (FIV)</td>
<td>tRNA$_{\text{Lys}^3}$</td>
</tr>
<tr>
<td>Mason-Pfizer monkey virus (MPMV)</td>
<td>tRNA$_{\text{Lys1/2}}$</td>
</tr>
<tr>
<td>Visna</td>
<td>tRNA$_{\text{Lys1/2}}$</td>
</tr>
<tr>
<td>Human spuma retrovirus (HSRV)</td>
<td>tRNA$_{\text{Lys1/2}}$</td>
</tr>
<tr>
<td>Moloney murine leukemia virus</td>
<td>tRNA$_{\text{Pro}}$</td>
</tr>
<tr>
<td>Spleen necrosis virus (SNV)</td>
<td>tRNA$_{\text{Pro}}$</td>
</tr>
<tr>
<td>Human T-cell leukemia virus I and II (HTLV)</td>
<td>tRNA$_{\text{Pro}}$</td>
</tr>
<tr>
<td>Bovine leukemia virus (BLV)</td>
<td>tRNA$_{\text{Pro}}$</td>
</tr>
<tr>
<td>Avian sarcoma and leukemia virus (ASLV)</td>
<td>tRNA$_{\text{Trp}}$</td>
</tr>
<tr>
<td>Avian myeloblastosis virus (AMV)</td>
<td>tRNA$_{\text{Trp}}$</td>
</tr>
</tbody>
</table>
tRNA\textsuperscript{Lys3} (Figure 1) is the primer used by HIV-1 and 2 for replication (Wain-Hobson et al., 1985; Ratner et al., 1985 and Raba et al., 1979). The 18 nucleotides at the 3' end of tRNA\textsuperscript{Lys3} are complementary to a sequence near the 5' end of the HIV genome (Figure 3 and Figure 4). The sequence is the primer binding site (PBS). When isolated from the virion, the 3'end of primer tRNA\textsuperscript{Lys3} is found annealed to the PBS. The PBS consists of nucleotides 183-200 of the HIV RNA genome. Furthermore, tRNA\textsuperscript{Lys3} can act as a functional primer for synthesis of the minus strand \textit{in vitro} starting at the PBS on HIV genome (Figure 2). This \textit{in vitro} DNA synthesis reaction requires the HIV nucleocapsid protein, as well as the RT and tRNA\textsuperscript{Lys3}. Furthermore, although several different tRNA species can be isolated from HIV virions, tRNA\textsuperscript{Lys3} is one of the main species.

3. Selection of primer tRNA from host cells: selective incorporation of tRNA\textsuperscript{Lys3} into virions

The primer tRNA found in retrovirus particles originates from the host cell tRNA population, from which it is selectively packaged during virus assembly (Figure 1). RT binds cellular tRNA\textsuperscript{Lys3} and both are packaged along with genomic RNA and other proteins into the virions formed in the cytoplasm of infected cells. There are more than 100 different species of tRNAs in the cells, but only a limited population is found in the virions. In HIV virions, tRNA\textsuperscript{Lys1,2,3} and tRNA\textsuperscript{Lle} are the major species incorporated in the viral particles (Jiang, et al., 1993). It is reported that HIV virions contain a subset of five to ten species of cellular tRNA, depending upon the cell type analyzed (Kleiman et al., 1991). Only tRNA\textsuperscript{Lys3} is tightly associated to the viral genome in wild type virus and
it is believed that tRNA\textsubscript{\text{Lys3}} is the primer tRNA for HIV RT. The tRNA\textsubscript{\text{Lys1,2}} isoacceptors found in the virions probably do not serve as primers. Their incorporation reflects the fact that they contain a recognition signal shared by all three tRNA\textsubscript{\text{Lys}} species (Jiang, et al., 1993). tRNA\textsubscript{\text{Lys1}} and tRNA\textsubscript{\text{Lys2}} differ by only one base in the anticodon stem, while the difference between these and tRNA\textsubscript{\text{Lys3}} is much larger. In COS-7 cells transfected with HIV-1 proviral DNA, the isoacceptors of tRNA\textsubscript{\text{Lys}} represent only 6\% of the low molecular-weight RNA isolated from the cytoplasm, but they represent 60 percent of the low-molecular-weight RNA isolated from virus particles. This observation suggests that tRNA\textsubscript{\text{Lys}} is selectively incorporated into HIV-1 particles.

Genomic RNA packaging is not required for the incorporation of primer tRNA into the virion (Jiang M. et al., 1993, Mak et al., 1994). However, selective packaging of the primer tRNA does not occur in RT-negative HIV-1 isolates (Mak et al., 1994). This demonstrates the importance of RT sequences in selective primer packaging. Gag-Pol polyprotein, the precursor of RT, is involved in viral assembly and is not cleaved until budding, Gag-Pol precursor can be inferred to be involved in the primer tRNA selection (Figure 1). This hypothesis is supported by the following findings of Mak and colleagues (Mak et al., 1994). First, selective tRNA\textsubscript{\text{Lys3}} incorporation and wild-type amounts of tRNA\textsubscript{\text{Lys3}} were maintained in a protease-negative virus unable to process Pr55gag and Pr160\textsuperscript{\text{Gag-Pol}} precursors. This finding indicated that precursor processing was not required for primer tRNA incorporation. Second, viral particles containing only unprocessed Pr55gag protein did not selectively incorporate tRNA\textsubscript{\text{Lys}}, while virions containing both unprocessed Pr55gag and Pr160\textsuperscript{\text{Gag-Pol}} proteins demonstrated selective tRNA\textsubscript{\text{Lys3}}
packaging. Third, studies with a proviral mutant containing a deletion of most reverse transcriptase sequence and approximately one-third of the integrase sequence in the Pr160Gag-Pol precursor resulted in the loss of selective tRNA incorporation and an eight fold decrease in the amount of tRNA Lys per two copies of genomic RNA (Mak et al., 1994). In mutant avian sarcoma and murine leukemia viruses that lack RT, the selection of incorporated tRNAs becomes nonspecific.

4. Interaction between the primer tRNA and genomic RNA for initiation of reverse transcription

The 3' 18 nucleotides of tRNA Lys are complementary to the primer binding site (PBS) of HIV. Studies have shown that a minimum degree of complementarity needs to be maintained for efficient reverse transcription (Kohlstaedt et al., 1992). There are more copies of tRNA Lys than copies of genomic RNA in each virion. Consequently, some of the primer tRNA is bound to the genome and some is free.

Besides the tRNA-PBS interaction, several other interactions were also found and these are illustrated in Figure 5. The PBS sequences of HIV and ALV (Avian Leukosis Virus) are not sufficient determinants for the primer tRNA identity. Virions produced from cells transfected with proviral DNAs that have mutational changes in the PBS sequence making it complementary to other tRNAs show slow initial replication kinetics in HIV (Das et al., 1995; Li et al., 1994; Wakefield et al., 1995) or ALV (Whitcomb et al., 1995). However, after extended in vitro culture, the mutant genomes eventually revert
Figure 5. Proposed regions of base pairing between tRNA$^{Lys}$ and the HIV-1 genome.

In addition to the PBS interaction, other regions in the viral genomic RNA may interact with the tRNA. These regions include the A-rich regions upstream (Isel et al., 1996, Jiang et al., 1993) and downstream (Lanchy et al., 1996) of the PBS, which interact with the anticodon loop (shown by the two arrows), as well as an interaction of the TψC loop with a U5 region upstream of the PBS (Aiyar et al., 1992, Aiyar et al., 1994). Adapted from Mak et al. (1997).
to the wild-type PBS sequences complementary to the natural primers and the virions grow at wild-type rates again. The A-rich loop was found to play a role in determining the identity of the primer tRNA used by HIV-1. When the PBS of the HIV-1 genome was substituted with a sequence complementary to tRNA$^{\text{His}}$ rather than tRNA$^{\text{Lys}}$, the initial rate of viral replication was low, but after extended *in vitro* culture the production rates approached those of wild-type virus. This increased replication efficiency was accompanied by the reversion of the tRNA$^{\text{His}}$ PBS to the wild-type tRNA$^{\text{Lys}}$, indicating that tRNA$^{\text{Lys}}$ is eventually selected as the primer (Wakefield *et al.*, 1995).

5. **Role of nucleocapsid protein**

Since tRNAs are highly structured molecules, cofactors are required for the 3' end to base pair with the PBS of the viral genome. HIV-1 nucleocapsid (NC) protein was found to facilitate the annealing of tRNA$^{\text{Lys}}$ to *in vitro*-transcribed genomic RNA sequences (de Rocquigny *et al.*, 1992; Huang *et al.*, 1997; Cen *et al.*, 1999) probably by unwinding the secondary structure of tRNA$^{\text{Lys}}$ (Khan and Giedroc, 1992). In addition to primer tRNA annealing, the NC protein is also involved in genomic RNA packaging and dimerization, and minus-strand strong-stop cDNA strand transfer (Darlix *et al.*, 1995; Guo *et al.*, 1997).
B. Hammerhead ribozymes

1. Definition of ribozymes

Ribozymes are RNAs that possess enzymatic properties: They catalyze the endoribonucleolytic cleavage of RNA molecules. Upon formation of a complex with their complementary target RNA molecule, they catalyze the degradation of the target via a cleavage reaction. In this way, the substrate RNA can be inactivated at a catalytic rate and with a high degree of substrate specificity.

2. Classification of ribozymes

Ribozymes were initially discovered in a group I self-splicing intron of the *Tetrahymena* pre-rRNA (Kruger *et al.*, 1982) and in the RNase P enzyme purified from *E. coli* (Guerrier-Takada, 1983). There are several classes of naturally occurring ribozyme structures. The *Tetrahymena* ribozymes were described as molecules capable of autocatalytic cleavage during the RNA splicing process in *Tetrahymena* (Cech, 1987) and belong to the *Tetrahymena* group I intron family. Guerrier-Takeda *et al.* (1983) elaborated a second class of ribozyme structure, ribonuclease (RNase) P. RNase P is an endoribonuclease involved in the 5' processing of pre-tRNA molecules resulting in the formation of the mature 5' end of tRNA molecules (see next section for more details about RNase P). The third class of ribozyme structure is the hairpin ribozymes. They were identified in the minus-strand of the satellite RNA of the tobacco ringspot viroid (Hampel and Tritz, 1989). Upon hybridization of the ribozyme catalytic domain consisting of 50 nucleotides with the substrate strand, a characteristic hairpin-like structure forms. Hence, the name hairpin ribozymes. The fourth group of ribozymes shares a two-dimensional...
structural motif known as the "hammerhead." The hammerhead ribozymes have a common secondary structure consisting of three stems connected by three single-stranded regions. They have been identified in plant viroids, virusoids and satellite viruses. Another class of catalytically active ribozyme motif was found in hepatitis delta virus (HDV). The HDV contains a circular single-stranded RNA genome. The genome has extensive intramolecular basepairing potential and a self-cleaving domain has been identified within the HDV genome (Wu et al., 1989; Perrotta et al., 1990). An RNA molecule encoded by an 881-nt mtDNA plasmid isolated from Neurospora was also found to have self-cleaving activity (Saville and Collins, 1990).

3. **Hammerhead ribozymes**

The hammerhead ribozyme is a very small RNA motif that may cleave at a specific phosphodiester bond to produce 2',3' cyclic phosphate and 5' hydroxyl termini (Hutchins et al., 1986; Forster & Symons, 1987). Cleavage by hammerhead ribozymes is magnesium-dependent and is site-specific. The hammerhead consists of three helices connected by three single-stranded regions.

In nature, hammerhead ribozymes are utilized by a number of small pathogenic plant-viroid and satellite RNAs for processing during rolling-circle replication (Symons, 1992) and they act "in cis" (on the same strand of RNA). Later, the hammerhead ribozyme was designed to work "in trans" [work against other RNA molecules (Uhlenbeck, 1987)]. The trans-acting hammerhead ribozyme has two antisense arms (stems I and III) and a catalytic domain with a flanking stem II as well as a loop section. In the hammerhead
motif, a specific set of three nucleotides is required at the cleavage site. The triplet is defined by NUH (where N is U, G, A, or C, and H is U, C, or A), with cleavage occurring 3’ to H. The GUC triplet is the most commonly used site in nature (Forster & Symons, 1987) and is cleaved most efficiently (Ruffner et al., 1990). This is the target site of choice in designing a new ribozyme. However, the optimal triplet depends also on the composition and nucleotide lengths of stem I and III. Because –UH- occur very frequently in RNA (once in every six nucleotides on average), the hammerhead ribozyme can be designed to target any RNA. The ribozyme hybridizes to the complementary sequences flanking the –UH- cleavage site within the target RNA. The complementary sequences form stems I and III. If these two stems are short enough, the cleavage products can dissociate from the ribozyme easily, allowing the ribozyme to bind and cleave another molecule. By repeating the cycles, one ribozyme molecule can be recycled to cleave many substrate molecules. The length and sequence of stems I and III affect the dissociation of the hammerhead ribozyme from the substrate and affect the catalytic properties of the ribozyme. The optimal lengths of each section of stems I and III are 9-12 nucleotides (Herschlag, 1991). Other factors that should be considered in optimizing the efficiency of hammerhead ribozymes include substrate secondary structure, creation of A-rich sequences in the hammerhead ribozyme that minimize U-G wobble pairs, and increased discrimination against non-targeted mRNAs (Herschlag, 1991; Bertrand et al. 1994).
4. **Factors affecting hammerhead ribozyme cleavage *in vitro***

Forster and Symons (1987) defined a consensus sequence as the catalytic core of hammerhead ribozymes. Single base changes in each of the 14 conserved sites show some effect on catalytic efficiency (Ruffner *et al.*, 1990). Mild to significant loss of catalytic activity can result from any change in the consensus sequence of the ribozyme catalytic core (Ruffner *et al.*, 1990).

Magnesium is needed to stabilize the 5' oxygen leaving group (Taira *et al.*, 1990) probably due to its ability to stabilize the tertiary structure of the hammerhead complex. Spermidine has been found to lower the concentration of magnesium required for hammerhead ribozyme cleavage, suggesting that spermidine can replace magnesium in stabilizing the active ribozyme structure (Dahm *et al.*, 1991).

5. **Potential therapeutic applications of hammerhead ribozymes**

Ribozymes can target specific RNA molecules and each molecule has the potential to cleave multiple copies of a given RNA. The hammerhead ribozyme is one of the smallest ribozymes known and can have great potential in gene therapy. The effects of hammerhead ribozymes have been shown to be due to catalytic activity rather than to a simple antisense effect.

Traditional pharmaceuticals inhibit protein function. However, ribozymes either disrupt the flow of genetic information from DNA to protein or may even directly disrupt RNA genomes, as in the case of RNA viruses. Diseases that result from undesirable
expression of RNA, such as neoplastic disorders and viral illnesses, are amenable to such a therapeutic approach. Hammerhead ribozymes have been studied as a therapeutic agent for many viral infections as well as malignancies and genetic diseases.

Rossi and colleagues described the first use of a hammerhead ribozyme to inhibit HIV-1 replication in tissue culture cells (Sarver et al., 1990). In this study, a ribozyme targeting the gag sequence was stably expressed in HeLa-CD4^+ cells and the p24 levels were reduced compared with the non-ribozyme expressing cells upon infection with HIV. Hammerhead ribozymes developed against the HIV-1 tat and tat/rev genes (Zhou et al., 1994) have been in clinical trials for several years. There have been reports of hammerhead ribozymes targeting other HIV genes including vif (Lorentzen et al., 1991), int (integrase) (Sioud et al., 1991) and the 5' UTR leader sequences (Weerasinghe et al., 1991). Each of these ribozymes resulted in delays or reduction in virus expression.

Hammerhead ribozymes can be used for other viral infections such as the chronic management of chronic hepatitis B (HBV) infections (Wen et al., 1999; Kim et al., 1999; Hsieh and Taylor, 1992; Netter et al., 1993).

Since ribozymes can distinguish RNA transcripts that differ from the wild-type transcripts by only one nucleotide, they can be designed to eliminate mutated transcripts while leaving wild-type transcripts intact. One example is the mutant H-ras gene implicated in bladder carcinoma targeted by a hammerhead ribozyme (Tone et al., 1993; Kashani et al., 1992). Another example is the bcr-abl transcript caused by the t(9;22) translocation in chronic myelogenous leukemia (CML) (Wright et al., 1993; Shore et al., 1993).
1993). Other potential targets for ribozyme-based cancer therapeutics include the MDR-1 (multidrug resistance gene) transcripts, topoisomerase α, methylguanine methyltransferase, dihydrofolate reductase, bcl-2, bcl-x and certain cytokines such as interleukin 6 and 9.

6. Problems that underlie successful ribozyme applications

Development in the following areas are prerequisites for successful ribozyme-based strategies: 1) persistent high-level expression in transduced cells; 2) targeting of the ribozyme into the correct subcellular compartment and colocalization of the ribozyme with its substrate; and 3) appropriate folding of the ribozyme for catalytic activity; and, 4) stable intracellular maintenance of the ribozyme.

C. Promoters for expressing ribozymes

1. RNA polymerase II promoters

Hammerhead ribozymes can be expressed by insertion under transcriptional control of RNA polymerase II promoters (Pol. II promoters), that may be of viral origin, a retroviral long terminal repeat, or a strong endogenous promoter. Ribozymes can be inserted into the untranslated regions of genes transcribed by RNA polymerase II which have strong promoters, such as the SV 40 early promoter (Cameron et al., 1989), the CMV IE-1 promoter (Mahieu et al., 1994), a retroviral long terminal repeat (Koizumi et al., 1992) and the β-actin gene promoters (Sarver et al., 1990). A polyadenylation signal needs to be added to the 3’ end of the gene to enable transcription termination and the
addition of the poly(A) tail. The poly(A) tail, together with the m7G cap, can increase the stability of the transcripts and facilitate the transport of the transcripts to the cytoplasm. However, the RNA polymerase II promoter is generally not suitable for production of short RNAs (Sanfacon et al., 1990). Extra sequences need to be added to both ends of the hammerhead ribozymes and several hundred nucleotides between the promoter and the terminator are needed to ensure effective transcription and termination at the correct sites. However, the extra sequences may have undesirable effects on cleavage activity (Chowrira et al., 1994; Cameron and Jennings, 1989). Expression of the ribozymes under the RNA polymerase II promoter does have the advantage of the availability of tissue-specific as well as regulatable promoters. When ribozyme expression is desired only in certain tissues or when expression needs to be turned on or off within a given tissue, RNA polymerase II promoters are the promoters of choice.

2. RNA polymerase III promoters

RNA polymerase III promoters (Pol III promoters) direct transcription of a variety of small nuclear and cytoplasmic RNAs that are abundant in all cell types. RNA polymerase III promoters have been widely used to express short RNA molecules, including antisense sequences, ribozymes and RNA decoys. The promoters used include the human snRNA U6 gene promoter, adenovirus-associated RNA 1 (Ad VA1) gene promoter, as well as tRNA (mainly tRNA\textsuperscript{Met} and tRNA\textsuperscript{Val}) promoters. Advantages of Pol III promoters over Pol II promoters include: 1) RNA Pol III transcribed transcripts are more abundant than poly(A)\textsuperscript{+} RNAs transcribed from Pol II promoters (Palmer et al., 1990); 2) RNA Pol
III-transcribed genes can be ubiquitously expressed; and 3) Pol III transcripts are short, thereby minimizing the possibility that the ribozyme activity may be masked in a long transcript.

D. The processing of tRNAs

Transfer RNAs are small (about 76 nucleotides) molecules that possess a cloverleaf secondary structure and maintain a compact tertiary L-shaped structure. They are transcribed as larger precursors with redundant 5’ end leaders and 3’ end trailers and must be processed through a series of steps by different enzymes to yield the mature tRNAs. These processing pathways, which differ in different systems, include 5’ and 3’ removal of extra sequences, the addition of CCA to the 3’ end, nucleotide modifications at specific residues, and in some tRNA gene transcripts, splicing of intervening sequences (Deutscher, 1984; Westaway and Abelson, 1995).

1. 5’ processing of tRNAs

Ribonuclease P (RNase P) is the endonuclease required for generating the mature tRNA 5’-end. Because of its role in the biosynthesis of tRNA, it is an essential enzyme for cell growth. This enzyme is present in representatives of all three domains of life (Archaea, Bacteria and Eucarya) as well as in mitochondria and chloroplasts. RNase P cleaves precursor tRNAs (pre-tRNAs) specifically by phosphodiester hydrolysis. The cleavage is endonucleolytic and the products of pre-tRNA cleavage retain 3’ OH and 5’ phosphate groups (Guerrier-Takada, 1983). Divalent metal ions (preferably Mg$^{2+}$) are
absolutely required for activity. Cellular RNase Ps are complex enzymes composed of essential RNA and protein subunits (Darr et al., 1990). The structural and functional organization of RNase P differs significantly across phylogenetic domains. The RNA component of bacterial RNase P is catalytically active in the absence of the protein subunit and is considered to be a ribozyme. In the case of nonbacterial RNase P, ribozyme activity has not been demonstrated although the RNA component is essential for catalytic activity (Frank and Pace, 1998).

There are about 60 precursor tRNA substrates for RNase P. Nevertheless, there is no sequence homology around the cleavage site in any of these. In the case of one precursor tRNA isolated, RNase P was found to cut at the 5' side of the first nucleotide of the tRNA domain and the upstream sequences were released. In other natural RNase P substrates in *E. coli*, such as 4.5 S RNA or 10S RNA, RNase P cuts at completely different sequences, but at similar sites in the proposed secondary structures (Altman, 1995).

2. **3' processing of tRNAs**

The 3' trailer of mammalian pre-tRNA is removed by the 3' processing endoribonuclease (3' tRNase) which cleaves the RNA immediately downstream of the discriminator nucleotide (which is the unpaired nucleotide 3' of the last base pair of the aminoacyl acceptor stem) onto which CCA residues are added to produce mature tRNAs. The CCA sequence is required for aminoacylation.
In prokaryotes, the 3' trailers of tRNAs are removed by a redundant family of 3'-exonucleases (reviewed by Deutscher, 1995). The CCA sequence is transcriptionally encoded. The 3'-exonucleases which remove the 3'-trailer can produce a mature tRNA that is ready for aminoacylation. The CCA-adding enzyme, tRNA nucleotidyl transferase (tNtase), is also present in prokaryotes. However, it is not required for viability but rather principally performs a repair function in prokaryotes (Deutscher, 1995).

In eukaryotes, precursor tRNAs are subject to 5' and 3' processing in the nucleus (Figure 6). The 3' processing endoribonuclease (3'-tRNase) is responsible for trimming the 3' trailer. 3'-tRNase can endonucleotically remove the 3' trailer by cleaving at the 3' side of the discriminator base (Solari & Deuscher, 1983; Castaño et al., 1985; Frendewey et al., 1985). 3'-exonuclease may sometimes be involved (Furter et al., 1992; Yoo & Wollin, 1997). In addition, the CCA-adding enzyme, tRNA nucleotidyl transferase (tNtase), is essential for eukaryotic viability because the CCA sequence is not transcriptionally encoded in eukaryotic tRNA genes (Sprinzl et al., 1998). CCA must be added by the tNtase.

It was established that mature tRNA (tRNA-3'+ CCA) is not a 3'-tRNase substrate (Nashimoto, 1997; Nashimoto et al., 1999). The 3'-tRNase is also extremely poorly inhibited by tRNA+CCA, ensuring that the 3'-tRNase can remain free to process precursor tRNAs with 3'end trailers and tRNAs can progress to aminoacylation. The
Figure 6. Substrate and products in the eukaryotic tRNA end-processing pathway.

A: Precursor *Drosophila* tRNA\textsuperscript{His} has a 16-nt 5' end leader and a 36-nt 3' end trailer. B: The 5' end leader has been catalytically removed by RNase P, producing the 3'-tRNase substrate. C: The 3' end trailer has been endonucleotically removed by 3'-tRNase, producing the tNtase substrate. D: tNtase has added CCA to the 3' end of substrate tRNA, using CTP an ATP as additional substrates (adapted from Mohan *et al.*, 1999).
active site of 3'-tRNase was postulated to have evolved to make an especially poor fit with tRNA+CCA (Mohan et al., 1999; Nashimoto, 1995).

E. The transport of tRNAs

Despite the short sequence of tRNAs, the biogenesis of functional and mature tRNAs is amazingly complicated. In addition to removal of the 5' leader and 3' trailer sequences and CCA addition, the tRNA molecules also undergo a complex set of base modifications by a series of enzymes that recognize specific features of tRNA structure. A subset of pre-tRNAs also contain intervening sequences (introns) and these have to be removed by a dedicated set of tRNA splicing enzymes. The processed tRNAs then must be exported from the nucleus to the cytoplasm and must be aminoacylated before they can participate in protein synthesis (see chapters in Söll et al., 1995 for reviews).

Transport of protein and RNA molecules through the nuclear pore has recently shown to be mediated by energy dependent receptors that are saturable and recognize specific signals on the various cargo molecules (reviewed in Izaurralde and Adam, 1998; Mattaj and Englmeier, 1998; Weis, 1998). The receptors are recycled to initiate additional rounds of transport after the cargoes are delivered. All nuclear import and export receptors thus far identified fall into the importin β superfamily and bind to the GTPase Ran (Fornerod et al., 1997; Görlich et al., 1997), regardless of the differences in the transported substrates. The substrate associates with the receptor on one side of the nuclear pore envelope and dissociates on the other. The GTPase Ran imparts directionality of either the import or the export process by acting as a molecular switch for receptor-cargo interaction (see below for more details of exportin-t receptor).
Export of tRNAs to the cytoplasm is no exception. Mature tRNAs were recently found to be recognized by a specialized receptor called exportin-t. This receptor has been identified as the receptor responsible for mediating the export of tRNAs from nucleus to the cytoplasm (Arts et al., 1998a, Kutay et al., 1998). Exportin-t was found to shuttle rapidly between the nucleus and cytoplasm and binds tRNA in a RanGTP-dependent manner. Ran binds GTP but the GTP-bound form can change to a GDP form by GTP hydrolysis and nucleotide exchange. This is catalyzed by the GTPase activating protein RanGAP1 that is only present in the cytoplasm. Thus, RanGTP is depleted from the cytoplasm. In the nucleus, RanGTP is generated by the nucleotide exchange factor RCC1. Therefore, RanGTP is thought to exist in higher concentration in the nucleus than in the cytoplasm. This gradient leads to loading of tRNA in the nucleus and, after passing through the nuclear pore complex, unloading in the cytoplasm. The correct tRNA shape and the TψC loop were recently reported to be critical for exportin-t binding (Lipowsky et al., 1999; Arts et al., 1998b). For reviews on tRNA export, see Simos and Hurt, 1999 and Wolin and Matera, 1999.

F. Goals, hypothesis, and objectives

Various tRNAs are used by different retroviruses as primers for initiating reverse transcription (Table 1). The primer employed by HIV is host tRNA^{Lys3}. The primer tRNA^{Lys3} can be recruited from the host cell cytoplasm and become encapsidated during packaging of HIV virions. This recruitment process has been shown to be mediated by interaction of tRNA^{Lys3} with the reverse transcriptase (RT) sequence in the precursor polyprotein Pr160^{Gag-Pol} (Mak et al., 1994).
Work in this laboratory (Sarver et al., 1990; Taylor et al., 1991 and 1992; Bertrand et al., 1994; Zhou et al., 1994 and 1996; Westaway 1995 and 1998; see Rossi, 1999 for review) and other laboratories (Lorentzen et al., 1991; Sioud et al., 1991; Weerasinghe et al., 1991) showed that ribozymes targeting different sequences of HIV can inhibit the infection of tissue cultured cells by HIV (see more details in section B (Hammerhead ribozymes) of this chapter).

Since tRNA can be used to express ribozymes and is packaged into HIV virions during encapsidation, we hypothesized that a ribozyme sequence could be inserted downstream of the tRNA^{Lys3} gene and used for inhibition of HIV replication. We therefore designed the ribozyme to target immediately upstream of the primer binding site (PBS) of HIV-1. The expression of such a construct inside cells would give rise to chimeric tRNA^{Lys3}-ribozyme transcripts. Computer RNA folding predicted that the chimeric transcripts could maintain their native tRNA structure. Thus we hypothesized that chimeric transcripts would be capable of interacting with the precursor of RT, the Pr160^{Gag-Pol} polyprotein, and become packaged into virions. The tRNA moiety in such a construct is expected to function both as an expression cassette as well as a carrier for bringing the ribozyme into virions. If the chimeric tRNA^{Lys3}-ribozymes are expressed at sufficient levels, they would also function as decoys of the natural primer tRNA^{Lys3} and competitively bind to the RT sequence of Pr160^{Gag-Pol} during packaging, thereby reducing the copy number of tRNA^{Lys3} packaged.
To test the above strategy, several problems had to be addressed, some of which were experimentally examined before I came into the laboratory. These problems were:

1. Are transcripts from the chimeric tRNA_{Lys}^\text{Ly}-ribozyme construct capable of binding reverse transcriptase, the presumed prerequisite for the chimera to be packaged into HIV virions? Computer RNA folding modeling suggested a positive answer. Chimeric tRNA_{Lys}^\text{Ly}-ribozyme with a G deletion in the TwC stem can bind to the RT with an affinity similar to that of wild type tRNA_{Lys}^\text{Ly} (Westaway et al., 1995).

2. Is the construct effective in inhibiting HIV? Chimeric tRNA_{Lys}^\text{Ly}-ribozyme with a deletion of one G in the TwC stem was demonstrated to be effective in reducing the infectivity of a viral stock that was produced from transiently transfected cells bearing the chimeric gene (Westaway et al., 1995). Ribozyme with one G deleted was also able to be exported into the cytoplasm, a prerequisite also for the ribozyme to get packaged into the virions and inhibit HIV.

To help achieve the laboratory's goal of an effective chimeric tRNA_{Lys}^\text{Ly}-ribozyme approach for therapeutic treatment of HIV infection, I identified the following objectives for my research.

