STRATEGIES FOR ISOLATION AND EXPRESSION OF RIBOZYMES FOR USE IN HIV GENE THERAPY

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
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This thesis is dedicated to the memory of my

father,

Fernando Menguito Medina.

You may be far away,

but…

You are always remembered.

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Ad	adenovirus
AT	antitrypsin
bp	base pair
CA	capsid
CAK	CDK-activating kinase
CAT	chloramphenicol acetyltransferase
CMV	cytomegalovirus
CRM1	chromosomal region 1 maintenance
CTD	carboxy-terminal domain
DAI	double-stranded RNA-activated inhibitor
DC	double copy
DIS	dimer initiation site
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
Env	envelope
ER	endoplasmic reticulum
Gag	group specific antigen
HBS	HEPES-buffered saline
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid
HIV	human immunodeficiency virus
HMW	high molecular weight
HPV	human papillomavirus
HSV	herpes simplex virus
IGF-II	insulin-like growth factor
IN	integrase
IP	interfering protein
IPTG	isopropylthio-β-D-galactoside
IR	interfering RNA
kb	kilobase
LB	Luria-Bertani
LMW	low molecular weight
LTR	long terminal repeat
M-tropic	macrophage-tropic
MA	matrix
ME	mercaptoethanol
MES	2-N-Morpholinoethanesulfonic acid
m.o.i.	multiplicity of infection
MMLV	Moloney murine leukemia virus
NC	nucleocapsid
Nef	negative effector

neo	neomycin
NES	nuclear export signal
NLS	nuclear localization signal
nt	nucleotide
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cell
PBS	primer binding site
PIPES	piperazine-N,N`-bis (2-ethanesulfonic acid) disodium salt
Pol	polymerase
PPT	polypurine tract
PR	protease
ψ	packaging signal
P-TEFb	positive transcriptional elongation factor b
RAC	Recombinant DNA Advisory Committee
RRE	Rev-response element
Rev	regulator of expression of virion proteins
Rev M10	*trans* dominant Rev mutant protein
RT	reverse transcriptase
SC	single copy
sn	small nuclear
SIV	simian immunodeficiency virus
T-tropic	T cell-tropic
TAK	Tat-associated kinase
Tat	*trans*-activator of transcription
TAR	*trans*-activation response
TDM	*trans* dominant mutant
tk	thymidine kinase
TM	transmembrane
VA	virus associated
Vif	virion infectivity factor
Vpr	viral protein R
Vpu	viral protein U
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Design, Characterization and Testing of $tRNA_3^{Lys}$-based Hammerhead Ribozymes*

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Transfusion Science (1996) 17:109-120

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ABSTRACT

Ribozymes are small, catalytic RNA molecules which possess endoribonuclease activities. They can be expressed as part of tRNA genes because of the strong tRNA promoter and the stable tRNA structure. Thus, a ribozyme which is inserted into a tRNA molecule will be produced in large quantities and will be protected from RNase degradation in cells. $tRNA_3^{Lys}$ was used for constructing HIV-1 RNA-specific tRNA-ribozymes. This tRNA is the primer used by HIV-1 for reverse transcription, and is co-packaged within the virus particles. By using the recombinant $tRNA_3^{Lys}$, the virus may be tricked into packaging the tRNA-ribozymes. Being in such close proximity, the ribozymes have the potential to bind and cleave HIV-1 RNA within the virus particles.

A hammerhead ribozyme targeted against the HIV-1 *env*-coding region was expressed as part of the anticodon loop of human $tRNA_3^{Lys}$ without sacrificing tRNA stability or ribozyme catalytic activity. These tRNA-ribozymes were isolated from a library which was designed to contain linkers (sequences connecting the ribozyme to the anticodon loop) of random sequence and variable length. The linkers on either side of the ribozyme ranged in length from 0 to 20 nucleotides. Active tRNA-ribozymes were selected *in vitro* by placing the ribozyme and its target *in cis* so that *cis* cleavage can occur. The tRNA-ribozymes that possessed ideal combinations of linkers were expected to recognize the *cis* target site more freely and undergo cleavage. The cleavage products containing active tRNA-ribozymes were separated from uncleaved target RNA due to size difference and used for reverse transcription followed by PCR. *In vitro* selected tRNA-ribozymes were cloned in the pGEM4Z plasmid and characterized.

$tRNA_{AC}$-Rz (0/0), tRNAAC-Rz (2/0), $tRNA_{AC}$-Rz (4/0), $tRNA_{AC}$-Rz (8/0) and $tRNA_{AC}$-Rz (10/0) were selected from this library. These tRNA-ribozymes were stable, possessed cleavage efficiencies lower or similar to the linear hammerhead ribozyme, and could be transcribed by a cell extract containing RNA polymerase III. Retroviral vectors expressing tRNA-ribozymes were tested in a human CD4+ T cell line and were shown to inhibit HIV-1 replication. These $tRNA_3^{Lys}$-based hammerhead ribozymes should therefore prove to be valuable for both basic and applied research.

INTRODUCTION

I. HIV VIRION

HIV-1 belongs to the Lentivirinae subfamily of viruses within the Retroviridae family. The HIV-1 genome consists of a 9.2 kb RNA with nine open reading frames (reviewed by Frankel and Young 1998, and Joshi and Joshi 1996, Fig. 1*A*). It possesses the *gag* (**g**roup specific **a**nti**g**en), *pol* (**pol**ymerase), and *env* (**env**elope) genes common to all retroviruses. The products of these genes are packaged within the virus particles (Fig. 1*B*). The *gag* gene gives rise to a Gag ($Pr55^{gag}$) precursor polyprotein which is subsequently processed into four proteins: MA (**ma**trix or p17), CA (**ca**psid or p24), NC (**nu**cleo**ca**psid or p9), and p6. MA proteins line the inner surface of the lipid bilayer envelope that originated from the host cell. CA proteins form a capsid, which surrounds two copies of the viral RNA genome that are closely associated with the NC proteins. NC proteins bind to the ψ signal and promote the encapsidation of genomic RNA during viral assembly. The p6 protein is important for incorporation of the Vpr protein during viral assembly, and contains the L (late) domain important for a late step in budding. The *gag* and *pol* genes give rise through a ribosomal frameshift to a Gag-Pol ($Pr160^{gag\text{-}pol}$) precursor polyprotein which is subsequently processed into the Gag proteins and three proteins which provide enzymatic functions: PR (**pr**otease or p10), RT (**r**everse **t**ranscriptase or p66/p51 heterodimer), and IN (**in**tegrase or p31). The *env* gene gives rise to two proteins: SU (**su**rface or gp120) and TM (**tm**transmembrane or gp41). The *gag* and *env* structural proteins make up the core of the virion and outer membrane envelope, respectively.

In addition, HIV-1 contains six genes coding for accessory proteins. These proteins include Tat (***t**rans*-**a**ctivator of **t**ranscription), and Rev (**r**egulator of **e**xpression of virion proteins), which are involved in the regulation of viral gene expression. The Vif (virion infectivity factor) protein

FIG. 1. **Schematic diagram of the HIV-1 genetic map and virion.** *A*, The HIV-1 genome possesses nine open reading frames which express the *gag*, *pol*, *env*, *tat*, *rev*, *env*, *vpu*, *vpr*, and *vif* genes. *B*, HIV-1 possesses a lipid envelope which it acquires as it buds from its host cell. The lipid bilayer of HIV-1 contains viral envelope proteins: gp41 which is a transmembrane protein and gp120 which is anchored onto gp41 *via* non-covalent interactions. Underneath the lipid bilayer are the MA proteins. CA proteins form a capsid, which surrounds two copies of the viral RNA genome that are closely associated with the NC proteins. The virion contains viral protease, reverse transcriptase, and integrase, which are required for viral maturation, reverse transcription, and integration, respectively. In addition, the Vpr protein and cellular $tRNA_3^{Lys}$ are packaged by the virus. The $tRNA_3^{Lys}$ is used as a primer during reverse transcription.

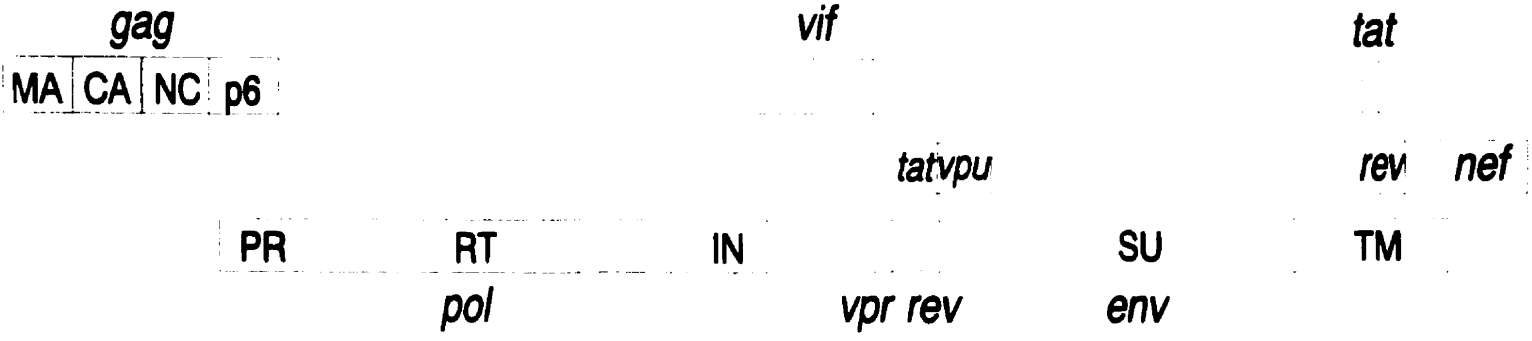

gag
vif
tat
MA
CA
NC
p6
tatvpu
rev
nef
PR
RT
IN
SU
TM
pol
vpr rev
env

Figure 1A.

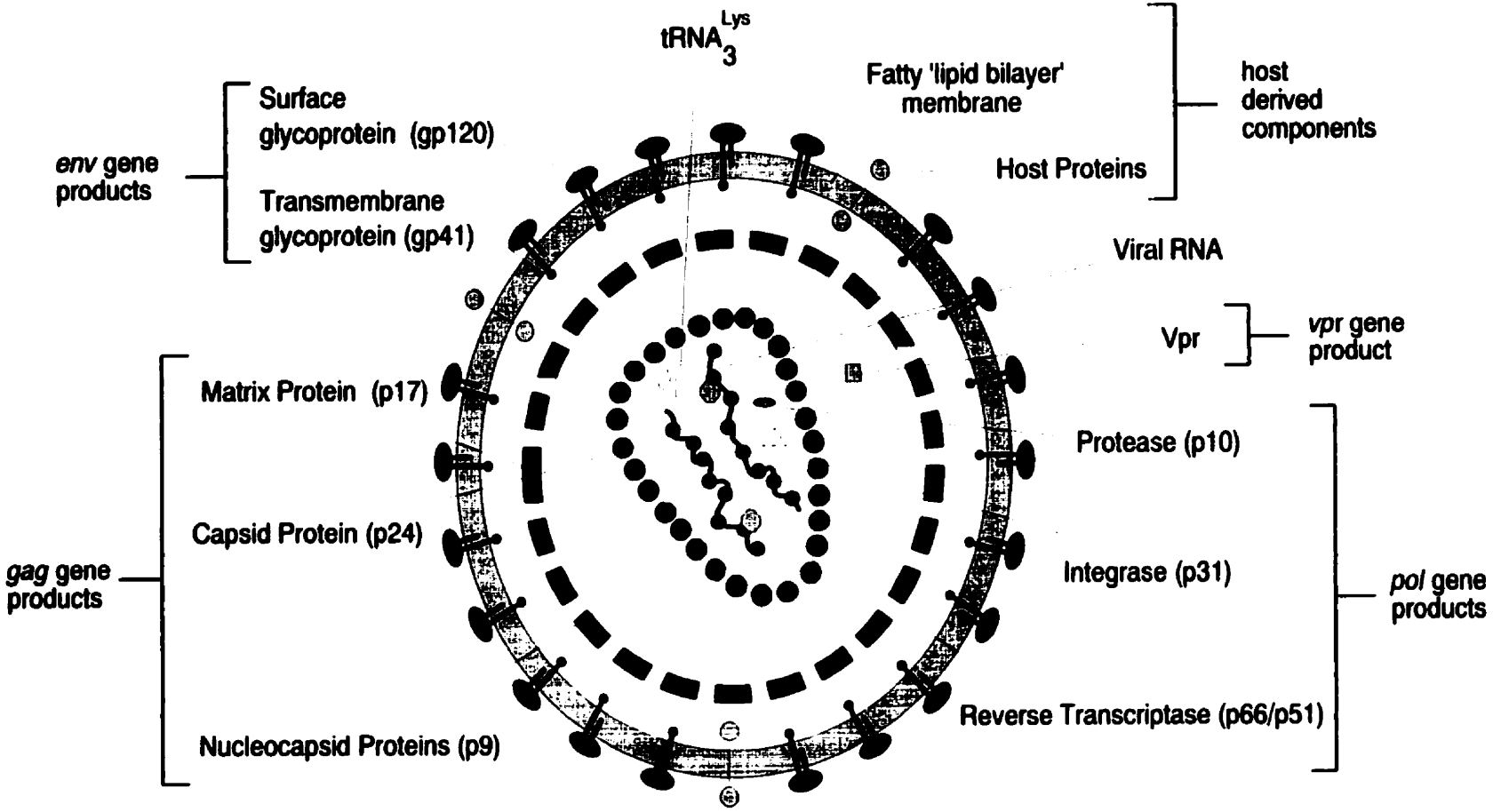

tRNA3Lys
Fatty 'lipid bilayer' membrane
host derived components
Host Proteins
Surface glycoprotein (gp120)
env gene products
Transmembrane glycoprotein (gp41)
Viral RNA
Vpr
vpr gene product
Matrix Protein (p17)
Protease (p10)
Capsid Protein (p24)
Integrase (p31)
gag gene products
pol gene products
Reverse Transcriptase (p66/p51)
Nucleocapsid Proteins (p9)

Figure 1*B*.

is important for the production of highly infectious mature virions, while Vpu (viral protein **u**) enhances the release of virus particles. The Vpr (viral protein **r**) is co-packaged within the released virion and plays a role in regulating nuclear import of the HIV-1 pre-integration complex. The Nef (**n**egative **ef**fector) protein contributes to reduction of CD4 and MHC class I molecules on the cell (reviewed by Trono 1995, and Emerman and Malim 1998).

II. HIV-1 REPLICATION CYCLE

HIV-1 preferentially infects macrophages and T cells, which express the primary receptor, the CD4 molecule. HIV-1 also utilizes chemokine receptors as co-receptors. Macrophage (M)-tropic and non-syncytium inducing viruses bind to CCR5 (Alkhatib et al 1996, Deng et al 1996, Dragic et al 1996), while the T cell (T)-tropic and syncytium inducing viruses bind to CXCR4 (Feng et al 1996). Additional coreceptors have been identified for HIV which include CCR2, CCR3, CCR8, STRL33/BONZO, BOB/GPR-15, V28/CX3CR, APJ and US28 (reviewed by Cairns and D'Souza 1998, and Proudfoot et al 1999).

Upon binding of the SU protein to the CD4, a conformational change ensues which exposes the variable V3 loop of SU and a highly conserved bridging sheet, which forms the binding site for the chemokine coreceptors (Kwong et al 1998, Wyatt and Sodroski 1998). The TM protein then undergoes a conformational change which results in exposure of an N-terminal hydrophobic "fusion" peptide. This results in fusion of the viral and cellular membranes, and entry of the viral core into the cell (reviewed by Hunter 1997). Following entry into the cell, the two copies of the HIV-1 genomic RNA are reverse transcribed within the viral core into a double stranded proviral DNA (Fig. 2). Negative strand DNA synthesis initiates from the 3' end of a host $tRNA_3^{Lys}$ primer, which possesses 18 3'-terminal nucleotides that are complementary to the PBS (**p**rimer **b**inding **s**ite) near the 5' end of the genomic RNA. The PPT (**p**olypurine **t**ract) serves as a primer during

FIG. 2. **Replication cycle of HIV-1.** Upon binding of the virus to the CD4 receptor and chemokine coreceptors, the viral nucleoprotein complex containing genomic RNA as well as MA, RT, IN, and Vpr proteins, are transported to the nucleus. Upon entry, the HIV-1 genomic RNA is reverse transcribed by RT into a double stranded DNA. The resulting double-stranded proviral DNA is then integrated into the host genome by IN protein. During the early phase of gene expression, fully-spliced, 2 kb mRNAs are produced which give rise to Tat, Rev and Nef proteins. Upon translation in the cytoplasm, the Tat protein by virtue of its NLS enters the nucleus, binds to the TAR element located at the 5' end of the nascent viral transcripts, and increases production of viral mRNAs approximately 100-fold. The Rev protein which contains an NLS enters the nucleus and binds to the RRE region which results in the transport of 4-5 kb singly-spliced and 9.2 kb unspliced RNAs to the cytoplasm. Translation of the singly-spliced RNA produces the Vpu, Vpr, Vif, and Env proteins. Translation of the unspliced RNAs produces the Gag and Gag-Pol polyproteins. The Env polyprotein is translated at the endoplasmic reticulum, and processed by a cellular protease in the Golgi apparatus into the SU and TM glycoproteins during transit to the cell surface. Cellular $tRNA_3^{Lys}$ is also packaged and serves as a primer during subsequent reverse transcription. An immature virus particle buds from the cell surface that undergoes a morphologic change. During the late stages of budding, or shortly thereafter, the Gag and Gag-Pol polyprotein precursors present in these particles undergo proteolytic processing by PR thus resulting in mature, infectious virus particles.

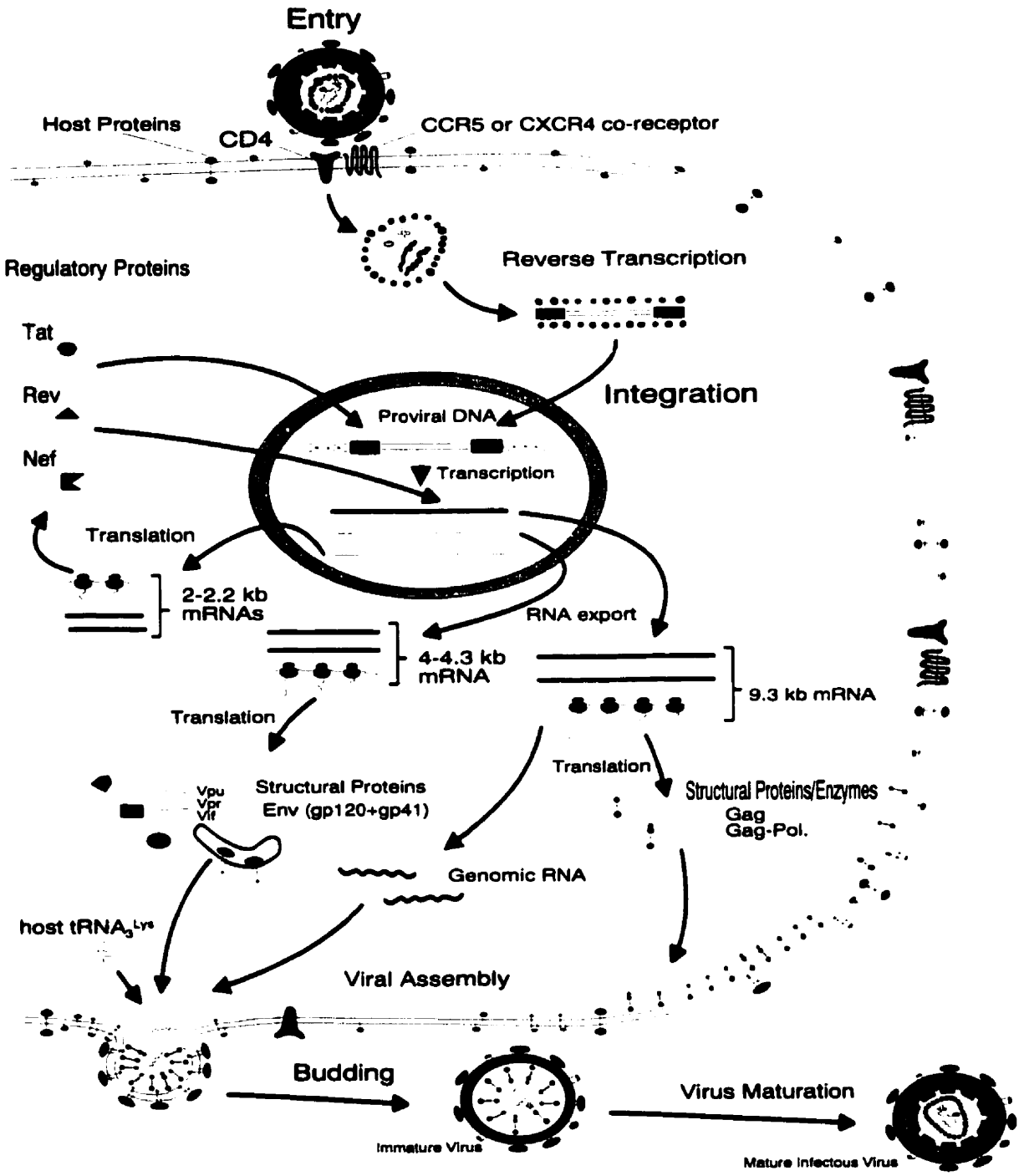

Entry
Host Proteins
CD4
CCR5 or CXCR4 co-receptor
Regulatory Proteins
Reverse Transcription
Tat
Rev
Nef
Integration
Proviral DNA
Transcription
Translation
2-2.2 kb mRNAs
RNA export
4-4.3 kb mRNA
9.3 kb mRNA
Translation
Translation
Vpu
Vpr
Vif
Structural Proteins
Env (gp120+gp41)
Structural Proteins/Enzymes
Gag
Gag-Pol.
Genomic RNA
host tRNA3Lys
Viral Assembly
Budding
Immature Virus
Virus Maturation
Mature Infectous Virus

Figure 2.

the positive strand DNA synthesis (reviewed by Telesnitsky and Goff 1997). The resulting proviral DNA contains a 5' and a 3' LTR (long terminal repeat), each consisting of a U3, R, and U5 region, which flank the viral genes. The U3 region contains a promoter for RNA polymerase II, enhancer sequences, and *cis*-acting elements, which demonstrate both positive and negative regulatory properties. It also contains one of the attachment sites for integration. The R region contains the TAR element, the transcription start site, the 3' processing site, and the polyadenylation site. The U5 region contains the other attachment site for integration (reviewed by Brown 1997).