1. To establish the encapsidation and packaging of the chimeric tRNA_{Lys}^\text{Ly}-ribozymes.

2. To enhance the expression of the chimeric transcripts by using dual RNA polymerase III promoters: human U6 small nuclear RNA gene promoter (extragenic) and tRNA intragenic promoter (intragenic) and test the feasibility of using hybrid promoters by quantitatively analyzing the expression. The level of expression from such a system is hard to predict because there may be steric hindrance between transcription factors bound to the different promoter elements. The expression level may be higher, the
same or even lower than expression from tRNA promoter alone. The initiation of transcripts may change due to the steric hindrance.

3. To establish enhanced HIV inhibition by the hybrid U6/tRNA promoter expression cassette if the level of expression is increased.

4. To determine the intracellular localization of transcripts made from the chimeric tRNA$^{1\text{ys}3}$-ribozymes under the tRNA promoter alone and under the dual promoters.

5. To determine the stability of the transcripts in transient and stable expression systems.

6. To express the chimeric tRNA$^{1\text{ys}3}$-ribozymes in stable cell lines either by transfection or transduction of ribozymes cloned into AAV vectors.

7. To compare active with inactive ribozymes to establish the benefit of ribozyme versus antisense effects in HIV inhibition.

8. To compare transcription from constructs with a wild type versus a mutant B box in the tRNA gene.
II. MATERIALS AND METHODS

A. Materials

1. Cells

Human embryonic kidney 293 cells were obtained from the American Type Culture Collection (Rockville, MD).

2. Reagents

Fetal calf serum was purchased from Irvine Scientific, Inc., (Irvine, CA). G418 was obtained from GIBCO/BRL-Life Technologies, Inc. (Gaithersburg, MD). MEGAscript™ Kit and T7-MEGA ShortScript Kit were purchased from Ambion Inc. (Austin, TX).

Oligonucleotides were synthesized by the DNA Core Facility of the Center for Molecular Biology and Gene Therapy of Loma Linda University (Loma Linda, CA) or DNA/Peptide Core Facility of the City of Hope Medical Center (Duarte, CA).

3. Solutions

*Deionized formamide:* Formamide was melted at room temperature and 50ml was deionized with 5 g AG 501 X 8 resin (BioRad) by stirring for 30 min at room temperature. The solution was filtered twice with Whatman #1 paper, aliquotted and stored at −20°C.

*Denhardt’s 50 X stock:* 1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin. The solution was then filtered through a disposable 0.22 micro Nalgene filter.
10 X MOPS: 0.2 M 3-(N-morpholino) propanesulfonic acid, 5mM sodium acetate, 5 mM EDTA, pH 7.0.

DEPC water: 0.1% diethyl-pyrocarbonate was added to double-distilled water overnight. The treated water was then autoclaved.

RNA loading buffer: 80% (vol/vol) formamide, 1 mM EDTA, pH 8.0, 0.1% bromophenol blue, 0.1% xylene cyanol.

20 X SSPE: 3.6 M NaCl, 0.03 M NaH$_2$PO$_4$, 0.02 M EDTA, pH 7.4.

20 X SSC: 3.0 M NaCl and 3.0 M sodium citrate, pH 7.0.

10 X TBE: 0.4 M Tris-HCl, pH 8.0, 0.4 M boric acid and 0.012 M EDTA, pH 8.3

TE buffer: 0.01 M NaOH, 0.01 M Tris-HCl, pH 8.0, 1 mM EDTA and 0.1% SDS

TBE loading buffer: 20% glycerol, 80% 10XTBE, 0.4% bromophenol blue and 0.4% xylene cyanol.

4. Miscellaneous Materials

The dNTPs and NTPs were purchased from Pharmacia (Piscataway, NJ). QIAprep Miniprep Kit, QIAprep Maxiprep Kit, QIAquick Gel Extraction Kit, QIAquick PCR Purification Kit and QIAquick Nucleotide Removal Kit were purchased from Qiagen Inc. (Chatsworth, CA). The pBluescriptIISK(+) plasmid was purchased from Stratagene (San Diego, CA). Plasmids pTZU6+1 and pAAVU6+1 were constructed by Edourad Bertrand (Bertrand et al., 1997).
B. Methods

1. Restriction enzyme digestion

Plasmid DNA or PCR products were digested with restriction enzymes from New England Biolabs (Beverly, MA) or Boehringer Mannheim Corporation (Indianapolis, IN). Appropriate amount of DNA in 20 to 50 µl volumes was incubated with 1 to 5 U enzyme/µg DNA at 37°C or at the manufacturer’s suggested temperature for 1 to 4 h. After digestion, the reaction mixture was analyzed by agarose gel electrophoresis.

2. Ligation of DNA

T4 DNA ligase (New England Biolabs) was used for ligation. Approximately 400 ng of total DNA was used in 10 to 20 µl reaction volumes with vector-to-insert ratios between 1:2 to 1:5. Ligation reactions were incubated at 16°C for at least 15 h.

3. Preparation of competent cells and transformation (DMSO/PEG method of Cung and Miller, 1988)

Preparation of competent cells: Bacteria were grown to the early log phase (OD600 of 0.3-0.6) in LB broth or terrific broth and then collected by centrifugation at 1000 X g for 10 min at 4°C. Bacteria were resuspend in 1/10 volume of transformation and storage buffer (TBS) in LB broth pH 6.1 [10% PEG (MW 3350), 5% DMSO, 10mM MgCl₂ and 10mM MgSO₄, filter sterilized (0.22 micron), stored at 4°C]. Bacteria were then incubated on ice for 10 min and frozen in prechilled tubes at −70°C for later use.

To clone the chimeric tRNA^{Lys}_3-ribozymes into the AAVU6+1 vectors. I used commercially available Max Efficiency Stbl2 Competent Cells from Life Technologies.
Plasmids with repeat sequences may not be stable in regular bacterial strains. This bacterial strain has the potential to stabilize certain plasmids. AAV vectors have two inverted terminal repeats (ITRs) and could remain more stable in this strain.

Bacterial transformation: One hundred microliters of competent cells were incubated with 100 pg of DNA for 5-30 min at 4°C. Then 0.5-0.9 ml of SOC medium was added and the cells were incubated at 37°C in a shaker for 60 min. Cells were plated on antibiotic plates and incubated at 37°C. (Expected transformation efficiency: \(2 \times 10^8\) transformants/microgram of DNA.)

4. **Frozen bacterial of stocks**

In screw cap tubes, 930 μl rich-grown culture (16-18 h of culture) were mixed with 70 μl of DMSO. After mixing, the tubes were transferred to dry ice for 10 to 20 min, then stored at −70°C.

5. **Plasmid preparation**

When plasmid preparations were used for identifying clones with the correct inserts, minipreps of plasmids were prepared with the QIAprep Miniprep Kit (Qiagen). When plasmid preparations were used for transfections, maxipreps were prepared with the Qiagen Plasmid Maxi Kit. Minipreps and Maxipreps were done according to the manufacturer’s instructions.
6. **Agarose gel electrophoresis of DNA**

DNA samples of appropriate quantity were mixed with 1/6 volume of 6X sample loading buffer. 0.8-2% (wt/vol.) agarose was dissolved in TE buffer. For visualizing smaller fragments (100-200 bp, such as the chimeric tRNA\(^{\text{Lys3}}\)-ribozymes), 2% agarose gels were usually used. For bigger fragments, 1% agarose gels were usually used. The DNA samples were loaded in wells and current was applied (approximately 90-100 V for 30-45 min). Thereafter, gels were removed from the electrophoresis apparatus (BioRad) and incubated in TE buffer containing ethidium bromide (0.5 µg/ml final concentration) for 15 min. DNA bands were observed and analyzed using an ultraviolet transilluminator or an AlphaImager\textsuperscript{TM} 2000 system (Alpha Innotech Corporation).

7. **Denaturing acrylamide gel**

For most applications related with the chimeric tRNA\(^{\text{Lys3}}\)-ribozymes, a 6% denaturing acrylamide gel was used. For a total volume of 60 ml gel, add together 25.2 g urea, 9 ml of 38% acrylamide/2% bisacrylamide, 6 ml of 10 X TBE and 24 ml of water.

For a minigel, 5-7 ml of gel solution was normally used. The gel was polymerized with 8 µl of TEMED and 40 µl of 10% ammonium persulfate. The samples were electrophoresed at about 200 volts for about 20 min.

For a medium-sized gel, 30 ml of gel solution was used. The gel was polymerized with 30 µl of TEMED and 144 µl of ammonium persulfate. After a prerun, the gel was loaded with samples and run at 25 mA for 1 to 2 hr.
8. Native (non-denaturing) acrylamide gel

Thirty milliliters of 4% 60:1 acrylamide native (non-denaturing) gel was prepared as follows.: 4 ml 30% 60:1 acrylamide/bisacrylamide, 0.75 ml 10 X TBE, 3 ml 50% glycerol were mixed together with water to a total volume of 30 ml. Then 30 μl TEMED and 144 μl 10 ammonium persulfate were added to catalyze gel formation. The gel was electrophoresed at 4°C at 250 V for about 3 hr for this medium sized gel.

9. Radioactive oligonucleotide probe labeling

Oligonucleotides were diluted to 5 pmol/μl. A 10 μl reaction was setup as follows: 2 μl (10 pmol) of oligonucleotide, 1 μl 10X T4 PNK buffer, 6 μl of water, 0.5 μl of [γ-32P ATP] and 0.5 μl of T4 PNK kinase were mixed. The reaction were then incubated at 37°C for 30 min. Then the kinase were heat inactivated at 90°C for 2 min. The tube was placed on ice and 10 μl water was added to a final concentration of 0.5 pmol/μl.

10. Primer extension

1) Oligonucleotide primer was labeled as described in method 9 (above).

2) Annealing (hybridization): 1 μl (0.5 pmol) of 32P-labeled primer was mixed with 5-50 μg of total RNA as follows: 1 μl labeled oligo, 1 μl RNA, 6 μl water and 3 μl 5X AMV RT buffer. After the mixture was heated to 80°C for 3 min, it was cooled to 37°C over a 90 min period.

3) Primer extension: To the 11 μl annealing mixture, 3 μl 5X dNTP mix (5x dNTP solution: 2mM each dNTP in water) was added. Then1 μl of AMV RT was added and the reaction was incubated at 37°C for 30 min. At the end, an equal volume of
sample loading buffer was added and the reaction was then heated to 90°C for 2 min and chilled on ice. The reaction was then analyzed in a denaturing acrylamide gel (load about 4-5 μl sample per lane).

11. In vitro transcription

*In vitro* transcription was carried out with MEGAscript, MEGAscript or MAXIscript kits (Ambion) according to the manufacturer’s instructions. Alternatively, *in vitro* transcription was carried out as follows. An appropriate amount of DNA template was used (about 1 μg for a 3-4 kb plasmid). A 20 μl reaction was prepared from an appropriate volume of DNA template, 2 μl 10X NEB RNA polymerase buffer, 1 μl of NTP mix (10 mM each of ATP, CTP and GTP, 0.5 mM final concentration), 1 μl of 1 mM UTP (0.05 mM final concentration), 1 μl of 32P-UTP and 1 μl of T7 or T3 RNA polymerase (NEB, 50 u/μl) and water to make the total volume 20 μl. The reaction was incubated at 37°C for 2 hr.

12. In vitro transcription for preparing radioactive RNA transcripts

1) Preparation: Linearize DNA with a restriction enzyme downstream of the desired transcripts. MEGAscript kit (Ambion) may be used for this purpose. Make a ‘transcription mix’ with enough volume for number of reactions desired. Mix 2 μl each of ATP, CTP, GTP, and 10X transcription buffer for each reaction planned. Add 1/8 vol. (0.25 μl) of UTP (1/8 of 2 μl times number of reactions planned). Dispense 8.25 μl into each reaction tube.
2) *In vitro* transcription: Into 8.25 μl of the ‘transcription mix’, add 1 μg of linearized plasmid, 2 μl of [α-32P]-UTP (3000 Ci/mmol), 2 μl of the Enzyme mix of T7 or T3 MEGAscript kit (Ambion) or appropriate amount of T7 or T3 polymerase and add water to a final volume of 20 μl. Incubate at 37°C for 2-2.5 hr. Add 1 μl of DNase I; incubate at 37°C for 15 min.

3) Purification: Separate the *in vitro* transcribed RNA with free nucleotides in a 6% denaturing acrylamide gel. Expose the gel for 5 min on an X-ray film to determine the position of the desired transcripts. Cut out the corresponding gel piece and crush into very fine pieces on a clean Petri dish. Elute the gel pieces overnight in 350 μl of elution buffer (0.5M NH₄OAc, 1mM EDTA, 0.1% SDS) at 25-37°C. Pour the eluted contents into Costar Spin-X centrifuge filter unit. Centrifuge for 3 min at maximum speed using a microcentrifuge. Add 1 μl of glycogen (Boehringer), 1/10 volume 3M NaAc and 2.5 volume ethanol to the supernatant. Centrifuge and wash the pellet with 70% ethanol. Resuspend in 20 μl RNase free water or TE buffer. Measure an aliquot for cpm and OD to estimate the specific activity and concentration of the transcripts.

13. Chimeric tRNA^{Lys3}-ribozyme-RT binding assay

*In vitro* transcribed chimeric tRNA^{Lys3}-ribozyme transcripts from WW and MW were transcribed and labeled with 32P-UTP as described above. Radioactive labeled transcripts from tRNA^{Lys3} and tRNA^{Val} were obtained in the same way and used as controls. The molar concentrations of the transcripts were calculated for HIV RT (NIH Research Reagents Repository) and for these transcripts. The activity of the HIV RT was tested in a primer extension assay. The binding was carried out at the following condition: 20mM
Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 5% glycerol. A 5X binding buffer was prepared. The binding reactions were carried out as follows: 5X binding buffer 2 μl, RNase inhibitor (Boehringer Mannheim) 0.5 μl, RNA transcripts 0.4 pmol - 2pmol (about 7000 cpm) and HIV RT 2 pmol were added together. The binding reactions were left at room temperature for 1 hour. The binding reactions were separated in a 4%, 60:1 acrylamide native (non-denaturing) gel, as previously described. The gel was then dried, subjected to autoradiography at -80°C and then analyzed.

14. Preparation of radioactive labeled size markers

(according to Shawn Westaway, personal communication)

Plasmid DNAs with known sizes were digested with restriction enzymes (an example is pBSSK+ digested with Hpa II). To 1 μg of precut markers or commercially available DNA ladders, 2 μl of 10X Klenow buffer (minus DTT), 2 μl of 0.1 M DTT, 1 μl Klenow (Boehringer Mannheim, 2 U/ml) and 1 μl [α-32P] dCTP at 10 μCi/μl were added. Then water was added to a total volume of 20 μl. The reaction was incubated at 37°C for 45 min. The markers were purified by phenol extraction and ethanol precipitation, then dissolved in 40 μl water. The radioactivity of a 1 μl sample was determined (cpm should be 500,000 to 1,000,000 cpm per μl). The samples were diluted 10-fold to 50,000 to 100,000 cpm/μl and 1 – 5 μl per lane were loaded onto the appropriate gel system.

15. PCR (polymerase chain reaction)

In a 50 μl reaction, add appropriate amount of template DNA, 5 μl of 10 X PCR buffer, 5 μl of 2.5 mM dNTPs, 50 pmol each of 5’ and 3’ primer and then add water to a
final volume of 49 μl. Add 50 μl mineral oil on top and preheat the samples to 94°C and add 1 μl Taq polymerase. The PCR reactions were carried out at 94°C, 40°C and 72°C for 30 cycles and an additional extension was usually performed for 10 min at 72°C.

16. RT-PCR

When total RNA from transiently transfected cells were used, the following procedure was followed. Total RNA was diluted and 0.1 μg total RNA was used as template. In a 50 μl reaction, 15 or 30 pmol of 5’ and 3’ primers, 5 μl 10 X PCR buffer, 8 μl of dNTP mix (1.25 mM each of dATP, dGTP, dCTP and dTTP; 0.25 mM final concentration) were added. Then an add appropriate amount of water was added to make the total volume to 49 μl. Heat sample to 90°C and let cool to room temperature over a 10 to 15 min period (some primers may give better result with a slower cooling). Also a quick (1 to 3 min) cooling may be sufficient. Put all samples on ice and add 1 μl (1 U/μl or 2 U/μl) AMV RT (For each sample, one control reaction without adding RT is necessary to rule out amplification from contaminating DNA templates). Transfer all tubes to a 37°C (37°C to 55°C) water bath and incubate 3 to 5 min. Then transfer to 95°C, incubate 5 min. Put back on ice and add 1 μl Taq. The PCR reactions were carried out at 94°C, 51°C and 72°C for 30 cycles and analyzed the products on an agarose gel.

17. Design and construction of chimeric tRNA<sup>lys</sup>-ribozymes

The original DNA fragments used for cloning into pBluescript were synthesized from annealing of several oligonucleotides and amplification by a PCR. The sequence of the DNA fragments for making the original clones is shown in Figure 1, with mutations
Figure 1. Sequence of the DNA fragments obtained by the PCR reactions used for cloning of the chimeric tRNA\textsuperscript{Lys3}-ribozyme constructs into the pBluescript.
indicated by arrows. The construction of the chimeric tRNA\textsubscript{Lys}\textsuperscript{3}-ribozymes was performed as follows. Gene fragments of the chimeric tRNA-ribozymes were synthesized by polymerase chain reactions (PCR) from oligonucleotides with \textit{Sal} I and \textit{EcoRI} restriction sites at the 5' and 3' ends, respectively. The following oligonucleotides were used (mutations introduced were shown in boldface letters):

(A) 5'-GTCTGATGCTCTACCGACTGAGCTATCCGGCGGTCCGAC- 3' 
(B) 5'-GTAGAGCATCAGACTTTTAATCTGAGGGTCCAGGTTTC- 3' 
(C) 5'-GTAGAGCATCAGACCTTTAATCTGAGGGTCCAGGCTAC- 3' 
(D) 5'-GCGCCCGAAAGGGGACTTGAACCCCTGGGACCT- 3' 
(E) 5'-GCGCCCGAAGGGGACTTGTAGGCTGGGACCT- 3' 
(F) 5'-CCCTGTTCGGGCGCCACTGCTAGACTGATGATCGGCCTGAGG- 3' 
(G) 5'-CGGAATTCCGCGGAAAAATGGCAAATTTGCCTCTACGGACTCAT- 3' 
(H) 5'-CGGAATTCTCCGCGGAAAAATGGGAAAATTTTCCTCCTACGGAACACTCAT- 3' 
(I) 5'-ACGCGTCGACGCCGGGATAGCCTCAGTTCGCG- 3'

Four annealing and extension reactions were performed with 5 pmol each of the following oligonucleotides: A, B, D, F, G for wild type tRNA\textsubscript{Lys}\textsuperscript{3}-wild type ribozyme (WW), A, C, E, F, G for mutant tRNA\textsubscript{Lys}\textsuperscript{3}-wild type ribozyme (MW), A, B, D, F, H for wild type tRNA\textsubscript{Lys}\textsuperscript{3}-mutant ribozyme (WM) and A, C, E, F, H for mutant tRNA\textsubscript{Lys}\textsuperscript{3}-mutant ribozyme (MM). The mixture was denatured at 94°C for 2 min, annealed at 40°C for 2 min and extended at 72°C for 10 min in the presence of 250 mM dNTPs and 2 U Taq polymerase in 1X Taq polymerase buffer (Boehringer).
Aliquots (5μl) from each of the extension reactions were used as templates for subsequent PCRs. Fifty picomoles of oligonucleotides I and G were used as the 5’ and 3’ primer for WW and MW, and oligonucleotides I and H for WM and MM. The PCRs were carried out for 30 cycles (94°C for 1 minute, 40°C for 1 minute and 72°C for 2 min) in the presence of 250 mM dNTP and 2 U Taq polymerase. The PCR products were purified with phenol/chloroform extraction, precipitated with ethanol in the presence of 0.3 M sodium acetate, dissolved in TE buffer and analyzed on 2% agarose gel. PCR products were digested with Sall and EcoRI, and cloned into the same sites in pBluescriptSK (+) (Stratagene, La Jolla, CA) to create the plasmid pWW, pMW, pWM and pMM. pΔGTRz is also a chimeric tRNA-ribozyme with a deletion of one G at the ΨΨC stem loop junction of tRNA^Lys3 (Figure 1), which was introduced fortuitously and was described previously (Westaway et al., 1998; the plasmid was named pΔGtRNA^Lys3-ribozyme in this thesis).

Plasmids pWW, pMW, pWM and pMM were digested with Sall and PstI and tRNA^Lys3-ribozyme fragments were gel purified and ligated into the pTZU6+1 vector (Bertrand et al., 1997) between the Sall and PstI sites downstream of the U6+1 promoter, which contains the entire U6 promoter sequence plus one nucleotide of the U6 snRNA structural gene. These constructs represent the dual U6/tRNA promoters series of plasmids pU6WW, pU6MW, pU6WM and pU6MM.

Plasmids pWW, pMW and pWM were digested with Sall and SpeI and the tRNA^Lys3-ribozyme fragments were gel-purified and ligated into the pAAV U6+1 vector (Bertrand...
et al., 1997) between the SalI and SpeI sites downstream of the U6+1 promoter. These ligations created the plasmids pAAVU6WW, pAAVU6MW and pAAVU6WM.

pU6ΔGTRz was constructed from pAAVU6ΔGTRz (Westaway et al., 1997; renamed pAAVU6+1ΔGtRNA^{Lys3}-rbz). pAAVU6ΔGTRz was digested with SalI and XbaI and the ΔGtRNA^{Lys3}-ribozyme fragment was gel-purified and ligated into the pTZU6+1 vector (Bertrand et al., 1997) between the SalI and XbaI sites downstream of the U6+1 promoter.

pU6Rz was constructed from the following two oligonucleotides:

5' -ACGCGTCGACCTGCTAGACTGATGAGICCGTGAGGACGAA-3'
5' -TGCGTCTAGACGCGGAAAAATGGCAAATTTCGTCCTCACG-3'

These two oligonucleotides have 12 bases of complementarity at their 3' ends. These were annealed and amplified by the polymerase chain reaction (PCR) as described previously. The final PCR product was analyzed and purified with a Nucleotide Removal Kit (Qiagen), digested with SalI and XbaI and ligated into the SalI and XbaI sites of pTZU6+1 vector.

18. Cell culture and transfections

Human 293 cells were grown in DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, and 1 mM sodium pyruvate. Cells were plated at 70% confluence in a 100 mm tissue
culture dish the day prior to transfection. The medium was changed 3 hr before transfection. Cells were transfected with the CellPhect Transfection Kit (Pharmacia) according to the manufacturer’s instructions. 10 μg of chimeric ribozyme plasmids were cotransfected with 1 μg of pTKGH (human growth hormone hGH gene under the control of the HSV TK promoter). 48 hr later, the concentration of growth hormone in the supernatant was measured using the HGH-TGES 100T Kit (Nichols Institute Diagnostics) according to manufacturer’s instructions in order to normalize transfection efficiencies (see below). Alternatively, 5 μg of a plasmid expressing GFP (green fluorescent protein) was cotransfected for normalizing transfection efficiency.

19. Use of hGH fusion genes as internal controls for normalizing transfection efficiency

A pTKGH plasmid was cotransfected with chimeric tRNA_{Lys}^3-ribozyme constructs in transfection experiments for normalizing transfection efficiency. 100 μl samples of culture media were taken before isolation of total RNA. The samples were assayed for growth hormone level using a HGH-TGES 100T Kit (Nichols Institute Diagnostics). A standard curve was obtained from assay of standard reagents. The GH levels of unknown samples were obtained by comparison with the standard curve and the transfection efficiencies were normalized accordingly. The assay procedure and standard curve derivation were done according to manufacturer’s instructions.
20. Total RNA extraction

Total RNA from cultured cells was isolated and purified using RNA STAT-60 (TEL-TEST “B”, Inc.) according to manufacturer’s instructions. The isolated RNA was subjected to DNase I treatment to remove the template DNA.

21. Stable cell lines expressing chimeric tRNA\textsuperscript{Lys3}-ribozymes

Human embryonic kidney 293 cells were transfected with pAAVU6WW, pAAVU6MW or pAAVAGTRz. These constructs contain a \textit{neo} gene expressing aminoglycoside phosphotransferase and allowing selection with G418. After transfection, cells were allowed to grow under nonselective conditions and to double twice. The cells were diluted 1:15 into selective medium containing 800 µg/ml G418 (Mediatech, Inc., Herndon, VA). The cells were fed with fresh medium twice a week. Single colonies were picked and maintained in selective medium after 2 weeks.

22. Frozen stocks of stable transformants

Prepare freezing medium; complete medium supplemented with 10% to 20% (v/v) fetal bovine serum (FBS) and 5% to 10% (v/v) DMSO, 4°C. Harvest cells and wash with complete medium. Resuspend cells in 4 ml of 4°C freezing medium and place on ice. Count cells and dilute as necessary to get a final cell concentration of 10\textsuperscript{6} to 10\textsuperscript{7} cells/ml. Pipet 1 ml aliquots of cell suspension into cryovials and place vials 1 hour to overnight at \textdegree\textminus70°C. Then transfer to liquid nitrogen.
23. Northern hybridization

The following protocol was followed for Part C of Chapter III. 48 hr after 293 cells were transfected with chimeric tRNA\(^{\text{Ly3}}\)-ribozymes, total RNAs were isolated and purified using RNA STAT-60 (TEL-TEST "B", Inc.) according to manufacturer’s instructions. The isolated RNAs were subjected to DNase I treatment, then 10 \(\mu\)g of total RNA and \textit{in vitro} transcribed RNAs (as a size marker) were fractionated on a 6% acrylamide denaturing gel and transferred to a Hybond\(^{\text{TM}}\)-N\(^+\) nylon membrane (Amersham) by electroblotting. The RNA was then crosslinked to the membrane with a Stratagene UV Stratalinker. The membrane was prehybridized in 6X SSPE, 7% SDS, 5X Denhardt’s at 48°C for 3 hr. 10 pmol of oligonucleotide was labeled with [\(\gamma\)-\(^{32}\)P]ATP using T4 polynucleotide kinase (New England Biolabs). The probe was added to the prehybridization solution and hybridization was carried out overnight at 48°C. Membranes were washed and exposed to both Fuji X-ray film and a Phosphoimager screen and subsequently analyzed with ImageQuant Tools software (Molecular Dynamics). The first probe used was specific for tRNA and had the following sequence: TGGCGCCCGAACAGGGAC. After hybridizing with this probe, the same membrane was stripped in 200 ml 0.1X SSC, 0.5% SDS at 95°C and rehybridized under the same conditions with a ribozyme-specific probe: TGGCAATTTTCGTCCTCAGG. The membrane was stripped again and then probed with another probe specific for endogenous U6 snRNA which is used as an internal control. The quantitation of the signals obtained by the Phosphoimager was normalized with U6 snRNA to account for loading variation and with growth hormone concentration to account for transfection efficiency variation.
Alternatively (Part B of Chapter III), 10 μg of total RNA and *in vitro* transcribed RNA (as a size marker) were fractionated on a 1% formaldehyde-agarose gel and transferred to a Hybond™-N⁺ nylon membrane (Amersham) by the method of Sambrook (Sambrook *et al.*, 1989). The RNA was then crosslinked to the membrane with a Stratagene UV Stratalinker. The membrane was prehybridized in 6XSSPE, 7% SDS, 5X Denhardt's at 48°C for 3 hr. 10 pmol of oligonucleotides were labeled with $^{32}$P using T4 polynucleotide kinase (New England Biolabs). The probes were added to the prehybridization solution and hybridization was carried out overnight at 48°C. Membranes were washed and exposed with both Fuji X ray film and a Phosphoimager screen and subsequently analyzed with ImageQuant Tools software (Molecular Dynamics). One probe used was a specific antisense sequence of the ribozyme TGGCAAATTTCGTCCTCACGG. After hybridizing with this probe, the same membrane was stripped in 200 ml 0.1X SSC, 0.5% SDS at 95°C and rehybridized under the same condition with another probe specific for the endogenous U6 snRNA which served as an internal control. The quantitation of the signals obtained by phosphoimager was normalized with U6 snRNA for loading variation and growth hormone concentration for transfection efficiency variation.

24. **Labeling of riboprobes for fluorescent in situ hybridization**

Templates for making DIG-labeled riboprobes were synthesized by polymerase chain reaction from two oligonucleotides. T7 and T3 promoter sequences are included at the 5' and 3' ends, respectively. The sequences of the templates for making the probes were as
follows: 1) ribozyme specific probe:
TAATACGACTCACTATAGGGctgetagactgatgagtccgtgaggacgaaatttgccaCCCTTTAGT
GAGGGTTAATT; 2) tRNA<sup>Lys</sup><sub>3</sub> specific probe:
TAATACGACTCACTATAGGGgcccggatag ctcaagtcggt agagcatcag acttttaatc tgagggtcca
gggttcagt ccctgttcgg gcgcacCCCTTTTAGTGAGGGTTAATT and 3) U6 snRNA specific
probe:
gtgctcgtcgcggcagcatacatatgaaattggaacgatacagagaagattagcatggcaccccttgccgagaaggtgacacgc
acctggaga cgttccatattttCCCTTTGAGGGTTAATT. Promoter sequences are in
uppercase and sequences of structural genes are in lowercase. Antisense and sense probes
were prepared and labeled with digoxigenin-11-UTP by in vitro transcription using T3
polymerase and T7 RNA polymerase, respectively. Transcription reactions were
performed using the Genius 4 Kit (Boehringer). The DIG-labeled riboprobes from these
transcriptions were purified and yields of probes were estimated according to
manufacturer’s instructions [The Genius 4 Kit was used in combination with DIG Wash
and Block Buffer Set (Boehringer) as described in Genius<sup>TM</sup> System User’s Guide For
Membrane Hybridization (Boehringer)].