A viral nucleoprotein complex containing reverse-transcribed DNA, RT, IN, Vpr, and possibly MA proteins (Gallay et al 1996), is then transported to the nucleus. Transport of the nucleoprotein complexes to the nucleus is mediated by the Vpr protein through its N-terminal amphipathic α -helices that localizes Vpr to the nuclear pores (Popov et al 1998). The double-stranded proviral DNA is then integrated into the host genome by IN protein. IN first removes two 3' nucleotides from each strand of the linear proviral DNA. Then, the 3' ends are covalently joined to the 5' ends of the target DNA. Unpaired nucleotides at the viral 5' ends are removed and joined to the target site 3' ends, generating an integrated provirus flanked by five base pair direct repeats of the target site DNA (reviewed by Brown 1997).

Once integrated, HIV-1 proviral gene expression is controlled by both cellular and viral proteins. Viral RNA is transcribed from the 5' LTR promoter, which possesses binding sites for several transcription factors including NF-κB, and Sp1. The full length 9.2 kb viral RNA is then differentially spliced to give rise to various HIV-1 mRNAs (reviewed by Rabson and Graves 1997). During the early phase of gene expression, fully-spliced, 2 kb mRNAs are produced that encode the Tat, Rev and Nef proteins. Upon being translated in the cytoplasm, the Tat protein by virtue of its NLS returns to the nucleus. The Tat protein increases the processivity of RNA pol II

and results in 100 to 1000-fold increase in LTR-directed transcription. It also enables complete transcription of the 9.2 kb viral genome. Tat protein binds to a pre-initiation complex and interacts with TFIIH, a multisubunit complex that is bound to nonphosphorylated RNA pol II (reviewed by Jones 1997). The CAK (CDK-activating kinase), associated with TFIIH, phosphorylates the RNA pol II carboxy-terminal domain (CTD) and assists in promoter clearance (Cujec et al 1997). The Tat protein then binds to a Tat-associated kinase (TAK), also known as P-TEFb (positive transcriptional elongation factor b), which is composed of a catalytic subunit, CDK9 (previously called PITALRE), and a regulatory subunit, cyclin T1 (reviewed by de Falco and Giordano 1998). After transcription of the *trans*-activation responsive (TAR) element located at the 5' end of the viral transcripts, Tat protein repositions CDK9 near the RNA pol II CTD. This results in hyperphosphorylation of the RNA pol II CTD, and converts it from an initiating (RNA pol IIa) to an elongating (RNA pol IIo) enzyme (Mancebo et al 1997, Ping and Rana 1999).

One of the early proteins to be produced, the Rev protein possesses both a nuclear localization signal (NLS), and a nuclear export signal (NES). These two signals enable Rev proteins to shuttle between the nucleus and the cytoplasm. By virtue of its NLS, Rev proteins return to the nucleus after being translated in the cytoplasm, and bind to the Rev responsive element (RRE) located in the *env* gene. The Rev NES remains exposed on the surface of the ribonucleoprotein complex, and interacts with a nucleoporin-like protein (hRip/Rab) located at the nuclear pore through the chromosomal region 1 maintenance (CRM1) protein, a nuclear export receptor (reviewed by Hope 1999). This results in transport of singly-spliced, 4-5 kb mRNAs encoding Vpu, Env, Vif, and Vpr, and the unspliced 9.2 kb genomic RNA encoding Gag and Gag-Pol precursor polyproteins to the cytoplasm. The unspliced and spliced mRNAs are then translated into viral structural and maturation proteins.

Env proteins are translated from a singly-spliced RNA at the endoplasmic reticulum (ER). The amino terminal region of Env contains a signal peptide which associates with a signal recognition particle (SRP). The SRP docks onto the SRP receptor present in the ER membrane. As Env proteins are translocated to the lumen of the ER, the signal peptide is cleaved by host signal peptidase. During translation, the Env proteins are also glycosylated by the addition of preassembled oligosaccharides at 30 sites, the majority of which are in gp120. These modifications enhance the stability of glycoproteins by protecting it from enzymes and reducing their immunogenicity. Glycosylation may also provide the hydrophilicity necessary to drive proper folding of the protein (reviewed by Swanstrom and Wills 1997).

The hydrophobic carboxy terminal region of Env prevents it from being released into the lumen of the ER. Several chaperone proteins present in the ER prevent Env proteins from forming aggregates with other unrelated proteins, and prevent the cysteine residues of Env from forming disulfide bonds with an unrelated molecule. They stabilize the folding and assembly competent states of Env until the individual subunits can locate one another. These proteins also prevent incompletely assembled oligomers form leaving the ER. Multimerization of Env is important to provide rigidity to its structure during transport to the membrane by allowing hydrophobic domains to be sequestered within the protein core. After leaving the ER, the Env polyprotein is processed by a cellular protease in the Golgi apparatus into the SU and TM proteins. Additional modifications to the sugar compositions of Env also take place, resulting in a heterogeneous population of Env proteins (reviewed by Swanstrom and Wills 1997).

The glycosylated Env polyproteins could potentially become trapped in the ER by forming complexes with CD4 molecules. The Vpu protein promotes CD4 degradation by proteasomes through associations between the C-terminal cytoplasmic tail of Vpu and the cytoplasmic tail of CD4 (Tiganos et al 1997). Vpu proteins also downregulate cell surface expression of MHC class

I proteins, which is thought to protect infected cells from recognition and killing by cytotoxic T lymphocytes. Similar to Vpu, Nef also reduces the levels of cellular CD4 and downregulates expression of MHC class I molecules (reviewed by Marsh 1999). CD4 degradation by Nef protein is accomplished by promoting endocytosis from the cell surface to lysosomes.

The Gag and Gag-Pol polyproteins are cotranslationally modified by the addition of myristate at their N-terminal region. The viral core is assembled from the Gag and Gag-Pol polyproteins, which interact with each other, and two molecules of the full length viral RNA containing the ψ signal. The ψ signal, located within the *gag* and *env*-coding regions, is responsible for viral RNA encapsidation by the Gag protein. The viral RNAs dimerize by a kissing loop mechanism through the dimer initiation site (DIS, reviewed by Paillart et al, 1996). The I (interaction) domains located in the NC sequences of Gag protein is the major region of Gag-Gag interaction, while two Cys-His boxes, also located within the NC, play a central role in the selective packaging of the RNA genome. Vpr is incorporated into viral particles through an interaction with p6 (Bachand et al 1999). The N-terminal myristate group of the Gag and Gag-Pol precursor polyproteins, and the basic residues located within the first 50 amino acids of the MA protein, allow targeting of the precursors to the plasma membrane. The Env glycoproteins are retained on the viral surface through an interaction between MA sequences and the TM portion of Env (reviewed by Freed 1998). Immature virus particles bud from the cell surface which subsequently undergo a morphologic change. During the late stages of budding, or shortly thereafter, the Gag and Gag-Pol polyprotein precursors present in these particles are processed by PR thus resulting in mature, infectious virus particles (reviewed by Swanstrom and Wills 1997).

Virions also package tRNA molecules which are enriched for the primer, $tRNA_3^{Lys}$, during reverse transcription (Kleiman et al 1991, Jiang et al. 1993). Each virion contains eight molecules of cellular $tRNA_3^{Lys}$ (Mak et al 1994) for the two molecules of HIV-1 genomic RNA.

RT protein, in the form of the Gag-Pol precursor polyprotein, is directly involved in the selection and packaging of primer tRNA (Mak et al 1994). The signals on $tRNA_3^{Lys}$ that allow it to be packaged are not known. Nevertheless, the anticodon of $tRNA_3^{Lys}$ is not essential for packaging since a mutant $tRNA_3^{Lys}$ possessing a modified anticodon could still be packaged within HIV-1 virions (Huang et al 1994, Huang et al 1996, reviewed by Mak and Kleiman 1997). Similarly, $tRNA_1^{Lys}$ and $tRNA_2^{Lys}$ which possess a different anticodon were also shown to be packaged with the same efficiency as $tRNA_3^{Lys}$ (Huang et al 1994, Jiang et al 1993).

III. INTERFERING MOLECULES THAT MAY BE USED TO INHIBIT HIV-1 REPLICATION *VIA* GENE THERAPY

A. Interfering Proteins

Interfering proteins (IPs) block the HIV-1 replication cycle by interfering with the function of HIV-1 regulatory or structural proteins or lead to the selective death of HIV-1-infected cells. Trans-dominant mutants (TDMs) of viral proteins are capable of interfering with the function of WT viral proteins (Liem et al 1993). RNases fused to viral proteins such as Tat (Melekhovets and Joshi 1996) or Gag (Singwi et al 1999) could specifically degrade HIV-1 RNA. Suicide proteins, such as the herpes simplex virus (HSV) *tk* (thymidine kinase) gene, could be expressed under control of the HIV-1 LTR promoter, such that upon HIV-1 infection, transduced cells undergo metabolic suicide in the presence of an appropriate prodrug (Marcello and Giaretta 1998). Intracellularly expressed single-chain antibodies trap viral proteins in the cytoplasmic compartments, resulting in inhibition of HIV-1 replication (Mhashilkar et al 1999).

B. Interfering RNAs

Various interfering RNAs (IRs) which can be delivered to cells for therapeutic purposes include sense RNAs, antisense RNAs, and ribozymes. Sense RNAs acting as decoys contain sequences which are identical to protein binding domains within the genomic RNA. They are able to compete with the target RNA for binding to proteins and thus block these proteins from their intended function. Sense RNAs that contain HIV-1 RNA sequences can interact with specific viral RNAs/proteins and should, upon competition with HIV-1 RNA for binding to these viral RNAs/proteins, prevent virus replication (Joshi et al 1991, Cohli et al 1994). On the other hand, antisense RNAs contain sequences complementary to portions of the target RNA. Antisense RNAs that are complementary to a specific region of HIV-1 RNA, could, upon hybridization with target RNA sequences, disrupt reverse transcription, processing, translation, and/or packaging of the RNA (Ding et al 1998). Also, the double-stranded antisense-target RNA hybrids may be subject to RNase 1 degradation (reviewed by Sorrentino and Libonati 1997), resulting in a permanent loss of the target RNA.

Ribozymes are small catalytic RNA molecules which can be designed to specifically pair with a target RNA sequence and cleave the phosphodiester backbone at a specified location (reviewed by Castanotto et al 1992). The naturally occurring hammerhead ribozymes are found in the avocado sunblotch viroid as well as the satellite RNAs of lucerne transient streak and tobacco ringspot viruses (Forster and Symons 1987). These RNA molecules normally cleave *in cis*, but can be designed to cleave a target sequence *in trans* (Haseloff and Gerlach 1988). The catalytic domain mediates cleavage of a target RNA, resulting in two RNA cleavage products: a 5' product with a 2', 3' cyclic phosphate and a 3' product with a 5' hydroxyl group. The conserved regions of the hammerhead catalytic domain associated with the cleavage reaction have been described. They consist of three branched RNA helices that flank the cleavage site, and of two single

stranded regions with highly conserved sequences (Haseloff and Gerlach 1988). The minimal structural requirements for the design of *trans*-acting ribozymes have been elucidated such that it is now possible to target ribozymes against any given RNA. Three criteria must be fulfilled for ribozyme-mediated *trans*-cleavage reaction. (i) The ribozyme must contain 11 of the 13 conserved nts found in naturally occurring cleavage domains (Uhlenbeck, 1987) (ii). The cleavage site within the target RNA must be immediately preceded by NUX (reviewed by Birikh et al 1997), with N being any nucleotide and X being any nucleotide except G (iii) The ribozyme and sequences immediately surrounding the cleavage site within the target RNA are bound together by antisense sequences composed of at least 8 nts which flank the catalytic domain (Fig. 3).

Hairpin ribozymes have also been designed to cleave in *trans*. The target site occurs immediately before a GUC triplet and is preceded by a BN sequence (BN↓GUC) with B being any nucleotide except A. Similar to the hammerhead ribozyme, cleavage results in 5' products containing a 2', 3' cyclic phosphate and 3' products with a 5' hydroxyl group. The hairpin ribozyme sequence must contain 50 nts which form four helices, two of which are involved in hybridization with sequences flanking the cleavage site within the target RNA, and five single-stranded loops, one of which is the cleavage site in the target RNA (reviewed by Hampel 1998).

IV. INTERFERENCE SITES WITHIN THE HIV-1 REPLICATION CYCLE

Various points within the HIV-1 replication cycle may be targeted using interfering RNAs and interfering proteins. The following lists the interference sites which could be targeted using a ribozyme.

FIG. 3. **Diagram of a hammerhead ribozyme.** The minimal structural requirements for *trans*-acting ribozymes include the conserved nucleotides found in naturally-occurring cleavage domains (boxed nucleotides), a cleavage site (⇩) within the target RNA which is immediately preceded by an NUX sequence, and antisense sequences which flank the catalytic region and allows hybridization to the target RNA. N, either C, U, G, or A; X, either C, U, or A.

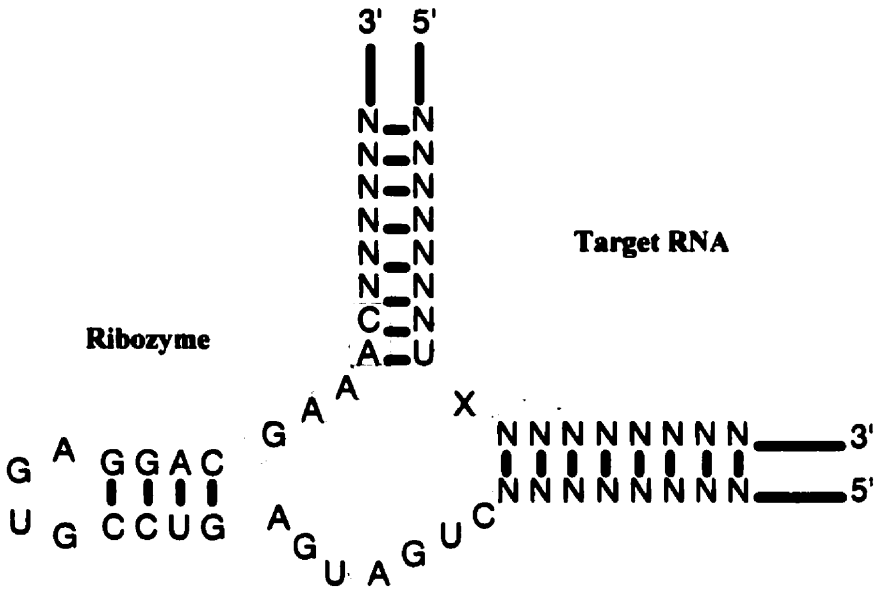

3'
5'
N N
N N
N N
N N
N N
N N
C N
A U
Target RNA
Ribozyme
A
G A
X
G A G G A C
U G C C U G
A G U A G U C
N N N N N N N N 3'
N N N N N N N N 5'

Figure 3.

A. Interference with HIV-1 replication

HIV-1 replication takes place using viral RT and cellular $tRNA_3^{Lys}$ which binds to the HIV-1 PBS *via* an 18-nt sequence complementarity. Interference at the level of reverse transcription might be achieved by expressing ribozymes against regions surrounding the PBS, which should inhibit elongation.

B. Interference with *trans*-activation of HIV-1 gene expression

The TAR element located near the 5' end of all HIV-1 mRNAs is required for Tat-mediated *trans*-activation of HIV-1 gene expression. Ribozymes could be designed against the TAR and/or the *tat*-coding region to inhibit *trans*-activation.

C. Interference with HIV-1 translation

The 5' terminal 287-nts of all HIV-1 mRNAs are identical. Thus, viral mRNA translation could be inhibited by designing ribozymes against the 5' leader sequence, which would cleave the 5' terminal portion of all mRNAs containing this sequence. Alternatively, translation of specific mRNAs could be inhibited by designing ribozymes to specific coding regions.

D. Interference with HIV-1 late gene expression

Rev-RRE interaction is required for late viral gene expression. Ribozymes could be designed against the RRE sequence present in singly-spliced and unspliced viral RNAs. Absence of these mRNAs would prevent late gene expression. Alternatively, expression of Rev proteins may be prevented by designing ribozymes against the *rev*-coding region. Absence of Rev proteins would prevent transport of singly-spliced and unspliced viral RNAs to the cytoplasm.

E. Interference with the packaging and release of infectious HIV-1 particles

Several viral proteins including Gag, Gag-Pol, Env, and Vif are required for the assembly, release, maturation, and infectivity of virus particles. Therefore, ribozymes directed against these coding regions could interfere with the release of infectious virus particles by cleaving the mRNAs coding for Gag, Gag-Pol, Env, and Vif proteins. Alternatively, interfering RNAs may be co-expressed with $tRNA_3^{Lys}$ to enable co-packaging of ribozymes, sense or antisense RNAs within virus particles.

V. METHODS OF DELIVERY

A. Exogenous Delivery

One method of administering ribozymes to target cells is by using liposome carriers (reviewed by Simoes et al 1999). These confer protection against degradation prior to ribozyme uptake by cells or tissues, since encapsulation prevents association with serum proteins. Cationic lipid formulations favor fusion of the liposome with the plasma membrane, increasing the chances of endocytosis of the ribozymes. An advantage of exogenous delivery is the capability to utilize chemically modified ribozymes that show increased stability *in vitro*. However, the main disadvantage of exogenous delivery is the requirement for frequent infusions to maintain an effective intracellular concentration. Long-term expression of ribozymes may thus be achieved by utilizing retroviral vectors for delivery into target cells.

B. Endogenous Delivery

1. *In vivo* transduction

At present, transduction of retroviral vectors *in vivo* is limited by a number of factors. Retroviral vectors produced in certain packaging cell lines are unstable in the bloodstream

(DePolo et al 1999). Also, targeted retroviral vectors have not yet been developed (reviewed by Buchholz et al 1999). Thus, introduction of a retroviral vector within a patient may result in inadvertent transduction of non-target cells. Currently, targeted retroviral vectors which would deliver genes only to specific cells are being developed by altering the gene encoding the envelope glycoprotein (Buchholz et al 1998, Fielding et al 1998, Chadwick et al 1999), such that they would bind to cell-surface receptors.

2. *Ex vivo* transduction

Delivery of genes for HIV-1 gene therapy is usually performed by transduction *ex vivo*. Lymphocytes are isolated from peripheral blood collected from patients. These are then transduced with retroviral vector particles expressing the therapeutic gene. The transduced cells, selected due to the marker gene present in the retroviral vector, are amplified and transplanted back to the individual (Fig. 4). This delivery method has been used to introduce genes into CD4+ lymphocytes and macrophages.

VI. RETROVIRAL VECTORS TO DELIVER ANTI-HIV-1 GENES TO TARGET CELLS

A. Delivery vector

There are several viral vectors in current use for gene therapy. However, the most popular vector system is the retroviral vector. The majority of RAC (Recombinant DNA Advisory Committee) approved protocols employ retroviral vectors (72%) to deliver the selected gene to the target cells (Orkin and Motulsky 1995). Infection by retroviral vectors leads to high efficiency transduction of dividing mammalian cells through stable integration of proviral DNA into the

FIG. 4. **Overview of HIV-1 gene therapy.** Bone marrow stem cells or peripheral blood lymphocytes (PBLs) from patients are collected, and transduced using retroviral vector particles. By including a selectable marker gene in the retroviral vector, cells which contain the therapeutic gene can be selected. These are then amplified as appropriate and transplanted back to the patient. Upon differentiation, the progeny cells will hopefully contain and express the anti-HIV-1 gene. With the help of support therapies, it is anticipated that patients will eventually reach a stable asymptomatic state.

ANTI-HIV-1 GENE THERAPY

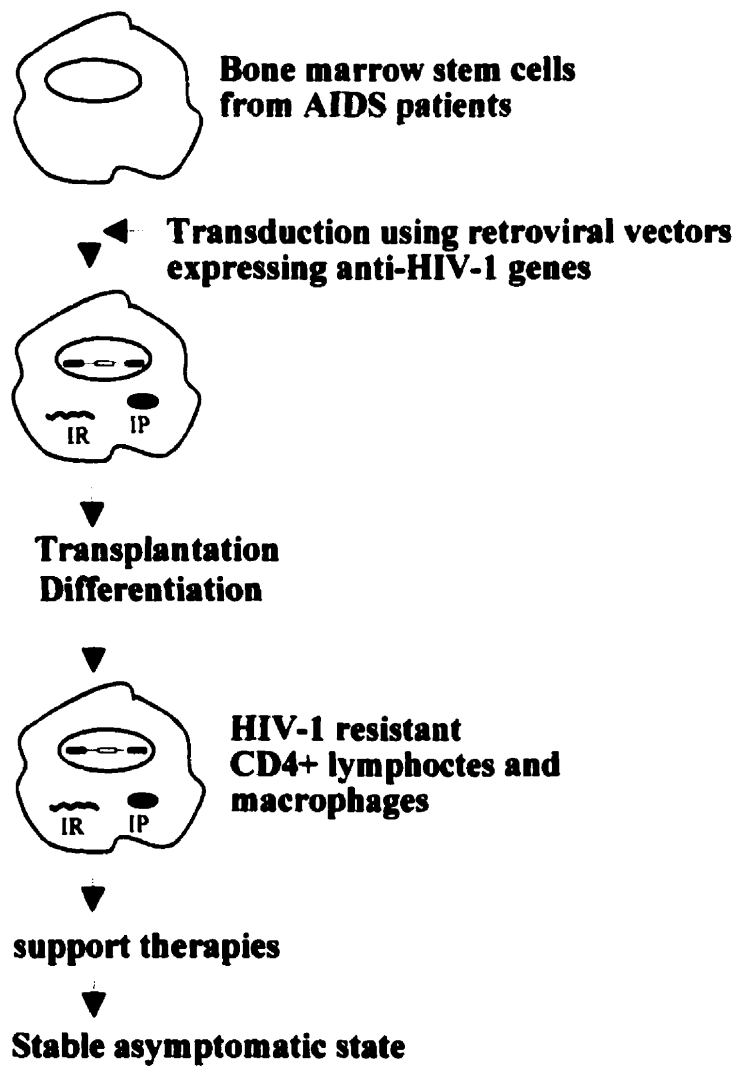


Figure 4.

cellular genome. Retroviral vectors are therefore ideal for gene transfer into mammalian cells in culture.