25. Fluorescent in situ hybridization (FISH)

Cells were plated at 70% confluence on coverslips treated with 0.5% gelatin and after
24 hr, transiently transfected with the constructs as described above. 48 hr later, the cover
slips were treated with PBS containing 4% paraformaldehyde and 10% acetic acid for 10
min at room temperature, washed with PBS twice and then permeabilized with 70%
ethanol overnight at 4°C. Prior to hybridization, cells were rehydrated with 2 X SSC,
50% formamide for 5 min at room temperature. 25 ng of digoxigenin-labeled probes were mixed with 40 μg carrier tRNA in 20 μl formamide and heated at 85°C for 1 min. The mixture was then added to 20 μl 2X hybridization solution (4X SSC, 20% dextran sulfate, 0.02% RNase-free BSA and 2 mM vanadyl ribonucleoside complex). Hybridization was carried out at 37°C overnight and the coverslips were washed twice in 0.1X SSC, 50% formamide for 30 min at 42°C. The coverslips were treated with a sheep anti-digoxigenin antibody (Boehringer) at 1:200 dilution in AB buffer (3X SSC, 10% formamide) in the presence of 0.1% RNase free BSA, 2 mM vanadyl ribonucleoside complex for 30 min at 37°C. They were then washed twice in AB buffer and treated with a donkey anti-sheep IgG antibody conjugated to FITC (Sigma) at 1:150 dilution in the same solution as the first antibody, for 30 min at 37°C, and subsequently washed once with 2X SSC. The coverslips were then counterstained with Propidium Iodide/Antifade (Oncor) reagent, which would stain the cell nucleus red, and mounted onto micro slides. Fluorescence microscopy was performed using an Olympus BX50 microscope. Slides were examined by successive exposure of the sample with a FITC filter to visualize the probe, Cy3 filter to visualize the propidium iodide stained nuclei, and dual (FITC and Cy3) filter to visualize the probe in the context of red-stained nuclei to determine the subcellular localization of the probed RNA transcripts. Micrographs were taken with a 3 CCD video camera system DEI-750 (Optronics Engineering) using the Image-Pro PLUS software (Media Cybernetics).
26. HIV-1 antiviral assays

Human 293 cells were cotransfected with ribozyme plasmids and HIV-1 DNA pNL4-3 as described above. The DNA mixture contained 4 µg control or test plasmid, 1 µg human growth hormone (HGH) gene pTKGH (as a transfection efficiency control) and 0.1 µg pNL4-3. After 2 days, supernatants were collected and the RT activity was assayed. CEM cells were infected with the supernatants from the transfection with equalized RT counts. The p24 level in culture media from these infections was assayed and compared after six and seven days.

27. Total RNA isolation

Total RNA was isolated and purified using RNA STAT-60 (TEL-TEST “B”, Inc.) according to the manufacturer’s instructions. When isolated from transiently transfected cells, RNA was subjected to DNase I treatment to remove contaminating plasmid DNA.

28. Establishment of stable cell lines expressing chimeric tRNA^Lys^3-ribozymes:

Human embryonic kidney 293 cells were transfected with pAAVU6WW, pAAVU6MW and pAAVA\text{GTRz}. These constructs contain a Neo\textsuperscript{R} gene expressing aminoglycoside phosphotrasferase which allows selection with G418. After transfection, cells were allowed to grow under nonselective conditions for two doublings. The cells were then split 1:15 into selective medium containing 800 µg/ml G418 (Mediatech, Inc., Herndon, VA). Cells were replaced with fresh media containing G418 twice a week. Single colonies were picked and maintained in selective medium after 2 weeks.
29. **Half-life determination of *in vivo* expressed ribozymes**

Intracellular stability of the transcripts from different constructs was compared by measuring the half-life of the RNAs after halting the transcription with actinomycin D. Half lives were determined in transiently transfected 293 cells and in stable cell lines (see above). In transient transfections, 293 cells were transfected with 10 μg pU6WW, pU6MW, pU6ΔGTRz or pU6Rz and 1 μg of pTKGH (normalizing transfection efficiency). Stable cell lines AAVU6WW, AAVU6MW and AAVΔGTRz were also used. 48 hr after transient transfection or 24 hr after splitting the stable cell lines, 10 μg/ml of actinomycin D (Sigma, St Louis, MO) was added to the culture medium. Cells were harvested and total cellular RNA was isolated at 0, 0.5, 1, 2, 4 hr (8 hr also for transient transfections) after actinomycin D treatment. The RNA samples were analyzed by Northern hybridization with a ribozyme-specific probe and a U6 snRNA-specific probe. Quantitation was normalized to the transfection efficiency and the U6 snRNA internal control, taking into account the published half-life of endogenous U6 snRNA.

30. **Preparation of cell extracts with tRNA processing activity**

Human embryonic kidney 293 cells were grown to 80% confluency, collected and rinsed three times in phosphate-buffered saline (PBS), and two times the cell pellet volume of Hypotonic Buffer (10 mM Tris, pH 7.5, 10 mM KCl, 1.5mM MgCl₂, 1.4 mM β-mercaptoethanol) was added. Cells were incubated on ice for 10 min and then homogenized with a Dounce (20 strokes). One-tenth volume of S20 Spin Buffer (230 mM Tris, pH 7.5, 1.275 M KCl, 40 mM MgCl₂, 6.4 mM β-mercaptoethanol) was added. The extracts were centrifuged at 20,000 x g for 10 min at 4°C, and supernatants were
transferred to fresh tubes, stored on ice, and subsequently used fresh in the processing assays (see below).

31. Chimeric tRNA^{Lys3}-ribozyme transcript processing assays in cell extracts

Chimeric tRNA^{Lys3}-ribozymes were transcribed and labeled with [α-\(^{32}\)P]-UTP by \textit{in vitro} T7 RNA polymerase transcription using linearized plasmids as templates. The transcripts were precipitated to remove unincorporated radioactive UTP. One-sixth (5 μl, approximately 50,000 cpm) of each transcription reaction was incubated in a 50 μl reaction in tRNA 3'-end processing buffer (Nashimoto, 1995; 10 mM Tris, pH 7.5, 1 mM DTT, 3.2 mM spermidine, 40 U RNasin) with or without spermidine, and with or without 10 μl 293 cell extract, for 30 min at 37°C. Incubations were phenol extracted, ethanol precipitated and resuspended in 15 μl water. One third (5 μl) of each reaction was then combined with an equal volume of formamide loading dye, heated to 65°C for 5 min and analyzed in a denaturing 10% acrylamide gel (8M urea). The gel was dried and autoradiographed with a BioMax (Kodak) screen at -70°C overnight.

32. Commonly used calculations

1) Total pmol of an oligonucleotide = (OD 260 X 90 X 10^3)/length.

2) Molar concentration of \textit{in vitro} transcribed chimeric tRNA^{Lys3}-ribozyme and tRNA^{Lys3} transcripts:

Measure OD and calculate the concentration (W μg/μl) of the RNA samples. The molar concentration (mol/μl) of the samples equals (W X 10^{-6} g/μl)/ [length (n.t.) of the transcripts X 330 g/mol]. The length of the chimeric tRNA^{Lys3}-ribozyme transcripts is 114 nucleotides and the length of the tRNA^{Lys3} is 76 nt.
III. EXPERIMENTAL RESULTS

A. Virion Encapsidation of tRNA3Lys-Ribozyme

Chimeric RNAs Inhibit HIV Infection

Shawn K. Westaway¹, Laurence Cagnon², Zongli Chang³, Shirley Li⁴, Haitang Li⁵,
Garry P. Larson⁶, John A. Zaia⁷, And John J. Rossi⁸

¹Postdoctoral fellow who constructed pAAVU6+1ΔGtRNA3Lys-rbz and the control constructs ptRNAValPBS-rbz/mrbz, established and analyzed the stable cell lines for expressing ΔGtRNA3Lys-rbz, and participated in the RT-PCR assay of viral RNA.

²,⁴Postdoctoral fellow and researcher who did anti-HIV assays involving the manipulation of infectious HIV-1 agent.

³Graduate student who constructed four constructs: ptRNA3Lys-rbz/mrbz and pU6tRNA3Lys-rbz/mrbz, established the much higher expression efficiency of the U6/tRNA expression cassette than the tRNA expression cassette alone in expressing chimeric tRNA-ribozymes. The latter two constructs using the U6/tRNA expression cassette were then effectively utilized in anti-HIV and virion encapsidation assays.

⁵Laboratory technician who performed the RT-PCR assay with the postdoctoral fellow Shawn Westaway¹.

⁶Former member of the laboratory who constructed the construct p ΔGtRNA3Lys-rbz and pPBS-rbz and carried out the in vitro assays of the chimeric ribozymes.

⁷Graduate committee member of the graduate student³ and principal investigator of the researcher Shirley Li⁴ who supervised and guided part of the work.

⁸Student’s mentor and graduate committee chairperson who supervised the work and preparation of the manuscript.

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Virion Encapsidation of tRNA$_3^{\text{Lys}}$-Ribozyme

Chimeric RNAs Inhibit HIV Infection

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ABSTRACT

Retroviruses require a specific host cellular tRNA primer for initiation of first strand DNA synthesis. This primer is bound by viral proteins and copackaged into virions. We have exploited this property in the design and testing of an antiviral ribozyme fused to tRNA$_{3}^{Lys}$, the primer utilized for lentiviral replication, including human immunodeficiency virus (HIV-1 and HIV-2). The chimera consists of tRNA$_{3}^{Lys}$ covalently attached to a hammerhead ribozyme which is targeted to the region immediately upstream of the Primer Binding Site of the HIV-1 genome. The tRNA-ribozyme chimeric transcript is catalytically active in vitro and is efficiently bound by HIV reverse transcriptase with an affinity similar to that of tRNA$_{3}^{Lys}$. We have expressed the chimeric RNAs from either the tRNA$_{3}^{Lys}$ intragenic RNA Polymerase III promoter or from a human U6 snRNA promoter. The U6 promoter results in up to 10-fold enhanced expression of the tRNA-ribozyme. Most importantly, the tRNA$_{3}^{Lys}$-ribozymes are encapsidated in HIV-1 virions such that they are effective in substantially reducing the level of infectious virus produced from cells cotransfected with HIV-1 proviral DNA. These results demonstrate the feasibility of using this novel strategy to reduce HIV infectivity and more generally indicate the potential power of utilizing the retroviral primer tRNAs as tools for expressing and delivering ribozymes and other anti-retroviral RNAs to the virion capsid.
INTRODUCTION

Reverse transcriptase (RT) is the viral enzyme responsible for copying single-stranded RNA to double-stranded DNA in all retroviruses, including Human Immunodeficiency Virus-1 (HIV-1), the causative agent of Acquired Immunodeficiency Syndrome (AIDS). The currently used inhibitors of HIV act by blocking various replication functions. These inhibitors become ineffective over time due to the rapid mutation of the virus (Richman, 1993; Schinazi et al., 1992). HIV RT is extremely error-prone, misincorporating a nucleotide at the rate of 1 in 6900 for RNA-dependent DNA polymerization or 1 in 5900 for DNA-dependent DNA polymerization (Ji and Loeb, 1992). These mistakes occur throughout the HIV genome, although there is evidence to indicate that the excessive mutation rate is not totally random but may be linked to regions of unusual or highly unordered RNA structure (Schinazi et al., 1994). Some of these mutations allow the RT itself to overcome the effects of anti-AIDS drugs, such as the block of polymerization caused by AZT (3'-azido-3'-deoxythymidine). In order for anti-HIV therapeutic agents to be effective, they must target a well-conserved and essential function of the virus.

We have exploited the replication cycle of HIV-1 in a strategy which fuses a therapeutic ribozyme to the cellular tRNA\textsubscript{3\,lys} required for viral replication. HIV RT uses this tRNA\textsubscript{3\,lys} as the primer to initiate synthesis (for reviews, see Varmus and Brown, 1989; Temin, 1992). HIV RT binds the host primer tRNA and both are packaged along with the viral genomic RNA and other proteins into HIV virions in the cytoplasm of infected cells. Though tRNA\textsubscript{1,2\,lys} are also packaged into HIV virions (Jiang et al., 1992; Jiang et al., 1993), tRNA\textsubscript{3\,lys} is the primer utilized for HIV replication (Roy et al., 1982;
Craig et al., 1989). Eighteen nucleotides of the 3'-end of human tRNA$_{3}$-Lys are complementary to the Primer Binding Site (PBS) of HIV-1 and HIV-2 RNA (Wain-Hobson et al., 1985; Ratner et al., 1985), and are used for priming of first-strand DNA synthesis. The specificity of this primer-target interaction is exemplified by the fact that tRNA$_{1,2}$-Lys, which differ from tRNA$_{3}$-Lys in 5 of the 18 positions complementary to the PBS, cannot serve as primers for reverse transcription, and are not found tightly associated with the genome (Jiang et al., 1992; Jiang et al., 1993; Mak et al., 1994; Kohlstaedt and Steitz, 1992; Das et al., 1995).

Current approaches employed to colocalize a ribozyme to its target RNA take advantage of viral genome characteristics, and include tethering ribozymes to viral packaging signals (Sullenger and Cech, 1993). We developed a unique approach for directing a hammerhead ribozyme to its target by attaching it to the 3'-end of human tRNA$_{3}$-Lys. The hammerhead ribozyme, which occurs naturally in the genome of several plant satellite RNAs (Symons, 1992), can be functionally separated into substrate binding arms, and the active site, thus effectively allowing site-specific cleavage to occur in trans (Uhlenbeck, 1987). A potential target for the ribozyme is the Primer Binding Site (PBS) of HIV RNA, located just 3' to the 5'-long terminal repeat (5'-LTR). If this region is cleaved, it will prevent copying of viral RNA into DNA and subsequently block infection. In addition, the PBS is highly conserved among HIV and SIV variants, since priming is accomplished solely from the tRNA$_{3}$-Lys bound to this site.

tRNA$_{3}$-Lys is complementary to the 18 nucleotides of PBS RNA, bound by HIV RT (Kohlstaedt and Steitz, 1992), co-packaged in the infectious virion, and required to initiate viral replication. We reasoned that fusing a ribozyme to the host cellular
tRNA$_{\text{Lys}}$ primer could result in incorporation of the ribozyme into the virion. Packaging of tRNA$_{\text{Lys}}$-ribozymes would occur via interactions with HIV RT, nucleocapsid proteins, and the viral RNA, resulting in colocalization to its target, consequent cleavage of the target, and inhibition of HIV replication. Most importantly, using the tRNA primer to deliver a ribozyme to the viral RNA target circumvents HIV's genetic variability problem because the tRNA, the PBS target, and HIV RT would each have to mutate at the same time in the host and the virus to accomplish escape tRNA$_{\text{Lys}}$-primed first strand DNA synthesis.

**MATERIALS AND METHODS**

**Construction of ribozymes and substrate**

The ΔGtRNA$_{\text{Lys}}$-ribozyme (ΔGtRNA$_{\text{Lys}}$-rbz) was constructed by the polymerase chain reaction (PCR) as described (Dillon and Rosen, 1990), with the following synthetic oligonucleotides in the PCR reactions:

(1) 5'-GACTCGAGCCCGGATAGCTCAGTCGGTAGAGCATCAGACITTTAATCTGAGGGTCCAGG-3' (2) 5'-GCCGCCGAACAGGGACTTGAACCCTGGACCCTCAG-3'
(3) 5'-CCCTGTTCGGGCGCCACTGCTAGACTGATGAGTCCGTGAGGACG-3'
(4) 5'-GTCCCGCGGAAAAATGGCAAAATTTCTCCTACCGG-3'

50 pmol each of #1 and #2 were combined, denatured at 94°C for 3 min, cooled to 25°C, and extended at 72°C for 10 min in the presence of 125 μM dNTP's, 2.5 mM MgCl$_2$, and 2 Units Taq polymerase in 1X Taq polymerase buffer. The same reaction was carried out for oligonucleotides #3 and #4. Reactions were diluted to 100 μl. 50 pmol each of the outside PCR primers (#1 and #4) were combined with 1 μl of the #1/#2 reaction (0.5
pmol), 1 μl of the #3/#4 reaction, 125 μM dNTP’s, 2.5 mM MgCl₂, and 1 Unit Taq polymerase in 1X Taq polymerase buffer. The PCR was carried out for 25 cycles at 94°C, 2 min; 55°C, 2 min; 72°C, 3 min. The PCR products were separated on a 6% nondenaturing acrylamide gel (0.5X TBE) and the 134-bp expected product was excised, crushed, and eluted overnight in 10 mM EDTA at 37°C. Supernatants were extracted once with phenol, once with chloroform, ethanol precipitated with 0.5 M NaCl, and the pellet was resuspended in 20 μl TE. The PCR product was digested with SacII and XhoI, phenol extracted, ethanol precipitated, ligated into XhoI/SacII cleaved and dephosphorylated pBluescriptSK(+) vector (Stratagene) and transformed into MC1061 E. coli cells to create the plasmid pΔgtrNA₃Lys-rbz. This construct has a deletion of one G at the TΨC stem-loop junction, just at the beginning of the tRNA B box internal promoter, caused inadvertently during the PCR used for gene synthesis (see Fig. 1). The PBS-rbz was constructed by PCR using the following synthetic oligonucleotides:

(5') 5'-GACTCGAGTCCCTGTTCGGGCGCCACTGCTAGACTGATGAGTCCGTGAGGACG-3'

and #4 above. 20 pmol of each oligonucleotide were incubated under the PCR conditions as described above and cloned into pBluescriptSK(+) vector to create the plasmid pPBS-rbz. Plasmid pTrNA-SUB was constructed by cloning the 148 bp HindIII/SalI fragment (HIVXHB2 coordinates 532 to 680) into the HindIII/SalI sites of pTZ19U (Pharmacia Biotech).

Human tRNA₃Lys-rbz or a mutant ribozyme version, containing a single point mutation in the catalytic core which eliminates ribozyme cleavage in vitro (Ruffner et al., 1990) (tRNA₃Lys-mrbz), and containing the correct wild-type sequence for tRNA₃Lys was
constructed by the PCR as above, with the following synthetic oligonucleotides in the PCR reactions:

(1) 5'-ACGCGTCGACGCCCAGATAGCTCAGTCGG-3'
(2) 5'-GTCTGATGCTCTACCGACTGAGCTATCCGGCGTGCAGCC-3'
(3) 5'-GTAGAGCATCAGACTTTTAAATCTGAGGGGCTCCAGGGTTC-3'
(4) 5'-GCGCCCGAACAGGGACTTTGAACCTTGACCTGACCCT-3'
(5) 5'-CCCTGTTCGGGGCCACCTGCTAGACTGATGCCTCGTGAGG-3'

and either (6) or (7) for wild-type or mutant ribozyme, respectively (single mutation is shown in bold):

(6, 7) 5'-CGGAATTCCGCGGAAAATGGCAAATT(C to T)GTCCTCACGGACTCAT-3'

The two tRNA31-ys-ribozymes (tRNA31-lys-rbz and tRNA31-lys-mrbz) were cloned into the Sal I and EcoRI sites of pBluescriptSK+ (Stratagene) to yield ptRNA31-lys-rbz and ptRNA31-lys-mrbz. These two constructs were also cloned downstream of a U6+1 snRNA promoter cassette in the plasmid pTZU6+1 (Bertrand et al., 1997) to yield pU6tRNA31-lys-rbz and pU6tRNA31-lys-mrbz.

tRNAVal-ribozymes (ptRNAValPBS-rbz and ptRNAValPBS-mrbz) were cloned by the PCR of plasmid pPBS-rbz with a 5'-primer containing a XhoI restriction site and a 3'-primer containing a MluI restriction site and either wild-type or mutant ribozyme sequences to yield PBS-rbz or PBS-mrbz PCR duplexes. These were digested and cloned into the compatible sites of ptV5 (kindly provided by A. Hampel) downstream of the tRNAVal sequence. Intracellular expression of these chimeric RNAs is driven by the

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tRNA\textsuperscript{Val} intragenic RNA Polymerase III promoter, and transcription terminates at the four thymidine residues at the 3'-end of the ribozyme.

The ΔGtRNA\textsubscript{3-Lys-rbz} sequence was PCR amplified from pΔGtRNA\textsubscript{3-Lys-rbz} with 5'- and 3'-primers containing flanking Sal I sites, cloned into the Sal I site of AAVU6+1 vector (Bertrand \textit{et al.}, 1997), to create pAAVU6+1ΔGtRNA\textsubscript{3-Lys-rbz}. Fig. 1a is a diagram of the different constructs bound to the HIV PBS target. Fig. 1b is a schematic of the constructs and their names as used in the text and figures.

\textbf{In vitro transcriptions}

1.6 µg plasmid ptRNA-SUB was linearized with 10 Units EcoRI in 1X BRL React Buffer 3 for 2 hrs. at 37°C, phenol/chloroform and chloroform extracted, ethanol precipitated, and used for transcription. EcoRI-linearized ptRNA-SUB yielded \textit{in vitro} T7 RNA Polymerase transcripts (performed as below) of 162 bp. All \textit{in vitro} cleavage and gel shift assays were done with tRNA\textsubscript{3-Lys}, PBS-rbz, or ΔGtRNA\textsubscript{3-Lys-rbz} transcripts derived from the PCR amplification of the pΔGtRNA\textsubscript{3-Lys-rbz} plasmid with the appropriate 5'-primer containing an 18-nucleotide sequence encoding the bacteriophage T7 promoter. For the PCR duplex product T7: tRNA\textsubscript{3-Lys} (98 nt), the PCR primer pairs were:

(6) 5'-TTAATACGACTCACTATAGGGCCGGATAGCTCAG-3'
(7) 5'-TGGCGCCCGAACAGGG-3'

For the PCR duplex product T7:ΔGtRNA\textsubscript{3-Lys-rbz} (136 nt), the PCR primer pairs were: #6 above and
For the PCR duplex product T7:PBS-rbz (78 nt), the PCR primer pairs were:

(9) 5'-TTAATACGACTCACTATAGGGTGCTCAGTCGC-3' and #8 above.

The PCR reactions contained 2 ng pΔGtRNA₃²Lys-rbz plasmid, 100 pmol of each primer, 0.2 mM dNTP's, 2.5 Units Taq DNA Polymerase, in 50 µl of 1X Taq Polymerase buffer. Reactions were carried out for 35 cycles at 94°C, 2 min; 40°C, 2 min; and 72°C, 3 min.

Transcriptions of the PCR duplexes were carried out using the Ambion T7 Megascript Kit in a 20 µl volume for 4-6 hr at 37°C according to the manufacturer's protocol with the following modifications: 3 µl of the PCR duplex and an additional 50 Units T7 polymerase were added, and 1 µl [α-³²P]UTP (3000 Ci/mmol) was added for radioactive transcripts. RNase-free DNase incubations were performed according to the manufacturer. Transcripts were purified on 6% polyacrylamide (30:1 acrylamide:bisacrylamide), 7 M urea, 1X TBE gels, eluted, and ethanol precipitated. *In vitro* transcriptions of plasmid templates were performed as above except 0.5 µg of plasmid linearized downstream of inserts was used as template.

**In vitro ribozyme cleavage reactions**

1 pmol of radiolabeled HIV substrate RNA transcript was incubated with 1 pmol PBS-rbz, ΔGtRNA₃²Lys-rbz, or tRNA₃²Lys transcripts in 50 mM Tris, pH 7.5 at 37°C for 1 hour. If present, Mg²⁺ was at a final concentration of 20 mM. Products of the cleavage reaction were separated on 6% PAGE, 7 M urea, 1X Tris-borate-EDTA gels and exposed to autoradiographic film for 18 hrs. at -70°C with an intensifying screen.
**In vitro binding of RT to tRNA or tRNA-rbz**

To measure the specificity of binding of tRNA\(^{3'\text{LyS}}\) or AGtRNA\(^{3'\text{LyS}}\)-rbz to HIV-1 RT, binding assays were performed at 25°C for 30 min in 10 µl reactions containing 100 nM radiolabeled tRNA\(^{3'\text{LyS}}\) or AGtRNA\(^{3'\text{LyS}}\)-rbz transcripts, 40 mM Tris, pH 8.3, 60 mM NaCl, 6 mM MgCl\(_2\), 5 mM dithiothreitol, and 100 nM AMV, MoMuLV, or HIV RT (p66/p51 heterodimer obtained from NIH AIDS repository). 1 µl loading buffer (30% Ficoll, 1X TBE, 0.05% bromphenol blue and xylene cyanol) was added and samples were electrophoresed under non-denaturing conditions at 4°C in a 4% polyacrylamide gel (60:1 acrylamide:bisacrylamide) with 0.5X TBE buffer. To demonstrate competition between tRNA\(^{3'\text{LyS}}\) and AGtRNA\(^{3'\text{LyS}}\)-rbz for binding to HIV-1 RT, 4 to 20 nM nonradiolabeled tRNA\(^{3'\text{LyS}}\) or LiGtRNA\(^{3'\text{LyS}}\)-rbz was added after preincubation of HIV RT with 1 pmol radiolabeled AGtRNA\(^{3'\text{LyS}}\)-rbz or tRNA\(^{3'\text{LyS}}\), respectively.

**Transcription of RNA in vivo**

Human embryonic kidney cells (293 cells) were transiently transfected with 10 µg of ptRNA\(^{3'\text{LyS}}\)-rbz, ptRNA\(^{3'\text{LyS}}\)-mrbz, or pU6tRNA\(^{3'\text{LyS}}\)-rbz using the CellPhect Transfection Kit (Pharmacia). 1 µg pTKGH was cotransfected with the samples for quantitation of transfection efficiency (HGH-TGES 100T Kit, Nichols Institute). Transfection efficiency was measured at 20%. After 2 days, RNA was prepared from transfected cells by the RNA STAT-60™ reagent according to the manufacturer’s directions (Tel-Test “B”, Inc., Friendswood, TX). Total RNA (25 µg) was resolved on 1% formaldehyde-agarose gels, blotted, and hybridized first to a probe specific for the ribozyme moiety, then washed and
hybridized to a U6 snRNA probe as a loading control. The oligonucleotide probes were kinased in 10 μl of 70 mM Tris, pH 7.6, 10 mM MgCl₂, 5 mM DTT, 150 μCi [γ-³²P]ATP, 10 Units T4 Polynucleotide Kinase (New England Biolabs), at 37°C for 30 min. Blots were exposed to autoradiograph film overnight.

**HIV transfection and infection of cultures**

293 cells were transiently transfected with HIV-1 proviral DNA (pNL4-3, Adachi et al., 1986) and ribozyme or control plasmids at a ratio of 1:10 HIV:ribozyme using the Cell-Pfect Kit (Pharmacia). Transfection efficiencies were estimated at 20% using the pTKGH human growth hormone standard. Supernatants were collected three days post transfection and the RT activity of the supernatant was determined (Goldstein et al., 1990). The different supernatants were standardized according to their RT activity, and used to infect the T cell line CEMX174. From 3 to 10 days after infection, supernatants were collected and assayed for HIV RT activity and HIV p24 antigen (Coulter) according to the manufacturer’s instructions. Virion production peaked in a control culture at days 6-7. Infections were done in triplicate and results were averaged.

**RT-PCR of viral RNA**

Virions were prepared from supernatants of 3-day post-transfected 293 cells. 500 μl of cell-free supernatant were loaded onto a 10-ml Sepharose A column (SIGMA), and eluted in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl. RNA was isolated from virions in fractions containing the peak of RT activity and p24 antigen using 1 ml of RNA
STAT-60™ reagent for 200 μl eluted virus (Tel-Test "B", Inc.). RNA was DNase treated by incubation in 50 mM Tris, pH 7.7, 5 mM MgCl₂, with 15 Units RNase-free DNase I (Boehringer Mannheim) in 30 μl volumes at 37°C for 1 hr, followed by two phenol/chloroform extractions, one chloroform extraction and ethanol precipitation. RNA from approximately 4 μl of cell supernatant was used for reverse transcription-PCR (RT-PCR). Samples were heated to 90°C in 50 μl reactions with 30 pmol each 5’ and 3’ primers, 1X PCR buffer (Boehringer Mannheim) and 0.2 mM dNTP’s, and allowed to cool slowly to room temperature. 1 Unit AMV Reverse Transcriptase (Life Sciences) was added, samples were incubated at 37°C for 5 min, and the PCR was subsequently performed with 2.5 Units Taq DNA Polymerase (Boehringer Mannheim) for 30 cycles at 94°C, 1 min; 42°C, 1 min; and 72°C, 2 min Products were first electrophoresed on acrylamide gels, gel purified and sequenced. Products were also electrophoresed on agarose gels, blotted, probed with a ribozyme-specific probe, and autoradiographed as described above.

Establishment and Analysis of Stable Cell Lines Expressing tRNA₃Lys-rbz

Plasmid pAAVU6+1ΔGtRNA₃Lys-rbz or pAAVU6+1 vector alone was transfected into human 293 cells using the Cell-Pfect Kit (Pharmacia). Clones were selected in G418 (Mediatech, Inc.) by virtue of the Neo⁵ gene encoded in the plasmid. Total RNA was prepared from subconfluent clonal cultures using the RNA STAT-60™ reagent (Tel-Test “B”, Inc.). Total RNA was resolved on 1.5% agarose-formaldehyde gels, and passively blotted to Zeta-Probe membranes (BioRad). In vitro T7 RNA polymerase transcribed tRNA₃Lys-rbz was used as a size marker and positive control. Membranes were
hybridized with $^{32}$P-kinased probes specific to either tRNA$_{3}^{\text{Lys}}$ or to the ribozyme moiety, washed and autoradiographed as described above.

**RESULTS**

**Design of a chimeric tRNA-ribozyme targeted to HIV RNA**

The primer for HIV RT must have a 3'-hydroxyl at the end of the 18 nucleotide region of complementarity to the PBS to allow for initiation of reverse transcription (Shimada *et al.*, 1994). We wished to eliminate the possibility that a chimeric tRNA$_{3}^{\text{Lys}}$-rbz transcript could support the initiation of replication, therefore we constructed the tRNA-rbz with the ribozyme moiety at the 3'-side of the tRNA (Fig. 1a). Though there has to be a limited amount of complementarity between the 3'-flanking arm of the ribozyme and its target RNA in order to bind and cleave, it would not be expected that RT would recognize or bind to the ribozyme moiety. However, the tRNA$_{3}^{\text{Lys}}$-rbz should bind RT via its tRNA$_{3}^{\text{Lys}}$ moiety and bind to the PBS of HIV RNA via complementary base pairing. This should result in packaging into the virion, where it can inhibit replication through ribozyme cleavage or by blocking HIV RT polymerization. Our strategy assumes that the tRNA moiety of the tRNA$_{3}^{\text{Lys}}$-rbz will retain its natural conformation *in vivo* as well as *in vitro*, and that RT will bind the tRNA$_{3}^{\text{Lys}}$-rbz with the same affinity as tRNA$_{3}^{\text{Lys}}$.