Retroviral vectors are produced by replacing the viral genes required for replication with the desired genes to be transferred. The proviral DNA sequences of the retrovirus which are required *in cis* for viral gene expression include the 5' and 3' LTRs (for viral RNA synthesis and proviral DNA integration), the Ψ signal (to allow encapsidation of the viral RNA into virions), the PBS and polypurine tract (PPT) which are both utilized during reverse transcription. The *gag*, *pol* and *env* structural genes of the retrovirus can be deleted and supplied *in trans* by a helper virus which has been transfected in a packaging cell line (Fig. 5). A therapeutic gene and a gene encoding a selectable marker may be cloned in place of the structural genes. Retroviral vectors such as those based on Moloney murine leukemia virus (MMLV) can take inserts up to 8 kb.

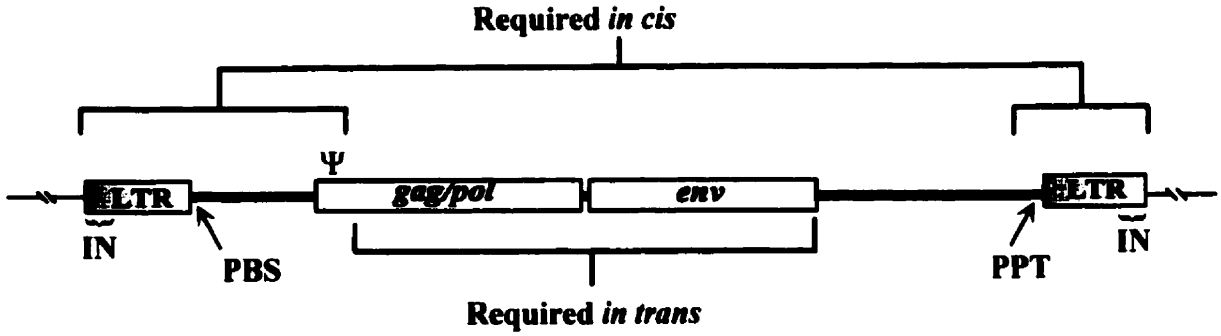
The retroviral vector, pUCMoTiN, used in this study contains a *tk* promoter driving neomycin phosphotransferase (*neo*) gene expression. The HIV-1 Tat mRNA leader sequence, which includes the TAR element, was cloned in place of the 5' leader sequence of the *neo* gene. This modification was shown to allow both constitutive and Tat-inducible expression of interfering RNAs. Various interfering RNAs are cloned in this vector as part of the 3' untranslated region of the *neo* gene and are therefore expressed as part of the retroviral vector RNA expressed from the 5' LTR, and the *neo* mRNA expressed from the *tk* promoter.

B. Packaging cells

For the MMLV-based vectors, the packaging cell line determines if the vector is ecotropic or amphotropic. Upon transfection of packaging cells with retroviral vectors containing the therapeutic gene, vector particles are produced which possess the ability to infect and integrate

FIG. 5. **Structure of MMLV-based retroviral vector.** *A*. The proviral sequences which are required *in cis* for MMLV gene expression, packaging, reverse transcription, and integration include the 5' and 3' LTRs, Ψ signal, PBS/PPT, and attachment sites, respectively. *B*. The *gag*, *pol* and *env* genes which code for structural proteins are deleted in the retroviral vector and replaced with an interfering RNA (IR) or interfering protein (IP), and a gene encoding a selectable marker (*neo*). P, internal promoter.

A. Moloney Murine Leukemia Virus



B. MoMuLV-based Retroviral Vector

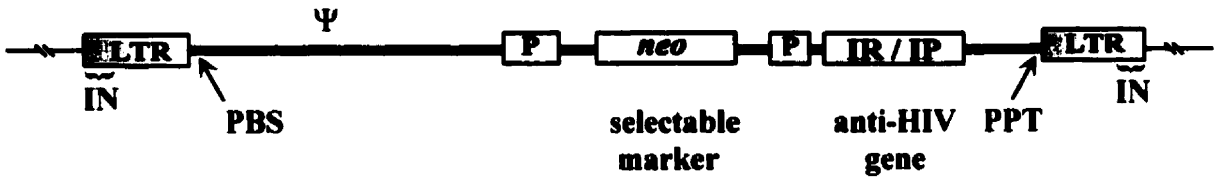


Figure 5.

into the host cell genome. But because the *gag*, *pol* and *env* genes had been deleted, the vector particles are only capable of one round of infection and are therefore replication-defective.

The retroviral vector constructs are used to transfect an ecotropic packaging cell line, such as ψ-2, which release retroviral vector particles that are then used to infect an amphotropic packaging cell line such as PA317. Gag, Pol, and Env, required for viral assembly, infection, replication, and integration, are produced in these cell lines from helper plasmids expressing an RNA that lacks the MMLV ψ signal. The amphotropic vector particles released from PA317 cells are used to infect human $CD4^+$ lymphoid cell lines, and PBLs.

C. RNA pol II-driven expression cassettes

Sites that have been targeted for ribozyme cleavage within the HIV-1 RNA include the 5' leader sequence (Weerasinghe et al 1991, Heidenreich and Eckstein 1992, Dropulic et al 1992), and the *gag* (Sarver et al 1990), *pro*, *RT*, *IN* (Sioud and Drlica 1991), *vif* (Lorentzen et al 1991), *tat* (Jackson et al 1998), *tat/rev* (Bauer et al 1997), *rev* (Michienzi et al 1996, Michienzi et al 1998), *env* (Chen et al 1992, Paik et al 1997), and *nef* (Larsson et al 1996)-coding regions. Ribozymes have also been developed against the mRNAs coding for CCR5 coreceptors (Gonzalez et al 1998, Goila et al 1998) to block virus entry in cells.

Successful cleavage *in vivo* was first demonstrated by Sarver et al (1990), who showed that ribozymes could protect cells from HIV-1 infection. CD4+ HeLa cells were constructed which stably expressed a ribozyme targeted against the *gag*-coding region. Upon challenge with HIV-1, these cells showed a marked reduction of HIV-1 replication. When RT-PCR analysis was performed on total RNA isolated from transduced cells expressing the ribozyme, significantly less uncleaved RNA relative to cleaved RNA was observed. The cells also contained up to 100 times less HIV-1 proviral DNA sequence than the infected, untransduced control.

Ribozyme cleavage specificity was tested using a ribozyme designed against an RNA containing the HIV-1 *gag*-coding region (Rossi et al 1990). Increasing amounts of total RNA extracted from uninfected or HIV-infected cells were added to a *trans* cleavage reaction containing the target RNA. Total cellular RNA from uninfected cells had no effect on the cleavage reaction, whereas inclusion of RNA from HIV-infected cells resulted in inhibited cleavage of the labeled *gag* substrate. When the amount of ribozyme was increased, substrate competition for ribozyme-mediated cleavage was overcome. Thus, ribozyme cleavage of the synthetic *gag* RNA was sequence-specific and HIV-1 RNA produced in infected cells was cleaved by the anti-*gag* ribozyme.

Other studies using ribozymes have confirmed the specificity of ribozyme cleavage of its target RNA (Sioud and Drlica 1991), and the reduction of target RNA levels in cells (Dropulic et al 1992). Furthermore, ribozymes were shown to result in greater inhibition of virus replication compared to antisense RNAs (Homann et al 1993). Incorporation of the hammerhead catalytic domain into an antisense RNA against the 5' leader/*gag* region resulted in 95% inhibition of virus replication compared to the antisense RNA (Homann et al 1993). No inhibition was observed in cells expressing the inactive form of the ribozyme.

A ribozyme against the 5' leader sequence of HIV-1 (Weerasinghe et al 1991) expressed under control of an internal simian virus (SV) 40 or cytomegalovirus (CMV) promoter delayed virus production up to 14 days in MT4 cells compared to the parental vector. Tat-inducible expression of the ribozyme by the HSV *tk*-HIV TAR fusion promoter inhibited virus production for up to 22 days post infection. Upon comparison of five hammerhead ribozymes targeted against highly conserved sequences within the *gag*, *pro*, *RT*, *tat*, and *env*-coding regions of HIV-1 RNA, the ribozymes targeted against the *pro* and *env*-coding regions were found to completely inhibit virus production in stably transduced cells for 12 and 15 days, respectively (Ramezani and Joshi 1996).

Virus replication was almost completely inhibited in cells expressing a multimeric ribozyme targeted against nine highly conserved sites within the *env*-coding region. No viral RNA or protein could be detected in the cells and in their culture supernatants for up to 60 days after infection (Ramezani et al 1997). A hammerhead ribozyme against the *tat*-coding region inhibited HIV-1 replication more than 80% compared with the control-vector transduced cells. Transduced PBLs expressing the ribozyme possessed greater viability compared to vector-transduced cells (Wang et al 1998). Another anti-*tat* ribozyme reduced Tat activity by 85-95%. CD4+ PBLs expressing this ribozyme were able to resist HIV infection for up to 20 days (Jackson et al 1998).

D. RNA pol III-driven expression cassettes

At low interfering RNA to target RNA ratios, specific and efficient inhibition of target RNAs did not occur. This was overcome by transcribing ribozymes under control of promoters expressing tRNAs, U6 sn (small nuclear) RNA, and Ad (adenovirus) VA (virus associated) RNAs. These promoters are used by RNA pol III, which produces high amounts of small, compact RNA molecules that possess greater stability in the intracellular environment.

1. Design of RNA pol III-driven expression cassettes

a. Promoter, processing site, and terminator

Transfer RNA and Ad VA RNA genes possess intragenic promoters (Fig. 6) consisting of boxes A and B that are highly conserved (reviewed by Willis 1993, and Geiduschek and Tocchini-Valentini 1988). Box A is located 10-20 bp downstream of the transcription start site. The distance between boxes A and B is 30-60 bp. The U6 snRNA promoter (Fig. 6) consists exclusively of sequences located upstream of the U6 snRNA-coding region (reviewed by Kunkel and Pederson 1989). The basal promoter contains a TATA box at position -30 and a proximal

FIG. 6. **Cloning sites in tRNA-, Ad VA RNA-, and U6 snRNA-promoter based expression cassettes.** The black arrows correspond to the regions that have been used for cloning interfering RNAs. The small arrow (P) in the tRNA cassette corresponds to the 3' processing site. Brackets correspond to the U6 snRNA-coding region. A and B, highly conserved sequences in tRNA and Ad VA RNA intragenic promoters. PSE, proximal sequence element. TATA, TATA Box. Ter, RNA pol III terminator.

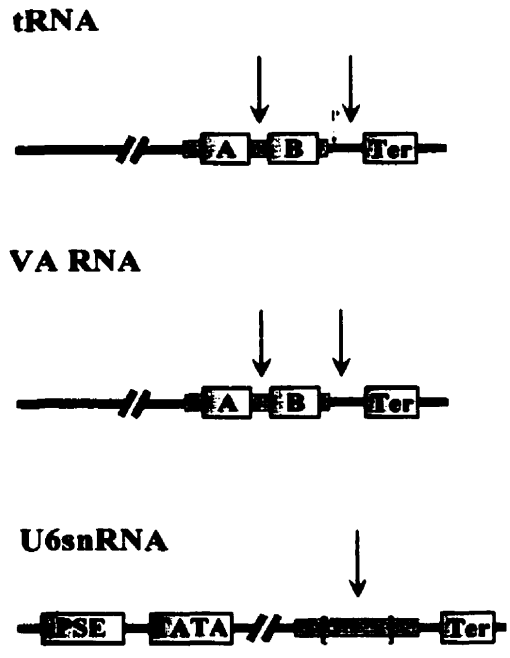


Figure 6.

sequence element (PSE) at position -60. The tRNA 3' processing site, located downstream of box B, allows the release of interfering RNA molecules; removal of this processing site has been shown to produce chimeric RNAs (Adeniyi-Jones et al 1984). Thus, tRNA promoters are used to express chimeric RNAs that may or may not be processed. Ad VA RNA promoters are used to express chimeric RNAs that are not processed (reviewed by Pruzan and Flint 1995), whereas the U6 promoter is used to express interfering RNAs with much of the U6 snRNA deleted (reviewed by Kunkel and Pederson 1989). The RNA pol III terminator consists of 4-6 T residues (reviewed by Willis 1993, and Geiduschek and Tocchini-Valentini 1988). For tRNA-, Ad VA RNA- and U6 snRNA-based expression cassettes, interfering RNA genes must be cloned before the terminator. T residues must be excluded from the sequences encoding interfering RNAs as they may lead to premature transcription termination; inclusion of two T residues upstream of the transcription terminator was shown to result in premature transcription termination during Ad VA RNA (Prislei et al 1997) and U6 snRNA (He and Huang 1997) synthesis.

b. Cloning sites within the RNA pol III-driven expression cassettes

Promoters for $tRNA_i^{Met}$, $tRNA^{Val}$, $tRNA_3^{Lys}$, $tRNA^{Pro}$, $tRNA^{Tyr}$, $tRNA^{Ala}$, Ad VA RNA, and U6 snRNA have all been used to express interfering RNAs (Table 1*A-C*). The regions in these genes that have been used for cloning various interfering RNAs are shown in Fig. 6.

For tRNA promoters, two possible cloning sites may be used. Interfering RNA genes may be cloned between boxes A and B, as the region between these boxes is not essential for promoting tRNA transcription. Alternatively, interfering RNA genes may be cloned downstream of box B, before the transcription terminator. For tRNA expression cassettes producing chimeric RNAs that are processed to yield two separate RNAs, interfering RNA genes must be cloned downstream of the 3' processing site. In the case of Ad VA RNA promoters, interfering RNA genes may be

TABLE 1A.

tRNA promoter-driven interfering RNAs used in HIV gene therapy

Promoter	*Cloning site* ●	*IR expressed* ●	*Chimera* ●	*Target site*	*Vector Plasmid* ●	*Cells*	*Intracellular Intravirion Localization* ●	*Inhibition* ●	*Ref*
$tRNA_i^{Met}$	BT	HH Rz	Yes	U5	DC FR	Jurkat	n.d.	> 28 d	Peng et al 1999
				tat	SC F	COS, CEM	Nucleus	7 d	Bertrand et al 1997
				tat, tat rev	DC F	CEM	n.d.	> 20 d	Zhou et al 1996
				leader sequence	DC F	MT2, CEM SS	n.d.	n.d.	Thompson et al 1995
				RRE	DC F	SupT1, PBMC, H9/IIIB	n.d.	> 30 d	Duan et al 1997
		S	Yes	U3,R,U5	DC F	Jurkat	Cytoplasm	> 140 d	Peng et al 1997
		Decoy	Yes	RRE	DC F	CEM SS	Cytoplasm	n.d.	Ilves et al 1996
				TAR, RRE	Plasmid	293	Nucleus	> 60 %	Good et al 1997
				TAR	DC F	CEM SS	n.d.	>24 d	Sullenger et al 1990a
				TAR	DC F	CEM SS	n.d.	17 d	Lee et al 1995
				RRE	DC F	CEM SS	n.d.	17 d	Lee et al 1992
				RRE	DC F	CEM SS	n.d.	> 40 d	Lee et al 1994
		AS	Yes	*tat, rev*	DC FR	Jurkat	n.d.	> 28 d	Peng et al 1999
				U3,R,U5,ψ	DC F	Jurkat	Cytoplasm	> 140 d	Peng et al 1997
				tat, rev	DC F, SC F	Jurkat	Cytoplasm	> 140 d	Peng et al 1996
				TAR	DC R	SupT1	n.d.	14, >28	Chuah et al 1994
				tat, rev	DC F	MT4	n.d.	6 d	Junker et al 1994
$tRNA^{Val}$	BT	HP Rz	No	U5	SC F	HeLa	n.d.	88 %	Yu et al 1993
				U5	SC R	Molt4/8	n.d.	> 40 d	Yamada et al 1994
						PBLs	n.d.	> 10 d	Leavitt et al 1994
						CD34+	n.d.	> 20 d	Li et al 1998
						CD34+	n.d.	6 d	Yu et al 1995a
						Jurkat	n.d.	> 35 d	Yamada et al 1994b
				rev env	SC R	Molt4/8	n.d.	> 36 d	Yamada et al 1994a
				U3/R	SC R	CEM/174	n.d.	> 43 d	Heusch et al 1996
						CD34+	n.d.	> 30 d	Rosenzweig et al 1997
		HP Rz-Decoy	No	U5 Rev SLII	SC R	Molt4/8 Jurkat/HIV-1	n.d	> 40 d	Yamada et al 1996
				U5 Rz-Rev SLII, *rev env* Rz- Rev SLII	TC (SC R, DC R)	Molt4/8	n.d	> 27 d	Gervaix et al 1997a
						CD34+	n.d.	> 28 d	Gervaix et al 1997b
		HH Rz	Yes	PBS	Plasmid	293	Cytoplasm, Virus	50 %	Westaway et al 1998
				U5	SC F	H9	Cytoplasm	> 11 d	Koseki et al 1999
		Decoy	n.d	TAR	Plasmid	HeLa	Cytoplasm	40 %	Koseki et al 1998
		Mz	Yes	*tat*	Plasmid	HeLa	Cytoplasm	> 90%	Kuwabara et al 1999
$tRNA_3^{Lys}$ ●	BT	HH Rz	Yes	PBS	Plasmid	293	Cytoplasm, Virus	>90 %	Westaway et al 1995, 1998
$tRNA_3^{Lys}$	BT	AS	Yes	TAR	DC R	MT2	Cytoplasm, Virus	17 d	Lu et al 1997
$tRNA^{Pro}$	AB	AS	Yes	*tat*	DC F	Jurkat	Nucleus	14 d	Biasolo et al 1996

TABLE 1B.

Ad VA RNA promoter-driven interfering RNAs used in HIV gene therapy

Promoter	*Cloning site●*	*IR expressed●*	*Chimera●*	*Target site*	*Vector Plasmid●*	*Cells*	*Intracellular Intravirion Localization●*	*Inhibition●*	*Ref*
Ad VA1	BT	HH Rz	Yes	U5	Plasmid	*X. laevis* oocytes	Cytoplasm	n.d	Prislei et al 1997
				gag	Plasmid	COS	Cytoplasm, Nucleus	n.d.	Barcellini-Couget et al 1998
				R	Plasmid	Jurkat, COS	n.d.	< 66%	Ventura et al 1993, 1994
		HP Rz	Yes	U5	SC F	HeLa	n.d.	< 88 %	Yu et al 1993
					SC R	CD34+	n.d.	> 10 d	Yu et al 1995a
				pol	SC R	Jurkat, Molt4/8	n.d.	< 35 d	Yu et al 1995b
		Decoy	Yes	RRE	SC FR, DC FR	CEM	Cytoplasm	n.d.	Ilves et al 1996
		AS	Yes	*rev*	Plasmid	CEM	n.d.	> 93 d	Cagnon et al 1995

TABLE 1C.

U6 snRNA promoter-driven interfering RNAs used in HIV gene therapy

Promoter	*IR expressed*	*Chimera*	*Target site*	*Vector Plasmid*	*Cells*	*Intracellular Intravirion Localization*	*Inhibition*	*Ref*
U6	Decoy	Yes	RRE TAR	SC FR DC FR	CEM SS	Nucleus (RRE)	n.d	Ilves et al 1996
		Yes	TAR	Plasmid	HeLa	Nucleus	60 %	Koseki et al 1998
U6+19	HH Rz	Yes	*tat*	SC F	COS, CEM	Nucleus 60-80%	7 d	Bertrand et al 1997
U6+19	Decoy	Yes	TAR-2	Plasmid	U937	n.d	> 6 d	Browning et al 1999
U6 +1 U6+19 U6+27	HH/HP Rz AS Decoy	Yes	U5 U5 TAR/RRE	Plasmid	293	Nucleus	> 75 %	Good et al 1997

●AB, between boxes A and box B; BT, between box B and RNA pol III terminator
●HH, hammerhead; HP, hairpin; Rz, ribozyme; Mz, minizyme; S, sense, AS, antisense
●Use of the entire tRNA gene is assumed to result in a processed tRNA transcript
●Retroviral vector design: SC, single copy, DC, double copy, TC, triple copy; F, forward, R, reverse
●n.d., not determined
● n.d., not determined; d, days
●Also driven by U6

cloned either between boxes A and B or in the central domain located between box B and the transcription terminator. When using the U6 snRNA promoter, only one cloning site may be used. The first 27 (Good et al 1997) and the last 18 (He and Huang 1997, He et al 1998) nts of the U6 snRNA possess a stable secondary structure and, therefore, may be included to enhance interfering RNA stability; inclusion of the rest of the U6 snRNA is not essential for gene expression.

c. Level of expression and RNA stability

Compared to RNA pol II promoters, RNA pol III promoters generally allow greater transcript accumulation through higher level expression and increased stability. A $tRNA_i^{Met}$-ribozyme reached a concentration of 250-25,000 copies per cell (Baier et al 1994), while $tRNA_i^{Met}$ and U6 snRNA promoter-driven ribozymes accumulated to ~50,000 copies per cell (Bertrand et al 1997). A $tRNA_i^{Met}$-decoy RNA represented 3-5% of total polyadenylated RNAs. (Sullenger et al 1990a). Also, a $tRNA^{Tyr}$-ribozyme was expressed in ~150-fold excess over RNA pol II-driven RNAs (Perriman et al 1995).

Expression cassettes based on the tRNA and Ad VA RNA promoters may potentially increase the stability of the chimeric RNAs due to the presence of tRNA and Ad VA RNA sequences. Transfer RNA chimeras have been shown to possess comparable stability to a wt tRNA in fetal bovine serum (Yuyama et al 1992). They were also shown to be very stable in *X. laevis* oocytes, as they could be detected in the nucleus for up to two days post-microinjection (Cotten and Birnstiel 1989). Accumulation (Zakharchuk et al 1995,Cagnon et al 1995) and half-life (Cagnon et al 1995) of a chimeric Ad VA RNA were similar to that of wt Ad VA RNAs expressed from plasmids. However, cloning immediately upstream of the Ad VA1 RNA transcription terminator was shown to produce unstable molecules with shorter half-lives (Prislei et al 1997, Barcellini-

Couget et al 1998). A U6 snRNA-chimera was also found to accumulate at the same level as U6 snRNA (He et al 1998).

In an effort to improve $tRNA_i^{Met}$-ribozyme stability, expression cassettes were developed in which nucleotides were inserted to stabilize the 3' end of the transcripts by creating a stem involving the 3' region alone or both the 3' and the 5' regions (Thompson et al 1995). When comparing the intracellular levels of transcripts produced from these expression cassettes, transcripts containing an 18-bp stem between the 5' and the 3' regions were produced at the highest levels. Further improvements on this design included a base-paired region preceding the site of insertion, a bulge to separate the ribozyme from flanking tRNA sequences, and a shorter stem between the 5' and the 3' regions (Thompson et al 1995). Transcripts produced from this construct accumulated similarly to high levels but possessed greater *in vitro* cleavage activity.

d. Intracellular and intravirion localization

1) Intracellular localization

Transfer RNAs and Ad VA1 RNAs are transcribed in the nucleus and transported to the cytoplasm, while U6 snRNA is retained in the nucleus. Chimeric RNAs based on tRNA and Ad VA RNAs should therefore localize in the cytoplasm, while U6 snRNA chimeras should remain in the nucleus. Ad VA RNA and U6 snRNA chimeras localized as expected in the cytoplasm and nucleus, respectively. However, tRNA chimeras were detected in both the nucleus and the cytoplasm. Thus, depending on the target RNA, the appropriate expression cassette may be used to direct interfering RNA localization in the nucleus or in the cytoplasm. Subcellular localization of various chimeric RNAs was demonstrated directly by Northern blot, RT-PCR, *in situ* hybridization and primer extension analyses, or indirectly based on results which would not have been possible if the interfering RNA was absent. For example, cytoplasmic localization was

inferred by the absence of a cytoplasmic target RNA. With the recent isolation and characterization of the exportin-t protein responsible for tRNA transport (Wollin and Matera 1999, Arts et al 1998a, Arts et al 1998b, Kutay et al 1998), tRNA chimeras may be developed that bind to exportin-t and allow localization in the cytoplasm.

2) Intravirion localization

By virtue of the small confines of the virion, ribozymes targeted against a viral RNA are expected to cleave their target RNA more efficiently in a virus than in the cell. This concept has been demonstrated using a MMLV-based system (Sullenger and Cech 1993). The $tRNA_3^{Lys}$ promoter is particularly attractive for expressing therapeutic RNAs against HIV infection, since $tRNA_3^{Lys}$ is packaged by the virus and serves as a primer during reverse transcription (reviewed by Mak and Kleiman 1997). As such, co-expression of an interfering RNA with this tRNA should lead to co-packaging of the chimeric RNA within viral progeny.

Human 293 cells transfected with a plasmid containing HIV-1 provirus DNA and a tRNA-ribozyme expression vector produced progeny virus that packaged the $tRNA_3^{Lys}$-ribozyme (Westaway et al 1998). The infectivity of the progeny virus was also reduced. A modified $tRNA_3^{Lys}$, in which 7 nts of the sequences complementary to the PBS were replaced with antisense sequences to the TAR region of HIV-1, was also found to be packaged by the progeny virus (Lu et al 1997).

e. Cytotoxicity

Transfer RNA chimeras may be recognized by cellular proteins such as RNase P, tRNA nucleotidyltransferase, tRNA splicing enzymes, and aminoacyl tRNA synthetases. Association of tRNA chimeras with these proteins may pose potential toxicity to the cell by interfering with

RNA transcription and processing. The toxicity of various chimeras was therefore tested. Lack of toxicity of a particular chimera could be established if expression could be confirmed and yet cell viability was not altered. *In vivo* expression by RNA pol III promoters has been demonstrated using pulse-chase (Cotten and Birnstiel 1989), RNase protection (Gebhard et al 1997,Perriman et al 1995), and Northern blot (Du et al 1998, Gebhard et al 1997, Good et al 1997, Ilves et al 1996, Sullenger et al 1990a, Kawasaki et al 1996, Kawasaki et al 1998, Westaway et al 1998, Koseki et al 1999, Kuwabara et al 1999, Zhao and Lemke 1999, Kuwabara et al 1998a) analyses. Lack of toxicity was measured by ^{3}H-thymidine uptake (Yamada et al 1994b,Rosenzweig et al 1997) and trypan blue (Yamada et al 1994a) exclusion. Cells transduced with various tRNA chimeras have not yet displayed altered viability, compared to untransduced cells.

Ad VA RNAs are known to interact with cellular proteins, such as the La (systemic lupus erythematosus-associated) protein and DAI (double-stranded RNA-activated inhibitor) protein kinase (Mathews and Shenk 1991). The central domain located between box B and the RNA pol III terminator is involved in binding to DAI kinase, while the 3' stretch of U residues is the binding site of La protein. Toxicity resulting from expression of Ad VA RNA chimeras has not been reported (Yu et al 1995b, Li et al 1998). U6 snRNA is involved in splicing of pre-mRNAs in the cell. However, the potential toxicity of U6 snRNA promoter-driven chimeras is minimal as expected, since most of the U6 snRNA is deleted while designing the expression cassettes.

2. Applications Of RNA Pol III-Driven Expression Cassettes

Most gene therapy applications of tRNA-based expression cassettes were aimed towards HIV infections and cancer. These are summarized below.

a. HIV gene therapy

Promoters for $tRNA_i^{Met}$, $tRNA_3^{Lys}$, $tRNA^{Val}$, and $tRNA^{Pro}$ have been used to express interfering RNAs against HIV-1 RNA. Ribozyme, sense RNA, or antisense RNA-coding sequences were cloned either between boxes A and B or between box B and the RNA pol III terminator. The Ad VA1 RNA promoter has been used to express ribozymes and an antisense RNA, and the U6 snRNA promoter was used to express a sense RNA. For the Ad VA RNA promoter-based expression cassettes, the interfering RNA-coding sequences were cloned between box B and the RNA pol III terminator.

Sites targeted within the HIV-1 RNA include the *gag*, *pol*, *env*, *tat*, and *rev*-coding regions as well as U3, R, U5, ψ signal, 5' splice site, and PBS. Sites targeted within the SIVmac and HIV-2 RNAs include a sequence spanning the U3/R region.

1) Transfer RNA promoter-based expression cassettes (Interfering RNAs cloned between boxes A and B)

i. Hammerhead ribozymes

The $tRNA_i^{Met}$ promoter has been used for expressing hammerhead ribozymes. Sequences encoding ribozymes were cloned between boxes A and B in the anticodon loop. Transcripts containing various $tRNA_i^{Met}$-ribozymes in tandem that were targeted against one or multiple sites within the HIV-1 RNA have been tested for HIV-1 RNA cleavage *in vitro* (Ohkawa et al 1993b, Ohkawa et al 1993a). *Cis*-acting ribozymes were also inserted upstream and downstream of each $tRNA_i^{Met}$-ribozyme. Inclusion of *cis*-acting ribozymes was shown to enhance anti-HIV ribozyme activity *in vitro*. However, the advantage of this strategy in inhibition of HIV replication has not been demonstrated.

ii. Antisense RNA

The $tRNA^{Pro}$ promoter has been used to express an antisense RNA. Sequences encoding an antisense RNA were cloned between boxes A and B of the $tRNA^{Pro}$ promoter. A 20-nt antisense RNA to the HIV-1 *tat*-coding region replaced the 7-nt anticodon loop of $tRNA^{Pro}$ and the most adjacent nt-pair in the anticodon stem (Biasolo et al 1996). Upon infection with HIV-1, stably transduced Jurkat cells expressing this RNA were protected for up to day 14, compared to cells transduced with the vector alone or with a vector containing scrambled antisense sequences. Individual clones were protected for up to day 22 post-infection (Biasolo et al 1996).

iii. Combination RNAs

Various $tRNA_i^{Met}$-ribozymes expressed in tandem (with *cis*-acting ribozymes to liberate individual $tRNA_i^{Met}$-ribozyme monomers) were further modified to co-express decoy RNAs. TAR or RRE (Rev response element) sequences were added to the *cis*-acting ribozymes, such that each *cis*-acting ribozyme could also act as a decoy RNA (Yuyama et al 1994). The *cis*-acting ribozyme-decoy RNAs were shown to interact with HIV-1 Tat or Rev proteins *in vitro*.

2) Transfer RNA promoter-based expression cassettes (Interfering RNAs cloned between box B and RNA pol III terminator)

i. Hammerhead ribozymes

Promoters for $tRNA_3^{Lys}$, $tRNA_i^{Met}$ and $tRNA^{Val}$ have been used to express anti-HIV hammerhead ribozymes downstream of the tRNA. The $tRNA_3^{Lys}$ promoter has been used to express a ribozyme against the HIV-1 PBS. The $tRNA_i^{Met}$ promoter was used to express a ribozyme dimer targeting the HIV *tat*-coding region and a common site within the *tat* and *rev*-

coding regions. The $tRNA^{Val}$ promoter was used to drive expression of ribozymes against the HIV-1 U5 region and the *tat*-coding region.

Packageable ribozymes for HIV gene therapy were developed using the $tRNA_3^{Lys}$ promoter. When a plasmid expressing the $tRNA_3^{Lys}$-ribozyme targeted against the PBS within the HIV-1 RNA and pNL4-3 containing an infectious HIV-1 clone were co-transfected in 293 cells, progeny virus produced at day 6 post-infection was shown to possess a 6-fold reduced infectivity, compared to that produced from control cells expressing the U6 vector alone (Westaway et al 1998). The infectivity of the progeny virus was determined by an infectivity assay using supernatants from the transfected cells. Virus replication was determined by measuring the amount of HIV-1 p24 antigen produced by the infected cells. At day 7, the progeny virus was 200-fold and 25-100 fold less infectious than that released from cells transfected with plasmids expressing $tRNA^{Val}$ alone or a $tRNA^{Val}$-ribozyme, respectively. Cells expressing an inactive $tRNA_3^{Lys}$-ribozyme also produced progeny virus with reduced infectivity, suggesting that the $tRNA_3^{Lys}$-ribozyme may also act as an antisense RNA through binding to the PBS.

A ribozyme dimer targeted against the HIV-1 *tat*-coding region and a site common to the *tat* and *rev*-coding regions was expressed either under the control of the MMLV LTR promoter upstream of the *neo* gene, the human CMV promoter downstream of the *neo* gene, or the human $tRNA_i^{Met}$ promoter in the 3' LTR of the retroviral vector (Zhou et al 1996). Ribozymes under control of the MMLV promoter were produced at the highest levels. However, compared to an inactive ribozyme control, transduced CEM cells expressing the different constructs displayed the same level of resistance to HIV-1 for up to 20 days, implying that level of expression is not the absolute determinant of protection.

Three constructs were designed to allow $tRNA^{Val}$ promoter-driven expression of ribozymes targeted against the HIV-1 U5 region, which varied only in the sequences connecting the tRNA

to the ribozyme. It was predicted that Rz3, which possessed flanking sequences that could assume a loop structure and remained unbound to other sequences, would demonstrate the greatest activity. However, although *in vitro* cleavage activity confirmed this prediction, the half-life and intracellular activity of Rz3 in transiently transfected HeLa cells were less than that of Rz2 which had only one flanking arm available for hybridization to the target RNA. Since Rz2 and Rz3 were expressed from the same promoter, the increased effectivity of Rz2 must have been due to its increased stability. This was confirmed by half-life measurements in stable transformants and steady-state levels in transduced H9 cells (Koseki et al 1999). Rz2 inhibited HIV LTR-driven luciferase activity in transiently (>60%) or stably (97%) transfected HeLa cells (Koseki et al 1999). Rz2 also inhibited HIV-1 replication in H9 cells up to 99% on day 11 post-infection.

Minizymes are hammerhead ribozymes which possess a short oligonucleotide linker in place of the hammerhead stem loop region. Dimeric minizymes are composed of two minizyme monomers which combine to form a secondary structure similar to a conventional hammerhead ribozyme (Kuwabara et al 1998a). The $tRNA^{Val}$ promoter-driven dimeric minizymes have been designed to target two sites in the HIV-1 *tat*-coding region. Luciferase activity in LTR-Luc HeLa cells was inhibited by >90% when each minizyme was expressed from a single plasmid or two separate plasmids. Inhibition was not observed from each separate minizyme or from the inactive minizyme control. The rate of *tat* mRNA depletion in the cytoplasm of cells expressing the dimeric minizymes was shown to be faster than in those expressing the conventional ribozyme (Kuwabara et al 1999).

ii. Hairpin ribozymes

The $tRNA^{Val}$ promoter has mostly been used to develop hairpin ribozymes against various sites within the HIV-1, HIV-2 and SIVmac RNAs. These sites were located within the U5 region, the *rev* and *env*-coding region, and U3/R region.

The $tRNA^{Val}$ promoter-driven hairpin ribozyme against the HIV-1 U5 leader sequence inhibited HIV-1 HXB2 and HIV-1 MN replication for up to 35 days. Proviral DNA synthesis in cells transduced with the ribozyme-expressing vector was decreased 50-100-fold compared to cells transduced with the vector alone. Only HIV-1 HXB-2 replication was inhibited and not of HIV-2 KR which does not possess the ribozyme target site (Yamada et al 1994b). This $tRNA^{Val}$-U5 hairpin ribozyme protected human PBLs against challenge with a clinical isolate of HIV-1 for 10 to 25 days (Leavitt et al 1994). This ribozyme was also expressed in placental and umbilical cord blood CD34+ cells collected from newborns of HIV-seropositive mothers. Differentiated macrophage-like progeny cells were tested and found not to harbour HIV-1. Upon challenge of macrophage-like progeny cells with HIV-1 Bal (a macrophage-tropic strain), protection was observed for up to 20 days. Long-term protection (up to 35 days) was obtained when the HIV isolated from the mother was used. However, virus production in these experiments was low, with control reaching p24 antigen values only as high as 220 pg/ml, while cells expressing the $tRNA^{Val}$-ribozyme produced up to 170 pg/ml p24 antigen (Li et al 1998).

Molt4/8 cells expressing the $tRNA^{Val}$-ribozyme against an HIV-1 sequence overlapping the *rev* and *env*-coding region suppressed p24 antigen production until day 36 (0.01 m.o.i.), compared to cells expressing the inactive ribozyme control. Replication of HIV-1 SF2, which possessed a natural mutation in the ribozyme cleavage site, was not inhibited (Yamada et al 1994a).

Transduced CEM/174 cells expressing the $tRNA^{Val}$-hairpin ribozyme targeted against a sequence spanning the U3/R region of SIVmac and HIV-2 RNAs were protected against infection by SIVmac239 (an SIV strain that replicates in T cells) or HIV-2 (strains NIHZ and KR) for up to 40 days post-infection, compared to untransduced cells or cells expressing the HIV-1 U5 ribozyme (Heusch et al 1996). Rhesus CD34+ cells expressing this ribozyme gave rise to CD4+ T cells which were resistant to infection by SIVmac239 for up to 30 days post-infection and to macrophage-like cells which were resistant to SIVmac316 (an SIV strain which replicates in macrophages) for up to 20 days post-infection, compared to cells expressing the vector alone or the HIV-1 U5 ribozyme (Rosenzweig et al 1997).

iii. Sense and antisense RNAs

The $tRNA_i^{Met}$ promoter has been used to express RRE and TAR RNA decoys as well as antisense RNAs to the HIV-1 TAR region and the *tat* and *rev*-coding regions. This promoter was also used to express either sense or antisense RNAs to the HIV-1 U3, R, U5, and ψ signal. The $tRNA_3^{Lys}$ promoter was used to express an antisense RNA to HIV-1 TAR. The following results were obtained.

A $tRNA_i^{Met}$ promoter-driven 45-nt RRE decoy reduced HIV-1 replication by 90% for up to 17 days post-infection (Lee et al 1992). A 13-nt minimal RRE decoy was then developed (Lee et al 1994), which lacked binding sites for cellular factors. This minimal RRE decoy was stabilized by the addition of a stem loop at its 3' end and was expressed using a $tRNA_i^{Met}$ promoter with a functional 3' processing signal. This stabilized and processed minimal decoy inhibited HIV-1 replication for up to 40 days post-infection. Similarly, a 60-nt TAR expressed from the $tRNA_i^{Met}$ promoter protected cells against HIV-1 ARV2 and SIVmac251 for up to 20 days post-infection (Sullenger et al 1990a). Addition of hairpin sequences upstream and downstream of the TAR

decoy led to a 10- to 15-fold increase in transcript accumulation. Use of a $tRNA_i^{Met}$ promoter with processing sites to express this TAR decoy did not lead to inhibition in CEM cells for up to 10 days post-infection. However, the maximum amount of virus produced by these cells at day 17 was less than the amount of virus produced by control cells at day 24 (Lee et al 1995).

Expression of a $tRNA_i^{Met}$-antisense TAR RNA inhibited up to 70% of HIV-1 LTR-CAT (chloramphenicol acetyltransferase) gene expression in HeLa cells transfected with HIV-1 LTR-Tat construct (Chuah et al 1994). HIV-1 MN replication in transduced Sup T1 cells expressing $tRNA_i^{Met}$-antisense TAR RNA was inhibited 97-100% for up to 28 days (m.o.i., 0.00002) or 14 days (m.o.i., 0.0002). Co-expression in CEM cells of $tRNA_i^{Met}$-*tat* antisense and $tRNA_i^{Met}$-*rev* antisense RNAs resulted in >75% inhibition of HIV-1 replication on day 6 post-infection. These antisense RNAs were found to be more effective than the corresponding sense RNAs (Junker et al 1994).

In another study, antisense RNAs to HIV-1 *tat* and *rev*-coding regions were expressed under the control of various promoters. Antisense RNAs expressed under control of the MMLV LTR promoter accumulated at the highest levels compared to expression by the HIV-1 LTR promoter or by the $tRNA_i^{Met}$ promoter inserted either before the *neo* gene or within the U3 region in the 3' LTR (Peng et al 1996). There was little or no expression observed from the expression cassettes inserted in the 3' LTR. However, stably transduced cells expressing the $tRNA_i^{Met}$-antisense RNA from the 3' LTR inhibited HIV-1 replication the best and were protected for up to 20 weeks. Thus, the observed inhibition may have been due to transcripts expressed from the LTR promoter present upstream of the $tRNA_i^{Met}$ promoter, or from other mechanisms aside from level of expression, such as colocalization of the $tRNA_i^{Met}$-antisense RNA with its target RNA or increased stability of the $tRNA_i^{Met}$-antisense RNA.

The $tRNA_i^{Met}$ promoter was used to express sense RNAs containing HIV-1 R-U5, U3-R-U5, or mutant U3-R-U5 sequences and antisense RNAs against HIV-1 U3-R-U5 or U3-R-U5-ψ signal. Upon challenge with HIV-1 NL4-3, Jurkat cells expressing both antisense RNAs and the U3-R-U5 sense RNA did not produce any progeny virus as judged by p24 enzyme linked immunosorbent assay (ELISA, for 20 weeks), immunofluorescence (for 9 weeks), or RT-PCR (for 14 weeks) (Peng et al 1997). Both types of RNAs were designed to interfere with reverse transcription; however, this was not demonstrated experimentally.

In another study, the gene encoding $tRNA_3^{Lys}$ was mutated such that the 3' region of $tRNA_3^{Lys}$, which normally binds to the HIV-1 PBS, would instead bind to the TAR region. The mutant tRNA (tRNAtarD) should lead to the formation of defective, incomplete provirus DNA by false priming during reverse transcription (Lu et al 1997). The tRNAtarD was shown to compete with $tRNA_3^{Lys}$ for binding to reverse transcriptase and to prime reverse transcription at the TAR region. Stably transduced MT2 cells expressing tRNAtarD delayed HIV-1 replication for up to 8 days, compared to cells expressing vector sequences alone. It also inhibited Tat-mediated *trans*-activation of the *CAT* gene, suggesting that it can inhibit translation of TAR-containing mRNAs.

iv. Combination RNAs

A $tRNA_i^{Met}$-hammerhead ribozyme targeted against the U5 region was co-expressed with either *tat* or *rev* antisense RNAs (Peng et al 1999). The combined interfering RNAs were more potent at inhibiting virus replication when each RNA was expressed using a separate promoter (Peng et al 1999). Compared to cells expressing the chimeric $tRNA_i^{Met}$-U5 ribozyme-*tat* antisense RNA, transduced Jurkat cells which co-expressed a $tRNA_i^{Met}$-U5 ribozyme and a $tRNA_i^{Met}$-*tat* antisense RNA were protected 21 (m.o.i., 0.15)- to 32 (m.o.i., 0.05)-fold better. Similarly, cells expressing a $tRNA_i^{Met}$-U5 ribozyme and a $tRNA_i^{Met}$-*rev* antisense RNA were 4

(m.o.i., 0.15) to 7 (m.o.i., 0.05) fold better protected than those expressing a chimeric $tRNA_i^{Met}$-U5 ribozyme-*rev* antisense RNA. However, each interfering RNA (*tat* antisense RNA, *rev* antisense RNA, and U5 ribozyme) expressed individually under control of the $tRNA_i^{Met}$ promoter conferred levels of inhibition similar or greater to that of co-expressed U5 ribozyme-*tat* antisense RNA, or U5 ribozyme-*rev* antisense RNA. Presumably, the co-expressed interfering RNAs assumed a structure that obstructed the activity of each interfering RNA. Thus, interfering RNA genes, which are to be co-expressed together, must be carefully screened in order to prevent this occurrence.

The $tRNA^{Val}$ promoter-based expression cassettes expressing a hairpin ribozyme targeted against the HIV-1 U5 region and a site overlapping the HIV-1 *rev* and *env*-coding regions were modified to include a minimal RRE (SLII). A triple copy vector has also been developed which contained the $tRNA^{Val}$- U5 ribozyme-SLII expression cassette cloned in the 3' LTR and the other one between the two LTRs.