All the chimeric transcripts contain a polyuridine tract downstream of the 3'-guide sequence of the ribozyme to allow RNA Polymerase III-mediated termination of the transcript. This RNA Polymerase III termination signal has been shown to consist of a stretch of 4 uridines at the end of the transcript (Cozzarelli *et al.*, 1983; Campbell and
Setzer, 1992). An important *in vitro* observation is that the tRNA$_{Lys}$-rbz complexed with the HIV primer binding site is incapable of supporting initiation of DNA synthesis from the 3'-end (data not shown).

The various tRNA-rbzs were constructed using synthetic oligonucleotides, or by the PCR from existing plasmids (see Materials and Methods). The first tRNA$_{Lys}$-rbz has a deletion of one G at the T$\psi$C stem-loop junction, just at the beginning of the tRNA B box internal promoter (DGtRNA$_{Lys}$-rbz), caused inadvertently during the PCR used for gene synthesis. Two other constructs have the wild-type tRNA$_{Lys}$ sequence fused upstream of either a standard hammerhead ribozyme sequence (tRNA$_{Lys}$-rbz) or a mutant ribozyme (tRNA$_{Lys}$-mrbz) which is catalytically inactive *in vitro* (Ruffner *et al.*, 1990). These two constructs were also cloned downstream of a U6 snRNA promoter to increase levels of transcription *in vivo* (U6tRNA$_{Lys}$-rbz or U6tRNA$_{Lys}$-mrbz). As a control for localization, the tRNA$_{Lys}$ moiety was replaced by a human tRNA$\text{Val}$ gene (plasmid ptV5) containing mutations which prevent 3'-end processing (Hampel, 1998, in press). These constructs still contain the 18 nucleotides complementary to the PBS, followed by the hammerhead ribozyme or mutant ribozyme and terminator sequences (tRNA$\text{Val}$PBS-rbz or tRNA$\text{Val}$PBS-mrbz). An important aspect of these constructs is that they lack the majority of the tRNA$_{Lys}$ elements required for recognition and binding by HIV RT. The tRNA$\text{Val}$ gene provides the RNA Polymerase III promoter for *in vivo* transcription. Fig. 1a is a diagram of all the tRNA-ribozyme constructs and their HIV target, and Fig. 1b is a schematic of the different plasmid constructs along with their names as used in the text.
Synthetic tRNA<sub>3</sub>-lys-rbz transcript cleaves its synthetic HIV target RNA in vitro

Fig. 2 shows that both the ΔGtRNA<sub>3</sub>-lys-rbz and PBS-rbz transcripts effectively cleave the 162 bp HIV RNA substrate in vitro in the presence of Mg<sup>2+</sup>, yielding the expected cleavage products of 100 and 62 bp. Since the region of nucleotide sequence complementarity between the flanking sequences of the ribozyme and the HIV RNA substrate is quite long (7 and 26 base pairs), dissociation of the cleavage products is rate limiting, and ribozyme turnover is not expected. There is virtually no difference in cleavage efficiency between the PBS-rbz and the ΔGtRNA<sub>3</sub>-lys-rbz.

HIV-1 RT binds tRNA<sub>3</sub>-lys-rbz with an affinity similar to tRNA<sub>3</sub>-lys

Specific domains of tRNA<sub>3</sub>-lys which interact with HIV-1 reverse transcriptase have been identified (Barat <i>et al.</i>, 1989; Barat <i>et al.</i>, 1991; Sarih-Cottin <i>et al.</i>, 1992; Wöhrl <i>et al.</i>, 1993). The ΔGtRNA<sub>3</sub>-lys-rbz construct retains these sequences, but it was not known whether the single G deletion and the appended ribozyme would affect RT binding. To examine this interaction of RT with the ΔGtRNA<sub>3</sub>-lys-rbz, we carried out in vitro binding assays with HIV RT. An electrophoretic gel mobility shift assay was employed to compare the HIV RT binding of in vitro synthesized tRNA<sub>3</sub>-lys to the ΔGtRNA<sub>3</sub>-lys-rbz. Although differences have been discovered between the affinity of in vivo-isolated tRNA<sub>3</sub>-lys versus in vitro transcribed unmodified tRNA<sub>3</sub>-lys for HIV-1 RT (Barat <i>et al.</i>, 1991), we reasoned that a comparison of the relative binding affinities of RT with in vitro synthesized tRNA<sub>3</sub>-lys and ΔGtRNA<sub>3</sub>-lys-rbz would reflect the relative in vivo affinities.
Specific recognition of tRNA_{3}^{Lys} occurs via HIV-1 RT. In vivo, however, the GAG-POL precursor protein is involved in the primer selection process (Mak et al., 1994). In addition, alignment of the primer on the PBS may be facilitated by other viral proteins, such as the nucleocapsid protein, NCp7. The tRNA_{3}^{Lys}-ribozymes would, at a minimum, need to be selectively bound by HIV RT.

To examine whether or not the ΔGtRNA_{3}^{Lys} and the appended ribozyme moiety would affect the interaction with HIV RT, binding assays were performed (Fig. 3). This figure depicts an autoradiograph of native polyacrylamide gel electrophoresis of HIV RT complexed with tRNA_{3}^{Lys} or ΔGtRNA_{3}^{Lys}-rbz, demonstrating a shift in electrophoretic mobility upon binding by HIV RT. Both tRNA_{3}^{Lys} and ΔGtRNA_{3}^{Lys}-rbz interact with HIV-1 RT (p66/p51) while they do not interact with Avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (MoMLV) reverse transcriptases under identical conditions. These data demonstrate that both tRNAs share a similar secondary and tertiary structure which allows for the preferential recognition by HIV-1 RT. In addition, nonradiolabeled ΔGtRNA_{3}^{Lys}-rbz competes effectively and specifically with radiolabeled tRNA_{3}^{Lys} for binding to HIV RT (Fig. 4a), and vice versa (Fig. 4b). Nonspecific RNA does not compete with either tRNA_{3}^{Lys} or ΔGtRNA_{3}^{Lys}-rbz for binding (data not shown).

Native polyacrylamide gel electrophoresis was used to compare the binding affinities of HIV RT to tRNA_{3}^{Lys} and ΔGtRNA_{3}^{Lys}-rbz by titrating a fixed amount of each RNA with increasing amounts of HIV-1 RT. The ΔGtRNA_{3}^{Lys}-rbz binds to HIV RT with a similar dissociation constant as that of tRNA_{3}^{Lys}. Neither the one base deletion nor
the appended ribozyme alters the binding kinetics of the $\Delta$GtRNA$_{3-ys}$-rbz by HIV RT (Westaway et al., 1995).

**U6 snRNA promoter significantly increases transcription in vivo**

Evidence that HIV virions contain an average of eight copies of the tRNA primer per two copies of the genome (Mak et al., 1994) suggests that more of the tRNA-rbz construct could be packaged if we increased its intracellular concentration. We chose the upstream promoter element of the human U6 snRNA gene to drive transcription of the tRNA$_{3-ys}$-ribozymes and increase the levels of expression. The rationale for this approach is: 1) The preservation of the characteristics of an RNA Polymerase III transcript, for example, a discrete transcript start and endpoint; 2) The high level of transcription of the U6 snRNA gene compared to tRNA$_{3-ys}$; and 3) The 5’-coding sequence of the U6 gene is not included, thus preventing the tRNA-rbz transcript from being capped as a U6 snRNA transcript (Good et al., 1997).

To compare levels of expression of genes driven by tRNA$_{3-ys}$ promoter elements with genes driven by the U6 snRNA promoter, human 293 cells were transiently transfected with these constructs. 48 hr post-transfection, total RNA was prepared and analyzed. Northern blot analysis of total RNA was hybridized to a probe specific for the ribozyme. Cells transfected with the chimeric gene driven by the tRNA$_{3-ys}$ promoter showed poor expression levels in vivo, whereas transcription of the tRNA$_{3-ys}$-rbz by the U6+1 promoter resulted in an approximately 10- to 20-fold increase in transcription (Fig. 5, lanes 3 and 4). The difference in relative abundance of the U6+1 versus tRNA$_{3-ys}$ promoter-driven chimeric RNAs could be the result of transcript levels and/or differential
3'-end processing of the ribozyme from the tRNA moiety. This difference also indicates that the presence of a fully functional internal tRNA promoter does not interfere with transcription by U6 promoter elements. The blot was also hybridized to a probe specific for U6 snRNA, as a loading control (Fig. 5, lanes 1 and 2). Given the high levels of tRNA$_{3}$Lys-rbz expression obtained from the U6+1 promoter-driven constructs, we chose to use these constructs for subsequent HIV challenge experiments.

The tRNA$_{3}$Lys-rbz inhibits HIV replication by altering virion infectivity

Based on the recognition of the ΔGtRNA$_{3}$Lys-rbz by HIV RT in vitro and its efficient expression in transfected cells, we predicted that tRNA$_{3}$Lys-rbz transcripts would be incorporated into viral particles. These particles should be defective, since the ribozyme moiety might be expected to cleave the genome and prevent the initiation of reverse transcription prior to, or soon after entry into a cell. To test the ability of the tRNA$_{3}$Lys-rbz to reduce viral infectivity, we assayed the infectivity of viral particles produced from cells expressing the chimeric constructs according to the procedure diagrammed in Fig. 6. First, human 293 cells were cotransfected with proviral HIV-1 DNA and the various tRNA-rbz plasmids. Since human 293 cells have no CD4$^+$ receptor, reinfection cannot occur, and HIV undergoes only a single round of replication. Three days after transfection, when virion production peaked, supernatants were collected and assayed for HIV reverse transcriptase activity. No significant differences in RT levels were detected in supernatants from cells transfected with the tRNA-rbz genes versus the U6 or tRNA$^\text{Val}$ vector alone control (data not shown). These results suggest little if any ribozyme activity in these cells.
In order to evaluate whether or not the tRNA-rbz affected viral infectivity by rendering virions defective for subsequent infection, an infectious assay using supernatants from the transfected 293 cells was carried out. Viral stocks were prepared from the cotransfected 293 cells and used to infect CD4+ human T-cells (CEMX174). Supernatants from this infection were analyzed for RT activity and p24 antigen production from 3 to 10 days post infection. HIV RT levels correlated well with p24 antigen results. When p24 antigen production from cells infected with virions produced from the U6-tRNA\textsubscript{3Lys}-rbz transfected cells was compared to the p24 antigen production from cells infected with virions produced from cells transfected with U6 vector alone, a 5 to 6-fold reduction could be measured, indicating that the ability of the virus to infect had been significantly reduced. Fig. 7 is a graph of the p24 antigen in supernatants from day 6 post-infected CEMX174 T-cells. These results demonstrate that the presence of the tRNA\textsubscript{3Lys-rbz} in 293 cells reduces the titer of infectious virions produced from these cells. A tRNA-rbz construct with a point mutation in the catalytic core of the ribozyme which abolishes cleavage \textit{in vitro} is also effective at reducing the titer of infectious virions produced from cells transfected with it. However, the wild-type tRNA-rbz is reproducibly more efficient at reducing titer than the tRNA-mutant-rbz construct.

Fig. 8 is a graph of p24 antigen production from CEMX174 T-cells, 7 days after the infection with virions from 293 cells transfected with the ΔGtRNA\textsubscript{3Lys-rbz}, U6tRNA\textsubscript{3Lys-rbz} or tRNAValPBS-rbzs. These results demonstrate that ribozymes of the same hybridization length as the tRNA\textsubscript{3Lys-ribozymes} but placed downstream of tRNA\textsubscript{Val} are much less inhibitory. The 50% level of inhibition demonstrated by the tRNA\textsubscript{Val} functional and mutant ribozymes reflects some reduction in viral infectivity, but it does
not approach the level of inhibition of the tRNA$_3$-lys-based ribozymes. The low level of inhibition of viral infectivity could be the result of either ribozyme cleavage by the functional ribozyme or a block in initiation of reverse transcription by the mutant ribozyme. Most importantly, inclusion of the tRNA$_3$-lys-moiety results in up to 200-fold reduction in infectivity over the control, and 25- to 100-fold reduction in infectivity when compared to tRNA$^{\text{Val}}$ chimeras (Fig. 8). This clearly demonstrates the importance and utility of the colocalization imparted by the specific binding of HIV RT to the tRNA$_3$-lys moiety of the chimeric RNAs.

Detection of RNA in virions

The above data also suggested that a percentage of the viral particles from the 293 cells initially transfected contained ribozyme-specific transcript. To examine the quantity and types of RNA present in the virions isolated from 293 cells cotransfected with HIV and various tRNA-ribozymes, virions were pelleted, purified on Sepharose columns, and fractions were assayed for presence of p24 antigen. p24 peak fractions were pooled, and viral RNA was extracted and depleted for any contaminating cellular DNA. RT-PCR was performed on the viral RNA to detect packaged ribozymes and separated on acrylamide or agarose gels. Product purified from acrylamide gels was sequenced and the sequence verified the presence of tRNA$_3$-lys-rbz or tRNA$_3$-lys-mrbz (data not shown). Agarose gels were blotted, and probed with a ribozyme-specific probe. The results (shown in Fig. 9) clearly demonstrate the presence of the tRNA-rbz in the virions produced from tRNA$_3$-lys-rbz transfected 293 cells. The packaging of the tRNA$_3$-lys-ribozyme in virions also indicates that at least a portion of the chimeric transcripts are not 3'-'end processed and
are localized to the cytoplasm in transiently transfected cells. It is not certain whether the nonprocessing tRNAValPBS-ribozyme is capable of nucleocytoplasmic transport. However, some cytoplasmic localization must occur in the transient cotransfection assay, as we have detected the inclusion of the tRNAValPBS-ribozyme in purified virions by RT-PCR (data not presented), consistent with our 50% inhibition using tRNAValPBS-ribzs in the antiviral assay above. Since retroviruses are known to nonselectively package non-primer tRNAs (Jiang et al., 1992; Jiang et al., 1993), it is not surprising that we detected low amounts of the tRNAVal chimera. Once packaged into the virion, the antiviral effect of the tRNAValPBS-ribozymes may be the consequence of their hybridization to the PBS region.

**Establishment of stable cell lines expressing tRNA$_3^{\text{Lys}}$-rbz**

We wished to determine if tRNA-ribozyme expression persisted in cell lines or if expression was toxic to the cell. We therefore transfected human 293 cells with the ΔGtRNA$_3^{\text{Lys}}$-rbz driven from a U6+1 snRNA promoter incorporated into an Adeno-Associated Viral Vector (AAV, Bertrand et al., 1997). Cells were selected on G418, allowed to expand, and clonally selected. Clones selected from pAAVU6+1ΔGtRNA$_3^{\text{Lys}}$-rbz-transfected cells grew at the same rate as clones selected from pAAVU6+1 vector alone, indicating there was no toxicity from the plasmid or transcripts with or without the ribozyme. Total RNA was prepared from the G418 resistant clones and analyzed by Northern blot for the presence of ΔGtRNA$_3^{\text{Lys}}$-rbz transcripts. Fig. 10 is an autoradiograph of a Northern blot probed with a radiolabeled oligonucleotide specific for the ribozyme moiety, and indicates the presence of intact transcript (lanes D5 and D6). Blots probed with an oligonucleotide specific for tRNA$_{3^{\text{Lys}}}$ confirmed equal amounts of
RNA were loaded (data not shown). This result demonstrates that transcription of this chimeric tRNA-ribozyme from the U6 snRNA promoter persists upon integration in stably transfected 293 cells. Furthermore, this tRNA-like molecule is not 3'-end processed as are wild-type tRNA precursor transcripts.

**DISCUSSION**

In this work, we have shown *in vitro* that a chimeric tRNA₃Lys-ribozyme RNA cleaves its HIV-1 RNA target and is bound by HIV RT with an affinity similar to that of wild-type tRNA₃Lys. Previously, we reported that the ΔGtRNA₃Lys-rbz RNA is transcribed *in vivo*, generating an intact chimeric RNA which is localized cytoplasmically (Westaway *et al.*, 1995). Interestingly, this chimeric ΔGtRNA₃Lys-rbz is not efficiently 3'-end processed as are most pre-tRNA transcripts, and the ribozyme moiety remains intact downstream of the tRNA molecule. This lack of apparent processing may be due in part to the overexpression of the chimera during transient transfections, the missing G near the B box of the tRNA, which could effect overall structure and hence recognition by 3'-end processing enzymes, or the fact that the CCA is already encoded in the DNA of the clone at the 3'-end of the tRNA at the junction between the tRNA and ribozyme. In eukaryotes, this CCA moiety is added post-transcriptionally after 3'-end processing. Alternatively, or in conjunction, the ribozyme structure could impede normal 3'-end processing. However, the cytoplasmic localization of ΔGtRNA₃Lys-rbz indicates that lack of apparent 3'-end processing does not interfere with nucleocytoplasmic transport. The cytoplasmic
localization also suggests that the tRNA\textsubscript{3}\textsuperscript{Lys}-ribozyme of the tRNA-rbz is folding correctly, since nucleotide changes in tRNAs that greatly alter secondary and tertiary structures have been shown to interfere with nucleocytoplasmic transport (Tobian \textit{et al.}, 1985). This result is important in that the tRNA-rbz localizes to the cellular compartment where viral packaging begins. It is not known from which subcytoplasmic compartment the viral proteins recruit the tRNA\textsubscript{3}Lys\textsuperscript{Lys} primer. It is also unknown whether this primer is recruited before aminoacylation, after aminoacylation and before recruitment by the translational machinery, or after the deacylated tRNA leaves the ribosome. The tRNA could be recruited in a deacylated state, or could be aminoacylated, and the amino acid removed during or after packaging.

HIV RT activity of supernatants from 293 cells transiently transfected with tRNA\textsubscript{3}Lys-ribozymes and HIV proviral DNA is not affected by the presence of the tRNA-rbz. This suggests that the tRNA\textsubscript{3}Lys-rbz either acts during or after viral packaging, and is ineffective in binding and cleaving viral mRNAs made prior to virion assembly.

p24 antigen production in supernatants from T-cells infected with virions produced from 293 cells transiently transfected with tRNA\textsubscript{3}Lys-ribozymes is reduced 5- to 200-fold over the controls. In addition, we have detected the presence of tRNA\textsubscript{3}Lys-rbz in the population of virions packaged from the 293 cells. We have also observed that viral particles produced from 293 cells transfected with tRNA\textsubscript{3}Lys-rbz and HIV proviral DNA have morphological defects when examined by electron microscopy (data not shown). The reduced infectivity of these virions is marked and reproducible. Ribozymes with a point mutation that abolish cleavage \textit{in vitro} are also effective in reducing HIV virion infectivity, but are somewhat reduced in this capacity compared to the wild-type. The
The most straightforward explanation for these results is that the tRNA$_{3}^{\text{Lys}}$-mrbz may be acting as a decoy (i.e., binding RT and preventing necessary interactions with other HIV RNAs or proteins), or as an antisense RNA, blocking initiation of reverse transcription. In fact, the result with mutant ribozyme inhibition of viral infectivity supports the hypothesis that these chimeric tRNAs can be encapsidated and aligned on the primer binding site. The same holds true for the wild-type ribozyme, but this does not preclude the possibility that wild-type ribozymes are cleaving the viral RNA as well. We have observed reduced levels of HIV RNA in virions produced from cells co-transfected with HIV proviral DNA and the chimeric ΔGtRNA$_{3}^{\text{Lys}}$-rbz (data not shown). Nevertheless, the inhibitory effect of the mutant ribozyme suggests that the mechanism for the functional ribozyme could be a mixture of cleavage and antisense activities. Importantly, stable expression of the U6-driven ΔGtRNA$_{3}^{\text{Lys}}$-rbz in 293 cells does not appear to be cytotoxic. The presence of the ribozyme prevents aminoacylation, thus the tRNA$_{3}^{\text{Lys}}$-rbz cannot interfere with the translation machinery.

The results presented here demonstrate that chimeric tRNA$_{3}^{\text{Lys}}$-rbz RNAs can be effective anti-HIV therapeutic agents. Their effectiveness is most probably the result of colocalization with the antiviral target RNAs via the normal packaging and primer selection pathways used for tRNA$_{3}^{\text{Lys}}$. Recently, one group described anti-HIV inhibitory activity of an altered tRNA$_{3}^{\text{Lys}}$ primer which directs priming from the TAR region of HIV (Lu et al., 1997). It would thus appear that such an approach could also be used to direct a ribozyme to cleave at a site other than within the region adjoining the primer binding site.
The work described here is part of our overall effort to utilize a ribozyme based gene therapy approach for the treatment and prevention of HIV infections. A strategic goal of this program is the simultaneous use of multiple ribozymes targeted to different regions of HIV RNA at different steps in the viral life cycle. The approach described in this study takes advantage of a critical part of the viral life cycle, virion assembly, to target a ribozyme to genomic virion RNA. It is anticipated that the chimeric tRNA3^Lys-rbz will complement the standard ribozyme targeting approaches to provide a complete intracellular immunity to HIV infection. This same approach can be used with other retrovirally caused diseases in both man and animals which use specific cellular transfer RNAs as primers for reverse transcription.

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A

HIV-1 RNA

5'

UGGAAA

ACCUGU UA

G

U

AGA/CAGUUGGCGLCCAGAACAGGAC

A

G

A

G

3'

UGGV'A

AGAUGUCACCUCGGGCUUGUCCUCG

A

G

A

G

Ribozyme Cleavage Site

Primer Binding Site

OR

G

A

C

U

G

A

G

Ribozyme

sometimes

G

G

C

C

U

U

tRNA^LYS^3

tRNA^VAL-IAC

B

PLASMID NAME

pPBS-rbz

ΔGtRNA^3^lys-rbz

ptRNA^Lys^-rbz/mrbz

TZU6+1 Vector

ptRNAValPBS-rbz/mrbz

ptRNAVal Vector (ptV5)

pAAVU6+1 Vector

pAAVU6+1ΔGtRNA^3^lys-rbz

KEY:

Plasmid sequences

AAV Vector sequences

Anti-PBS sequences

Ribozyme sequence

3'-stabilization sequence

ΔGtRNA^lys^ sequence

tRNAVal sequences

U6+1 promoter

tRNA^lys^ sequence

TTTTT Transcription terminator

82
Figure 2. *In vitro* cleavage of HIV substrate RNA by ΔGtRNA$_{3}^{Lys}$-rbz and PBS-rbz.

MW markers are indicated to the left of the diagram. Substrate (A) and two cleavage products (B and C) are indicated to the right. Lane 1, substrate alone, incubated in the presence of Mg$^{2+}$. Lanes 2, 3, and 4: Substrate incubated with PBS-rbz, ΔGtRNA$_{3}^{Lys}$-rbz, or tRNA$_{3}^{Lys}$, respectively, in the absence of Mg$^{2+}$. Lanes 5, 6, and 7: Same as lanes 2, 3, and 4, in the presence of Mg$^{2+}$. 
Figure 3. tRNA\textsubscript{3}\textsuperscript{Lys} and ΔGtRNA\textsubscript{3}\textsuperscript{Lys-rbz bind to HIV RT. Binding of tRNA\textsubscript{3}Lys or ΔGtRNA\textsubscript{3}Lys-rbz is specific for HIV RT.

Lanes 1-4 contain radiolabeled tRNA\textsubscript{3}Lys; lanes 5-8 contain radiolabeled ΔGtRNA\textsubscript{3}Lys-rbz. The type of RT added is labeled above the figure. S indicates the position of the substrate and B indicates the bound complex.
Figure 4. Native gel electrophoresis demonstrates competition of tRNA\textsubscript{3Lys} and ΔGtRNA\textsubscript{3Lys-rbz} complexed to HIV RT.

Panel \textit{a}, lanes contain 1 pmol radiolabeled tRNA\textsubscript{3Lys} complexed with HIV RT. Panel \textit{b}; lanes contain 1 pmol radiolabeled ΔGtRNA\textsubscript{3Lys-rbz} complexed with HIV RT. In both panels: lane 1, no HIV RT; lanes 2 and 3, no competitor, with 1 pmol and 10 pmol HIV RT, respectively; lanes 4 and 5, 1 pmol HIV RT with 4 and 20 μM unlabeled tRNA\textsubscript{3Lys} competitor, respectively; lanes 6 and 7, 1 pmol HIV RT with 4 and 20 μM unlabeled ΔGtRNA\textsubscript{3Lys-rbz} competitor, respectively. S indicates the position of the substrate, while B1 and B2 indicate two different migrations of bound complexes.
Figure 5. U6 snRNA promoter increases expression of tRNA-ribozymes.

Two constructs, driven by either a tRNA<sub>3</sub>-Lys promoter (ptRNA<sub>3</sub>-Lys-rbz) or fused downstream of the human U6 snRNA promoter (pU6tRNA<sub>3</sub>-Lys-rbz) were transiently transfected into 293 cells and total RNA was analyzed by Northern blot with a U6 snRNA probe as a loading control (lanes 1 and 2) or a ribozyme-specific probe (lanes 3 and 4). Lanes 1 and 3 are from ptRNA<sub>3</sub>-Lys-rbz transfected cells, and lanes 2 and 4 are from pU6tRNA<sub>3</sub>-Lys-rbz transfected cells. The arrow at the far right indicates where \textit{in vitro} transcribed tRNA<sub>3</sub>-Lys-rbz migrates (not shown).
Figure 6. Flow chart of method for testing anti-HIV ribozymes for inhibition of HIV. See text for explanation.
Figure 7. **p24 antigen production from virions isolated from infected T-cells.**

The T cell line CEMX174 was infected, in triplicate, with virions isolated from supernatants of three-day post-transfected 293 cells transiently transfected with the constructs listed and proviral HIV DNA, using normalized HIV RT activity. T-cell supernatants were collected and assayed for p24 antigen (pg/ml). A day 6 post-infection timepoint is shown with p24 antigen levels for each of the viral populations used to infect the T cells. Corresponding mean values for each triple infection are shown above their respective error bars.
Figure 8. Graph of p24 antigen production at day 7 as in Fig. 7, but for different viral populations used to infect the T cells.

Corresponding mean values for each triple infection are shown above their respective error bars.
RNA isolated from fractionated virions from supernatants of transfected 293 cells were subjected to RT-PCR using 5’ and 3’ primers specific for the tRNA\textsubscript{3Lys-ribz}. Products were electrophoresed on an agarose gel, transferred to a nylon membrane and hybridized to a radiolabeled probe specific for the ribozyme. Lanes are RNA from cells transfected with: 1) pΔGtRNA\textsubscript{3Lys-ribz}; 2) pU6tRNA\textsubscript{3Lys-ribz}; 3) ptRNAValPBS-ribz; 4) ptRNAValPBS-mrbz; and 5) ptV5 (ptRNAVal). Lane labeled marker is a RT-PCR of \textit{in vitro} transcribed tRNA\textsubscript{3Lys-ribz} as a size marker and positive control. + and - indicate the presence or absence of AMV reverse transcriptase. Lanes 3-5 are negative, since PCR with tRNA\textsubscript{3Lys-ribz}-specific primers would not be expected to amplify tRNAVal-ribzs.
Figure 10. Northern blot analysis of total cellular RNA demonstrates nontoxicity and expression of $\Delta$GtRNA$_3^{1\text{ys}}$-rbz.

Total cellular RNA isolated from stably transfected clonal cultures of 293 cells transfected with pAAVU6+1 vector alone (lanes B3 and A4), or pAAVU6+1$\Delta$GtRNA$_3^{1\text{ys}}$-rbz (lanes D5 and D6) was resolved on an agarose formaldehyde gel, blotted and probed with a probe specific for the ribozyme moiety. Lane designated Marker is in vitro transcribed tRNA-rbz used as a size marker and positive control.
B. Expression of chimeric tRNA\textsuperscript{Lys3}-ribozymes under juxtaposed U6 snRNA gene and tRNA gene promoters in human cell cytoplasm and efficient HIV inhibition

**Introduction**

Human immunodeficiency virus (HIV) is the causative agent of acquired immune deficiency syndrome (AIDS). In its life cycle, the HIV single-stranded RNA genome is reverse transcribed into double-stranded proviral DNA by reverse transcriptase (RT). Proviral DNA is then transported into the nucleus and integrates into the host chromosome. Like all retroviruses, HIV also employs one species of host tRNA as the primer for initiating the reverse transcription. The primer employed by HIV-1 is host native tRNA\textsuperscript{Lys3}, which is packaged into virions from the cytoplasm during virion encapsidation by the action of the polyprotein Pr160\textsuperscript{Gag-Pol}, the precursor of RT (Mak et al., 1994, 1997). The nucleotide sequence at the 3' end of tRNA\textsuperscript{Lys3} is complementary to 18 nucleotides of viral sequence near the 5' end of the RNA genome, called the primer binding site (PBS). During the initiation of reverse transcription, these 18 nucleotides are annealed to the HIV genome and prime the synthesis of the first strand of DNA.

The interaction of HIV RT with host tRNA\textsuperscript{Lys3} could potentially be exploited to bring RNA-based therapeutics including ribozymes, antisense RNAs and RNA decoys into the virions. Ribozymes are catalytic RNA molecules capable of cleaving target RNA molecules at specific sites. While ribozymes also function as antisense molecules, the fact that these can cleave and then recycle themselves to other target molecules provide an advantage over standard antisense molecules.
We attached a hammerhead ribozyme to the 3' end of the mature tRNA\textsubscript{Lys}\textsuperscript{3} after the CCA tail. The ribozyme target is upstream of the PBS on the HIV-1 genome. Because tRNA genes possess internal RNA polymerase III promoters, such a construct should express chimeric tRNA\textsubscript{Lys}\textsuperscript{3}-ribozyme transcripts at high levels from the internal tRNA promoter. The chimeric tRNA\textsubscript{Lys}\textsuperscript{3}-ribozyme was found to be catalytically active \textit{in vitro} and bind to HIV-1 RT with an affinity similar to wild-type tRNA\textsubscript{Lys}\textsuperscript{3} (Westaway \textit{et al.}, 1995, 1998). The chimeric tRNA\textsubscript{Lys}\textsuperscript{3}-ribozyme could be detected inside the virions after being cotransfected with HIV proviral DNA into 293 cells. The HIV virions produced from the 293 cells were found to be less infectious than virions from control cells transfected with only HIV DNA (Westaway \textit{et al.}, 1998).

One important aspect of the \textit{in vivo} function of ribozymes is that they should be expressed at high levels to increase the ribozyme-to-substrate ratio to ensure maximal substrate inhibition (Cotten and Birnstiel, 1989). Therefore, higher level intracellular expression of chimeric tRNA\textsubscript{Lys}\textsuperscript{3}-ribozymes is essential and is expected to enhance the inhibition of viral replication by the ribozymes. In addition, it is also expected that a higher level of expression of the chimeric tRNA\textsubscript{Lys}\textsuperscript{3}-ribozymes would lead to more incorporation into virions, and concurrently less incorporation of endogenous tRNA\textsubscript{Lys}\textsuperscript{3}. This expectation is based upon the suggestion of a limited number of molecules of Pr\textsubscript{160}\textsuperscript{Gag-Pol} for interacting with the tRNA\textsubscript{Lys}\textsuperscript{3} (Huang \textit{et al.}, 1994).

Transfer RNAs including tRNA\textsubscript{Met} and tRNA\textsubscript{Val} have been widely used in gene therapy approaches as \textit{in vivo} expression cassettes to produce a high level of short therapeutic RNA molecules, such as ribozyme and antisense molecules. Among many characteristics of tRNA expression cassettes, the most important advantage is that they produce short RNA molecules at high efficiency. They possess internal RNA polymerase
III promoters and can express ribozymes at levels at least an order of magnitude more than RNA polymerase II (Pol II) promoters (Cotten and Birnstiel, 1989).

While expression of the chimeric tRNA\textsubscript{Lys}\textsuperscript{3}-ribozymes under the tRNA\textsubscript{Lys}\textsuperscript{3} promoter is effective in HIV inhibition (Westaway \textit{et al.}, 1998), our goal has been to increase the expression level further. The human U6 small nuclear RNA (snRNA) is a very abundant species of RNA involved in spliceosome formation during pre-mRNA splicing, reaching as high as 0.4-0.5 $\times 10^6$ copies per cell (Sauterer, 1988; Weinberg and Peuman, 1968). The U6 promoter drives transcription of short RNA molecules at levels higher than expression from tRNA promoters (Ileves \textit{et al.}, 1996). Both U6 and tRNA promoters belong to the Pol III promoter class and transcripts from these two promoters do not have a 3' polyA tail added after transcription as do mRNA transcripts from RNA polymerase II promoters. However, one important difference exists between the U6 and tRNA promoters—the U6 promoter is positioned upstream of its structural gene, while the tRNA promoter is intragenic, embedded inside the structural gene. This difference makes it possible to juxtapose these two Pol III promoters in tandem as an expression cassette for enhancing the expression level of RNA-based molecules.

In an attempt to enhance the expression level of the ribozymes and hence boost the HIV inhibition, we adjoined the U6 promoter upstream of the chimeric tRNA\textsubscript{Lys}\textsuperscript{3}-ribozymes. In these constructs, two promoters are connected in tandem, one extragenic U6 promoter and another intragenic tRNA promoter. Using combinations of different promoter elements of both U6 and tRNA promoters (A and B box without the rest of the tRNA gene introduced into the coding sequence of \textit{Xenopus} U6 gene), Parry and Mattaj (1990) demonstrated that there could be different interactions between the two promoter elements. However, the feasibility of using juxtaposed U6 extragenic and tRNA intragenic promoters as an expression cassette for expressing short therapeutic RNAs has
never been tested in mammalian cells. There could be additive, synergistic or antagonistic effects on the expression level.

Another factor that is important in determining the feasibility of using tRNA or U6 promoters in expressing short genetic materials like ribozymes for therapeutic inhibition of HIV is that they have to be exported to the cytoplasm, where they can colocalize with their target and be packaged into virions. Despite reports of nuclear retention of transcripts expressed from the U6 promoter, it is likely that the nuclear retention is a function of the transcripts per se rather than a function of the promoter. With tRNALys\textsuperscript{3} as part of the chimera, we expect that the tRNA moiety could facilitate the export of the chimeric molecules expressed from the dual U6/tRNA promoters. The tRNA moiety of the chimera is expected to retain the tRNA secondary and tertiary structure, while a CCA appended to the 3'end of the tRNALys\textsuperscript{3} also helps to mimic the mature tRNA 3'-end. Therefore, the tRNA moiety of the chimera is expected to facilitate the export of these transcripts into the cytoplasm.

We demonstrated that juxtaposing the U6 snRNA promoter and tRNA promoter could result in higher levels of expression of chimeric tRNALys\textsuperscript{3}-ribozymes than that obtained from the tRNA promoter alone. Additionally, the transcripts can be transported to the cytoplasm. These constructs were shown to inhibit HIV-1 infectivity effectively. The hybrid U6/tRNA expression cassette can be valuable in applied research using RNA-based therapeutics and could contribute to basic research in RNA polymerase III promoters as well.
Results

Design of chimeric tRNA-ribozymes under intragenic tRNA promoter and U6 snRNA promoter

tRNA genes have intragenic promoter elements, called the A and B boxes (Fig. 1A). tRNA expression cassettes (Fig. 1C) have been widely used for expressing RNA-based therapeutics including chimeric tRNA-ribozymes. Different anti-HIV chimeric tRNALys3-ribozymes, pWW, pMW, pWM, pMM and pΔGTRz were designed (Fig. 2). The 3' 18 nucleotides of tRNALys3 are complementary to the HIV-1 primer binding site (PBS). The hammerhead ribozyme targeting 8 nucleotides upstream of the PBS is fused to the 3'end of the tRNALys3 gene.

Specific single mutations in the B Box of the tRNA (GCTACAAAGTCC instead of the wild type B box GGTTCAAGTCC) were shown in vitro to abolish transcription from the tRNA intragenic promoter (Murphy et al., 1983). The sequences of the chimeric tRNA-ribozymes have 33 nucleotides of homology with the sequences of HIV-1 and this could result in inhibition of HIV replication through an antisense effect. To compare if the resulting inhibition of HIV-1 was due to ribozyme cleavage or an antisense effect, we introduced a mutation in the catalytic core of the ribozyme moiety (Fig. 2), which would abolish the ribozyme activity of the transcripts (Ruffner et al., 1990).

Different constructs with different combinations of the wild type and mutant forms of the tRNALys3 and ribozyme were synthesized and cloned (Fig. 1B). These were synthesized by PCR from synthetic oligonucleotides and cloned between the Sall and EcoRI sites of pBluescript II SK, in between the T7 and T3 promoters. These include
Figure 1. Structure of the tRNA gene, the human U6 snRNA gene, and the tRNA expression cassette or the hybrid U6/tRNA promoter expression cassette driving the expression of chimeric tRNA-ribozyme sequence.

(A) The tRNA gene has intragenic promoter elements, the A and B box, for driving the expression of tRNA molecules. (B) The human U6 snRNA gene has an upstream distal sequence enhancer (DSE), proximal sequence element (PSE) and TATA box as promoter elements. (C) The tRNA expression cassette is widely used for the expression of ribozymes. Our constructs pWW, pMW, pWM and pMM use this cassette. (D) The hybrid U6/tRNA expression cassette, i.e., the human U6 snRNA promoter (Bertrand et al., 1997) adjoined upstream of the tRNA promoter, is developed here for expressing the ribozyme sequence. The constructs in this series include pU6WW, pU6MW, pU6WM and pU6MM. The structural sequence of the U6 snRNA is not included. However, the whole sequence of the tRNA gene is incorporated and the transcripts would incorporate the tRNA sequence and the ribozyme sequence in a chimeric molecule, as in the tRNA expression cassette. (A-D) All the genes and expression cassettes use a run of Ts as the termination signal.
Figure 2. Structure of the chimeric tRNA\textsuperscript{Lys3}-ribozymes and their proposed interaction with the HIV genome RNA.

The chimeric tRNA\textsuperscript{Lys3}-ribozymes are shown base-paired to the flanking sequence of the AUC cleavage site indicated on the HIV RNA. The 3' end 18 nt of the tRNA moiety is complementary to the primer binding site (PBS) of HIV. The ribozymes target 8 nt upstream of the PBS. The 5 uridine residues at the 3' end of the ribozyme serves as an RNA polymerase III terminator. The G*A mismatch at the end of the ribozyme/HIV RNA duplex was introduced to prevent premature transcription termination, because the perfectly matched ribozyme sequence would have a stretch of four uridines. The wild type sequence of tRNA and ribozyme is present in the WW construct. The G-to-A change in the catalytic core of the ribozyme, present in the WM and MM constructs (Westaway \textit{et al.}, 1998) is inactive for cleavage in vitro (see text). The U to A and G to C changes in the B box of the tRNA sequence, present in MW and MM constructs, were shown to abolish transcription from the tRNA promoter in vitro. One deletion in the TPC stem, immediately upstream of the B box, is present in the \textit{ΔGTRz} as described previously (Westaway \textit{et al.}, 1998). All the constructs in the tRNA expression cassette series, WW, MW, WM, MM and \textit{ΔGTRz}, are also cloned under the U6 promoter, giving rise to the U6/tRNA expression cassette series U6WW, U6MW, U6WM, U6MM and U6\textit{ΔGTRz}.
pWW, pMW, pWM and pMM. Another construct named pΔGTRz has a single G deletion in the TΨC stem loop junction, immediately upstream of the B Box of the tRNA gene.

U6 snRNA is an abundant RNA in eukaryotic cells (Fig. 1B). The U6 snRNA promoter is extragenic. Therefore, we could place our chimeric tRNA_{Lys}^3-ribozymes downstream of the U6 snRNA promoter and attempt to drive expression of tRNA_{Lys}^3-ribozymes by two promoters in tandem (Fig. 1D). Only one nucleotide from the structural U6 RNA gene was included downstream of the U6 promoter, excluding the capping signal, which is important for nuclear localization of the U6 snRNA (Good et al., 1997). These constructs are named pU6WW, pU6MW, pU6WM and pU6MM and pU6ΔGTRz.

Enhanced expression of chimeric tRNA_{Lys}^3-ribozymes under the hybrid U6/tRNA promoter

One important aspect for enhancing the efficiency of a therapeutic ribozyme is the expression level. The higher the expression level, the higher the ribozyme to substrate ratio, and the more effective the ribozyme targeting. Earlier studies showed that the chimeric tRNA_{Lys}^3-ribozymes could be packaged into HIV virions during encapsidation and subsequently inhibit HIV infectivity (Westaway et al., 1998). We expected that if we increased the intracellular levels of expression of the chimeric tRNA_{Lys}^3-ribozymes, more copies of these molecules would be incorporated into the virions during virion encapsidation, and consequently lead to more efficient inhibition of HIV-1. This was suggested by a study which showed that each virion contains 8 copies of tRNA_{Lys}^3 and the total number of copies of tRNA_{Lys}^3, tRNA_{Lys}^1 and tRNA_{Lys}^2 was fixed at 20 copies per virion (Huang et al., 1994). When human cells were transfected with a plasmid containing both HIV proviral DNA and a human tRNA_{Lys}^3 gene, which would largely
increase cytoplasmic concentration of tRNA^Lys^3, the tRNA^Lys^3 copies incorporated into virions more than doubled (from 8 to 17 copies). However, the total number of tRNA^Lys isoacceptors remained at 20 copies with a concurrent drop in the copy numbers of tRNA^Lys^1, 2 from 12 to 3 copies. This was suggested to be due to either limited space in the virions or a limited number of molecules of Pr160^Gag-Pol, which is responsible for recruiting and packaging the tRNA^Lys^3 primers (Huang et al., 1994). Therefore, it can be inferred that if we increase the levels of cytoplasmic expression of the chimeric tRNA^Lys^3-ribozymes, we should be able to increase packaging of the chimeric tRNA-ribozymes into virions, thereby enhancing the inhibition of HIV replication.

It was therefore of interest to compare the expression levels of tRNA promoter driven with levels from juxtaposed U6 promoter / tRNA promoter driven transcription. There was the possibility that the promoter elements present in the intragenic tRNA promoter would compete or interfere with the upstream promoter elements of the U6 gene (Parry et al., 1990). Therefore we sought mutations in the B box of the tRNA which could decrease this interference by decreasing or eliminating transcription from this promoter. We began by comparing the relative abundance of transcripts from the construct with (pMW, pMM and pU6MW) and without (pWW, pWM and PU6WW) the mutations in the B Box.

The constructs harboring the tRNA promoter alone or the adjoining U6 and tRNA promoters were transiently transfected into 293 cells. A plasmid pTKGH for expressing growth hormone was cotransfected to normalize transfection efficiencies. After 48 hours, total RNAs were isolated and analyzed by Northern hybridization. The ribozymes were detected using a probe specific for the ribozyme sequence (Fig. 3A). The membrane was stripped and rehybridized with another probe specific for U6 snRNA to normalize for
Expression Levels of Chimeric tRNA-rbz under Different Promoter Elements
RNA loading (data not shown). Quantitation of the expression levels (Fig. 3B) was carried out by Phosphoimager analysis.

Expression from the chimeric tRNA ribozymes with mutant B box, i.e., MW and MM, was the lowest (Fig. 3A, lanes 4, 6) but not totally absent, which was different from expectations. Little or no expression from tRNAs with single mutations in the B box was demonstrated in \textit{in vitro} assays (Murphy \textit{et al.}). The presence of the U6 promoter upstream of the chimeric tRNA-ribozymes enhanced the expression of the transcripts by nearly 3 fold in U6WW (Fig. 3A, lanes 1 vs 3) and by almost 10 fold in U6MW (Fig. 3A, lanes 2 vs 4). The highest expression level is seen in U6MW in which the U6 promoter was adjoined upstream of the tRNA moiety with mutations in the B box, even higher than expression from U6WW in which the tRNA with wild a type B box was joined downstream of the U6 promoter (Fig. 3A lanes 1 and 2). The difference in expression between U6MW and U6WW is nearly 2 fold (Fig. 3C, ratio c). Compared with the expression from WW (wild type tRNA-ribozyme without the U6 promoter), the construct U6MW yielded an expression level approximately 4 times higher.

\textbf{Subcellular localization of the tRNA\textsuperscript{Lys3}-ribozyme transcripts expressed from the tRNA promoter alone or the U6 and tRNA promoters in tandem}

For ribozymes to work inside cells, they have to be able to colocalize with their target RNA molecules (Bertrand \textit{et al.}, 1997; Good \textit{et al.}, 1997; Sullenger \textit{et al.}, 1993). It is known that endogenous tRNAs are transcribed in the nucleus and exported into the cytoplasm while endogenous U6 snRNA remains exclusively in the nucleus (Fig. 4A). Because the chimeric tRNA\textsuperscript{Lys3}-ribozymes are expected to maintain the tRNA shape
and structure, we expected that our chimeric tRNA\textsuperscript{Lys3}-ribozyme transcripts would localize in the cytoplasm. Since our U6 driven tRNA\textsuperscript{Lys3}-ribozymes do not contain any U6 snRNA localization signals, transcription from U6 should not affect intracellular localization of a heterologous gene product.

The intracellular localization of the chimeric tRNA-ribozymes transcripts were detected by fluorescent \textit{in situ} hybridization. The probes were antisense riboprobes produced and labeled with digoxigenin by \textit{in vitro} transcription from DNA fragments containing the T3 promoter. A probe specific for the ribozyme moiety with no sequence complementarity with the tRNA\textsuperscript{Lys3} was used to detect the transcribed tRNA\textsuperscript{Lys3}-ribozymes (Fig. 4 B, C, D). Specific probes for the U6 snRNA and tRNA\textsuperscript{Lys3} were employed to detect the intracellular localization of these two endogenous RNA species as nuclear and cytoplasmic localization controls (Fig. 4 A). 293 cells were transiently transfected with the constructs, fixed after 48 hours and then subjected to \textit{in situ} hybridization. The probes were subsequently detected by FITC labeled antibodies against the digoxigenin-11-dUMP tag. The slides were counterstained with propidium iodide to stain the nucleus red.

The lower panels of Fig. 4A show the intracellular localization of endogenous U6 snRNA. In agreement with one previous report (Good \textit{et al.}, 1997), the U6 snRNAs were found in large numbers of evenly distributed punctate loci outside the nucleoli, shown as hollow red spots. Another report showed that U6 snRNAs were localized to small numbers of punctate loci only (Carmo-Fonseca \textit{et al.}, 1992). Endogenous tRNA\textsuperscript{Lys3} was shown to be localized exclusively to the cytoplasm (Fig. 4A upper panels). Expression of ΔGTRz was found to be cytoplasmic (Fig. 4B), as reported previously by nuclear-cytoplasmic fractionation (Westaway \textit{et al.}, 1995). This is in agreement with most reports
about cytoplasmic localization of transcripts from tRNA expression cassettes (see one exception in the discussion). Chimeric tRNA_Lys3-ribozymes expressed under the U6 promoter (U6MW and U6WW) were found to be localized homogeneously in both the nucleus and cytoplasm (Fig. 4 C, D).

**Chimeric tRNA-ribozyme mediated inhibition of HIV-1 corresponds with tRNA-ribozyme expression levels**

We previously demonstrated that the chimeric tRNA-ribozymes can inhibit HIV-1 by virion encapsidation (Westaway et al., 1998). Here, we checked and compared the efficiency of these chimeric tRNA-ribozymes expressed under the hybrid U6/tRNA promoter for their ability to inhibit HIV-1 infectivity. The fact that these transcripts can be detected in the cytoplasm suggests that they should be able to inhibit HIV-1 by being packaged into the virions from the cytoplasm (Westaway et al., 1998). The level of HIV inhibition should correspond with their expression levels since the higher the level of expression, the more competitive they would be in getting packaged into the virions. If HIV-1 infectivity is being reduced by the transcripts, it is an indirect indication that the transcripts have been exported into the cytoplasm. We also wanted to compare chimeras with wild type versus mutant ribozymes with respect to HIV-1 inhibitory activity. If the constructs with mutant ribozyme exhibit HIV inhibition, this is likely to be due to an antisense or a decoy effect in which the tRNA_Lys3 moiety competitively interacts with reverse transcriptase or its precursor Pr160^{Gag-Pol} during reverse transcription or encapsidation.

Human 293 cells were cotransfected with HIV-1 proviral DNA and plasmids which can express tRNA_Lys3-ribozymes under the U6 promoter, or a negative control plasmid

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pTZU6+1 (Bertrand et al., 1997), which has the U6 promoter but lacks any chimeric tRNA_{Lys}^3-ribozyme sequences. Because these chimeric tRNA-ribozymes have been shown to work by incorporation into virions (Westaway et al., 1998), the virions produced from the supernatant of these cotransfections are expected to have reduced replication capability during reinfection of other CD4+ cells. Because 293 cells are CD4 negative, virions produced from these cotransfected 293 cells do not reinfect neighboring cells. We collected supernatants that contain virions that may have packaged chimeric tRNA_{Lys}^3-ribozymes and assayed for RT activity. No significant difference in RT activity was observed in these samples compared with that from the pTZU6+1 vector control, suggesting that little if any ribozyme inhibition occurred in the cytoplasm prior to encapsidation. We then used viral stocks prepared from the supernatant, which were equalized for RT counts, to reinfect CEM cells, which are CD4 positive. On day 6 and day 7, the p24 antigen levels from the supernatant were assayed and compared (Fig. 5).

The best inhibition of HIV-1 p24 level was observed in U6MW, the construct with highest intracellular expression level. There was a 14-20-fold reduction of p24 level on day 7 and day 6 respectively compared with that of the pTZU6+1 vector control. There was a 5-6-fold p24 reduction when U6WW was used. The constructs with mutant ribozymes also showed HIV-1 inhibition. For U6MM, p24 level showed a reduction by 3 to 4-fold on day 7 and day 6 respectively. This inhibition is presumably due to an antisense or decoy effect. The mutant tRNA_{Lys}^3-ribozyme could anneal to HIV RNA and inhibit initiation of replication by an antisense effect. The mutant tRNA_{Lys}^3-ribozyme may also interact with Pr160_{Gag-Pol} and interfere with packaging of endogenous tRNA_{Lys}^3, or bind to RT inside virions and hinder the interactions of RT with native tRNA_{Lys}^3 and PBS. There is a 5-fold difference in HIV inhibition when U6MW (with wild type ribozyme) and U6MM (with mutant ribozyme) are compared. Because they
Figure 5. HIV inhibition by chimeric tRNA\textsuperscript{Lys3}-ribozymes expressed from the hybrid U6/tRNA promoter expression cassettes.

293 cells were cotransfected with HIV-1 pNL4-3 proviral DNA and chimeric tRNA\textsuperscript{Lys3}-ribozyme constructs containing hybrid U6/tRNA promoter cassettes. Supernatants were collected and measured for RT counts. Supernatants with equalized RT counts were then used to infect CEM cells. After 6 and 7 days, p24 levels in the media of the CEM cells were measured and compared.
have the same promoter elements, their expression levels in vivo should be the same. This observation suggests a significant action of ribozyme cleavage activity on the HIV RNA target, which most likely occurs in the virions (Westaway et al., 1998).

Discussion

We reported that anti-HIV chimeric tRNA\textsuperscript{Lys\textsubscript{3}}-ribozymes could be detected inside the virions obtained from cells cotransfected with HIV DNA and the chimeric tRNA-ribozyme constructs and the virions were found to be defective with reduced infectivity in subsequent infection assays (Westaway et al., 1998). During encapsidation of HIV-1 virions, endogenous tRNA\textsuperscript{Lys\textsubscript{3}} molecules are recruited from the host cytoplasm by interacting with Pr\textsubscript{160}gag-pol, the precursor of reverse transcriptase (Mak et al., 1994). In the chimeric constructs, the ribozymes were fused to the 3' end of tRNA\textsuperscript{Lys\textsubscript{3}} and presumably brought into HIV virions during encapsidation through interactions with Pr\textsubscript{160}Gag-Pol. It should be noted that these chimeric tRNA\textsuperscript{Lys\textsubscript{3}}-ribozymes were found to have binding affinities to reverse transcriptase similar to wild type tRNA\textsuperscript{Lys\textsubscript{3}} (Westaway et al., 1998). In an effort to enhance the expression of the chimeric ribozymes and hence improve their competitiveness with endogenous tRNA\textsuperscript{Lys\textsubscript{3}} for packaging into HIV virions, we cloned the chimeric tRNA\textsuperscript{Lys\textsubscript{3}}-ribozymes under the human U6 snRNA promoter. We studied the expression of anti-HIV chimeric tRNA\textsuperscript{Lys\textsubscript{3}}-ribozymes from the juxtaposed U6 promoter and tRNA promoters, the intracellular localization of the transcripts and the effectiveness of these constructs in HIV inhibition.
The feasibility of using such an approach for expression of short RNAs from juxtaposed U6 and tRNA promoters depends upon whether or not there is promoter interference. There may be steric hindrance between factors bound to the U6 promoter and tRNA promoter elements (Parry et al., 1990; Fig. 1D). For the tRNA promoter, transcription factor TFIIIC recognizes the B box and binds to a region spanning both the B and A box. This binding enables TFIIIB, the positioning factor, to bind to a sequence surrounding the startpoint. One component of TFIIIB, the TATA Binding Protein (TBP), is responsible for directly interacting with RNA polymerase III. For the upstream U6 promoter, the TATA element is important for determining the initiation site. TATA is bound by a factor that contains the TBP, the subunit that actually recognizes the sequence. These proteins specifically recruit RNA polymerase III. For both tRNA and U6 promoters, the TBP and its associated proteins help to correctly position RNA polymerase III at the startpoint. When the U6 promoter is adjoined upstream of the tRNA gene, there could be some steric hindrance caused by the positioning of these transcription factors within close proximity to each other. Such hindrance may compromise the effectiveness of such an expression system. Nevertheless, it is also possible that in our constructs such hindrance either does not occur or is not a significant factor in transcriptional efficiency from each of the two promoters.

Parry and Mattaj (1990) introduced just the consensus A and B boxes of a tRNA gene into the coding sequence of a Xenopus U6 snRNA gene and studied the effect on transcription. The constructs were microinjected into Xenopus oocytes and transcription levels were studied by RNA isolation and primer extension. When both an A and a B box were inserted into the U6 snRNA gene and combined with the complete U6 promoter, with 26 nucleotides as the distance between the TATA element of the U6 promoter and the A box of the tRNA promoter, the resulting construct was transcriptionally inactive.
This antagonistic consequence was thought to be due to a steric hindrance that could block transcription initiation. When the TATA element-A box distance was increased to 33 or 42 nt, the constructs were strongly transcribed (Parry et al., 1990). In our study, the whole tRNA sequence with its internal A and B box was appended to the end of the U6 promoter. The TATA element-A box distance is 36 nucleotides. The results obtained in Xenopus oocytes are in agreement with our results and our constructs are able to initiate transcription and show high level expression.

The second problem that may potentially undermine the use of juxtaposed Pol III promoters is the intracellular localization of the transcripts. In order for ribozymes to work, they have to be able to colocalize within the same cellular compartment as their target (Bertrand et al., 1997, Sullenger et al., 1993). Because HIV encapsidation occurs in the cytoplasm, the expressed tRNA\text{Lys}^3-ribozymes have to be exported to the cytoplasm for them to be effective in competing for packaging into virions. We engineered the tRNA\text{Lys}^3-ribozymes such that they have a CCA at the end of the tRNA moiety. The added CCA should make the tRNA\text{Lys}^3-ribozymes mimic mature tRNA and therefore evade the action of the 3' processing endoribonuclease (3'-tRNase) which is responsible for the 3'-processing of pre-tRNAs (Mohan et al., 1999). Computer modeling also predicts that the tRNA moiety of the chimeric tRNA\text{Lys}^3-ribozymes would maintain all the secondary structure and properties of the mature tRNA, but aminoacylation can not occur due to the presence of the ribozyme at the 3' end. These native structural properties are likely to assist in recognition by the tRNA export system and facilitate export of the chimeric ribozymes.
U6 and tRNA promoters enhance expression when placed in tandem

U6 snRNA promoter and tRNA promoters can drive the expression of small RNA molecules individually. In this research, we studied the juxtaposition of the extragenic U6 snRNA and intragenic tRNA promoters in expressing chimeric tRNA^{Lys3}-ribozymes targeting HIV-1 PBS for therapeutic purposes. It was found that the dual U6/mutant tRNA promoter in U6MW gave the highest levels of expression. Why is expression from this construct higher than expression from the U6 promoter upstream of the wild type B box (U6WW)? There are two possibilities. First, in pU6MW the mutations in the B box are in the T\textsuperscript{TF}C loop and T stem. Changes in this loop or reduced base-pairs in the T stem have been reported to reduce 3' processing of tRNA molecules by the 3' processing endoribonuclease (3'-tRNase; Nashimoto, 1996 and Nashimoto \textit{et al.}, 1999). A second possibility is that there are antagonistic interactions between promoter elements when the wild type B box is used. In other words, transcription factors bind to the different promoter elements in both the U6 and tRNA promoters. This may lead to steric hindrance of transcriptional initiation in one of the two promoters. Since transcript levels from U6MW were greater than those observed from U6WW (Fig. 2), and the only difference between these two constructs lies in the tRNA promoter which harbors two point mutations in the B box, there may be less promoter interference with this construct. However, it is also possible that the transcripts with the mutant B box are more stable or are 3'-processed to a lesser extent than those with a wild type B box. This could also contribute to the higher intracellular level of U6MW over U6WW.

Subcellular localization of the chimeric tRNA^{Lys3}-ribozymes

After being transcribed in the nucleus, the chimeric tRNA^{Lys3}-ribozymes expressed under any promoter have to be exported to the cytoplasm for them to function in
inhibition of HIV replication. For a ribozyme utilizing the tRNA^{Lys3} as a carrier to get packaged into the virions, this requirement is essential.

Exportin-t was recently identified as a receptor responsible for mediating the export of tRNAs from the nucleus to the cytoplasm (Arts et al., 1998a, Kutay et al., 1998). Exportin-t was found to shuttle rapidly between the nucleus and cytoplasm and binds tRNA in a RanGTP-dependent manner. Ran binds GTP, but the GTP-bound form can change to a GDP form by GTP hydrolysis and nucleotide exchange. This is catalyzed by the GTPase activating protein RanGAP1 that is only present in the cytoplasm. Thus, RanGTP is depleted from the cytoplasm. In the nucleus, RanGTP is generated by the nucleotide exchange factor RCC1. Therefore, RanGTP is thought to exist in higher concentration in the nucleus than in the cytoplasm. This gradient leads to loading of tRNA in the nucleus and after passing through the nuclear pore complex, unloading in the cytoplasm. It was recently reported that the correct tRNA shape and the T\textsuperscript{\Psi}C loop are critical for exportin-t binding (Lipowsky et al., 1999; Arts et al., 1998b).

There are reports which showed that small RNA molecules including ribozymes expressed from a tRNA gene can be expressed and localized in the cytoplasm (Koseki et al., 1999; Ilves et al., 1996; Westaway et al., 1995; Gebhard et al., 1997). We demonstrated in this work that expression of chimeric tRNA^{Lys3}-ribozymes under a tRNA promoter alone or a juxtaposed U6 external and tRNA internal promoter both lead to detection of transcripts in the cytoplasmic compartment (Fig. 4), where they are available for encapsidation into HIV virions.