The $tRNA^{Val}$-U5 ribozyme-SLII expression cassette inhibited virus replication better than those expressing the ribozyme alone, the inactivated ribozyme plus SLII, or the inactive ribozyme alone (Yamada et al 1996). In cocultivation experiments, virus production from $tRNA^{Val}$-U5 ribozyme-SLII RNA-expressing cells remained low for 40 days post-infection, whereas virus production in control cells began on days 25-30. After 7h infection, the amount of provirus DNA in cells transduced with the $tRNA^{Val}$-U5 ribozyme-SLII expression cassette was 1/7 and 1/3 of the provirus DNA in cells transduced with expression cassettes producing the inactive ribozyme with SLII or the ribozyme alone, respectively (Yamada et al 1996). Thus, the $tRNA^{Val}$-U5 ribozyme-SLII RNA seems to interfere with HIV replication upon virus entry prior to integration.

The triple copy vector expressing both $tRNA^{Val}$-U5 ribozyme-SLII RNA and $tRNA^{Val}$-*rev/env* ribozyme-SLII RNA produced a higher amount of RNA in Molt4/8 cells, compared to a single copy vector expressing the $tRNA^{Val}$-U5 ribozyme or a double copy vector expressing the $tRNA^{Val}$-U5 ribozyme-SLII RNA (Gervaix et al 1997a). Also, this vector conferred resistance to Molt4/8 cells against challenge with five HIV-1 clades (A, B, C, D and E) for up to 27 days post-infection (Gervaix et al 1997a). The activity of this triple copy vector was then demonstrated in monocyte/macrophage-like cells derived from transduced CD34+ cells of 5 HIV-1 infected individuals (Gervaix et al 1997b). Infection with HIV-1 Bal strain resulted in decreased p24 antigen production for up to 28 days, compared to control cells expressing the vector alone. Unchallenged cells did not produce p24 antigen, thus ruling out any endogenous HIV production by the cells (Gervaix et al 1997b).

In addition to co-expressing various interfering RNAs, an interfering RNA may also be co-expressed with an mRNA encoding a protein. A vector was designed to co-express a $tRNA_i^{Met}$-ribozyme targeted against HIV-1 RRE and an mRNA coding for a single chain variable fragment against HIV-1 Rev protein (Duan et al 1997). This vector was able to protect SupT1 cells and peripheral blood mononuclear cells (PBMCs) against challenge with HIV-1 NL4-3 and a primary HIV-1 isolate, respectively. A 0.5 to 1.0 log decrease in virus replication was observed, compared to SupT1 and PBMCs expressing the single chain variable fragment alone. The combination vector was also tested for inhibition of virus replication in H9 cells chronically infected with HIV-1 IIIB. Virus production was inhibited by 75-84%, compared to 40-50% inhibition by a vector expressing a *trans* dominant mutant Rev (M10) and 18-27% inhibition by a vector expressing the $tRNA_i^{Met}$-ribozyme alone (Duan et al 1997).

3) Ad VA RNA promoter-based expression cassettes

i. Hammerhead ribozymes

The Ad VA1 RNA promoter has been used to express hammerhead ribozymes against the HIV-1 R region and against two sites within the HIV-1 5' leader sequence. Both ribozymes were cloned between box B and the RNA pol III terminator.

An Ad VA1 RNA promoter-driven expression cassette was designed to produce a 242-nt RNA containing a hammerhead ribozyme targeted against the HIV-1 R region (Ventura et al 1993). This RNA failed to cleave HIV-1 RNA *in vitro*. This expression cassette was then modified to include the ribozyme target site upstream of the ribozyme. *Cis* cleavage of the primary transcript produced from this expression cassette resulted in a shorter, 92-nt RNA, which displayed ribozyme activity *in vitro* (Ventura et al 1993). However, both types of ribozymes suppressed CAT activity in Jurkat, COS, and JP220 cells expressing pLTR-CAT and pLTR-Tat (Ventura et al 1994).

In another study, Ad VA1 RNA promoter-driven expression cassettes were designed to express monomeric hammerhead ribozymes targeted against the HIV-1 5' leader sequence (Prislei et al 1997). Sequences encoding ribozymes were cloned either by replacing part or most of the central domain region of Ad VA1 RNA as this region does not influence transcription. The ribozymes were active against the HIV target RNA *in vitro*. Vectors expressing these expression cassettes were then tested in *X. laevis* oocytes. Ribozymes produced in oocytes microinjected with these vectors could be immunoprecipitated with La antibodies, suggesting that they maintained the overall organization of the VA1 ribonucleoprotein particle. Oocyte extracts containing proteins and RNAs were also used for *trans* cleavage reactions *in vitro* using a labeled HIV RNA substrate. The activities of the ribozymes present in the extracts were higher than the ones obtained from *in vitro* transcribed ribozymes, indicating that the extracts contained certain

proteins that enhance ribozyme catalytic activity (Prislei et al 1997). Sequences encoding a ribozyme targeted against the HIV-1 5' leader sequence were also cloned at the 3' end of the central domain, just before the terminator sequence. A stem-loop structure was added at the 3' end of the ribozyme to stabilize the transcript. However, the transcript was shown to be trimmed. This RNA did not associate with the La protein and possessed a short half-life in the nucleus and in the cytoplasm (Prislei et al 1997). Thus, for efficient expression, cloning within the Ad VA1 central domain should be preferred.

ii. Hairpin ribozymes

The Ad VA1 RNA promoter was used to express ribozymes against the HIV-1 *pol*-coding region and the U5 region. The $tRNA^{Val}$ promoter was also used to direct expression of the ribozyme targeted against the U5 region.

Stably transduced Jurkat and Molt4/8 cells expressing an Ad VA1 RNA promoter-driven hairpin ribozyme against the HIV-1 *pol*-coding region were protected upon challenge with HIV-1 HXB2 (m.o.i., 0.01) for up to 12 (Jurkat cells) and 16 (Molt4/8 cells) days. The ribozyme also inhibited HIV-1 MN (m.o.i., 0.001) replication in Jurkat cells for up to 5 weeks post-infection (Yu et al 1995b).

Compared to the RNA pol II-driven β-actin promoter, expression of a ribozyme against the 5' HIV-1 U5 leader sequence from $tRNA^{Val}$ promoter or Ad VA1 RNA promoter yielded greater inhibition of HIV replication (Yu et al 1993). Upon transduction of CD34+ cells derived from human fetal cord blood, progeny macrophage-like cells expressing the $tRNA^{Val}$-ribozyme or Ad VA1-ribozyme were protected against challenge with HIV-1 Bal strain (Yu et al 1995a).

iii. Antisense RNA

A 28-nt antisense RNA to *rev*, expressed as part of the central domain of Ad VA1 RNA (between box B and the RNA pol III terminator) represented up to 3% of cellular mRNA levels in CEM cells. HIV-1 replication in cells expressing this antisense RNA was inhibited for the 3-month period tested (Cagnon et al 1995).

4) U6 snRNA promoter-based expression cassettes

Sense RNA

A vector allowing the U6+19 promoter-driven expression of HIV-2 TAR RNA decoy diminished *trans*-activation of *CAT* gene expression from pHIV-2-LTR-CAT in Jurkat cells when cotransfected with a vector expressing HIV-1 or HIV-2 *tat* gene (Browning et al 1999). When cotransfected with HIV-1 pNL4-3 in 293 cells, the TAR RNA led to decreased virus production in a dose dependent manner. Similar results were obtained in U937 cells upon co-transfection with pHXB3 (Browning et al 1999).

b. Cancer gene therapy

Promoters for $tRNA_i^{Met}$, $tRNA^{Val}$, Ad VA RNA, and U6 snRNA have also been used to express ribozymes, sense RNA and antisense RNAs for cancer gene therapy. Sites that have been targeted include mRNAs coding for *lck* and *fyn* (Baier et al 1994), *b2a2* (Shore et al 1993, Kuwabara et al 1997a, Kuwabara et al 1998b), *c-erbB-2*, (Wiechen et al 1998), IGF-II (insulin-like growth factor) (Xu et al 1999), and EGFR (epidermal growth factor receptor) (He et al 1998). The *E7* gene of human papillomavirus (HPV type 16) has also been targeted as infection by this virus is associated with cervical carcinoma (He and Huang 1997).

VII. CLINICAL TRIALS OF INTERFERING RNAs AGAINST HIV-1

Several interfering RNAs and interfering proteins have advanced to phase I and II clinical trials. Phase I clinical trials address the safety and feasibility of an anti-HIV-1 ribozyme gene therapeutic approach, while phase II trials address whether ribozymes can impact on AIDS disease course (reviewed by James 1998). The first published report of a gene therapy study involved insertion of an altered *rev* gene that produces a TDM, Rev M10, into CD4+ T cells of HIV-infected patients. This TDM suppresses HIV replication by competing with the function of normal Rev protein. The cells expressing Rev M10 demonstrated a 4- to 5-fold selective survival advantage. Retroviral-mediated transfer of the Rev M10 gene into CD34+ peripheral blood stem cells from HIV-1 infected adults has been performed. However, no transduced cells could be detected in the circulation of patients after infusion (Bonyhadi et al 1997).

Therapeutic effect resulting from gene transfer into human hematopoietic stem cells, which are the ideal targets of HIV-1 gene therapy, have not yet been achieved. Delivery systems, as well as improved interfering RNAs and interfering proteins, therefore need to be further developed. The only tRNA-ribozyme currently in clinical trial is a $tRNA^{Val}$ hairpin ribozyme (see below). However, hammerhead ribozymes are more suitable for gene delivery since, compared to the hairpin ribozyme catalytic domain (50 nts), the hammerhead catalytic domain is smaller (22 nts), is better characterized, and imposes fewer restraints on the target site (reviewed by Hampel 1998, and Tanner 1999).

A. Ribozymes

1. Transfer of an anti-U5 ribozyme gene into CD4+ peripheral blood mononuclear cells of HIV-1 positive adults

A phase I clinical trial of a $tRNA^{Val}$-U5 hairpin ribozyme has been implemented wherein CD8-depleted PBMCs from six asymptomatic HIV-1 seropositive individuals were transduced

with a murine retroviral vector lacking or expressing a $tRNA^{Val}$-U5 hairpin ribozyme (Wong-Staal et al 1998). Vector-transduced cells could be detected by PCR in all subjects up to 24 weeks, while ribozyme-transduced cells were detected in only one patient. This clinical trial is currently in progress.

2. Transfer of an anti-*tat* ribozyme gene into CD4+ cells of HIV-1 discordant twins

A phase I clinical trial was set up involving 4-6 pairs of identical twins discordant for infection with HIV-1 (Cooper et al 1999, reviewed by Macpherson et al 1999). CD4+ PBLs from the uninfected twin were transduced with a murine retroviral vector either lacking or expressing a ribozyme targeted against the HIV-1 *tat*-coding region. Genetically marked cells will be detected by PCRs. Results have not yet been reported.

3. Transfer of anti-*tat* and anti-*rev* ribozyme genes into CD34+ peripheral blood stem cells of HIV-1 positive adults

CD34-enriched cells from the peripheral blood of five subjects were transduced with a murine retroviral vector lacking or expressing ribozyme sequences against the HIV-1 *tat* and *rev*-coding regions (Bauer et al 1997, reviewed by Engel and Kohn 1999). Cells from peripheral blood samples of two patients contained vector sequences in PBMCs and/or bone marrow at 6 months, but with a low frequency (< 1/100,000). Transduced cells could not be detected at 9 or 12 months post-transfusion (Zaia et al 1998). Two patients were negative for vector sequences, while one was positive only at 4 months. Thus, transduction and engraftment of long-lived stem cells did not take place.

In another phase I study, mobilized CD34+ stem cells from seven HIV-1-positive individuals were transduced with either the control murine retroviral vector or a ribozyme vector targeted

against the HIV-1 *tat*-coding region (Amado et al 1999, reviewed by Macpherson et al 1999). In four patients, transduced cells were detected in bone marrow and peripheral blood granulocytes at four weeks, and in peripheral blood T cells and monocytes at twelve weeks (Amado et al 1998). The study is still underway to determine whether transduced cells expressing ribozymes possess a selective survival advantage.

4. Transfer of anti-*tat* and anti-*rev* ribozyme genes into CD34+ peripheral blood stem cells of adults with HIV-1 and lymphoma

In this phase II study, peripheral blood stem cells from five HIV+ patients undergoing chemotherapy and stem cell transplantation for non-Hodgkin's lymphoma were transduced with a murine retroviral vector lacking or expressing ribozymes against the HIV-1 *tat* and *rev*-coding regions (Zaia FDA STUDY 2, reviewed by Engel and Kohn 1999). Initial results showed detectable levels of vector-derived transcripts in PBMCs and granulocytes. Enduring engraftment of transduced CD34+ cells and production of ribozyme-expressing progeny cells remain to be determined.

B. Antisense RNA

Transfer of antisense TAR and TDM Rev protein genes into CD4+ cells of HIV-1 discordant twins

In a current phase I/II clinical trial, CD4+ lymphocytes obtained from the uninfected identical twin of an HIV-infected patient were transduced with a murine retroviral vector expressing both a marker gene (*neo*) and anti-HIV-1 genes (antisense TAR and TDM Rev, Morgan and Walker 1996, Bechtel NIH/00783). This trial is currently underway, and results have not yet been reported.

C. Decoy RNA

Transfer of an RRE decoy gene into CD34+ cells from the bone marrow of HIV-1-positive children

Cells from the bone marrow of four HIV-1 infected children were transduced with a murine retroviral vector encoding the *neo* gene, or encoding both an RRE decoy and the *neo* gene (Kohn et al 1999, reviewed by Engel and Kohn 1999). Transduction efficiencies as determined by PCR, and by measuring the percentage of G418-resistant hematopoietic colonies, ranged from 7%-30%. However, cells expressing the RRE decoy were detected in peripheral blood only on the day after cell infusion. Transduced cells in peripheral blood samples that contain vector sequences one year after infusion occurred at a frequency of 1/100,000.

RATIONALE FOR PROJECT

I. Cloning within the anticodon loop

We hypothesized that a $tRNA_{AC}$-Rz (ribozyme cloned within the anticodon loop of a tRNA), as opposed to a tRNA-Rz (ribozyme cloned downstream of a tRNA), would provide a more compact and stable structure to the ribozyme due to its location within the tRNA. Since the tRNA itself possesses a compact and stable structure, it is likely to impart this phenotype to the ribozyme.

II. Optimization of the linker sequences connecting the ribozymes

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

Several studies have demonstrated that tRNA-ribozymes are not as active compared to a linear ribozyme. A $tRNA_i^{Met}$-ribozyme against perforin mRNA (Du et al 1998), a $tRNA_i^{Met}$-ribozyme against *lck* mRNA (Baier et al 1994), and $tRNA_i^{Met}$-based ribozymes against the HIV-1 leader sequence (Thompson et al 1995) demonstrated reduced catalytic activity *in vitro* compared to a linear, hammerhead ribozyme. The *in vitro* activity of a $tRNA_i^{Met}$-ribozyme against the *A2* gene of RNA coliphage SP (Yuyama et al 1992) was reduced by 30%, while a $tRNA^{Tyr}$-ribozyme against *CAT* mRNA cleaved its target RNA less efficiently (38%) than the linear ribozyme (75%,

Perriman et al 1995). The kinetic activity of the $tRNA^{Val}$-based ribozyme controls of the $tRNA^{Val}$-minizymes was also less than its linear counterpart (Kuwabara et al 1999).

The design may be improved by modifying the linker sequences in the anticodon loop. These are the sequences that connect the ribozyme in the anticodon to the rest of the tRNA sequence. In the previous designs, the lengths of the linkers were not designed, and in some cases were based solely on the availability of restriction enzyme sites in the anticodon loop. These designs must have caused steric hindrance that affected ribozyme activity. The tRNA-ribozyme structure must have been constrained to an extent that the ribozyme was unable to hybridize to its target. This may explain why the construct displayed a reduction in catalytic activity.

The length and sequence of linkers connecting the ribozyme to the tRNA sequences upstream (5' linker) and downstream (3' linker) of the ribozyme may therefore be optimized. To identify optimal lengths of 5' and 3' linkers, a selection strategy was used to isolate $tRNA_3^{Lys}$-based hammerhead ribozymes, expressed as part of the anticodon loop of human $tRNA_3^{Lys}$, that were able to cleave a target site *in cis* (Fig. 7). These $tRNA_3^{Lys}$-ribozymes were isolated from a library, which was constructed such that linker sequences connecting the ribozyme to the anticodon loop were of random sequence and variable length. The structure that contains the ideal combination of linkers should have been able to recognize the target RNA more freely and therefore display catalytic activity.

III. Use of $tRNA_3^{Lys}$ to express ribozymes

All $tRNA_{AC}$-Rzs developed to date have utilized the $tRNA_i^{Met}$ and $tRNA^{Tyr}$ genes. A better alternative for HIV gene therapy is $tRNA_3^{Lys}$. HIV-1 utilizes $tRNA_3^{Lys}$ as a primer for reverse transcription, which is co-packaged within virus particles. By using $tRNA_3^{Lys}$ to express ribozymes, the virus may be "tricked" into packaging these chimeric tRNAs. Being in such close proximity, the ribozymes will easily bind and cleave their target. For this reason, $tRNA_3^{Lys}{}_{AC}$-

FIG. 7. **Overview of selection strategy for $tRNA_{AC}$-Rzs.** A template DNA library containing the T7 promoter, the different $tRNA_{AC}$-Rzs with 5' and 3' linkers of variable length and sequences, and the ribozyme target site was transcribed *in vitro*. Active $tRNA_{AC}$-Rzs are expected to cleave the ribozyme target site provided *in cis*. The 5' cleavage products (containing active $tRNA_{AC}$-Rzs) were separated from the 3' products and uncleaved transcripts (containing inactive and less active $tRNA_{AC}$-Rzs) by denaturing PAGE. The 5' cleavage products were eluted from the gel and used for RT-PCR followed by cloning and characterization. Enlarged view depicts the modified tRNA anticodon loop containing the ribozyme hybridized to its target sequence. Nucleotides 5'-CUU-3' and 5'-UAA-3' are part of the original $tRNA_3^{Lys}$ anticodon. The dotted lines correspond to the 5' and 3' linkers, which varied in length and sequence. Ribozyme flanking sequences and the target sequences to which they bind are shown in small case. ⇩ denotes cleavage site. Ribozyme catalytic domain is shown in large case. → corresponds to the A to G mutation in the $tRNA_{AC}$-InRz.

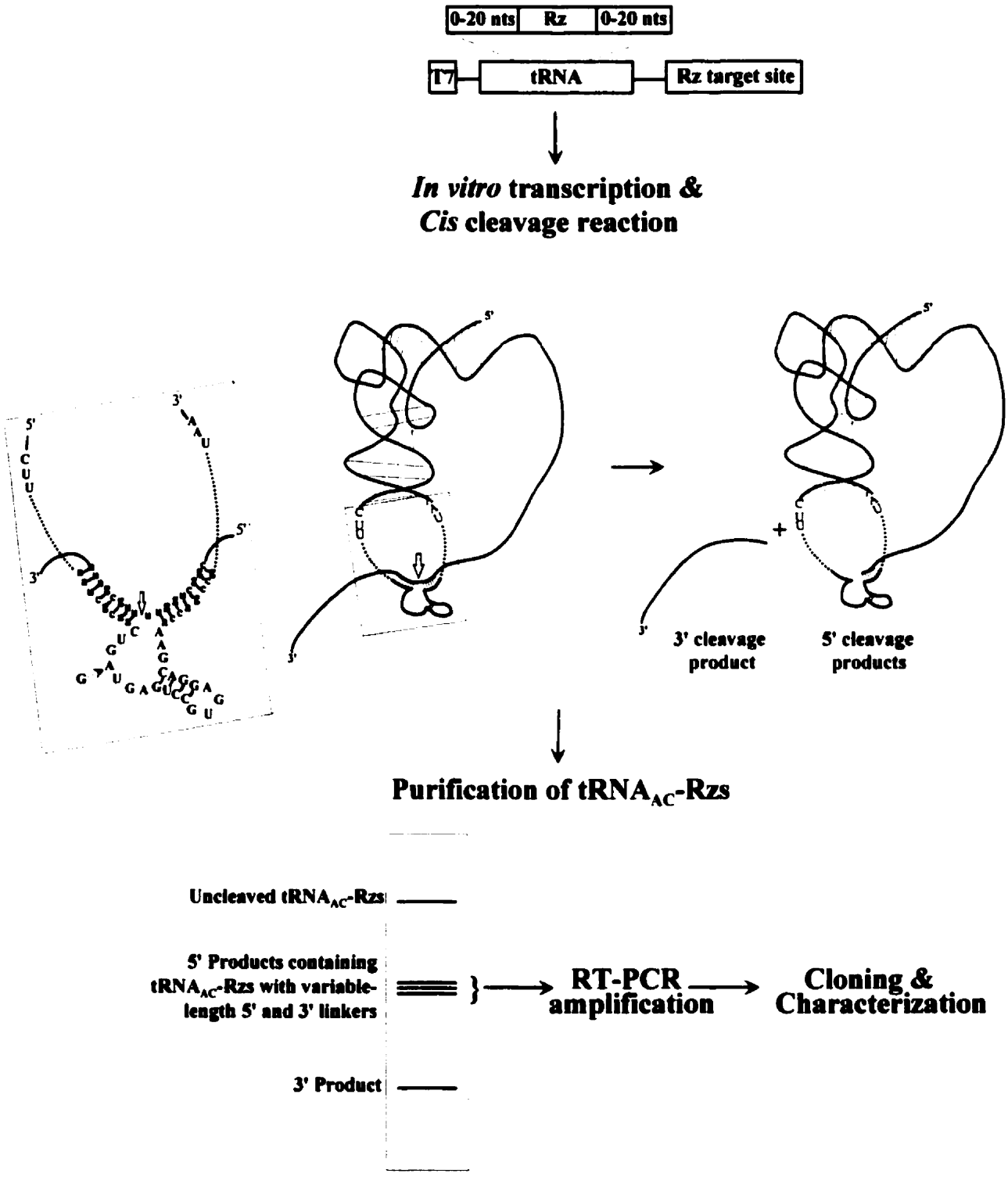

0-20 nts
Rz
0-20 nts
T7
tRNA
Rz target site
In vitro transcription &
Cis cleavage reaction
3' cleavage product
5' cleavage products
Purification of tRNA$_{AC}$-Rzs
Uncleaved tRNA$_{AC}$-Rzs
5' Products containing tRNA$_{AC}$-Rzs with variable-length 5' and 3' linkers
3' Product
RT-PCR amplification
Cloning & Characterization

Figure 7.

Rzs were developed in which a ribozyme targeted against human immunodeficiency virus (HIV)-1 *env*-coding region was inserted within the anticodon loop of human $tRNA_3^{Lys}$. The ribozyme inserted in the anticodon loop of $tRNA_3^{Lys}$ should not be spliced out since this tRNA does not contain introns (Sprinzl et al 1989). The $tRNA_3^{Lys}$ sequence was used with the intent of developing ribozymes which will cleave HIV-1 RNA both within the cell and HIV-1 progeny.

RESEARCH PROPOSAL

I. DEVELOP IMPROVED $tRNA_3^{Lys}{}_{Ac}$-Rz *VIA* SELECTION *IN VITRO*

A. Design and *in vitro* selection strategy

B. Template DNA library encoding different $tRNA_{AC}$-Rzs with 0-20 nt-long random sequence linkers

C. Controls

1. To confirm greater occurrence of *cis* over *trans* cleavage
2. To confirm that $tRNA_{AC}$-Rzs present in the library are able to cleave a target RNA in *trans*

D. *In vitro* transcription of DNA templates expressing $tRNA_{AC}$-Rzs and *cis* cleavage reaction

E. Cloning of selected $tRNA_{AC}$-Rzs

F. Characterization of selected $tRNA_{AC}$-Rzs

1. Electrophoretic mobility of $tRNA_{AC}$-Rzs transcribed *in vitro*
2. Identification of active $tRNA_{AC}$-Rzs allowing *trans* cleavage
3. Linker sequences of selected $tRNA_{AC}$-Rzs
   a. 5' and 3' linkers of each $tRNA_{AC}$-Rz
   b. 3' linkers of $tRNA_{AC}$-Rzs present in the template DNA library
4. Kinetics of *trans* cleavage by selected $tRNA_{AC}$-Rzs
5. Secondary structure of selected $tRNA_{AC}$-Rzs
6. Stability of selected $tRNA_{AC}$-Rzs
7. RNA pol III-driven transcription from the tRNA promoter

II. TEST RETROVIRAL VECTORS EXPRESSING $tRNA_{AC}$-RZS FOR INHIBITION OF HIV-1 REPLICATION IN A HUMAN CD4+ LYMPHOID MT4 CELL LINE

A. Development of retroviral vectors expressing $tRNA_{AC}$Rzs and various controls

B. *In vitro* cleavage activities of $tRNA_{AC}$-Rzs and various controls cloned within the retroviral vector

C. Development of pools of stable MT4 transductants expressing $tRNA_{AC}$-Rzs and various controls

1. Establish stable transductants expressing $tRNA_{AC}$-Rzs and various controls
2. Confirm the presence of proviral vector DNA within the cellular genome
3. Confirm the expression of $tRNA_{AC}$-Rzs and various controls

D. HIV-1 susceptibility of pools of stable MT4 transductants

MATERIALS AND METHODS

I. PLASMIDS

pUCMoTiN was previously constructed in our laboratory (Joshi et al 1993). It is a MMLV-derived retroviral vector which contains a *tk* promoter driving *neo* gene expression. Plasmids pSW2060 and pSW201 (obtained from S. Westaway and J. J. Rossi) contain the $tRNA_3^{Lys}$-coding region. pSW2060 also contains a T7 promoter upstream of $tRNA_3^{Lys}$. pHEnv (obtained from A. Panganiban) contains HIV-1 *env*-coding sequences. pM13-Lys,3 (obtained from J.D. Rosenblatt, Lu et al 1997) contains the $tRNA_3^{Lys}$ gene including sequences upstream and downstream of the coding region. pDTNβ (obtained from from J.L Whitton, Gebhard et al 1997) contains a truncated $tRNA_i^{Met}$ gene cloned between two divergently transcribed genes expressing β-galactosidase and *neo*. pGEM-Rz_{Env}-Env (see Appendix A) contains a ribozyme targeted against HIV-1 *env*, and the *env* target site, cloned *in cis* within the *lacZ*-coding region of plasmid pGEM-4Z

II. CELL LINES

The ψ-2 packaging cell line (Mann et al 1983) contains the *gag*, *pol* and *env* genes of an ecotropic murine leukemia virus. In the PA317 packaging cell line (Miller and Buttimore 1986), the ecotropic *env* gene was replaced with the amphotropic *env* gene. In addition, the 5' end of the 5' LTR has been deleted, and the SV40 polyadenylation site has replaced the PPT and the 3' LTR. MT4 cells (obtained from D. Richman) are human T cells isolated from a patient with adult T-cell leukemia and are transformed by nature with HTLV-1 (Pauwels et al 1987, Larder et al 1989). HeLa-CD4-LTR-β-gal cells, used for measuring the HIV-1 titer, express high levels of CD4, and contain one integrated copy of the HIV-1 LTR linked to the β-galactosidase gene

(Kimpton et al 1992). The MT4 cell line, HeLa-CD4-LTR-β-gal cell line, and HIV-1 strain NL4-3 were received through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

III. SYNTHESIS OF PCR PRIMERS

All primers used for construction of the $tRNA_{AC}$-Rz library were synthesized using the Millipore Expedite Nucleic Acid Synthesis System (Etobicoke, Canada) in a 0.05 μmole scale. The oligonucleotides were cleaved from the column by passing 1 ml of 30 % NH_4OH along the column at least 20 times using 5 ml slip-tip syringes. After a 45 min incubation at 37°C, the solution was passed through the column for another 20 times and the column was further incubated at 37°C for 45 min. The solution was collected in a screw-cap tube, heated overnight at 55°C, lyophilized, and the precipitated primer was resuspended in water. Primers were also produced by GSD (Toronto, Canada), ACGT (Toronto, Canada) and HSC Biotech (Toronto, Canada).

IV. 5' END LABELING OF PRIMERS AND RNAs

RNAs (50-200 pmol) were first dephosphorylated using calf intestinal phosphatase (Boehringer Mannheim; Laval, Canada) in the presence of 1 mM $ZnCl_2$, 1 mM $MgCl_2$, and 10 mM Tris-HCl (pH 8.3) for 1 h at 50°C. The mixture was extracted using an equal volume of phenol:choloroform:isoamyl alcohol (25:24:1). RNA was precipitated using three volumes of 100 % EtOH and 75 mM NaAc. The RNA was then resuspended in water and 5' end-labeled using polynucleotide kinase (Pharmacia Biotech Inc; Baie d'Urfé, Canada) in the presence of 10 mM Tris-Ac (pH 7.5), 10 mM MgAc, 50 mM KAc and [γ-^{32}P] ATP ((3000 Ci/mmol; Amersham Canada Ltd.; Oakville, Canada) for 30-120 min at 37°C. Primers (50 pmol) and 100 bp DNA

ladder (1 µg, Life Technologies; Burlington, Canada) were labeled without prior dephosphorylation.

V. PURIFICATION OF DNA AND RNA

A. By agarose gel electrophoresis

After running PCR products and DNA restriction fragments in 1-2 % agarose gels, bands were viewed under long wave UV light (365 nm) and excised from the gel with a blade. The DNA was electroeluted using elution blocks by running for 30 min at 70 V in elution buffer (0.02 M Tris-HCl [pH 8.0], 5 µM NaCl and 120 nM EDTA [pH 8.0]). The DNA which precipitates with 100 µl of 7.5 M NH_4Ac in the tunnel of the elution block was collected and precipitated overnight using 1 ml of absolute EtOH at -20°C. The DNA was then collected after centrifugation for 20 min at 13000 x *g*, and the pellet was resuspended in water.

B. By polyacrylamide gel electrophoresis (PAGE)

After running primers or RNAs on 8-10 % - 8 M urea polyacrylamide gels, the DNA and RNA bands were excised and transferred to 1.5 ml Eppendorf tubes. The gel was crushed using a plastic pestle and the nucleic acid was eluted overnight by shaking vigorously in 100 µl elution buffer (0.3 % SDS, 0.14 M NaCl, 0.05 M NaAc, pH 5.1) in a total of 400 µl. The supernatant was extracted once using an equal volume of phenol:choloroform:isoamyl alcohol (25:24:1), and the nucleic acid was precipitated using 2 volumes of 100 % EtOH and 200 mM NaAc.

C. By Sephadex

pUCMoTiN-based plasmids were purified using homemade spin columns before transfection. A 1 ml syringe was plugged with glass wool and filled with Sephadex G-50 (Pharmacia Biotech

Inc.; Baie d'Urfé, Canada) suspended in TE buffer (pH 7.6). The syringe was placed in a 15 ml tube and spun for 3 min at 300 x *g* to remove the buffer. The syringe was again filled with slurry, and the step repeated until the Sephadex reached the 1 ml level of the syringe. The syringe was placed in a new 15 ml tube, and the sample was applied to the column. The column was centrifuged for 3 min at 300 x *g*, and the eluate was collected. STE buffer (0.1 M NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) was then applied to the column, and the column was again centrifuged for 3 min at 300 x *g*. The two eluates were pooled, and the DNA was EtOH precipitated.

VI. PCRs

PCRs were performed in a Perkin-Elmer Cetus Thermal Cycler using up to 20 ng of DNA template, 1.5 mM $MgCl_2$, 200 μM of dNTPs, 0.2 μM of each forward and reverse primer and 1-5 units of *Taq* or *Pfu* polymerase in a final volume of 10-100 μL. The sample was overlaid with mineral oil and subjected to 30 cycles of denaturation (1 min, 95°C), annealing (1 min, 56°C), and extension (1 min, 72°C). Labeled PCR products were obtained by using a 5' end- labeled primer.

For overlapping PCR(s), products (20-50 ng each) of preliminary PCRs were first EtOH precipitated and purified by agarose gel electrophoresis. The bands were excised from the gel, electroeluted and EtOH precipitated before serving as templates in subsequent PCRs. Conditions used were same as regular PCRs.

TABLE 2A.

Primers used for the construction of the template DNA library

Primer	Length	Sequence	Description
a^+	17	CGAGGCCCTTTCGTCTC	binds upstream of $tRNA_3^{Lys}$ in SW2060, used for construction of the template DNA library
a^-	34-54	ACTCATCAGTTGCGATT$[N]_{0\text{-}20}$-AAGTCTGATGCTCTACC	for insertion of linker sequences upstream of the anticodon; $[N]_{0\text{-}20}$ denotes 0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 nt-long random (A, T, C or G) sequence linkers
b^+	34-54	GAGGACGAAACCAGCCG$[N]_{0\text{-}20}$-TAATCTGAGGGTCCAGG	for insertion of linker sequences downstream of the anticodon; $[N]_{0\text{-}20}$ denotes 0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 nt-long random (A, T, C or G) sequence linkers
b^-	34	TCAAAAAAGGTACCCCGCCGTGG CGCCCGAACAG	binds at 3' end of $tRNA_3^{Lys}$, overlaps with c^+ primer
c^+	38	CGGGGTACCTTTTTTGAATTCGTA GCGGGAGAATGATA	binds upstream of the target site in pHEnv, overlaps with b^- primer
c^-	17	GTCCGTGAAATTGACAG	binds downstream of the target site in pHEnv
d^+	38	*AATCGCAA***CTGATGAGTCCGTGA GGACGAA***ACCAGCCG*	contains Rz catalytic domain (bold) and flanking sequence complementary to HIV-1 *env* (italics)

TABLE 2B.

Primers used for characterization

Primer	Length	Sequence	Description
AR50	50	ATATCATATG**TAATACGACTCAC TATAG**GGCGAGTGCAGAAAGAAT ATGC	binds upstream of the target site in pHEnv, contains a T7 promoter (bold)
Cat^+	22	CTGATGAGTCCGTGAGGACGAA	contains Rz catalytic domain
d^-	38	*CGGCTGGT***TTCGTCCTCACGGA CTCATCAG***TTGCGATT*	contains the antisense sequences of primer d^+; Rz catalytic domain (bold) and flanking sequence complementary to HIV-1 *env* (italics)
MM12	18	AACCAGCCGGGGCACAAT	for preparing the template used for transcription of the 5' cleavage product marker, binds just before the ribozyme target site in the template DNA library,
MM13	18	TGGCGCCCGAACAGGGAC	binds at 3' region of $tRNA_3^{Lys}$
MM15	51	CGCGGATCCTAATACGACTCACT ATAGGGCGACGGCGGGGTACCTT TTTTG	contains a T7 promoter, binds right after the CCA tRNA sequence of the template DNA library, used to amplify the template for transcription of the Rz target sequence which was used for *trans* cleavage by the pool of $tRNA_{AC}$-Rzs
MM18	52	ATATATATA*GGATCC***TAATACGAC TCACTATAG**ggcccggatagctcagtcg	contains *Bam*H I (italics), T7 promoter (bold) and tRNA (lowercase) sequences, used for PCR to generate DNA for cloning in pGEM4Z or *in vitro* transcription
MM19	32	ATATATATA*GGATCC*gcccggatagctca gtc	contains *Bam*H I (italics) and tRNA (lowercase) sequences, used for PCR to generate DNA for cloning in pUCMoTiN
MM20	39	ATATATATA*ATCGATGGAGCTC*aaaa aa*GGTACC*CCGCC	contains *Cla*I, *Sst* I, *Kpn* I sites (italics) and RNA pol III terminator (lowercase), used for RT-PCR , for PCR to generate DNA for cloning in pUCMoTiN/pGEM4Z or *in vitro* transcription
MM21	35	ATATAT*ATCGAT*aaaaaa**CGGCTGG TTTCGTCCTC**	contains *Cla*I (italics), RNA pol III terminator (lowercase) and Rz_{Env} (bold) sequences, used for PCR to generate DNA for cloning Rz_{Env} into pUCMoTiN
MM22	29	ATATAT*AGATCT*gtggcgcccgaacaggg	contains *Bgl* II (italics) and tRNA (lowercase) sequences, used for PCR to generate DNA for cloning $tRNA_3^{Lys}$ into pUCMoTiN-Rz_{Env}
MM23	17	GACGCCGGCTGGATGAT	binds upstream of Mo-F in pUCMoTiN, used with T7-Mo-R for generating the template used for transcribing the probe for RNase protections
Mo-F	17	GATGGCCGCTTTGGTCC	binds upstream of *Bam*H I in pUCMoTiN
Mo-R	17	GCTCGTACTCTATAGGC	binds downstream of *Cla*I in pUCMoTiN
T7-Mo-F	44	**ATATATATATAATACGACTCACT ATAGG**ATGGCCGCTTTGGTCC	Mo-F containing the T7 promoter (bold)
T7-Mo-R	44	**ATATATATATAATACGACTCACT ATAGG**CTCGTACTCTATAGGC	Mo-R containing the T7 promoter (bold)
T7	23	CGAAAT**TAATACGACTCACTATA**	binds to T7 promoter (bold)
Usp	17	GTAAAACGACGGCCAGT	used for sequencing and for amplifying the template DNA library

A. PCRs for synthesis of template DNA library encoding different $tRNA_{AC}$-Rzs with 0-20 nt-long random sequence linkers

Template DNA library encoding $tRNA_{AC}$-Rzs containing various linkers was constructed by overlap PCRs. To investigate the effects of linkers on the activity of RNA_{AC}-Rzs, two sets of primers were designed. The a^- set of primers consists of 11 individually synthesized primers containing 0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 nt-long linkers (Fig. 8*A*) which bind upstream of the anticodon. The b^+ set of primers consists of 11 individually synthesized primers containing 0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 nt-long linkers (Fig. 8*A*) which bind downstream of the anticodon. Equimolar mixtures of primers a^- and b^+ (each containing the 11 different primers) were prepared. The primers within each set possess the same 17 nts, which are complementary to tRNA sequences, and enable them to hybridize to the template during PCR. The primers within each set also contain the same 17 nts, which make up a portion of the ribozyme and its flanking sequences. The primers differ in the length of the sequence located between the anticodon loop and the ribozyme flanking sequence. This varies in length from 0-20 nts. The a^- primers are reverse primers which can be used in a PCR with primer a^+. The b^+ primers are forward primers which can be used in a PCR with primer b^-. Primer b^- also contains 17 nts which are complementary to primer c^+. Primer d^+ contains the entire ribozyme catalytic domain, and 8 nt flanking sequences complementary to either side of the cleavage site (Fig. 8*A*). pSW2060 was used to amplify the T7 promoter and 5' half of $tRNA_3^{Lys}$, and pSW201 was used to amplify the 3' half of $tRNA_3^{Lys}$. pSW2060 was used as a PCR template with a^+ (forward primer which binds upstream of the T7 promoter) and a^-. pSW201 was used in a PCR with b^+ and b^- (reverse primer which binds at the 3' end of $tRNA_3^{Lys}$). The HIV-1 target sequence was amplified from the pHenv plasmid using c^+ and c^- (primers which flank the ribozyme target site, Fig. 8*A*).

FIG. 8. **Overlap PCRs to generate the template DNA library containing the T7 promoter, the different $tRNA_{AC}$-Rzs and the ribozyme target sequence.** *A*, The secondary structures of a^-, b^+ and d^+ primers are shown. The dotted lines in a^- and b^+ primers correspond to the 5' and 3' linkers, respectively, which are of random sequence and range in length from 0 to 20 nts. The a^- (left-hand side of the tRNA diagram), b^+ (right-hand side of the tRNA diagram), and d^+ primers are shown. A, B and C are products of regular PCR cycles. The templates used for products A (pSW2060) and B (pSW201), both containing $tRNA_3^{Lys}$, were amplified using a^+/a^- and b^+/b^- primer pairs, respectively. The template for product C (pHEnv) was amplified using c^+/c^- primers. This product contains the $tRNA_{AC}$-Rz target site. *B*, PCR products B and C were used as overlapping templates to generate product D (lanes D' are re-amplifications of product D). Product D was PCR amplified using d^+/c^- primers to generate product E. Products A and E were then used as overlapping templates with a^+/c^- primers to generate product F, the $tRNA_{AC}$-Rz template DNA library. This library consists of a 988-1028 bp DNA population which contains the T7 promoter, the $tRNA_{AC}$-Rzs ($tRNA_3^{Lys}$ with random-sequence, variable-length 5' and 3' linkers connecting the ribozyme to the anticodon loop) and the ribozyme target site. The dotted lines correspond to the nucleotides incorporated by *Taq* polymerase after annealing of the overlapping templates. M, ϕx174-DNA *Hae* III marker.

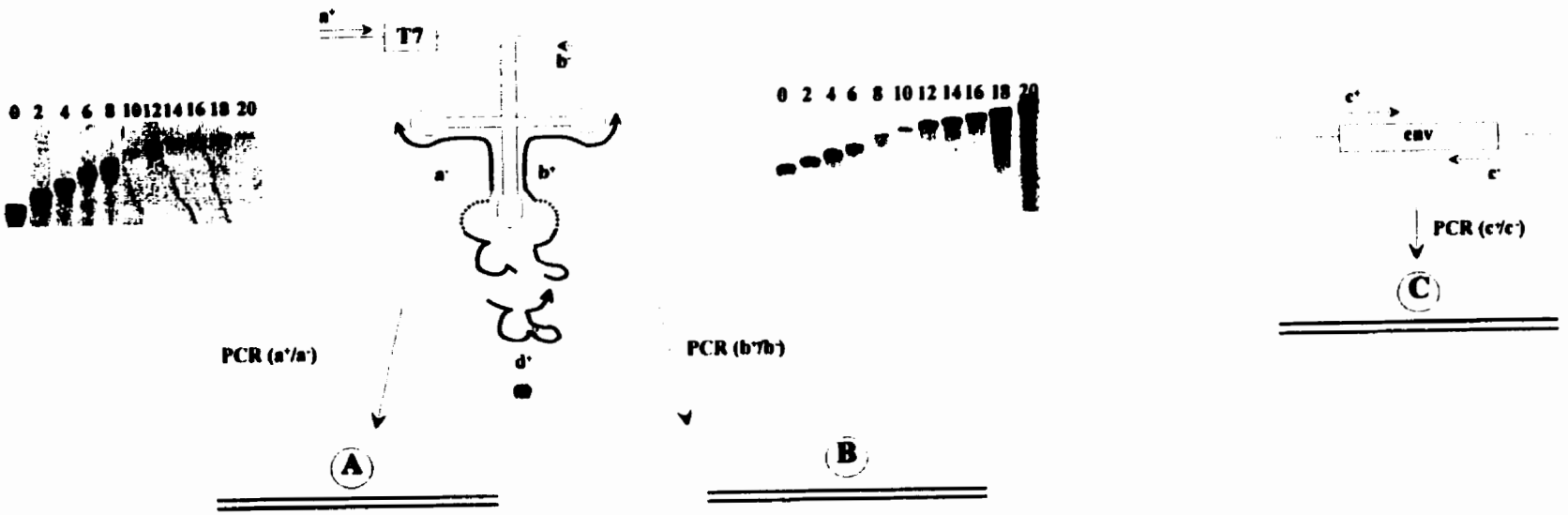

0 2 4 6 8 10 12 14 16 18 20
a'
T7
b'
a'
b'
d'
PCR (a'/a')
A
0 2 4 6 8 10 12 14 16 18 20
PCR (b'/b')
B
c'
cmv
c'
PCR (c'/c')
C

Figure 8A.