Constructs with deletions involving the 3' terminal regions up to +62 in the coding sequence of the human tRNA^{Met} gene were created to produce an unprocessed co-
transcript of tRNA and downstream sequences (Adeniyi-Jones et al., 1984). There have been reports of nuclear localization for transcripts expressed from such a tRNA Met promoter with the 3' sequence deleted after the B box (Good, 1997; Koseki et al., 1999). Another study showed 70% nuclear and 30% cytoplasmic localization from the same tRNA Met cassette (Du et al., 1998). Yet another report showed cytoplasmic expression from this cassette (Gebhard et al., 1997). While there was not a consensus when the 3'deleted tRNA Met cassette was used, there seems to be an agreement when the whole tRNA sequence is used (Ilves et al., 1996; Koseki et al., 1999; Westaway et al., 1995 and this study). As suggested by recent findings in tRNA transport (Lipowsky et al., 1999), the correct tRNA shape is important for export through the exportin-t/RanGTP pathway. In 3' - deleted tRNA Met, the tRNA molecule is not able to maintain its shape and nuclear retention makes more sense, which is in agreement with most reports (Good, 1997; Koseki et al., 1999; Du et al., 1998).

As suggested by computer modeling, the tRNA moieties in chimeric tRNA Lys3-ribozymes are expected to maintain their wild type tRNA shape. In addition, the expressed chimeric tRNA Lys3-ribozyme transcripts are expected to maintain a mature 5'-end, while the fate of the 3'-end deserves further study. With a CCA inherently expressed from the genes, we expect that the 3'end is going to be stable because it was shown that mature tRNA containing the 3' terminal CCA is not a substrate for 3'-tRNase, the enzyme responsible for the 3' processing of pre-tRNAs (Mohan et al., 1999). At least a significant proportion of our chimeric tRNA Lys3-ribozyme remains intact (Fig. 3A) and a large proportion of these intact molecules can be exported into the cytoplasm (Fig. 4), evading the 3' processing which normally occurs in the nucleus.
It was reported that tRNAs lacking the CCA at the 3' end are much less efficient in exportin-t/RanGTP binding and therefore have deficient export (Lipowsky et al., 1999). Although unremoved 3' extensions decrease affinities of tRNAs with exportin-t/RanGTP (Lipowsky et al., 1999), it is possible that the decreased binding could be due to the absence of CCA in these tRNAs. Therefore, it is possible that tRNAs like the chimeric tRNA\textsuperscript{Lys}\textsuperscript{3}-ribozymes with CCA at 3'end of tRNA and with ribozyme as a 3' trailer after the CCA could still bind exportin-t and get exported into the cytoplasm. There have been several reports that transcribed tRNAs with 3' trailers for therapeutic purposes expressed from tRNA cassettes could be exported to the cytoplasm (Koseki et al., 1999; Ilves et al., 1996 and this study).

Aminoacylation of tRNA before export has been recently suggested to be a mechanism of proofreading for tRNAs (Lund & Dahlberg, 1998). Nevertheless, nonacylated tRNAs have been shown to bind exportin-t efficiently (Arts et al., 1998a, 1998b, Lipowsky et al., 1999) and there have been reports showing that nonaminoacylated or poorly aminoacylated tRNAs can be exported efficiently (Lipowsky et al., 1999; Arts et al., 1998b). The chimeric tRNA\textsuperscript{Lys}\textsuperscript{3}-ribozymes cannot be aminoacylated due to the presence of the ribozyme at the 3' end but this obviously did not abolish the export of these transcripts.

There is evidence which suggests that tRNAs may get exported through exportin-t independent pathways (reviewed in Wolin and Matera, 1999 and Simos and Hurt, 1999). It is possible that the chimeric tRNA\textsuperscript{Lys}\textsuperscript{3}-ribozymes may get exported through these pathways that are yet to be defined.
Short RNA transcripts (a stem loop structure, an antisense, a ribozyme, or an RNA aptamer) expressed under the U6 promoter with little or no U6 structural sequence, were found to be retained in the nucleus (Bertrand et al., 1997; Good et al., 1997). Our results demonstrate that the chimeric tRNA\textsuperscript{Lys\textsubscript{3}}-ribozymes expressed under the U6 promoter can be detected in the cytoplasm (Fig. 4). The promoter used was the same as one of the promoters used in Bertrand and Good's papers. The difference in subcellular localization is likely to be due to the nature of the transcripts, with tRNA characteristics helping to get the transcripts exported into the cytoplasm. There could be arguments that the cytoplasmic localization of transcripts from the dual U6/tRNA promoter could be a result of high level expression due to overwhelming expression from transient transfection that exceeds the nuclear transport pathway capacity of the nucleus, allowing the transcripts to "leak" from this compartment. However, this is unlikely because transcripts from several constructs which were expressed at levels of up to 1 million copies per cell did not leak out of the nucleus (Good et al; 1997).

**Efficient HIV inhibition**

The importance of intracellular colocalization of ribozymes with their targets has been well established (Sullenger and Cech, 1993; Bertrand et al., 1997). When expressed under the hybrid U6/tRNA promoter, the level of expression of the chimeric tRNA\textsuperscript{Lys\textsubscript{3}}-ribozymes increases greatly (Fig. 3), while the cytoplasmic export of the transcripts is maintained (Fig. 4). It is therefore conceivable that the increased expression would lead to increased packaging of the tRNA\textsuperscript{Lys\textsubscript{3}}-ribozymes, and resultant increased inhibition of HIV infectivity. As can be seen in Fig. 5, the inhibition levels corresponded well with the relative levels of expression of the various constructs. Antisense molecules or ribozymes expressed under the U6 promoter alone with no tRNA sequence have been
found to be retained in the nucleus and showed no inhibition of HIV gene expression (Good et al., 1997). Therefore, our findings once again highlight the importance of high level expression and intracellular colocalization of RNA therapeutics with their targets.

Constructs with active ribozyme moieties in the chimeric tRNA^Lys3-ribozyme (pU6WW and pU6MW) showed stronger inhibition of HIV than constructs with inactive ribozyme moieties (pU6WM and pU6MM; Fig. 5), demonstrating an advantage of the ribozyme over an inert antisense. The chimeric tRNA^Lys3-ribozymes can be packaged into the virions (Westaway et al., 1998), therefore the ribozymes are highly colocalized with their targets inside a very limited space within the virions. This contributes to a greater probability for the ribozymes to interact with their targets.

**General considerations**

RNA polymerase III promoters have been widely used in expressing short therapeutic molecules. These include human tRNA genes (tRNA^{Met}, tRNA^{Val}, tRNA^{Lys}), human U6 small nuclear RNA genes, and adenovirus-associated RNA 1 (Ad VA1) gene promoters. There were many reports of using tRNA^{Val}, tRNA^{Met} and tRNA^{Lys} for driving the transcription of small RNAs including ribozymes, antisense molecules and RNA decoys. Despite the finding that the presence of a 3' trailer leads to decreased affinity with exportin-t (Lipowsky et al., 1999) as discussed previously, several studies showed that the transcripts from the tRNA promoter with a 3' trailer can lead to cytoplasmic localization of transcripts. Although it was found that the U6 promoter led to higher level expression than tRNA promoters (Ileves et al., 1996), there were far fewer reports in using the U6 promoter for RNA expression presumably due to the nuclear localization of U6 snRNA and the finding that heterogeneous transcripts expressed from
the U6 promoter were also localized to the nucleus (Ilves et al., 1996; Bertrand et al., 1997; Good et al., 1997) and would not colocalize with mRNA or viral RNAs.

The finding that expression from juxtaposed U6 and tRNA promoters can be used to express small therapeutic RNAs at high levels has meaningful implications for developing expression cassettes for therapeutic RNA molecules (ribozymes, antisense molecules and RNA aptamers). Besides the advantage of higher levels of expression, transcripts expressed from juxtaposed U6 and tRNA promoters have the tRNA structure as part of the chimera. When properly designed, the properties of the tRNA important for export can be reasonably well preserved in the chimera and export of the chimera can be facilitated and ensured by the presence of tRNA as a part of the structure. Therefore, it is worthwhile to test the feasibility of juxtaposing the U6 promoter with other useful tRNA expression systems such as tRNA\textsuperscript{Val} or tRNA\textsuperscript{Met}, which have been and are presently utilized for expressing therapeutic RNAs. These two RNA species are more abundant than tRNA\textsuperscript{Lys} (\textit{in vitro} transcription experiments in human cell extract demonstrate a much higher level of transcription for tRNA\textsuperscript{Val} than for tRNA\textsuperscript{Lys}, Shawn Westaway, personal communication).
C. Transcription, stability and processing of chimeric
tRNA$^{\text{Lys3}}$-ribozymes

Introduction

Ribozymes are catalytic RNAs which can catalyze the endoribonucleolytic cleavage of RNA molecules (Cech, 1987). Upon formation of a complex with their complementary target RNA molecule, they catalyze the degradation and inactivation of the target RNA via a cleavage reaction. The hammerhead ribozyme, the smallest ribozyme identified (Guerrier-Takada et al., 1983; Kruger et al., 1982), has shown great potential in inhibiting specific gene functions. Hammerhead ribozymes have been designed to cleave a variety of target RNAs. Diseases with undesirable expression of RNAs such as malignant disorders, viral infections and genetic diseases are amenable to the actions of ribozymes (reviewed in Kiehntopf et al., 1995a and 1995b; Phylactou et al., 1998; Couture and Stinchcomb, 1996).

RNA polymerase III promoters have been widely used in expressing RNA-based therapeutics including ribozymes, antisense molecules and decoys. Advantages of Pol III promoters over Pol II promoters include 1) RNA Pol III transcribed transcripts are more abundant than poly(A)$^+$ RNAs transcribed from Pol II promoters (Palmer et al., 1990), 2) most RNA Pol III-transcribed genes are ubiquitously expressed in many cell types and 3) Pol III transcripts are short and avoid the possibility that the ribozyme activity may be
masked in a long transcript. The RNA polymerase III promoters that have been used include the human U6 small nuclear RNA gene promoter, adenovirus-associated RNA 1 (Ad VAI) gene promoter and tRNA gene promoters. tRNA gene promoters including tRNA\textsuperscript{Met} and tRNA\textsuperscript{Val} are the most commonly employed Pol III expression cassettes in the literature. The level of expression from the Pol III system is at least one order of magnitude higher than that obtainable from Pol II systems (Cotten and Birnstiel, 1989).

While native tRNA molecules are small (around 75 bp), they are transcribed as precursor tRNAs and go through complex maturation processes including 5’ processing, 3’ processing, splicing (for some tRNA species), base modifications, CCA addition, aminoacylation and export into the cytoplasm. Eukaryotic pre-tRNAs are 5’ processed by RNase P, 3’ processed by the 3’ processing endoribonuclease (3’ tRNase), and exported through interaction with the tRNA export machinery with exportin-t as the export receptor (Arts \textit{et al.}, 1998a, Kutay \textit{et al.}, 1998). A CCA sequence is added to the 3’ end of the tRNA molecule before it is exported. Because the tRNA promoters are intragenic, the transcripts produced from our tRNA expression cassettes are chimeric molecules with the tRNA as part of the RNAs. The exogenous RNA sequences in the tRNA chimerics should not be easily processed from the tRNA molecule. The chimeric transcripts should also be exported into the cytoplasm because the great majority of RNA therapeutics target cytoplasmic molecules. However, it has been reported that expression of exogenous sequences under a tRNA promoter allowed the liberation of the expressed molecule as a separate molecule upon tRNA processing (Lee \textit{et al.}, 1995). Attempts to enhance the stability of the chimeric transcripts (Adeneyi \textit{et al.}, 1984; Cotten and
Birnstiel, 1989; Koseki et al., 1999) led to cytoplasmic (Koseki et al., 1999), or nuclear localization of the transcripts (Good et al., 1997; Bertrand et al., 1997; Koseki et al., 1999; Cotten and Birnstiel, 1989) when the tRNA expression cassette was modified. Nuclear localization of the transcripts when the target molecules are cytoplasmic would certainly greatly discount the effectiveness of these RNA-based therapeutics.

Because tRNA promoters are widely used in RNA-based gene therapy endeavors, studies on proper tRNA expression cassette design should be undertaken to achieve high level expression of stable transcripts with proper subcellular localization before using the promoters in practical applications. We developed effective anti-HIV chimeric tRNA\textsubscript{Lys3}-ribozymes, in which a ribozyme targeting immediately upstream of the primer binding site of HIV genome was fused to the 3' end of tRNA\textsubscript{Lys3}. When expressed under the tRNA\textsubscript{Lys3} promoter in cells cotransfected with HIV proviral DNA, the chimeric tRNA\textsubscript{Lys3}-ribozyme can be encapsidated into virions and reduce the infectivity of those virions (Westaway et al., 1998). We also developed a U6/tRNA expression cassette, in which the human U6 snRNA is adjoined upstream of the tRNA\textsubscript{Lys3} promoter for expressing the chimeric tRNA\textsubscript{Lys3}-ribozymes. The hybrid U6/tRNA Pol III promoter cassette expresses the chimeric tRNA\textsubscript{Lys3}-ribozymes at much higher levels than expression from the tRNA promoter alone. The transcripts were exported into the cytoplasm and enhanced inhibiting activity against HIV was observed (Chapter III, Part B).

To investigate further the feasibility of using such chimeric U6 and tRNA promoters in tandem for transcribing ribozymes or other tRNA chimerics for practical applications,
we first studied the transcripts made from such constructs and compared them with transcripts expressed from the tRNA$^{\text{Lys}3}$ promoter alone. We found the major species of transcript is intact and is the same size regardless of promoter used. The relative level of expression of these transcripts was demonstrated to be close to that of endogenous tRNA$^{\text{Lys}3}$ in the construct with the highest expression level.

The effects of several modifications of chimeric tRNALys3-ribozyme structure on the stability of transcripts and the effect of increased stability upon inhibition of HIV infectivity were also studied. With an increased understanding of tRNA export and tRNA processing, more optimal designs for increasing stability and directing proper intracellular localization are becoming realities. According to recent developments in tRNA export and 3' processing of tRNA (Arts et al., 1998a and 1998b; Kutay et al., 1998; Lipowsy et al., 1999; Nashimoto et al., 1996; 1997; 1999), we hypothesized that several modifications in tRNA structure, including an encoded CCA in between the tRNA and the ribozyme, ΨC loop mutations and a deletion of one G in the T stem could lead to an increase in the steady-state level while retaining the export property of the transcripts. We expect these changes to increase the stability of the intact transcripts because similar changes were reported to greatly reduce the action of the 3' processing endoribonuclease (3' tRNase) (Nashimoto et al., 1996; 1997; 1999). One construct with a deletion in the T stem led to increased stability of transcripts in cell extracts and this chimeric tRNA$^{\text{Lys}3}$-ribozyme showed better inhibition of HIV infectivity.
Results

Construction of chimeric tRNA^Lys3^-ribozymes expressed under different promoters

We reported that chimeric tRNA^Lys3^-ribozymes could effectively inhibit HIV infectivity by virion encapsidation (Westaway et al., 1998). In these constructs, hammerhead ribozymes were fused to the 3' end of the tRNA^Lys3. As in native tRNA^Lys3, 18 nucleotides at the 3' end of the tRNA moiety of the chimera are complementary to the HIV-1 primer binding site (PBS). These ribozymes target immediately upstream of the PBS of the HIV genome.

In this study, several constructs with different modifications in the tRNA^Lys3 moiety of the chimeric constructs were studied. One construct named pWW contains a functional (wild type) ribozyme fused downstream of the wild type tRNA^Lys3 sequence (Fig. 1A). Another construct has mutations in the B box of the tRNA^Lys3 promoter. In replace of the wild type B box sequence (GGTTCAAGTCC), two mutations were introduced and gave rise to the sequence GCTACAAGTCC (Fig. 1B). This construct is named pMW, denoting mutant (M) tRNA and wild type ribozyme (W). The mutations were reported to abolish transcription from the tRNA internal promoter during in vitro experiments (Murphy et al., 1983). Although we found that these mutations significantly reduce expression in mammalian cells (293 cells), they did not completely eliminate transcription of the chimeric tRNA^Lys3^-ribozymes (Chapter III, Part B). Another chimeric tRNA^Lys3^-ribozyme construct pΔGTRz has a deletion of one G immediately 5' of the B
[A] CCA sequence upstream U6 promoter in U6WW

[B] CCA sequence upstream U6 promoter in U6MW

[C] CCA sequence upstream U6 promoter in U6.GTRz

[D] CCA sequence upstream U6 promoter in U6.Rz

WW and U6WW

MW and U6MW

U6.GTRz and U6.Rz
Human U6 snRNA gene and promoter

U6/tRNA expression cassette driving expression of chimeric tRNA\textsuperscript{Lys3}-ribozymes
box of the tRNA sequence in the T stem (Fig. 1C), and has been described previously (Westaway et al., 1998).

As mentioned earlier, the chimeric tRNA<sub>Lys</sub>-ribozyme transcripts may be subject to 3' processing by the 3' processing endoribonuclease. The 3' trailer of mammalian pre-tRNA is removed by the 3' processing endoribonuclease (3' tRNase) which cleaves the RNA immediately downstream of the discriminator nucleotide (the unpaired nucleotide 3' of the last base pair of the acceptor stem), onto which CCA residues are added to produce mature tRNAs. The CCA sequence is required for aminoacylation. We hypothesized that several modifications in these chimeric tRNA<sub>Lys</sub>-ribozyme constructs could reduce the processing of the transcripts. First, a CCA sequence is inserted in between the tRNA<sub>Lys</sub> and the ribozyme sequence in our design of chimeric tRNA<sub>Lys</sub>-ribozymes to make these mimic the end of mature tRNA. It was reported that tRNAs with a 3' trailer composed of CC, CCA or CCA plus one or two nucleotides were not processed by the 3' processing endoribonuclease at all and tRNAs with a 19 nt 3' trailer beginning with CCA were processed at very low efficiency only (Nashimoto, 1997; Nashimoto et al., 1999). Second, it was reported that changes or deletions in the TΨC loop could reduce the cleavage efficiency by 3' tRNase (Nashimoto, 1996 and Nashimoto et al., 1999). Transcripts from MW have two point mutations located in the TΨC stem and loop (Fig. 1B) and therefore could have reduced 3' processing and increased stability. Third, one and two base-pair deletions in the T stem were poorly processed by 3' tRNase (Nashimoto et al., 1999). pΔGTRz with a deletion of one G in the T stem immediately 5' of the B box would have one less base-pair in the T stem (Fig. 1C) and
should theoretically reduce the 3' processing of the transcripts. In pMW, the upstream mutation would also result in one less base-pair in the T stem and could also reduce the processing of the transcripts (Fig. 1B).

Chimeric tRNA\textsuperscript{Lys3}-ribozyme genes with restriction sites for adapting into cloning vectors were synthesized from different oligonucleotides as described previously (Materials and Methods, Chapter II). The chimeric tRNA\textsuperscript{Lys3}-ribozymes were subcloned into another vector pTZU6+1, downstream of the human U6 snRNA promoter and these gave rise to the constructs pU6WW, pU6MW and pU6\textDelta GTRz (Fig. 1A, B, C). The U6 promoter was shown to enhance expression of the chimeric tRNA\textsuperscript{Lys3}-ribozymes, with the highest expression level in pU6MW (Chapter III, Part B). The same ribozyme sequence without the tRNA sequence was directly cloned into pTZU6+1 vector under the U6 promoter, giving rise to pU6Rz (Fig. 1D and see Materials and Methods, Chapter II). This construct would allow the comparison of the stability of the ribozyme with or without the tRNA structure as part of the molecule, an important study only made possible by the use of an upstream U6 promoter. This construct also allows the comparison of expression levels from the hybrid U6/tRNA promoter and the U6 promoter alone. The gene structure of the hybrid U6/tRNA promoter driving the expression of the chimeric tRNA\textsuperscript{Lys3}-ribozymes is depicted in the lower panel of Fig. 1E. The gene structure of human U6 snRNA gene with promoter elements and structural gene sequence is shown in the upper panel of Fig. 1E for comparison.
To make stable cell lines and to study the stability of these chimeric ribozymes in these stable cell lines, we subcloned the chimeric tRNA\(^{\text{Lys3}}\)-ribozymes into an adeno-associated virus (AAV) vector with a neo gene. These constructs were termed pAAVU6WW, pAAVU6MW, pAAVU6WM and pAAVU6ΔGTRz. These were transfected into 293 cells and stable cell lines expressing these chimeric tRNA\(^{\text{Lys3}}\)-ribozymes under the dual U6/tRNA promoter were made by selection with G418.

**Qualitative differences in transcripts from the tRNA promoter alone and from the hybrid U6/tRNA promoters**

Previously, quantitative comparisons of expressions under tRNA promoters (pWW with wild type B box and pMW with mutant tRNA B box) and hybrid U6/tRNA promoters (WW and MW under the U6 promoter without the U6 structural gene sequence) were conducted (Chapter III, Part B). It was found that the construct pMW with the mutant tRNA B box led to decreased (not abolished) expression compared with the construct pWW with the wild type tRNA B box. However, expression from hybrid U6/mutant tRNA promoters in the construct pU6MW led to much higher expression than that obtained from the hybrid U6/wild type tRNA promoters in the construct pU6WW. This apparent paradox was explained in terms of steric interference caused by the binding of transcription factors to the U6 and tRNA promoters, with stronger interference between the wild type tRNA promoter and the U6 promoters. It was also suggested that the transcripts from pU6MW could be more stable due to mutations in the T\(\psi\)C stem which could lead to decreased processing by the 3'-' tRNase. However, the interference between the U6 and tRNA promoters was just inferred from the quantitative difference in
expression levels (Chapter III, Part B; Parry et al., 1990). In addition to the quantitative difference in expression, the steric interference from the two promoters could also result in qualitative changes in the initiation sites of the transcripts.

One of the objectives in this paper is to study the qualitative difference of the transcripts from the tRNA promoter alone and from the hybrid U6/tRNA promoters. Different constructs with different promoter elements including pMW, pWW, pU6MW and pU6WW, were employed. They were transiently transfected into 293 cells and after 48 hours, total cellular RNAs were isolated and purified. The total RNAs were then separated on a 6% denaturing polyacrylamide gel and subsequently electroblotted onto a nylon membrane. The membranes were then probed with an oligo specific for the tRNA (Fig. 2A). Following hybridization with the tRNA probe, the membrane was stripped and reprobed with a ribozyme-specific probe (Fig. 2B).

The tRNA-ribozyme is not present in the mock-transfected cells (lane 5, Fig. 2A and 2B). In constructs with tRNA promoters, i.e., pMW and pWW, there are differences in the amount of transcripts. In the tRNA promoter with mutant B box, there is a transcript X1, which is similar in size to the in vitro transcribed chimeric tRNA-ribozyme (Fig. 2B, lane 1 and lane 6) and another transcript X2 initiated about 60 bp upstream of X1. In

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1 In vivo expressed chimeric ribozymes have extra 5' sequences (6 bp restriction site). In lane 6, in vitro transcribed RNAs are used as RNA markers. The template for the chimeric ribozyme is a PCR product that does not contain the extra sequences.
### B.

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- X2 or X2 and X4
- X1 or X1 and X3

- 100 bp marker
- 10 bp ladder
pWW the transcripts X1 and X2 are similar in size with those expressed from pMW (Fig. 2B, lane 2). There exists yet another transcript X3 initiated about 5-6 bp downstream of the X1, which is expressed from the pWW but absent in transcripts from pMW (Fig. 2B, lanes 1 and 2).

In constructs with hybrid U6/tRNA promoters, U6MW and U6WW (Fig. 2, lanes 3 and 4), the major transcript X1 expressed from pU6MW and pU6WW are similar in size to those expressed from pMW and pWW (Fig. 2B, cf. lanes 3 and 4 with lanes 1 and 2). The transcript X3 from pU6WW is similar with the X3 transcript in WW (Fig. 2B, cf. lane 4 with lane 2). However, the upstream minor band X2 was shifted about 15 bases downstream (Fig. 2B, cf lanes 3 and 4 with lanes 1 and 2). Because the ribozyme probe is complementary to the 3' end of the transcript, the change in size is most probably due to a downstream shift of the 5' initiation site. This could be the result of interference between the transcription factors bound to the U6 and the tRNA promoter elements (Fig. 1E). In U6WW, another transcript X4 that is about 5 bases shorter than X2, is present below X2 (Fig. 2B lane 4). However, despite the interference that results in downstream initiation of a minor species of transcript, the two promoters enhance total expression when utilized in constructs such as pU6MW and pU6WW (Chapter III, Part B).

The X1 and X3 doublet present in pWW and pU6WW could be the result of differential initiation from transcription complexes bound to U6 promoter elements and tRNA promoter elements. If this is true, it may be a manifestation of interference between the two promoters and could explain the lower level of expression in U6WW compared
with U6MW (see Chapter III, Part B). The mutations in the B box may lead to different positioning of the transcription complex that does not allow interference between U6 and tRNA promoter elements and as a result only one major band is visible. On the contrary, there is a possibility that the mutations in the B box could favor synergism between the U6 and tRNA promoters (transcription complexes bound to the two promoters synergistically initiate from the same site). Alternatively, the shorter one of these two bands could be the result of processing by the tRNA 5’ processing enzyme RNase P. The mutation in the B box could possibly interfere with the processing by RNase P, resulting in the absence of the smaller band X3 of the doublet in lane 1 of Fig. 2B.

In the tRNA probed gel (Fig. 2A), a transcript of more than 40 bp is present. This could be a prematurely terminated transcript due to the presence of a run of four U’s in the anticodon loop of the tRNA moiety (Fig. 1). This string of U’s may constitute a weak Pol III termination signal (four T’s in the tRNA^{Lys3} gene). The band is also faintly visible in the mock transfected sample (Fig. 2A).

Relative intracellular levels of chimeric tRNA^{Lys3}-ribozymes and endogenous tRNA^{Lys3} and increased stability of transcripts with mutations in TΨC stem-loop

It was reported that HIV virions recruit tRNA^{Lys3} from the cytoplasm by the interaction of the precursor of reverse transcriptase, Pr160^{Gag-Pol} polyprotein with tRNA^{Lys3} (Mak et al., 1994). tRNA^{Lys1} and tRNA^{Lys2} are also packaged into HIV virions. Because of the limited space inside the virions or the limited number of molecules of
Pr160Gag-Pol, the number of molecules of tRNAs incorporated into the virions is limited (Huang et al., 1994). It has been shown that in cells cotransfected with HIV proviral DNA and a human tRNA<sub>lys</sub> gene, there is a large increase in the amount of cytoplasmic tRNA<sub>lys</sub> packaged into the virions with a concomitant decrease in the amount of tRNA<sub>lys</sub> and tRNA<sub>lys</sub> packaged, while the total amount of tRNA<sub>lys</sub> molecules remains the same (Huang et al., 1994). As we previously demonstrated, our chimeric tRNA<sub>lys</sub>-ribozymes get packaged into the HIV virions (Westaway et al., 1998). Therefore, it can be expected that if we increase the intracellular expression level of chimeric tRNA<sub>lys</sub>-ribozymes, the copy numbers of the chimera would increase while the number of tRNA<sub>lys</sub> packaged should decrease.

The intracellular expression levels of chimeric tRNA<sub>lys</sub>-ribozymes are compared with the level of intracellular tRNA<sub>lys</sub> in the transient transfection assay. 293 cells were transfected with different chimeric tRNA<sub>lys</sub>-ribozyme constructs and the levels of expression of the chimeric tRNA<sub>lys</sub>-ribozymes and the level of tRNA<sub>lys</sub> were determined by Northern hybridization with a probe specific to the tRNA<sub>lys</sub>, which detect both chimeric tRNA<sub>lys</sub>-ribozymes and tRNA<sub>lys</sub>. Because the chimeric tRNA<sub>lys</sub>-ribozymes can be processed to tRNA<sub>lys</sub> size (see later in Fig. 5), the tRNA<sub>lys</sub> band could be from native tRNA<sub>lys</sub> and processed tRNA<sub>lys</sub>-ribozyme. The result is shown in Fig. 2A. The levels of chimeric tRNA<sub>lys</sub>-ribozymes are compared with the tRNA<sub>lys</sub> level in the same sample by getting a chimeric tRNA<sub>lys</sub>-ribozyme to tRNA<sub>lys</sub> ratio (tc/t ratio; tc stands for tRNA chimeric and t stands for tRNA; Table 1). Because the tc/t ratio is obtained from two molecules within the same sample, no loading control is necessary.
**Table 1. tc/t analysis for evaluating chimeric tRNA\(^{L\text{ys}3}\)-ribozyme transcript stability and assessing relative chimeric tRNA\(^{L\text{ys}3}\)-ribozyme expression levels.**

tc stands for tRNA chimeric and t stand for tRNA. Because chimeric tRNA-ribozymes (or other tRNA chimerics fused to the 3' end of tRNA) can be processed to the size of tRNA (Fig. 7), more stable transcripts would have a higher tc/t ratio.

Summary of interpretation of tc/t analysis:

- Transcripts with equal stability and equal promoter strength should have the same tc/t ratio.
- Unstable transcripts with strong or weak promoter: low tc/t ratio.
- Stable transcripts with weak promoter: higher tc/t ratio than unstable transcripts.
- Stable transcripts with strong promoter: even higher tc/t ratio.
The ratio represents the relative amount of intracellular levels of chimeric tRNA\(^{\text{Lys}3}\)-ribozymes compared with tRNA\(^{\text{Lys}3}\) and a higher ratio is advantageous because it could lead to more molecules of chimeric tRNA\(^{\text{Lys}3}\)-ribozymes getting competitively packaged into the virions. It can be seen in U6MW that the level of chimeric tRNA\(^{\text{Lys}3}\)-ribozyme can be very close to the level of tRNA\(^{\text{Lys}3}\) (a tc/t of 0.74), although a portion of the tRNA\(^{\text{Lys}3}\) band may come from the processed chimeric tRNA\(^{\text{Lys}3}\)-ribozyme.