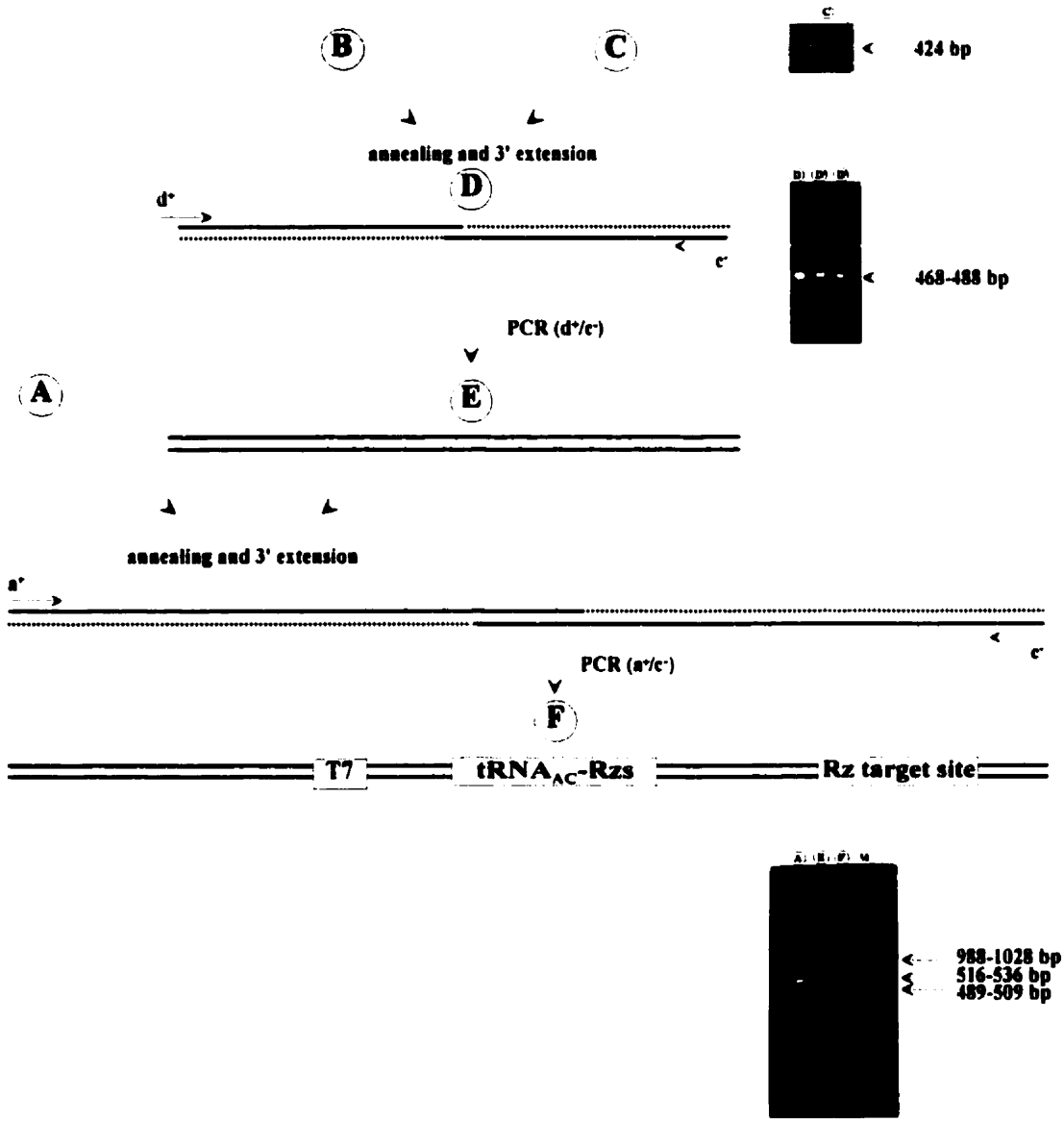

B
C
424 bp
annealing and 3' extension
D
d+
c-
468-488 bp
PCR (d+/c-)
A
E
annealing and 3' extension
a+
c-
PCR (a+/c-)
F
T7
tRNA$_{AC}$-Rzs
Rz target site
988-1028 bp
516-536 bp
489-509 bp

Figure 8*B*.

The a^+/a^- PCR product contains the T7 promoter, the 5' end of $tRNA_3^{Lys}$, the 5' region of the ribozyme, the 5' flanking sequence, and the 5' linkers. The b^+/b^- PCR product contains the 3' end of $tRNA_3^{Lys}$, the 3' region of the ribozyme, the 3' flanking sequence, and the 3' linkers (Fig. 8*A*). Because primer b^- was designed to overlap with primer c^+, PCR products resulting from the use of these primers would possess overlapping ends. These PCR products (b^+/b^- and c^+/c^-) were used as templates in an overlapping PCR using primers b^+ and c^-. The resulting PCR product was further amplified using d^+ and c^- primers. The product from this reaction was then used as an overlapping template, along with the a^+/a^- PCR product in a PCR using a^+ and c^- primers (Fig. 8*B*). This product was further amplified using Usp (forward primer which binds upstream of the T7 promoter) and c^- primer pair to generate enough DNA for *in vitro* transcription.

B. PCRs for confirmation of the variability of linkers in the template DNA library and in each cloned $tRNA_{AC}$-Rz

The 3' linker region of the template DNA library was amplified using d^+ (forward primer which contains the ribozyme and flanking sequence) and b^- (reverse primer which binds at the 3' end of $tRNA_3^{Lys}$) primer pairs, or cat+ (forward primer which contains the ribozyme catalytic domain) and MM13 (reverse primer which binds to the 3' region of $tRNA_3^{Lys}$) primer pairs. Both d^+ and cat+ were 5' end-labeled to visualize the PCR products. The 3 linker regions of each individual $tRNA_{AC}$-Rz was also amplified using d^+ and MM13 primer pairs. The $tRNA_{AC}$-Rzs including both 5' and 3' linker regions were amplified using primers MM18 (forward primer which binds at the 5' end of $tRNA_{AC}$-Rzs) and MM13.

C. PCRs to generate templates for *in vitro* transcription

All templates used for *in vitro* transcription were generated by PCR using a forward primer that contains a T7 promoter or binds upstream of a T7 promoter sequences present in a plasmid. These were then transcribed *in vitro* and used for *trans* cleavage reactions.

The template DNA library was PCR amplified to generate templates enabling T7 promoter driven transcription of $tRNA_{AC}$-Rzs using Usp (forward primer which binds upstream of the T7 promoter) and MM13 (reverse primer which binds to the 3' end of $tRNA_{AC}$-Rzs). The target RNA used to assess the *trans* cleavage ability of the pool of $tRNA_{AC}$-Rzs was amplified using MM15 (contains the T7 promoter sequence) and c^- (binds downstream of the ribozyme target site) primer pair.

To prepare linear ribozyme (Rz) containing the ribozyme target site *in cis*, Rz_{Env}-Env was amplified from pGEM-Rz_{Env}-Env (See Appendix A) using T7 (forward primer which binds to the T7 promoter) and Usp (reverse primer which binds downstream of the ribozyme target sequence).

To prepare RNA used for *trans* cleavage reactions, various $tRNA_{AC}$-Rzs, $tRNA_{AC}$-InRz and $tRNA_3^{Lys}$ were each PCR amplified from the respective pGEM-4Z or pUCMoTiN-based plasmids using MM18 (binds at the 5' end of $tRNA_{AC}$-Rzs and contains the T7 promoter sequence) and MM13 (binds at the 3' end of $tRNA_{AC}$-Rzs) primer pair. The linear Rz was PCR amplified from pGEM-Rz_{Env} using T7 (binds to the T7 promoter sequence) and MM21 (binds to the 3' end of the ribozyme) primer pair, and amplified from pUCMoTiN-Rz_{Env} using T7-Mo-F (contains the T7 promoter sequence) and MM21 primer pair. The downstream ribozyme, tRNA-

Rz, was amplified using MM18 and MM21. The target RNA was amplified from pHEnv using AR50 (binds upstream of the ribozyme target site and contains the T7 promoter sequence), and c⁻ (binds downstream of the ribozyme target site).

The DNA template for a 5' product marker was prepared by PCR using the template DNA library as a PCR template, T7 primer (forward primer which binds to the T7 promoter) and MM12 (primer which binds just before the ribozyme target site in the template DNA library).

The template, which was used for transcribing the probe used for RNase protection, was obtained by PCR amplification of $tRNA_{AC}$-Rz (0/0) sequences and surrounding retroviral vector sequences from the pUCMoTiN-$tRNA_{AC}$-Rz (0/0) plasmid. The plasmid was first digested with *Csp*45 I and used in a PCR with MM23 and T7-Mo-R primer pair.

D. PCRs to generate DNA used for cloning

For cloning in pUCMoTiN, $tRNA_{AC}$-Rz (0/0), $tRNA_{AC}$-Rz (2/0), $tRNA_{AC}$-Rz (4/0), $tRNA_{AC}$-Rz (8/0), $tRNA_{AC}$-Rz (10/0), and $tRNA_{AC}$-InRz sequences were PCR-amplified from the respective pGEM4Z plasmids using MM19 (contains *Bam*HI site) and MM20 (contains *Cla*I site). The $tRNA_3^{Lys}$ sequences were amplified from pSW201 plasmid using MM19 and MM22 (contains *Bgl* II site).

E. PCRs for confirming the identity of bacterial clones

Upon cloning of $tRNA_{AC}$-Rzs within the *Bam*HI and *Sst*I sites of pGEM-4Z, correct clones were screened by PCR using T7 (forward primer which binds to the T7 promoter) and Usp (reverse primer which binds downstream of the ribozyme) which flank the cloning site. Correct clones

demonstrated a PCR product (≥275 bp) larger than the PCR product obtained from the parental pGEM-4Z plasmid (134 bp).

pUCMoTiN-Rz_{Env} and pUCMoTiN-tRNA-Rz clones were screened by PCR using Mo-F and MM21 primer pair. The correct size products (79 nts and 162 nts) were observed in each case.

F. PCRs for confirming proviral DNA integration within the cellular genome

PCRs were performed to confirm the presence of proviral DNA within the cellular genome. Genomic DNA from transduced PA317 cells was used for PCR using Mo-F and Mo-R primer pair.

VII. RT-PCR

The RNA is first incubated with the reverse primer (100 ng) at 65°C for 10 min. After a 1 min incubation on ice, 75 units of RNA guard, 5 mM DTT, 125 mM dNTPs, and 400 units MMLV RT (Life Technologies; Burlington, Canada) were added in the presence of 1x RT buffer (Life Technologies; Burlington, Canada) containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$, to a total volume of 40 µl. The reaction mixture was incubated at 37°C for 1 h and then at 65°C for 10 min. 4 µl of the reaction mixture was then used in a PCR as described.

The 5' cleavage products obtained after *cis* cleavage of the pool of $tRNA_{AC}$-Rzs were reverse transcribed using MM20 primer (binds downstream of $tRNA_{AC}$-Rz sequences and contains the RNA pol III terminator, *ClaI* and *SstI* sites). The cDNA was used as a PCR template with MM18 (forward primer which binds at the 5' end of $tRNA_{AC}$-Rzs, contains a *Bam*HI site and T7

promoter) and MM20 primers and cloned within the *Bam*HI and *Sst*I sites of the pGEM-4Z plasmid.

To detect RNA pol II-transcribed RNAs, total RNA isolated from transduced PA317 and MT4 cells were reverse transcribed using Mo-R or T7-Mo-R primers which bind to retroviral vector sequences downstream of Rz_{Env}, tRNA-Rz, $tRNA_{AC}$-InRz, and $tRNA_{AC}$-Rzs. The cDNA was then used in PCRs with Mo-F primer, which binds to retroviral vector sequences upstream of Rz_{Env}, tRNA-Rz, $tRNA_{AC}$-InRz, and $tRNA_{AC}$-Rzs, along with Mo-F or T7-Mo-F primer. The RT-PCR products (10 µl) were then analyzed by 2 % agarose gel electrophoresis.

To detect RNA pol III-transcribed RNAs, low molecular weight (LMW) RNA isolated from transduced MT4 cells was reverse transcribed using MM22 primer (binds to the 3' end region of $tRNA_3^{Lys}$). The cDNA was then used in PCRs with MM19 primer (binds to the 5' end region of $tRNA_3^{Lys}$) and MM22 primer. The cDNA was also PCR-amplified using MM18 primer (contains the T7 promoter), and MM22 primer. The MM18/MM22 PCR product was transcribed *in vitro* as described and used for *trans* cleavage reactions. The presence of high molecular weight (HMW) RNA was detected by RT-PCR using Mo-F and T7-Mo-R primers.

VIII. PRIMER EXTENSION

Total RNA isolated from untransduced or transduced MT4 cells expressing $tRNA_{AC}$-Rz (0/0), $tRNA_{AC}$-Rz (2/0), $tRNA_{AC}$-Rz (4/0), $tRNA_{AC}$-Rz (8/0), $tRNA_{AC}$-Rz (10/0), $tRNA_{AC}$-InRz, tRNA-Rz, linear Rz, and MoTiN was first incubated with the MM13 primer (50 ng) at 65°C for 10 min. After a 1 min incubation on ice, 1x RT buffer (Life Technologies; Burlington, Canada) containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$, DTT (5 mM), dTTP (50

μM), dCTP (50 μM), dGTP (50 μM), dATP (5 μM), [α-^{32}P] dATP (3000 Ci/mmol; Amersham Canada Ltd.; Oakville, Canada), and MMLV RT (100 units; Life Technologies; Burlington, Canada) were added to a total volume of 10 μl. The reaction mixture was incubated at 37°C for 1 h and then at 65°C for 10 min. Internally labeled primer extension products were analyzed by 8 M urea-8 % PAGE followed by exposure to a phosphor screen.

Alternatively, total RNA isolated from transduced MT4 cells expressing $tRNA_{AC}$-Rz (0/0) or MoTiN was incubated with a 5'end-labeled d$^-$ primer, while transduced MT4 cells expressing $tRNA_{AC}$-Rz (10/0) or MoTiN was incubated with the MM13 primer for internal labeling. The mixtures were incubated at 65°C for 10 min, 58°C for 20 min, and room temperature for 10 min. The extension was then performed at 42°C for 30 min after addition of the rest of the reagents including MMLV RT.

IX. *IN VITRO TRANSCRIPTION* AND CO-TRANSCRIPTIONAL *CIS* CLEAVAGE

In vitro transcription of PCR products which possessed T7 promoters were performed in the presence of 40 mM Tris-Cl (pH 8.0), 25 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 1 mM of each NTP and 200 units of T7 RNA polymerase (Life Technologies; Burlington, Canada) in a 100 μl reaction volume with or without addition of [α-^{32}P] UTP (3000 Ci/mmol; Amersham Canada Ltd.; Oakville, Canada). After 2 h incubation at 37°C, the reaction was stopped by digesting the template DNA with 5 units of RQI RNase-Free DNase (Promega Corp.; Madison, USA) for 10 min. The mixture was extracted once with water-saturated phenol and the RNA precipitated with 3 volumes of 100 % EtOH with 0.3 M NaAc pH 5.1. The transcripts were then analyzed by 8 M urea-8 % PAGE followed by methylene blue staining. Labeled RNAs were

visualized by exposure to a phosphor screen and scanning by Storm phosphorimager (Molecular Dynamics; Sunnyvale, USA).

To select for active $tRNA_{AC}$-Rzs, co-transcriptional *cis* cleavage was performed by utilizing the conditions used for *in vitro* transcription using PCR DNA in a reaction mixture of 100 µl. *Cis* cleavage of the target sequence by the $tRNA_{AC}$-Rzs occurred under the condition used for transcription, without further incubation or addition of reagents. To visualize the product to be eluted, transcription was performed in the presence of a labeled [α-^{32}P] UTP.

To compare relative occurrence of *cis* and *trans* cleavage, *in vitro* transcription of Rz_{Env}-Env was performed for 30, 60, 90 and 120 min using the same amount of PCR DNA as template in the presence of a *trans* target RNA.

To demonstrate RNA pol III-driven transcription of $tRNA_{AC}$-Rzs, pGEM4Z-based plasmids containing $tRNA_{AC}$-Rzs, pM13-Lys,3 expressing $tRNA_3^{Lys}$, and pDTNβ expressing $tRNA_i^{Met}$ were each transcribed *in vitro* using HeLa nuclear extract (Promega Corp.; Madison, USA) following instructions provided by the supplier. Briefly, plasmids were incubated in the presence of [α-^{32}P] UTP, 3 mM $MgCl_2$, 1 µl of 25x NTP, and 4 µl of extract in 25 µl total for 1 h at 30°C. Stop buffer (175 µl) pre-incubated to room temperature was then added. The mixture was extracted with water-saturated phenol and EtOH precipitated overnight at –20 °C. The transcripts were analyzed by 8 M urea-8 % PAGE, and visualized by exposure to a phosphor screen and scanning by Storm phosphorimager (Molecular Dynamics; Sunnyvale, USA).

Alternatively, transcription was performed using MT4 cell extracts with 31 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 7.5), 100 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 500 μM each NTP and ½ total volume of MT4 cell extract in the presence of [α-^{32}P] UTP. The mixture was incubated at 37°C for 3 h, followed by phenol extraction. RNAs were precipitated using 3 volumes of 100 % EtOH and 100 mM NaAc.

X. *IN VITRO* CLEAVAGE REACTIONS *IN TRANS*

A. *In vitro* cleavage reactions using pooled $tRNA_{AC}$-Rzs

The pool of $tRNA_{AC}$-Rzs and target RNA were combined in a reaction mixture (10 μl) containing 40 mM Tris-Cl (pH 8.0) and 10 mM NaCl, heated to 65°C for 5 min and cooled to 37°C. The reaction was initiated by adding 20 mM $MgCl_2$. After incubation for 2 h at 37°C, the reaction was stopped by adding 5 mM EDTA.

B. *In vitro* cleavage reactions using individual $tRNA_{AC}$-Rzs

To determine which among the twenty-one selected $tRNA_{AC}$-Rzs were capable of *trans* cleavage, reactions (5 μl) were performed for 2 h at 37°C using equal amounts (2 μg) of each $tRNA_{AC}$-Rz, linear Rz and target RNA transcribed *in vitro* by T7 RNA polymerase. Reactions were also performed using [γ-^{32}P]-labeled target RNA (0.64 pmol) which was combined with each $tRNA_{AC}$-Rz, linear Rz and $tRNA_3^{Lys}$ (9.6 pmol). Initial rates of reaction by the $tRNA_{AC}$-Rzs were determined by performing *trans* cleavage at 10 min. A *trans* cleavage reaction was also performed without prior denaturation of the ribozyme and target RNA at 65°C.

To determine the kinetics of cleavage reactions, each $tRNA_{AC}$-Rz and linear Rz was incubated with [α-^{32}P]-labeled target RNA in a *trans* cleavage reaction (36 μl) containing 60 pmol of each

$tRNA_{AC}$-Rz and linear Rz and 6 pmol target RNA. Aliquots (containing 10 pmol of each $tRNA_{AC}$-Rz and linear Rz and 1 pmol target RNA) were taken at various time points.

To determine the kinetic constants of $tRNA_{AC}$-Rz (10/0) and linear Rz, each ribozyme (0.25 μM) was mixed with varying concentrations of target RNA (0.5 μM, 1 μM, 2 μM, 4 μM) and the *trans* cleavage reactions were performed as above for 5 min at 37°C. Vo and Vo/S were determined as described in (Yu et al 1995b). Vo = amount of product formed over time, S = substrate concentration. The Vo and Vo/S were calculated and plotted in an Eadie-Hofstee graph.

To confirm *trans* cleavage activity of RNA pol III-transcribed $tRNA_{AC}$-Rz (10/0), reactions (10 μl) were performed for 2 h at 37°C using unlabeled 333-nt target RNA (2 μg) and all of the resulting transcript obtained from transcription (25μl) of a plasmid expressing $tRNA_{AC}$-Rz (10/0) using HeLa nuclear extract. Linear Rz was used as a positive control for the *trans* cleavage reaction.

To confirm *trans* cleavage activity of $tRNA_{AC}$-Rzs cloned in pUCMoTiN, $tRNA_{AC}$-Rz and $tRNA_{AC}$-InRz were transcribed *in vitro* from PCR DNA templates amplified from each pUCMoTiN-based plasmid. All of the transcripts which resulted from each transcription reaction (25 μl) were used in *trans* cleavage reactions (5 μl) with the target RNA for 2 h at 37°C.

Cleavage products were analyzed by 8 M urea-8 % PAGE followed by methylene blue staining. *Trans* cleavage reactions which utilized a labeled target RNA were analyzed by 8 M urea-8 % PAGE followed by exposure to a phosphor screen and scanning by Storm phosphorimager (Molecular Dynamics; Sunnyvale, USA). The amount of target RNA cleaved was determined by measuring band intensities using ImageQuant software (Molecular Dynamics; Sunnyvale, USA).

XI. CLONING

Ligation reactions were performed using 1 unit of T4 DNA ligase (Life Technologies; Burlington, Canada), 1x T4 ligase buffer, 1000-fold excess of insert to vector DNA in a 10-20 µl volume. DNA inserts obtained by PCR amplification were digested with the appropriate restriction enzymes and were either purified or used directly after phenol:chloroform:isoamyl alcohol extraction (25:24:1) and EtOH precipitation. Incubations were performed at 23°C for 1 h or at 4°C for 2 days. The ligation mixture was pre-incubated for 5 min at 55°C to denature both vector and insert, followed by 10 min annealing at room temperature, before ligase was added. Ligation controls were included which consisted of the digested vector alone, T4 ligase and ligase buffer.

A. Cloning of $tRNA_{AC}$-Rzs in pGEM-4Z vector

PCR products resulting from amplification of the reverse-transcribed 5' cleavage products using MM18 and MM20 primers were digested with *Bam*HI and *Sst*I and cloned at the same sites within pGEM4Z. Plasmid DNA from thirty-four individual colonies were isolated and screened by PCR. Cultures of the twenty-three clones identified to be correct by PCR were then re-streaked on agar plates. Plasmid DNA isolated from single colonies were further screened by *Apa* I digestion. Twenty-one clones which possessed a single *Apa* I site lacking in the parental pGEM4Z plasmid demonstrated the correct restriction enzyme pattern.

B. Cloning of $tRNA_{AC}$-Rzs and controls in pUCMoTiN vector

PCR products resulting from amplification of $tRNA_{AC}$-Rzs and $tRNA_{AC}$-InRz from the individual pGEM-4Z plasmids using MM19 and MM20 primers were digested with *Bam*HI and *Cla*I and cloned at the same sites within the retroviral vector pUCMoTiN downstream of the *neo*

gene. Plasmid DNA isolated from ampicillin and kanamycin resistant colonies were screened by restriction enzyme analysis using *Sst*I. Clones expressing $tRNA_{AC}$-Rzs were also sequenced.