A higher tc/t ratio could also be a reflection of the stability or 3’ processibility of the chimeric tRNA\(^{\text{Lys}3}\)-ribozyme transcripts. An increased tc/t ratio could be due to decreased processing or degradation of the transcripts or increased promoter strength. Significantly higher tc/t ratios were observed in MW (a tc/t of 0.57) and U6MW (0.74) and very low ratios in WW (0.28) and U6WW (0.21). In MW, the increased ratio could not be the result of promoter strength change, because the mutations in the B box in the construct would result in a weaker promoter (Chapter III, Part B; Murphy et al., 1983). However, in U6MW, a much stronger promoter (the U6 promoter upstream of the mutant tRNA promoter) in the presence of stable transcripts (essentially the same transcripts as MW) does increase the tc/t ratio even further, indicating a role of both higher stability and higher promoter strength for the highest expression level of U6MW among these constructs (Chapter III, Part B). The tc/t ratio of WW is much lower than that of MW, despite of the fact that WW has a much stronger promoter than MW (Murphy et al., 1983). This could explain why the intracellular level of MW could reach a comparable, albeit lower level than WW (Chapter III, Part B). However, increased promoter strength in the face of unstable or easily processed transcripts is not likely to increase the tc/t ratio.
because the increased tRNA chimeric (tc) transcripts would rapidly be converted to tRNA (t) size. This is observed in U6WW; it has a stronger promoter than WW (Chapter III, Part B) but only has a tc/t ratio similar to that of WW. It can be concluded that the mutations in the B box of MW and U6MW result in somewhat more stable transcripts.

**Intracellular stability of transfected chimeric tRNA^{Lys}_3-ribozymes in transient transfection analysis**

The ribozyme structures were fused to the 3’ end of the tRNA sequence. We included a CCA sequence at the 3’ end of the tRNA sequence in between the tRNA and the ribozyme sequence. In prokaryotic cells, this sequence is encoded in the tRNA gene, while in eukaryotic cells this sequence is added to the 3’ end of the tRNA by the CCA-adding enzyme after 3’ end processing (Deutscher, 1990). We expect that this sequence could help to stabilize the tRNA-ribozyme structure because the transcripts made this way would mimic the mature tRNA structure.

It has been reported that mature tRNA with added CCA (tRNA-3’+ CCA) is not a substrate for 3’-tRNase. The 3’-tRNase is not inhibited by excess tRNA+CCA, ensuring that the 3’-tRNase can remain free to process precursor tRNAs with 3’ end trailers and tRNAs can progress to aminoacylation. It was postulated that the active site of 3’-tRNase might have a poor fit with tRNA+CCA (Mohan et al., 1999; Nashimoto, 1995). If this is the case, we expect that the tRNA+CCA structure could possibly enhance the stability of the ribozymes.
In order to study the contribution to stability of the tRNA+CCA structure in the chimeric tRNA\textsuperscript{Lys}\textsubscript{3}-ribozymes, we used constructs in which the chimeric tRNA\textsuperscript{Lys}\textsubscript{3}-ribozymes are inserted under the U6 promoter as well as a construct containing the riboyme without the tRNA structure, also under the U6 promoter. We also wanted to examine if the mutations in the B box, which are present in pU6MW, have any effect on the stability of the transcripts. Two assay systems were used for analyzing the intracellular stability of the transcripts.

In the first system, 293 cells were transiently transfected with pU6WW, pU6MW, pU6ΔGTRz and pU6Rz. To normalize for transfection efficiency, a non-related reference growth factor expressing plasmid pTKGH was cotransfected with constructs expressing chimeric tRNA\textsuperscript{Lys}\textsubscript{3}-ribozymes. After 48 hours, transcription was halted by adding actinomycin D to the culture medium. Total RNA was isolated at different time points. The RNA samples were then subject to Northern hybridization and analyzed for half-lives (Fig. 3 A, B). The endogenous U6 snRNA level was monitored as a loading control since its half-life is known to be 16-24 h (Sauterer \textit{et al.}, 1988; Terns \textit{et al.}, 1993; Noonberg \textit{et al.}, 1994). The loading was normalized according to the half-life-adjusted native U6 level. The half-lives of U6WW, U6MW, U6ΔGTRz and U6Rz were shown to be approximately 25 to 50 minutes (Fig. 3C). The use of a U6 promoter driven ribozyme sequence without the tRNA sequence allowed comparison of the stability of the chimeric tRNA\textsuperscript{Lys}\textsubscript{3}-ribozyme transcripts with stand-alone ribozyme transcripts. It was found that the half life of the stand-alone ribozyme is about 63 min (Fig. 3C).
Figure 3. Stability of ribozyme and chimeric tRNA\textsuperscript{Lys3}-ribozymes in transiently transfected 293 cells.

The half-lives of the ribozyme or chimeric ribozyme transcripts are determined by transiently transfecting 293 cells with different constructs (pU6WW, pU6MW, pU6ΔGTRz and pU6Rz). After 48 hours, transcription was halted by adding actinomycin D. Total RNA samples were isolated at different time intervals and the RNA samples were then analyzed and quantitated by Northern hybridization with ribozyme a specific probe.

A. Determination of half-lives of transcripts from pU6WW and pU6MW (Northern hybridization with a ribozyme specific probe).

B. Determination of half-lives of transcripts from pU6ΔGTRz and pU6Rz (Northern hybridization with a ribozyme specific probe).

C. The half-lives of the transcripts from different constructs were calculated and compared from quantitation of the ribozyme RNA levels remaining at different time points. Values indicate percent intact RNA remaining compared to time 0. Quantitation involves normalization of transfection efficiency and normalization of loading variations with U6 snRNA level probed with a U6 snRNA specific probe (data not shown). Half life of U6 snRNA was taken into account.

Note: Although the half-life of the U6Rz is a bit longer than the other chimeric transcripts, the intracellular level of U6ΔGTRz is much higher than that of U6Rz (B) probably due to the action of a stronger hybrid U6/tRNA promoter.
A.

![Image of gel electrophoresis for U6WW and U6MW samples, labeled with tRNA-ribozyme and 10bp ladder].

B.

![Image of gel electrophoresis for U6ΔGTRz and U6Rz samples, labeled with tRNA-ribozyme and 10bp ladder].
Half-lives of chimeric tRNA<sup>Lys<sub>3</sub></sup>-ribozymes in transient transfection

![Graph showing the half-lives of chimeric tRNA<sup>Lys<sub>3</sub></sup>-ribozymes.](image)

- U6WW
- U6MW
- U6ΔGTRz
- U6RZ

Percentage of ribozymes remaining vs. Hours after administration of actinomycin D.
In the second assay, the stability of the transcripts from U6WW, U6MW and U6ΔGTRz were analyzed under more natural, intracellular conditions in stable cell lines. Cell lines stably expressing U6WW, U6MW and U6ΔGTRz were established and we determined the half-life of the transcripts by halting intracellular RNA polymerase III transcription with actinomycin D. Endogenous U6 snRNA levels were monitored and used as a loading normalization control. The half-lives of the transcripts were determined to be similar with those obtained from transient transfections (Fig. 4). These results indicate that the absolute increase in chimeric tRNA\(^{\text{Lys3}}\)-ribozyme steady-state levels do not affect our half-life determinations, indicating a first-order process of transcript degradation.

One deletion in the T\(\psi\)C stem increases the stability of the chimeric tRNA\(^{\text{Lys3}}\)-ribozyme in cell extract

The construct with a deletion in the T\(\psi\)C stem (ΔG) may have increased resistance to 3’ processing due to the involvement of the stem in interaction with 3’ processing endoribonuclease (3’-tRNase; Nashimoto et al., 1999). We further compared the stability of transcripts from the ΔGTRz tRNA-ribozyme with transcripts from WW tRNA-ribozyme in cell extracts that harbor tRNA processing activities. The cell extract was prepared from 293 cells as described in Materials and Methods. In vitro transcribed chimeric tRNA\(^{\text{Lys3}}\)-ribozyme transcripts from ΔGTRz and WW were incubated with the
Figure 4. Stability of chimeric tRNA\textsuperscript{Lys3}-ribozymes in stable transformants.

Transformants stably expressing U6WW, U6MW and U6ΔGTRz were obtained by transfecting 293 cells with pAAVU6WW, pAAVU6MW and pAAVU6ΔGTRz and selecting clones with G418. The half-lives of the chimeric tRNA\textsuperscript{Lys3}-ribozyme transcripts are determined by halting transcription by adding actinomycin D. Total RNA samples were isolated at different time intervals. The half-lives of the transcripts from different constructs were then calculated and compared. Values indicate percent intact RNA remaining compared to time 0.
cell extract for 30 minutes. The ΔGTRz transcripts (147-149 bps) are longer than the WW transcripts (121-123 bps) because longer 5' polylinker sequence is present in the pΔGTRz construct (Fig. 5 lanes 2-4 vs. lanes 5-6). As suggested elsewhere, the in vitro system exhibits relatively slow processing kinetics (Adeniyi-Jones et al., 1983) and this allowed us to analyze the stability and processing of transcripts from different constructs. It can be seen that part of the pWW transcripts were processed to the approximate tRNA size (both ends processed, about 75 bps) and pΔGTRz transcripts were processed to 3' processed size (104-106 bps) only (and it can be seen that the transcripts from ΔGTRz have markedly reduced processing than transcripts from WW (compare lanes 5, 6 with lanes 2, 3 in Fig. 5).

**Increased stability resulted in stronger HIV inhibition**

Since transcripts from ΔGTRz show increased resistance to 3' end processing, we subcloned this ΔGTRz sequence under the U6 promoter in a dual U6/tRNA promoter design to maximize the intracellular level of the chimeric tRNA\textsuperscript{Lys3}-ribozyme. The anti-HIV effect of the construct pU6ΔGTRz was compared with that of pU6MW.

293 cells were cotransfected with proviral HIV DNA and chimeric tRNA\textsuperscript{Lys3}-ribozyme constructs (pU6MW, pU6ΔGTRz and pU6Rz). The infectivity of the supernatant with normalized RT counts was then checked as described before (Westaway et al., 1998). The results showed that the ΔGTRz transcript expressed under the dual U6/tRNA promoters had the highest HIV inhibition activity (Fig. 6). The inhibition is stronger than that obtained with pU6MW, in accordance with the increased resistance of
Figure 5. Processing of chimeric tRNA-ribozymes in cell extract.

Cell extract with tRNA processing activity was isolated from 293 cells as described in Materials and Methods. Chimeric tRNA\textsuperscript{Lys}\textsubscript{3} -ribozymes (WW and ΔGTRz) and tRNA\textsuperscript{Lys}\textsubscript{3} were transcribed and labeled with [α-\textsuperscript{32}P]-UTP by in vitro T7 transcription using linearized plasmids as templates. The transcripts were then incubated with (+) or without (-) cell extract in a 3'-end processing buffer (see Materials and Methods). The processing reactions were then separated on a 10% denaturing acrylamide gel.

ΔG: ΔGTRz. tRNA: tRNA\textsuperscript{Lys}\textsubscript{3}.
293 cells were cotransfected with proviral DNA, anti-HIV ribozyme constructs (pWW, pMW, pU6WW, pU6MW, pU6ΔGTRz, pU6Rz) or a vector control pTZU6+1. 48 hours later, supernatants were collected and the RT activity was assayed. CEM cells were infected with the supernatants from the transfection with equalized RT counts. The p24 level in culture media from these infections were assayed and compared after six and seven days. 10 μg anti-HIV ribozyme and 1 μg proviral DNA were used.
transcripts from pU6ΔGTRz to 3’ end processing. Interestingly, the ribozyme expressed under the U6 promoter alone exhibits same HIV inhibition effect, which may be mediated prior to HIV encapsidation.

**Discussion**

Ribozymes are potentially powerful tools for suppressing the expression of specific genes. They can be engineered to cleave other RNA molecules with high specificity (Haseloff et al., 1988; Uhlenbeck, 1987) and are therefore not likely to interfere with normal cellular functions. They also have the advantage of being less immunogenic than protein-based therapeutics. In addition to an antisense effect, ribozymes have additional benefits in that they can cleave the target and recycle to cleave additional molecules.

The successful development of a suitable expression cassette is one major challenge related to the use of RNA-based molecules including ribozymes, antisense RNAs, decoy RNAs and aptamers as therapeutic agents. tRNA promoters have been popularly used in the expression of different RNA-based therapeutics. However, the transcripts expressed from these cassettes can be subject to the action of the tRNA 3’ processing endoribonuclease (3’-tRNase) and the stability of these transcripts inside cells becomes crucial for their practical use in intracellular immunization.

Despite the popularity of tRNA expression cassettes, relatively little is known about the intracellular characteristics of the expressed transcripts. We previously demonstrated that an anti-HIV chimeric tRNA<sup>Lys<sub>3</sub></sup>-ribozyme cassette could be placed under the human U6 snRNA promoter, resulting in a much higher expression level than that from the
tRNA\textsuperscript{Lys3} promoter alone (Chapter III, Part B). Here, we qualitatively studied the transcripts from the tRNA promoter alone or dual U6/tRNA. Intact chimeric tRNA\textsuperscript{Lys3}-ribozymes expressed from the tRNA expression cassette and U6/tRNA expression cassette were detectable in cells. The lower level of expression when the wild type B box rather than the mutant B box of tRNA was put under the U6 promoter could be due to stronger interference between the wild type B box and the U6 promoter. Differences in resistance to 3' end processing could also contribute. Transcription factors bound to the different promoter elements of the two promoters (Fig. 1E) could sterically interfere with each other.

In this study, we analyzed the effect on resistance to 3' end processing of CCA at the 3' end of tRNAs harboring mutations in the B box or a deletion of one G residue in the T\Psi C stem. As mentioned in the introduction, the presence of the CCA at the end of tRNA molecules causes inefficient cleavage by the 3' processing endoribonuclease (Nashimoto, 1997 and Nashimoto et al., 1999). The constructs pU6WW, pU6MW, pU6ΔGTRz all have CCA encoded between the tRNA and the ribozyme sequence. The construct pU6ΔGTRz has a deletion of one G immediately 5' of the B box in the T stem and this deletion would lead to one reduced base-pair in the T stem, which could in turn lead to reduced processing by 3' tRNase (Nashimoto et al., 1999). The construct pU6MW has two base mutations in the B box present in the T stem-loop. The 5' mutation could also result in one less base pair in the T\Psi C stem and the 3' mutation is in the T\Psi C loop. Changes in this loop were also reported to reduce the processing activity of 3' tRNase (Nashimoto, 1996 and Nashimoto et al., 1999). One advantage of the U6/tRNA dual
promoter system is that the sequence of the B box can be changed to achieve higher
stability of the construct and the expression level is not likely to be affected since the U6
promoter predominates due to lack of promoter interference.

As reported in the literature, modifications in the tRNA expression cassette could lead
to nuclear retention of the transcripts. Constructs with a deletion of the last 11 bases of
the human tRNA\textsuperscript{Met} gene were developed to produce an unprocessed co-transcript of
tRNA and downstream sequences (Adeniyi-Jones et al., 1984). This is probably because
the deletion would lead to disrupted base-pairs in the acceptor stem and T stem which are
recognized by 3′-tRNase (Nashimoto, 1999). Nevertheless, transcripts from the 3′
deleted cassette were found to remain in the nucleus in two studies (Good et al., 1997;
Koseki et al., 1999) probably as a result of disrupted secondary and tertiary structure of
the tRNA recognized by the export machinery (Lipowsy et al., 1999; Arts GJ et al.,
1998b). Therefore, stability is increased but cytoplasmic export of the transcripts is
sacrificed. Another interpretation is that nuclear retention enhances stability. For most
gene inhibition applications of ribozymes, cytoplasmic export of the transcripts is
essential. Ribozymes retained in the nucleus can only be used against a very narrow
range of targets such as splice sites, introns or polyadenylation signals and would not be
desirable where the RNA to be cleaved is quickly exported to the cytoplasm. Our
constructs would maintain the secondary structure and overall shape of the tRNA and we
expect that the transcripts would have a reasonable chance of being transported into the
cytoplasm.
In the half-life determination assay, the half-life of the transcripts from pU6WW, pU6MW and pU6ΔGTRz were found to range from 25 minutes to 50 minutes (Fig. 3). These are somewhat shorter than the half-life of the ribozyme transcript without the tRNA moiety (about 63 minutes). However, the overall intracellular level of transcripts from the dual U6/tRNA cassettes (U6WW, U6MW and U6ΔGTRz) are much higher than the level of the ribozyme alone expressed from the U6 promoter (Fig. 3B). The half-lives of these are sufficiently long, in the context of the strong U6/tRNA expression cassette, to give rise to very high intracellular level of the chimeric tRNA\textsuperscript{Lys3}-ribozymes (Chapter III, Part B). The intracellular chimeric tRNA\textsuperscript{Lys3}-ribozyme level is comparable to that of tRNA\textsuperscript{Lys3} sized band in U6MW (Fig. 2A and Table 1) although tRNA\textsuperscript{Lys3} sized band could be native tRNA\textsuperscript{Lys3} or be from 3'-processed chimeric tRNA\textsuperscript{Lys3}-ribozyme. This stability in combination with the high level expression could make the chimeric tRNA\textsuperscript{Lys3}-ribozyme transcripts competitive for packaging into the HIV virions, contributing to their efficient HIV inhibition. Once inside the virions, these should function efficiently, because they are highly colocalized with their substrate. In addition, they should remain intact, because the 3' processing endoribonuclease and other processing enzymes are not specifically packaged into virions. In other words, the tRNA\textsuperscript{Lys3} expression cassette is advantageous in that the tRNA\textsuperscript{Lys3} moiety of the chimeric tRNA\textsuperscript{Lys3}-ribozymes facilitates the colocalization of the ribozyme with its target not only for enhanced cleavage action, but also for increased ribozyme stability.

The half-lives achieved are comparable to those obtained by strategies for enhancing stabilities of other tRNA chimerics. These changes in the expression cassette give rise to
transcripts with stabilities comparable to those obtained from other stabilizing strategies. In one study, chimeric tRNA\textsuperscript{Val}-ribozyme constructs were constructed by attaching the ribozyme sequence to the 3' end of the tRNA and making the 3' terminus of the ribozyme complementary to the 3' terminus of tRNA\textsuperscript{Val}. These were found to be exported to the cytoplasm. The half lives of the constructs were about 35 to 100 minutes (Koseki et al., 1999). In another study, constructs with a ribozyme sequence inserted into the anticodon loop of tRNA\textsuperscript{Lys}\textsubscript{3} with linkers were found to have half-lives of 50 to 80 minutes (Medina et al., 1999). In another study, constructs with a ribozyme inserted into the anticodon loop of tRNA\textsuperscript{Met} were found to have transcripts mainly localized to the nucleus (Cotton and Birnstiel, 1989). The transcripts have greater stability when assayed in a nuclear extract (data not shown in the paper). In another study, a tRNA\textsuperscript{Met} expression cassette was modified so that the 3' terminus of the transcript was complementary to the 5' terminus and this resulted in improved accumulation of transcripts by >100 fold (Thompson et al., 1995). Because the TΨC loop and the overall shape of tRNA which has been found to be important for the export of the tRNA molecules (Lipowsy et al., 1999; Arts et al., 1998b) is modified, the subcellular localization of these transcripts deserves further study. Can we adopt these strategies in our chimeric tRNA\textsuperscript{Lys}\textsubscript{3}-ribozyme? Obviously, the strategy of putting our ribozyme into the anticodon of tRNA is not feasible for our chimeric tRNA\textsuperscript{Lys}\textsubscript{3}-ribozyme because of the resultant nuclear localization of the transcripts. The strategy of making the 3' end of the ribozyme complementary to the 5' end of the tRNA would be difficult in our case because of the requirement for the 3' 18 nt of tRNA\textsuperscript{Lys}\textsubscript{3} to anneal to the PBS of HIV genome.
The half-life assay could be subject to some inaccuracies because the time that is required to process each sample could give rise to some errors. The tc/t ratio analysis could be more accurate because the ratio is obtained from the same lane (sample) and variations in loading would not affect the ratio. In such an analysis, the mutations in the B box were found to significantly increase the level of the transcripts. In a processing assay of transcripts made from pWW and pΔGTRz in cell extracts, the transcripts from pΔGTRz showed little if any processing, indicating a significant effect of the deletion of one G in the T stem on the stability of the chimeric tRNA\textsuperscript{Lys}\textsubscript{3}-ribozyme. This in vitro assay is much less error-prone, since the in vitro processing kinetics are slow (Adeniyi-Jones et al., 1983). When ΔGTRz is expressed under the strong U6/tRNA promoter, the construct showed stronger inhibition of HIV infectivity than pU6MW (Fig. 6). As reported earlier (Chapter III, Part B), transcripts from ΔGTRz and MW could be exported to the cytoplasm. This is probably due to the maintenance of the overall shape of the tRNA structure and other properties recognized by the tRNA export machinery.

As a result of increased promoter strength and increased transcript stability, the relative expression level of the chimeric tRNA\textsuperscript{Lys}\textsubscript{3}-ribozyme MW reaches a level close to that of tRNA\textsuperscript{Lys}\textsubscript{3}, which can consist of both native tRNA\textsuperscript{Lys}\textsubscript{3} and processed chimeric tRNA\textsuperscript{Lys}\textsubscript{3}-ribozyme. This gives the chimeric tRNA\textsuperscript{Lys}\textsubscript{3}-ribozyme transcripts a chance to be effectively packaged into the virions.

In conclusion, these modifications in tRNA-ribozyme expression cassettes, including the linear arrangement of 'tRNA+CCA+ribozyme' sequences, in combination with
mutations in the TΨC loop, or especially with the deletion of one G in the T stem, have been shown to give rise to a higher overall level of tRNA-ribozyme transcripts. The transcripts can be exported to the cytoplasm. When expressed under the U6/tRNA dual promoter, these result in cytoplasmic accumulation of very high levels of chimeric tRNA\textsuperscript{Lys3}-ribozyme transcripts that are efficient in HIV inhibition. These modifications may prove valuable in the design of other chimeric tRNAs for expressing RNA-based therapeutics in general. The tc/t ratio analysis is an easy and accurate way for determining stability of chimeric tRNA transcripts different in size from the original tRNA molecule. It could be easier to perform and interpret because the tc/t ratio is obtained within the same sample and inter-sample experimental and quantitative errors that could be present in cell extract processing assays can be avoided. It may be valuable for the analysis of other chimeric tRNA chimerics to obtain constructs with optimal stability in studies using any tRNA expression cassette for expressing RNA-based therapeutics (ribozymes, antisense RNAs, decoys and aptamer RNAs).
IV. GENERAL DISCUSSION

Ribozymes are catalytic RNAs that can catalyze the endoribonucleolytic cleavage of RNA molecules (Cech, 1987). Ribozymes form a complex with their complementary target RNA molecule and then catalyze the degradation and inactivation of the target RNA via a cleavage reaction. Hammerhead ribozymes have shown great potential in inhibiting specific gene functions and have been designed to target a variety of RNAs. Many diseases show undesirable expression of RNAs, such as malignant disorders, viral infections and genetic diseases and, therefore, are amenable to the actions of ribozymes (reviewed in Kiehntopf et al., 1995a and 1995b; Phylactou et al., 1998; Couture and Stinchcomb, 1996).

A number of groups have reported the use of ribozymes in inhibiting HIV-1 infection. In 1990, Rossi and colleagues first reported the use of a hammerhead ribozyme targeting the gag sequences to inhibit HIV-1 replication (Sarver et al., 1990). Hammerhead ribozymes against HIV-1 tat and tat/rev genes developed by this group (Zhou et al., 1994) have already been under evaluation in clinical trials for several years. For more details on the application of ribozymes to HIV infection, see a review by John Rossi (Rossi, 1999).

It can be conceived that ribozymes would work more efficiently if they can be localized to the same subcellular compartment with their targets inside the cells. The
importance of intracellular colocalization of ribozymes with their targets has been very well established (Sullenger and Cech, 1993; Bertrand et al., 1997; Pal et al., 1998). In particular, Rossi and colleagues demonstrated that a ribozyme was effective only when it was expressed cytoplasmically and colocalizes with the cytoplasmic targets (Bertrand et al., 1997).

Enhancing the colocalization of anti-HIV ribozymes with their targets and enhancing the efficacy of these ribozymes is one of the major goals of my research. In reported colocalization studies, the retroviral Psi (ψ) signal tethered to ribozymes was utilized as a colocalization strategy (Sullenger and Cech, 1993; Pal et al., 1998). In this study, we employed a novel colocalization strategy in which anti-HIV ribozymes were fused to the 3’ end of tRNA_{Lys}^{3} sequence. tRNA_{Lys}^{3} is the natural primer employed by HIV-1 for initiating reverse transcription. It is recruited by the virions during virion packaging through interactions with the polyprotein precursor of RT, Pr160^{Gag-Pol} from host cells (Mak et al., 1994). The tRNA_{Lys}^{3} moiety of the chimeric tRNA_{Lys}^{3}-ribozyme is expected to maintain the natural tertiary structure as demonstrated by computer modeling. The chimeric tRNA_{Lys}^{3}-ribozyme also showed similar binding affinity to RT (Westaway et al., 1998), the portion of Pr160^{Gag-Pol} which is responsible for interacting with tRNA_{Lys}^{3} (Mak et al., 1994). Therefore, we expect that the anti-HIV ribozymes can be brought into HIV virions through the interactions of the tRNA_{Lys}^{3} moiety with Pr160^{Gag-Pol} during virion encapsidation. Thus, the ribozymes can be expected to colocalize with the cleavage target site on HIV genome inside the virions, thereby, aiding in efficient viral inhibition..
In addition, the ribozymes fused to the 3' end of the tRNA\textsubscript{Lys} can be expressed from the internal promoters of tRNA\textsubscript{Lys}, generating chimeric tRNA\textsubscript{Lys}-ribozyme molecules.

The chimeric tRNA\textsubscript{Lys}-ribozymes could go through a complex series of processing and transport events which could inhibit incorporation into HIV virions. For the chimeric tRNA\textsubscript{Lys}-ribozymes to be effective at inhibiting HIV infection, they should, (1) be produced at highest possible levels tolerable to cells, (2) be exported into the cytoplasm, (3) be stabilized in cells and, (4) interact with the precursor of RT for packaging into virions. To maximize anti-HIV activities by the ribozymes, I studied the colocalization of ribozymes with their target, determined the encapsidation of the ribozymes into the virions, explored a novel hybrid Pol III promoter expression cassette in order to increase the intracellular expression level of the chimeric tRNA\textsubscript{Lys}-ribozymes and also studied the intracellular stability of the chimeric tRNA\textsubscript{Lys}-ribozyme transcripts. The chimeric tRNA\textsubscript{Lys}-ribozymes were also studied for capability to inhibit HIV infectivity.

The chimeric tRNA\textsubscript{Lys}-ribozyme was designed to target immediately upstream of the primer binding site of human immunodeficiency virus (HIV) and was fused to the 3' end of the tRNA\textsubscript{Lys} gene. It was found to be catalytically active \textit{in vitro}, and was efficiently recognized and bound by HIV-1 reverse transcriptase with affinities similar to tRNA\textsubscript{Lys}. The chimeric tRNA\textsubscript{Lys}-ribozyme could be detected in virions purified from cells cotransfected with the chimeric ribozyme and HIV proviral DNA, indicating that the chimeric tRNA\textsubscript{Lys}-ribozyme has been packaged into the virions. These virions were shown to have reduced infectivity in subsequent rounds of infection of CD4+ cells.
These results indicate that the natural primer of HIV, tRNA$^{\text{Lys}}$, can be used as a carrier to bring therapeutic ribozymes into virions, thereby colocalizing the ribozyme with their targets RNAs.

It is very important to maximize the ribozyme to substrate ratio to ensure maximal in vivo substrate inhibition by ribozymes (Cotten and Birnstiel, 1989). To achieve this goal, I inserted the chimeric tRNA$^{\text{Lys}}$-ribozymes under a human U6 small nuclear RNA promoter. In such constructs, two promoters were put in tandem, with an extragenic upstream U6 promoter and an intragenic tRNA promoter (A box and B box).

To compare the levels of expression of the chimeric tRNA$^{\text{Lys}}$-ribozymes under the U6 promoter and under the tRNA promoter alone, Human embryonic kid ney 293 cells were transiently transfected with the chimeric ribozyme plasmids and total RNAs were isolated and analyzed by Northern hybridization 48h later. The overall level of expression of the chimeric tRNA$^{\text{Lys}}$-ribozymes under the U6/tRNA dual promoter was found to be elevated relevant to expression from the tRNA internal promoter alone. The highest expression was observed from the dual U6/WtRNA promoters, which includes the U6 promoter upstream of a tRNA promoter harboring mutations in the B box (GCTACAAGTCC). This construct had more abundant expression than the dual U6/WtRNA promoters harboring the U6 promoter upstream of the tRNA promoter with wild-type B box (GGTTCAAGTCC).
The transcripts made from the tRNA promoter and the dual U6/tRNA promoters were also analyzed qualitatively. It was found that when the tRNA-ribozyme genes were placed under the U6 promoter, one species of the transcripts initiated downstream of the site normally utilized in the tRNA promoter. The lower level of expression observed when the wild type B box versus the mutant B box of tRNA$^{\text{Lys3}}$ was put under the U6 promoter could be due to interference between the wild type B box and the U6 promoter. Transcription factors bound to the different promoter elements of the two promoters could sterically hinder each other. Such interference could change the initiation site of transcription and could also adversely affect the levels of transcription. However, the entire chimeric tRNA$^{\text{Lys3}}$-ribozyme sequence is present in all transcripts. The 5' end processing of these transcripts should generate the same 5' ends but this was not the case. Despite this interference between promoter elements, the two promoters juxtaposed in tandem do provide enhancement of transcription. The expression levels from these hybrid U6/tRNA expression cassettes are much higher than from the tRNA or U6 promoter alone.

For the chimeric tRNA$^{\text{Lys3}}$-ribozymes to be packaged into virions, the transcripts have to be exported into the cytoplasm. There have been varying reports of the intracellular localization of transcripts made from chimeric tRNA genes. While several reports showed cytoplasmic localization of chimeric transcripts (Koseki et al., 1999; Ilves et al., 1996; Westaway et al., 1995; Gebhard et al., 1997), there have been reports of nuclear localization as well (Good, 1997; Koseki et al., 1999).
Because the tRNA moiety in the chimeric tRNA\(^{\text{Lys}}\)-ribozymes should maintain native tRNA folding, we expect that the tRNA could be exported to the cytoplasm. We also expect the transcripts from the dual U6/tRNA promoters to be localized to the cytoplasm, since the tRNA moiety is the determining factor for facilitating export.

By transiently transfecting cells with different constructs and detecting the transcripts with riboprobes complementary to the ribozyme sequence, we demonstrated that the transcripts from the hybrid U6/tRNA promoters could be exported into the cytoplasm. It was demonstrated that transcripts expressed under the internal tRNA promoter alone localized to the cytoplasm and transcripts expressed under U6 and tRNA promoter in tandem localized evenly to both the cytoplasm and nucleus. The cytoplasmic expression makes the ribozymes available for packaging during virion encapsidation. HIV inhibition data showed that HIV inhibition corresponds with expression level, with the greatest level of inhibition deriving from the construct with dual U6/MtRNA promoters. These findings with the U6/tRNA expression cassettes are very important because they demonstrated that two juxtaposed RNA polymerase III promoters, namely, the U6 and tRNA promoters, could be used in tandem to express small RNA therapeutic genetic materials much more efficiently, allowing cytoplasmic accumulation.

We also studied the effect of several modifications in the tRNA sequence of the tRNA expression cassette on the stability of transcripts. Natural pre-tRNAs are 5' processed by RNase P and 3' processed by 3' tRNase. A CCA sequence is added to the 3' end of native tRNA after the 3' trailer is processed by 3' tRNase. The chimeric tRNA\(^{\text{Lys}}\)-
ribozymes may also be subjected to processing by the 3' tRNase, making the chimeric tRNA\textsuperscript{Lys3}-ribozymes unstable. The chimeric tRNA\textsuperscript{Lys3}-ribozymes were designed to have a CCA sequence between the tRNA and the ribozyme moieties. A stabilization effect was expected because the CCA sequence in between the tRNA and the ribozyme could protect against 3'-processing endoribonuclease (3'-tRNase) activity. It has been reported that tRNAs with CCA at 3' ends are not substrates for the 3'-tRNase \textit{in vitro} (Mohan \textit{et al.}, 1999; Nashimoto, 1995). Constructs with the CCA sequence in between the tRNA and the ribozyme sequence were designed to have two point mutations in the TΨC stem-loop (MW and U6MW), or a one base deletion in the TΨC stem (ΔGTRz and U6dGTRZ). The point mutations in MW and U6MW would lead to a mutation in the TΨC loop and one less base pair in the TΨC stem. The deletion of one G in the TΨC stem would also lead to one less base pair in the TΨC stem. Changes in the TΨC loop and basepairing in the TΨC stem have been shown to reduce processing by the 3'-tRNase tRNase (Nashimoto, 1996 and Nashimoto \textit{et al.}, 1999).

The stability of the transcripts of chimeric tRNA\textsuperscript{Lys3}-ribozymes were studied by expressing them in Human embryonic kidney 293 cells and subsequently blocking transcription by exposure to actinomycin D. Total RNAs were isolated at different time points after treatment with actinomycin D and the chimeric tRNA\textsuperscript{Lys3}-ribozyme transcript levels were analyzed by Northern hybridization. The half-lives of the transcripts were analyzed. The half-lives of the constructs analyzed were from 25-50 min (Figure 3 of Chapter III, part C). The half-lives of these were sufficiently long to give rise to high intracellular levels of the chimeric tRNA\textsuperscript{Lys3}-ribozymes. The half-lives achieved are
comparable to those obtained by other strategies for enhancing tRNA chimeric stability (Koseki et al., 1999; Medina et al., 1999).

In a tc/t analysis, an analysis of the ratio of the intracellular level of tRNA chimerics (tc, chimeric tRNA^{Lys3}-ribozymes here) and tRNA (t, tRNA^{Lys3} here) suggests that the MW construct is more stable than WW (Figure 2 and Table 1 of Chapter III, Part C). The tc/t analysis proposed here can potentially be used in the screening of tRNA expression cassettes with increased transcript stability (Table 1 of Chapter III, Part C).

Compared with pWW, the construct pΔGTRz was shown to have reduced processing in a Human embryonic kidney 293 cell extract, which has active tRNA 3' processing enzyme activity. This chimeric tRNA^{Lys3}-ribozyme construct with one G deletion right at the 5' end of the B box could be more stable because the deletion leads to disruption of one base pair in the TψC stem, which was found to be related to the interaction of 3'-tRNase with tRNA (Nashimoto et al., 1999). This was supported by one HIV inhibition experiment in which this construct expressed under U6 promoter was found to have a stronger inhibition on HIV than the construct U6MW (Fig. 5, Chapter III, Part C).

tRNA processing, tRNA export as well as the encapsidation and role in initiating reverse transcription are all very complex processes. Likewise, the chimeric tRNA^{Lys3}-ribozymes are also inevitably involved in complex interactions with different cellular and viral components. An attempt to simplify these processes is outlined in Figure 1 with an
Mechanisms of HIV inhibition by the chimeric tRNA$^{\text{Lys}^3}$-Ribozymes

Figure 1. An overview of the mechanisms of HIV inhibition by the chimeric tRNA$^{\text{Lys}^3}$-ribozymes.
overview of the possible mechanisms of HIV inhibition by the chimeric tRNA\textsuperscript{Lys3}-ribozymes. These mechanisms are now discussed in greater depth.

The U6/tRNA expression cassette has an upstream U6 promoter and the intragenic tRNA promoter placed in tandem and resulted in much higher expression than the tRNA expression cassette alone. Therefore, U6 promoter acts synergistically with the tRNA promoter in enhancing the expression level of the chimeric transcripts. This is an important observation because U6 promoter and tRNA promoter have been traditionally utilized separately in expressing RNA therapeutics. The U6/tRNA expression cassette, therefore, has the potential to be used in expressing other RNA therapeutics including ribozymes, antisense and decoys.

The tRNA moiety in the tRNA\textsuperscript{Lys3}-ribozyme chimera plays several pivotal roles. On one hand, it works synergistically with the upstream U6 promoter in enhancing expression from the hybrid Pol III promoter. On the other hand, the tRNA moiety acts through a piggyback mechanism to bring the anti-HIV ribozyme into virions. Endogenous tRNA\textsuperscript{Lys3}, the primer for initiating reverse transcription, is brought into the virions through interaction with the precursor polyprotein of RT, Pr160\textsuperscript{Gag-Pol} (Mak et al., 1994). Our chimeric tRNA\textsuperscript{Lys3}-ribozymes showed affinities to RT and were therefore, expected to be incorporated into virions through a similar mechanism.

In addition, the ability of the transcripts from these constructs to be exported cytoplasmically (Part B of Chapter III) most likely lies in the tRNA moieties of the
transcripts. The receptor, exportin-t, for tRNA export has only recently been identified (Arts et al., 1998a, Kutay et al., 1998) and not much is yet known about the interactions between exportin-t and tRNA molecules. The correct tRNA shape and the TψC loop were recently reported to be critical for exportin-t binding (Lipowsy et al., 1999; Arts et al., 1998b). Notably the tRNA moieties in the chimeric tRNA\(^{\text{Lys}3}\)-ribozymes should maintain their tertiary structure as demonstrated by computer modeling and therefore are expected to maintain their affinity to exportin-t. The cytoplasmic export of the transcripts makes these available for encapsidation into HIV-1 virions, which was demonstrated in virions obtained in a cotransfection assay with proviral HIV-1 DNA and our constructs. Therefore, the tRNA\(^{\text{Lys}3}\) moiety plays a crucial role in colocalizing the ribozyme with its target on the HIV genome.

The tRNA\(^{\text{Lys}3}\) moiety in the chimera could also function as a decoy for RT. Because the chimeric transcripts have affinity with RT, they can potentially compete with natural tRNA\(^{\text{Lys}3}\) primer in interacting with RT or its precursor, Pr\(_{\text{160Gag-Pol}}\) during encapsidation and reverse transcription when expressed at high intracellular levels. The 3' end of the tRNA\(^{\text{Lys}3}\) moiety in the chimera is complementary to the PBS of HIV and, therefore, may have an antisense effect. The CCA sequence in between the tRNA moiety and the ribozyme, as well changes in the TψC stem-loop, may assist in stabilizing the chimeric transcripts.

The ribozyme moiety of the chimera plays a relatively simple role when compared to the U6 promoter sequence and the tRNA moiety. Constructs with active ribozyme
moieties showed stronger inhibition than constructs with inactive ribozyme moieties, suggesting active ribozyme cleavage of HIV-1 genomic RNA in the virion. The anti-HIV effects demonstrable in the constructs with inactive ribozymes are presumably due to the inherent antisense effect of the inactive ribozyme and the tRNA moiety, as well as the decoy effect from the tRNA moiety.

The intracellular stability of ribozymes has always been a great concern in practical applications. While our chimeric tRNA^{Lys3}-ribozymes are sufficiently stable to maintain the high level expression by the hybrid U6/tRNA cassette, their stability may deserve further improvement. While tRNA molecules are small (about 76 bp), they are involved in very complex interactions with different enzymes and translationally associated proteins. Some of the processes related to the use of tRNA expression vectors include 5' processing, 3' processing, intervening sequence splicing, base modifications and export into cytoplasm. The processes which would most likely affect the feasibility of using the tRNA expression vectors include 3' processing and export.

While there have been reports that ribozymes expressed from tRNA promoters can be stabilized several ways, these designs are very likely to affect other aspects of the intracellular fates of these transcripts. Stability may be increased at the expense of export properties due to changes in the overall shape of the tRNA-based transcripts. The overall shape (tertiary structure) of tRNA has been shown to be very important for interactions of tRNA with exportin-t, part of the tRNA export machinery (Lipowsky et al., 1999; Arts et al., 1998b). Ribozymes that remain in the nucleus would have a narrow range of targets.
including splice sites, introns or polyadenylation signals and would not be desirable for cleaving RNAs which are cytoplasmic. No comprehensive studies on this issue are available at the present time. Because tRNA promoters are very widely used in gene therapy, comprehensive studies addressing these issues should be carried out. Positive results from such studies would represent a big technical advancement for gene therapy applications using ribozymes and other small RNA based therapeutics.

A few approaches have been attempted for improving the stability of transcripts expressed from tRNA promoters. These include 3' 11 base deletion of mature tRNA, insertion of the ribozyme sequence into the anticodon loop of tRNAs, and incorporation of structures at the 3' terminus of the transcripts which hybridize to the 5' terminus. Constructs with a deletion of the last 11 bases of the human tRNA\textsuperscript{Met} gene were developed to produce an unprocessed co-transcript of a tRNA\textsuperscript{Met} and downstream sequences (Adeniyi-Jones \textit{et al.}, 1984). Nevertheless, transcripts from the 3' deleted cassette were found to localize to the nucleus in two studies (Good \textit{et al.}, 1997; Koseki \textit{et al.}, 1999). Another study showed 70% nuclear and 30% cytoplasmic localization from the same tRNA\textsuperscript{Met} cassette (Du \textit{et al.}, 1998). Yet another report showed cytoplasmic expression from the cassette (Gebhard \textit{et al.}, 1997). Since the recent work of Koseki \textit{et al.} (1999) confirmed the nuclear localization of transcripts from such a cassette using the same construct as Good \textit{et al.} (1997), nuclear localization seems a more likely destination of such transcripts. The deletions would disrupt the secondary structure of the acceptor stem and change the overall shape of the resulting transcripts. These transcripts are not likely to interact with the exportin-t, which would lead to nuclear retention. Therefore,
the nuclear localization of transcripts from such expression cassette is in accordance with the findings that the overall shape of tRNAs are crucial for the export of tRNA transcripts studies (Good et al., 1997; Koseki et al., 1999).

Another approach for inserting ribozymes or antisense in tRNAs involves placement into the anticodon loop of the tRNA sequence. Cotten and Birnstiel placed a ribozyme coding sequence between the A and B box of a Xenopus tRNA\textsuperscript{Met} gene (Cotten and Birnstiel, 1989). The tRNA-ribozyme transcripts produced inside frog oocytes had half-lives much shorter than tRNA\textsuperscript{Met}. There was little evidence to support a hypothesis that placing the ribozyme within the tRNA structure enhanced its stability because the tRNA-ribozyme transcripts appeared to decay more rapidly than tRNA\textsuperscript{Met}. However, it was claimed that transcripts from such a construct had a greater stability than the linear ribozyme when assayed in a nuclear extract. The expressed tRNA-ribozyme was also found to remain nuclear with only a small portion entering the cytoplasm.

Thompson et al. (1995) tried to insert a number of novel structural elements into a tRNA-ribozyme chimera. One construct was designed to have the 3' terminus of the transcript hybridize to the 5' terminus. This gave rise to more than a 100 fold accumulation of ribozymes expressed from a tRNA\textsuperscript{Met} promoter. In another study, utilizing chimeric tRNA\textsuperscript{Val} ribozymes, the last seven bases of the mature tRNA\textsuperscript{Val} were removed in order to block 3' end processing of the transcript (Koseki et al., 1999). These sequences were replaced by a linker followed by a ribozyme and additional linker sequences that hybridize to the 5' terminus of tRNA\textsuperscript{Val}. The transcripts from these were
exported to the cytoplasm. The half lives of transcripts from three of these constructs
were about 35, 40 and 100 min. The most stable construct inhibited HIV most efficiently
(Koseki et al., 1999). Although the cytoplasmic localization could be due to the
maintenance of the native shape of tRNA, the increased stability could be due to the
secondary structure of the ribozyme moiety, rather than the 3' seven base deletion of
mature tRNA$^{\text{Val}}$. This deletion was intended to block 3' processing and was replaced by a
linker that could restore the base pairs present in the native tRNA$^{\text{Val}}$.

One of our chimeric tRNA-ribozymes, the pΔGTRz, showed reduced processing in
cell extracts. This is in agreement with Nashimoto's result (1999), showing that the
number of base pairs in the TψC stem is one factor recognized by 3' tRNase. Further
deletions or modifications in the secondary structure or sequences recognized by 3'-
tRNase have the potential of leading to even greater stability. The selection of these
modifications should be guided by our ever-increasing understanding of the 3' processing
process. Care should also be taken not to disrupt the recognition sequence utilized by the
tRNA export receptor exportin-t. As our knowledge of the interactions between 3'
tRNase and exportin-t and the tRNA molecules increases, the design of transcripts with
increased stability should become more feasible. Modifications intended for increasing
transcripts with increased stability can be tested by using the tc/t analysis (Table 1 of
Chapter III, Part C).

The deletion of eleven bases at the 3' end of tRNA have been widely adopted to
increase the intracellular stability of transcripts expressed (see Chapter I for more
details). This would disrupt the secondary structure recognized by 3’-tRNase (Nashimoto, 1999) and lead to increased stability, but would also result in nuclear retention of transcripts (Good et al., 1997; Koseki et al., 1999) because the tRNA shape is recognized by the tRNA export machinery (Lipowsky et al., 1999; Arts et al., 1998b).

The ribozyme can be attached to the 3’ end of tRNA^lys3 and secondary structure can be incorporated to make the 3’ terminus hybridize with the 5’ end of the transcript. This would result in greater stability (Thompson et al. 1995; Koseki et al., 1999). However, our chimeric tRNA^lys3-ribozymes have a 3’ end of 18 nucleotides which are complementary to the HIV PBS sequence. The secondary structure is likely to make annealing of these 18 nt with the PBS difficult. If this approach is to be tried, the ribozyme should be targeted to other sites, making the 3’ end design more versatile for targeting other sites. Cytoplasmic localization of such transcripts appears to be possible (Koseki et al., 1999).

SELEX (Systemic Evolution of Ligands by EXponential enrichment) is a procedure for isolating high-affinity ligands to a target protein from a pool of variant nucleic acid sequences (Tuerk and Gold, 1990). We intend to modify the in vitro SELEX procedure and use what we call an in vivo SELEX procedure to select mutant chimeric tRNA^lys3-ribozymes with increased stability, efficient export and increased affinity for RT, and hence increased encapsidation. Mutations would be introduced into the chimeric tRNA^lys3-ribozymes by mutagenic PCR. These would be used in cotransfection of cells with proviral HIV DNA. The virions produced would preferably incorporate these variant
chimeric tRNA\textsuperscript{Lys³}-ribozyme mutants. This approach would select for decreased interaction with 3' tRNase, increased interaction with export machinery and increased interaction with RT simultaneously.

The dual U6/tRNA expression cassette for expressing the chimeric tRNA\textsuperscript{Lys³}-ribozymes has an additional advantage. Since the B box is likely to be involved in the 3' processing, it allows us to select for mutations in the B box which increase stability without compromising the transcription levels (with increased transcription levels as a matter of fact).

Lastly, I would like to propose some additional experiments that deserve further considerations

- Compare in greater details the expression levels from U6 promoter alone (without any tRNA sequences – the U6Rz construct) and U6/tRNA expression cassette to more definitively determine the superiority of the U6/tRNA expression cassette over the U6 or tRNA expression cassette alone.
- Compare the expression from U6ΔGTRz and U6MM, the two constructs with the highest stability.
- Compare the HIV inhibition capabilities of more constructs including constructs with tRNA\textsuperscript{Lys³} expression cassette alone, U6 promoter expression cassette alone and U6/tRNA expression cassette.
• Test the efficacy of the chimeric tRNA$^{\text{Lys}}_3$-ribozymes expressed from the AAV vector and retroviral vector in anti-HIV assays.

• Carry out in vivo SELEX (see above).

Below are a couple of more distantly related research areas that I think are important:

• Test the affinity of the chimeric tRNA$^{\text{Lys}}_3$-ribozymes with exportin-t and assay the export properties in the *Xenopus* microinjection system. This study is important because tRNA promoters have been widely used in expressing RNA therapeutics but little is known about the export properties and mechanisms.

• Use of tRNA$^{\text{Lys}}_1$ or tRNA$^{\text{Lys}}_2$ instead of tRNA$^{\text{Lys}}_3$ as a colocalization vehicle as well as a promoter. These can also be placed in the U6/tRNA expression cassette. This approach would allow targeting of additional sites on the HIV genome because these tRNAs would not interfere with the primer binding site, yet are incorporated into HIV-1 virions at high copy numbers (Huang *et al.*, 1994).
V. REFERENCES


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Nashimoto, M. 1996. Specific cleavage of target RNAs from HIV-1 with 5' half tRNA by mammalian tRNA 3' processing endoribonuclease. RNA 2:523-524.


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VI. APPENDIXES

1. List of constructs

Table 1. List of plasmids used/constructed

<table>
<thead>
<tr>
<th>Name</th>
<th>Parent Vector</th>
<th>Features</th>
<th>Constructed by</th>
</tr>
</thead>
<tbody>
<tr>
<td>pWW</td>
<td>pBlueScript SK+</td>
<td>Wild type tRNA(^1), wild type ribozyme(^2)</td>
<td>Z. Chang</td>
</tr>
<tr>
<td>pMW</td>
<td>pBlueScript SK+</td>
<td>Mutant tRNA(^3), wild type ribozyme</td>
<td>Z. Chang</td>
</tr>
<tr>
<td>pWM</td>
<td>pBlueScript SK+</td>
<td>Wild type tRNA, mutant ribozyme(^4)</td>
<td>Z. Chang</td>
</tr>
<tr>
<td>pMM</td>
<td>pBlueScript SK+</td>
<td>Mutant tRNA, mutant ribozyme</td>
<td>Z. Chang</td>
</tr>
<tr>
<td>pAGTRz</td>
<td></td>
<td></td>
<td>G.P. Larson</td>
</tr>
<tr>
<td>pTZ U6+1</td>
<td>pTZ U6+1</td>
<td></td>
<td>E. Betrand</td>
</tr>
<tr>
<td>pU6WW</td>
<td>pTZ U6+1</td>
<td>WW under U6 promoter</td>
<td>Z. Chang</td>
</tr>
<tr>
<td>pU6MW</td>
<td>pTZ U6+1</td>
<td>MW under U6 promoter</td>
<td>Z. Chang</td>
</tr>
<tr>
<td>pU6WM</td>
<td>pTZ U6+1</td>
<td>WM under U6 promoter</td>
<td>Z. Chang</td>
</tr>
<tr>
<td>pU6MM</td>
<td>pTZ U6+1</td>
<td>MM under U6 promoter</td>
<td>Z. Chang</td>
</tr>
<tr>
<td>pU6AGTRz</td>
<td>pTZ U6+1</td>
<td>AGTRz under U6 promoter</td>
<td>Z. Chang</td>
</tr>
<tr>
<td>pU6Rz</td>
<td>pTZ U6+1</td>
<td></td>
<td>Z. Chang</td>
</tr>
<tr>
<td>pAAVU6WW</td>
<td>pAAV U6+1</td>
<td>WW under U6 promoter in AAV vector</td>
<td>Z. Chang</td>
</tr>
<tr>
<td>pAAVU6MW</td>
<td>pAAV U6+1</td>
<td>MW under U6 promoter in AAV vector</td>
<td>Z. Chang</td>
</tr>
<tr>
<td>pAAVU6WM</td>
<td>pAAV U6+1</td>
<td>WM under U6 promoter in AAV vector</td>
<td>Z. Chang</td>
</tr>
<tr>
<td>pAAVU6MM</td>
<td>pAAV U6+1</td>
<td>MM under U6 promoter in AAV vector</td>
<td>Z. Chang</td>
</tr>
<tr>
<td>pAAVU6AGTRz</td>
<td>pAAV U6+1</td>
<td>AGTRz under U6 promoter in AAV vector</td>
<td>S.K. Westaway</td>
</tr>
</tbody>
</table>

Note:

1. Wild-type tRNA: tRNA gene with wild type B box sequence: GGTTCAAGTCC
2. Wild-type ribozyme: wild type ribozyme sequence with catalytic activity.
3. Mutant tRNA: tRNA gene with two mutations in B box sequence: GCTACAAGTCC. The mutations were reported to abolish transcription from the tRNA promoter \textit{in vitro} (Murphy \textit{et al.}, 1983).
4. Mutant ribozyme: one mutation in the catalytic core of the ribozyme sequence, which was reported to abolish ribozyme catalytic activity (Ruffner \textit{et al.}, 1990).
2. Testing of anti-retroviral tRNA$^{\text{Lys}}$-ribozymes in adeno-associated virus (AAV) vectors

Four different chimeric tRNA$^{\text{Lys}}$-ribozymes with different combinations of wild type tRNA promoter, mutant tRNA promoter, wild-type ribozyme and mutant ribozymes were cloned into an AAV vector under a human U6 snRNA promoter. These constructs include WW, MW, WM and MM [the first letter indicates wild type (W) or mutant tRNA promoter (M) and the second letter indicates wild type (W) or mutant ribozyme (M)]. The constructs with mutations in the tRNA promoter and/or in the catalytic core of the ribozyme serve as controls.

These anti-HIV chimeric tRNA$^{\text{Lys}}$-ribozymes were successfully constructed into the adeno-associated virus vector and the constructs were named pAAVU6WW, pAAVU6MW, pAAVU6WM and pAAVMMM. The construction was more sophisticated than originally contemplated and took much longer time than originally planned. The AAV vector has two homologous sequences, the inverted terminal repeats (ITRs). These two ITRs are unstable and the constructs often have small deletions which include parts of ITRs. These are undesirable for the vectors because they are important in proper packaging and expression of the vectors in the host cells.

The constructs from the first construction were found to have these deletions. In addition to checking for the correct inserts, they were checked via digestions with several restriction enzymes ($Bgl$ II, $Msc$ I and $Sma$ I). These enzymes cleave at restriction sites.
that are present in the ITRs and give rise to restriction digestion patterns that would suggest the intactness or presence of deletions in ITRs. The pattern of the digestion suggested the presence of small deletions in the ITRs. The construction was repeated and the constructs obtained were then transformed into a different bacterial strain. A commercially available bacterial strain called Max Efficiency Stbl2 Competent Cells (Life Technologies) was used. We expect that the bacterial strain may potentially stabilize the inverted terminal repeats (ITRs) in the AAV vector. The constructs were checked and found to have intact ITRs.

Instead of packaging the AAV vectors and analyzing the constructs in transduction assays as originally planned, we made stable cell lines in 293 cells by selecting with G418 for expression of the neo gene in the constructs. Several clones were picked and total RNA was isolated. The expression levels of the chimeric tRNA\textsuperscript{\text{Lys3}}-ribozymes were quantitated and compared in a Northern hybridization analysis. The intracellular localization of the transcripts was checked in a FISH (fluorescent in situ hybridization) assay. The FISH assay failed to detect the chimeric tRNA\textsuperscript{\text{Lys3}}-ribozyme in stably transfected cells, but they were detected in Northern hybridization (Figure 1). FISH analyses of the expression of these constructs following transient transfection detected the chimeric tRNA\textsuperscript{\text{Lys3}}-ribozymes in the cytoplasm. The clones with the highest expression levels (Figure 1) were used in analyses of the stabilities of the chimeric tRNA\textsuperscript{\text{Lys3}}-ribozymes in vivo. The half-lives of the constructs were analyzed and found to be about 30 minutes.
The stable clones with the highest expression levels were also studied in an anti-HIV assay. The assay failed to detect the levels of anti-HIV effects previously demonstrated in transient transfections. This could be due to the fact that the levels of expression from the AAV vectors in the stable cell lines were insufficient to elicit anti-viral activity. Therefore, enhanced expression in stable cell lines is a task that deserves more research efforts and is a source of future research efforts.

3. Compilation of additional data

1. Selection of clones of cells stably expressing chimeric tRNA\textsuperscript{Lys3}-ribozymes

![Image](image_url)

Figure 1. Selection of clones of cells stably expressing chimeric tRNA\textsuperscript{Lys3}-ribozymes.

293 Cells transfected with the plasmids pAAVU6WW, pAAVU6MW and pAAVU6WM are selected with G418 as described previously. The following clones were obtained: AAV U6+1 vector control: AAV; AAVU6WW: 1-p, 1-2, 1-4; AAVU6MW: 2-1p, 2-2p, 2-1, 2-2, 2-3, 2-4; AAVU6WM: 3-1, 3-2, 3-5.
Total RNA samples were isolated from these clones and the expression levels of the chimeric tRNA^{Lys3}-ribozymes checked by Northern hybridization with a wild type ribozyme-specific probe (left-upper panel and a mutant ribozyme specific probe (right upper panel). The same membrane was stripped and rehybridized with a U6 snRNA specific probe to normalize for loading variations (lower left and lower right panels). The membranes were quantified with a phosphoimager and analyzed with ImageQuant software. The clones were compared for expression levels. Figure 2 shows that clones 1-2 and 2-2 have the highest expression of WW and MW chimeric tRNA^{Lys3}-ribozymes. It is obvious from Figure 1 that clone 3-1 has the highest expression of WM.

**Selection of Highest Expressing Clones of Stably Expressed tRNA-Rbz**

![Graph showing normalized volume of RNA samples for different clones](image)

Figure 2. Selection of stable clones with highest expression level of chimeric tRNA^{Lys3}-ribozymes (AAVU6WW and AAVU6MW).
2. Binding assay of chimeric tRNA\textsuperscript{Lys3}-ribozymes and reverse transcriptase

The protocol for the experiment was described in Chapter II: Methods and Materials.

1) The activity of HIV RT was tested and compared with AMV RT in a primer extension assay (see Chapter II: Materials and Methods). In vitro transcribed chimeric tRNA\textsuperscript{Lys3}-ribozyme transcripts are used as templates. An oligonucleotide complementary to the 3' end of the chimeric tRNALys3-ribozyme is used as primer, which was labeled with \textsuperscript{32}P. Equal units of AMV RT (Promega) and HIV RT were used. The result is shown in Figure 5. The HIV RT was found to be active.

![Figure 3. Testing of activity of HIV RT in a primer extension assay.](image)

2) Several buffers were tested in the binding assay

1 X Buffer 1, the buffer I have been using, 20mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 5% glycerol; 1 X Buffer 2, a buffer recipe used by Dr. Gary Larson, 40 mM Tris, pH 8.3, 60 mM NaCl, 6 mM MgCl, 5 mM DTT; 1X Buffer 3, a recipe from Dr. John Francis which was used in another gel shift experiment, 0.01 M K2HPO4, 0.01 M NaH2PO4, 0.14 M KCl.
Figure 4. Testing of three buffers for the binding assay of chimeric tRNA\textsuperscript{Lys3}-ribozyme transcripts with reverse transcriptase.

Buffer 1 was found to be most efficient.

3) Binding of tRNA\textsuperscript{Lys3} with HIV RT and AMV RT (as a control)

Figure 5. Binding of tRNA\textsuperscript{Lys3} transcripts with HIV RT.

AMV RT was used as a control. AMV RT 1 was from Promega. AMV RT 2 was from Life Science. AMV RT stuck in the wells and therefore was not a good control.
Therefore, in the next experiment, transcripts of $\text{tRNA}^{\text{Val}}$ were transcribed and used as a control.

4) Binding assay of WW and MW with HIV RT

The binding of transcripts of WW and MW from U6WW and U6MW with HIV RT was compared with the binding of the transcripts of $\text{tRNA}^{\text{Val}}$. The sequence of the $\text{tRNA}^{\text{Val}}$ gene was as follows: GTTT CCG TAG TGT AGT GGT TAT CAC GTT CGC CTA ACA CGC GAA AGG TCC CCG GTT CGA AAC CGG GCG GAA ACA. A DNA fragment with the $\text{tRNA}^{\text{Val}}$ gene under a T7 promoter is constructed by polymerase chain reaction. This DNA fragment was used in in vitro transcription to obtain radiolabeled $\text{tRNA}^{\text{Val}}$ transcripts (see Materials and Methods, Chapter II).

![Figure 6. Binding assay of WW and MW with HIV RT.](image)
Figure 7. Binding assay of WW and MW with HIV RT.

Same gel as in Figure 6 with a longer exposure time. Two additional lanes were shown on the left.