The linear Rz control was amplified from pGEM-Rz_{Env} using T7 (forward primer which binds upstream of the *Bam*HI site in pGEM-4Z) and MM21 (reverse primer which contains a *Cla*I site). The linear Rz was cloned within the *Bam*HI and *Cla*I sites of pUCMoTiN to generate pUCMoTiN-Rz_{Env}. The downstream ribozyme control, downstream-Rz, was constructed by cloning $tRNA_3^{Lys}$ at a *Bam*HI site upstream of Rz_{Env} in pUCMoTiN-Rz_{Env}, resulting in pUCMoTiN-tRNA-Rz.

XII. TRANSFORMATION OF COMPETENT CELLS

Competent cells used for transformation were prepared as follows. A single colony of *E. coli* (DH5α) was picked from an agar plate and grown overnight in 10 ml of Luria-Bertani (LB) medium at 37°C. The overnight culture was then transferred to 500 ml of LB containing 15 mM $MgCl_2$ and grown until the $O.D._{600}$ reached 0.4-0.6. The cells were pelleted by centrifugation at 850 x *g* at 4 °C for 20 min, and were resuspended in 150 ml of Solution A containing 10 mM $MnCl_24H_20$, 50 mM $CaCl_2$, and 10 mM MES (2-N-Morpholinoethanesulfonic acid, pH 6.3). After a 20 min incubation on ice, the cells were recovered by centrifugation at 850 x *g* at 4°C for 15 min and resuspended in 30 ml of Solution A containing 15 % glycerol. Aliquots of the competent cells were stored in 1.5 ml tubes at $-70\,^0$C.

The ligation mix and plasmid controls were added to 25-100 µl of competent cells thawed on ice for 30 min. After a 30 min incubation on ice, the cells were heat-shocked for 2 min at 42°C, and then incubated on ice for 5 min. After adding 250-500 µl of LB, the cells were grown for 1 h at

37°C. The cell culture was then spread on LB agar plates containing the appropriate drug (50 μg/ml ampicillin and/or 30 μg/ml kanamycin), along with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, 800 μg), and isopropylthio-β-D-galactoside (IPTG, 0.4 μmol), and grown overnight at 37^0C.

XIII. LARGE-SCALE AND SMALL SCALE PLASMID DNA ISOLATION

Plasmid DNA used for analysis were isolated as described in Sambrook et al 1989. Single colonies were grown in 5 ml of LB containing the appropriate antibiotic overnight at 37°C. The cells (1.5 ml) were pelleted by spinning for 1 min at 11000 x *g*, and resuspended in 100 μl of ice-cold Solution I (25 mM Tris-Cl [pH 8.0], 10 mM EDTA [pH 8.0], 50 mM glucose). The cells were lysed by gently mixing with 200 μl of freshly prepared Solution II (0.2 N NaOH, 1 % SDS). After a 5 min incubation on ice, 150 μl of ice-cold Solution III (3 M KAc, 11.5 % CH_3COOH) was added. Cell debris and genomic DNA were precipitated by incubation on ice for 5 min, and pelleted by centrifugation for 10 min at 13000 x *g*. The supernatant was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), and collected by centrifugation at 13000 x *g* for 1 min. This step was repeated using an equal volume of chloroform:isoamyl alcohol (24:1). The DNA was precipitated from the supernatant by adding 0.3 M NaAc and two volumes of EtOH followed by 1 h incubation at -20°C. The DNA was then pelleted by centrifugation for 20 min at 13000 x *g*, washed using 500 μl 70 % EtOH, and resuspended in water.

Plasmid DNA used for transfection were isolated as follows. Single colonies were grown overnight in 10 ml of LB medium at 37°C in the presence of appropriate antibiotic. The overnight cultures were then transferred to 1L LB with antibiotic and grown overnight. The next day, cells were pelleted by centrifugation at 18000 x *g* at 4°C for 15 min, and were resuspended

in 14 ml of TE buffer (pH 8.0) containing 15 % sucrose. After transferring the cells in a 250 ml flask, 4 ml of 0.5 M EDTA (pH 8.0) and 2.25 ml of freshly prepared egg white lysozyme (12 mg/ml in STE buffer) were added. The cells were incubated at 37°C with gentle shaking (75rpm) for 30 min. After adding 2.25 ml of 10 % SDS, the incubation was continued for another 6 min. 6.5 ml of 5 M NaCl was then added, and the cells were incubated on ice for 4 h, with gentle shaking every 30 min. The viscous pellet was transferred to a 30 ml tube and centrifuged for 1 h at 250 x *g* at 4°C. The DNA was precipitated from the supernatant with two volumes of isopropanol overnight at -20°C. The next day, the DNA was pelleted by centrifugation for 15 min at 1100 x *g* at 4°C, dried, and resuspended in 9.4 ml TE buffer (pH 8.0). After adding 11 g of CsCl, and 100 µl of ethidium bromide (10 mg/ml), the mixture was centrifuged for 5 min at 650 x *g* at room temperature. The supernatant was collected and cleared of cellular debris by centrifugation for another 5 min at 650 x *g*. The supernatant was transferred to a quick seal tube and centrifuged at 270000 x *g* overnight. The next day, the DNA band was collected using a 5 ml syringe, and extracted several times with an equal volume of n-butanol saturated with 5 M NaCl in TE buffer (pH 8.0) until the bottom phase was clear. The supernatant was then collected, measured and 2 volumes of TE buffer (pH 8.0) was added. The DNA was precipitated by adding 2 volumes of cold 100 % EtOH, and incubating at –20°C for 1 h. The DNA was pelleted by centrifugation for 15 min at 18000 x *g*, and washed with 10 ml of 70 % EtOH. The DNA pellet was dried and resuspended in water.

For screening colonies by PCR, 100 µl of cell cultures were boiled for 10 min, and the supernatants were cleared of cell debris by spinning for 10 sec at 13000 x *g*. 1 µl supernatant was then used as a template in a 10 µl PCR.

XIV. PLASMID DNA SEQUENCING

Plasmid DNA was sequenced using the Pharmacia ^{T7}Sequencing Kit (Pharmacia Biotech Inc.; Baie d'Urfé, Canada) following instructions provided by the supplier. Briefly, 1.5-2 µg of plasmid DNA was denatured at 65°C using 1 M NaOH in the presence of the Usp primer (reverse primer which binds downstream of the ribozyme target sequence). The primer was allowed to anneal to the template by addition of 1 M HCl and 2 µl annealing buffer. The annealed template and primer was then labeled with [α-^{32}P] dCTP (3000 Ci/mmol; Amersham Canada Ltd.; Oakville, Canada) using T7 DNA polymerase. Aliquots of the labeled DNA were transferred into 4 tubes, each containing a mixture of all four nts (dATP, dGTP, dTTP, dCTP) and one type of dideoxynucleotide (either ddATP, ddGTP, ddTTP, ddCTP). After incubation at 37°C for 5 min, the reaction was stopped by adding 5 µl of stop solution containing 98 % formamide, 10 mM EDTA (pH 8.0), 0.025 % xylene cyanol, and 0.025 % bromophenol blue. The reaction products (3 µl) were then analyzed by 8 M urea - 6 % PAGE. Bands were visualized by using X-ray film (Kodak; Toronto, Canada).

XV. STABILITY MEASUREMENTS

MT4 cell lysate was prepared by a method described by Weil et al (1979). MT4 cells (5 x 10^7) were spun for 5 min at 800 x *g* and washed with PBS. The packed cell volume estimated to be 300 µl was washed with 1 ml hypotonic buffer containing 10 mM HEPES (pH 7.9), 10 mm KCl, 1.5 mM $MgCl_2$ and 0.5 mM DTT. The cell pellet was then resuspended in twice the packed cell volume (600 µl) of hypotonic buffer. The cells were transferred to a 2 ml Dounce tissue grinder (Kontes; Mississauga, Canada) and allowed to swell for 10 min on ice. Cells were lysed by 10-15 strokes of pestle B (Kontes; Mississauga, Canada). After addition of one-tenth volume (90 µl) of a solution containing 0.3 M HEPES (pH 7.9), 1.4 M KCl and 0.03 M $MgCl_2$, the lysate was

centrifuged at 100,000 x *g* for 60 min. The supernatant was then stored with 10 % glycerol in -70° C. Protein concentration was determined from a standard curve obtained by measuring the OD_{630} of protein standards (Bio-Rad; Richmond, USA).

To determine the amount of lysate to use, control experiments were performed using 10,000 cpm of $tRNA_{AC}$-Rz (0/0) incubated with 12.5 μg/ml, 125 μg/ml, 250 μg/ml, 500 μg/ml, and 1.25 mg/ml of cell lysate. The same Rz was also incubated without any lysate for 5 and 10 min to test for degradation in the absence of proteins.

The relative stability of labeled $tRNA_{AC}$-Rzs (16,400 cpm each) transcribed *in vitro* using T7 RNA polymerase was examined for up to 3 h at 30°C in the presence of MT4 cell lysates (500 μg/ml). Aliquots were taken at 0 min, 10 min, 20 min, 1 h, 2 h and 3 h, and the reactions were stopped by adding an equal volume of buffer containing 80 % formamide, 10 mM EDTA, 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue and 1 mg/ml yeast tRNA. Samples were kept on dry ice until the last incubation and then analyzed by 8 M urea-8 % PAGE followed by exposure to a phosphor screen and scanning by Storm phosphorimager (Molecular Dynamics; Sunnyvale, USA). The amount of intact RNA remaining was determined by measuring band intensities using ImageQuant software (Molecular Dynamics; Sunnyvale, USA).

Stability of $tRNA_{AC}$-Rz (0/0), $tRNA_{AC}$-Rz (2/0) $tRNA_{AC}$-Rz (4/0), $tRNA_{AC}$-Rz (8/0), $tRNA_{AC}$-Rz (10/0), and wt tRNA were also measured using 8000 cpms of each RNA. MT4 cell lysate (500 μg/ml) was added to all of the RNAs, which were incubated separately for 0, 10, 20 and 60 min at 30°C. After incubation, equal volume of buffer containing 10 mM EDTA (pH 7.5) in deionized formamide was added. Samples were then analyzed by 8 M – 8 % PAGE and either

exposed to an XAR X-ray film (Kodak; Toronto, Canada) or to a phosphor screen. Band intensities were quantified as described.

XVI. RNase PROTECTION ANALYSIS

The probe used for RNase protection was obtained upon *in vitro* transcription of the MM23/T7-Mo-R PCR product amplified from linearized pUCMoTiN-tRNA$_{AC}$-Rz (0/0). To set up the conditions for RNase protection, the antisense probe (80 pmol) and *in vitro* transcribed tRNA$_{AC}$-Rz (10/0) (160 pmol) were mixed in the presence of 29 µl of hybridization buffer containing 80 % formamide, 0.1 % SDS, 40 mM piperazine-N,N'-bis [2-ethanesulfonic acid] disodium salt (PIPES, pH 6.4), 1 mM EDTA and 400 mM NaCl. After 2 min incubation at 85°C, the mixture was vortexed briefly, spun, and incubated at 52°C overnight. The next day, 350 µl of RNase mixture containing 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 300 mM NaCl, 40 µg RNase A, and 2 µg RNase T1 was added. After incubation for 1 h at 30°C, the RNases were inactivated by addition of 20 µl 10 % SDS, and 10 µl of 10mg/ml proteinase K at 37°C for 30 min. The mixture was then extracted with an equal volume of water-saturated phenol, and the nucleic acids were precipitated with 100 % EtOH.

Total RNA (200-900 ng) from untransduced, and transduced MT4 cells expressing tRNA$_{AC}$-Rz (0/0), tRNA$_{AC}$-Rz (2/0) tRNA$_{AC}$-Rz (4/0), tRNA$_{AC}$-Rz (8/0), tRNA$_{AC}$-Rz (10/0), tRNA$_{AC}$-InRz, tRNA-Rz, linear Rz, and MoTiN were each used in RNase protections with the antisense probe under the conditions described above.

XVII. DEVELOPMENT OF ECOTROPIC AND AMPHOTROPIC RETROVIRAL VECTOR PARTICLES

The ecotropic ψ-2 packaging cell line was transfected with pUCMoTiN, pUCMoTiN-Rz, pUCMoTiN-tRNA-Rz, pUCMoTiN-$tRNA_{AC}$-InRz and each pUCMoTiN-$tRNA_{AC}$-Rz. Twenty-four hours prior to transfection, 2 x 10^5 ecotropic ψ-2 packaging cells were seeded in 60-mm plates. A solution containing 1-5 μg of DNA and 250 mM $CaCl_2$ in a total volume of 250 μl was added dropwise to 250 μl of 2x HEPES-buffered saline (HBS) containing 280 mM NaCl, 50 mM HEPES (pH 7.05) and 1.5 mM Na_2HPO_4. The mixture was incubated for 30 min at room temperature to allow precipitate formation. After a 30 min incubation at room temperature, the precipitate was added dropwise to the cell monolayer and incubated for 16 h at 37°C and 5 % CO_2. The cells were washed with PBS and allowed to grow under normal conditions for 1 day. The cells were then trypsinized, transferred to 100-mm plates, and grown in selective medium (α-MEM, 10 % FCS and 200 μg/ml G418) until colonies were clearly visualized. After determining the number of resistant colonies, the cells were washed with PBS, trypsinized, reseeded and amplified in selective medium.

The vector particles released from pools of ψ-2 transductants were used to transduce the amphotropic PA317 packaging cell line. Twenty-four hours prior to vector particle collection, the cells were washed and maintained in non-selective media. Vector particles were collected by filtering the culture medium through a 0.2 μm filter and stored at -70°C. 2 x 10^5 amphotropic PA317 packaging cells were transduced by using 500 μl of ecotropic vector particles and 8 μg/ml polybrene in 1 ml DMEM containing 10 % fetal calf serum. After 2 h, 4 ml of medium was added, and selection as described for the ψ-2 cells was applied. The amphotropic vector particles released from the pooled PA317 transductants were collected and frozen at -70°C.

XVIII. STABLE TRANSDUCTION OF HUMAN CD4$^+$ LYMPHOID MT4 CELL LINE WITH RETROVIRAL VECTOR PARTICLES EXPRESSING VARIOUS RIBOZYMES

MT4 cells (2 x 10^6) were transduced with 1 ml of amphotropic vector particles in the presence of 8 μg/ml polybrene for 2 h at 37°C and 5 % CO_2 in 60-mm petri dishes. Fresh RPMI-1640 (4 ml) was then added and the cells were grown overnight. The next day, the transduced cells were centrifuged for 5 min at 200 x *g* and resuspended in 10 ml of selective medium (RPMI-1640, 10 % calf serum, 2 mM L-glutamine, 1x antibiotic/antimycotic, 400 μg/ml G418) and transferred to 50 ml flasks. Half of the medium was removed and replaced with fresh selective medium every 3-4 days. The stable transductants were selected within 21-28 days. Pools of G418 resistant stable MT4 transductants lacking or expressing various $tRNA_{AC}$-Rzs and controls were then selected and analyzed without cloning.

XIX. TRANSDUCTION OF PERIPHERAL BLOOD LYMPHOCYTES

Human PBLs were isolated from blood obtained from a healthy donor using a Ficoll-Hypaque gradient (Pharmacia Biotech Inc; Baie d'Urfé, Canada). PBLs were washed once with PBS and grown at a density of 1 x 10^6 cells/ml in RPMI 1640 medium containing 10 % FBS, 20 units/ml IL-2 (Boehringer Mannheim; Laval, Canada), and 5 μg/ml phytohemagglutinin (Sigma; St. Louis, USA) for 24 h. The suspension cells were collected and grown for 2 more days. PBLs (1 x 10^6) cells were then mixed with amphotropic vector particles (1 ml), 20 units/ml IL-2, 16 μg/ml polybrene, and centrifuged for 1 h at 200 x *g*. After centrifugation, the cells were grown overnight. The transduction was repeated a total of 3 times. After the third transduction, the cells were grown for 1 day in RPMI 1640 medium containing 10 % FBS and 20 units/ml IL-2. Selective medium containing 1 mg/ml G418 was then added.

XX. GENOMIC DNA ISOLATION

Transfected ψ-2, or transduced PA317 and MT4 cells (5 mls cell suspension without counting) were washed with PBS twice and lysed using 350 µl Solution D containing 4 M guanidium isothiocyanate, 25 mM sodium citrate and 0.5 % sarcosyl, 350 µl phenol:choloroform:isoamyl alcohol (25:24:1) and 35 µl 3 M NaAc. The aqueous phase collected after spinning for 1 min at 13000 x *g* was further extracted with 350 µl chloroform:isoamyl alcohol (24:1). Nucleic acid was precipitated at -20°C for 30 min using 350 µl of ice-cold isopropanol.

XXI. CELLULAR RNA ISOLATION

Total RNA from stable MT4 transductants lacking or expressing various $tRNA_{AC}$-Rzs, $tRNA_{AC}$-InRz and other controls were isolated using the acid guanidium thiocyanate-phenol-chloroform extraction described by Chomczynski and Sacchi (1987). After lysing cells by vortexing in the presence of 700 µl Solution D, the cell lysate was extracted using 10 µl of 2-mercaptoethanol (ME), 700 µl water-saturated phenol, 280 µl chloroform:isoamyl alcohol (24:1) and 70 µl 2 M NaAc pH 4.0. The mixture was incubated on ice for 10 min and then centrifuged for 10 min at 1100 x *g*. The aqueous phase was collected and further extracted with 700 µl chloroform:isoamyl alcohol (24:1). After spinning for 5 min at 13000 x *g*, the nucleic acid was precipitated from the aqueous phase using 700 µl of isopropanol for 1 h at -20°C. The pellet was washed once with 500 µl of 70 % EtOH, dried and resuspended in 31 µl of water.

Any residual DNA was digested for 15 min at 37°C using 4 units of RNase-free DNase (Promega Corp.; Madison, USA) in the presence of 20 mM $MgCl_2$ and 10 mM DTT. The digestion mixture was then extracted with 300 µl Solution D, 5 µl 2-ME, 350 µl water-saturated phenol, 140 µl

chloroform:isoamyl alcohol (24:1) and 35 µl 2 M NaAc pH 4.0. After spinning for 5 min at 13000 x *g*, the aqueous phase was collected and further extracted with 700 µl chloroform:isoamyl alcohol (24:1). The nucleic acid was then precipitated from the aqueous phase using 700 µl of isopropanol for 1 h at -20°C. The pellet was washed once with 500 µl of 70 % EtOH, dried and resuspended in 20-40 µl of water.

Alternatively, cellular RNA was isolated using by using the RNA/DNA kit (Qiagen; Santa Clarita, USA) following instructions from the supplier. This kit allows sequential isolation of LMW RNA, HMW RNA and DNA from animal cells. MT4 cells were pelleted by centrifugation at 300 x *g* for 5 min, and lysed using 500 µl buffer QRL1 containing guanidium thiocyanate and 0.1 M 2-ME. The sample was vortexed for 10 sec and homogenized by passing 3-4 x through an 18-gauge needle. Proteins were precipitated by adding 500 µl buffer QRV1, and eliminated by centrifugation for 20 min at 15000 x *g*. The supernatant was collected, and the nucleic acids were precipitated using 800 µl ice-cold isopropanol for 5 min on ice. The nucleic acids were pelleted by centrifugation for 30 min at 15000 x *g*, and resuspended in 150 µl buffer QRL1 by heating for 3 min at 60°C followed by vortexing for 5 sec. After adding 1.35 ml buffer QRV2, the sample was centrifuged for 5 min at 5000 x *g* to remove undissolved particles. The sample was then applied to a Qiagen-tip that has been equilibrated with buffer QRE. After washing contaminants away using 2 ml buffer QRW, LMW RNA was eluted with 1 ml buffer QRW2 containing 750 mM NaCl, 50 mM MOPS (pH 7.0), and 15 % EtOH. HMW RNA was then eluted using 1 ml buffer QRU preheated at 45°C. LMW and HMW RNA were precipitated for 10 min on ice using equal volume of ice-cold isopropanol. RNAs were then pelleted by centrifugation for 30 min at 15000 x *g*, washed twice with 500 µl 70 % EtOH, dried and resuspended in water.

XXII. DETERMINATION OF HIV-1 TITER BY AN INFECTIVITY ASSAY

HeLa-CD4-LTR-β-gal cells were grown overnight in 12-well plates at 8 x 10^4 cells per well in DMEM with 10 % calf serum. The next day, the medium was removed, and the cells were incubated with 5 µl or 50 µl of the viral stock in a total volume of 300 µl in the presence of 20 µg/ml DEAE-dextran (Pharmacia Biotech Inc; Baie d'Urfé, Canada) for 2 h at 37°C. The plate was gently rocked every 30-45 min. After incubation, 1 ml DMEM with 10 % calf serum was added, and the cells were grown for 2 days. The cells were then fixed at room temperature for 5 min with 2 ml of a solution containing 1 % formaldehyde and 0.2 % glutaraldehyde in PBS. The cells were washed 3 x with PBS, and stained for 50 min at 37°C with 500 µl of a solution containing 4 mM $K_3Fe(CN)_6$, 4 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, and 0.4 mg/ml X-gal. After washing 3 x with PBS, the number of blue cells was counted using an inverted microscope at 100x magnification.

XXIII. HIV-1 SUSCEPTIBILITY OF STABLE MT4 TRANSDUCTANTS

The pools of stable MT4 transductants lacking or expressing various $tRNA_{AC}$-Rzs, $tRNA_{AC}$-InRz, tRNA-Rz, and linear Rz were each infected with HIV-1 strain NL4-3 (Adachi et al 1986) as described previously (64, 65, Cohli *et al.*, 1994). A 2 ml cell culture of actively dividing MT4 cells (2 x 10^6 cells/ml) expressing the ribozymes were infected with HIV-1 strain NL4-3 at a multiplicity of infection (m.o.i.) of 0.1 and 1 at room temperature for 2 h. The cells were pelleted, washed 3 times with PBS, resuspended in 3 ml of medium, transferred to 12-well plates and allowed to grow at 37°C. Every 3 days, a 1 ml sample containing cells and medium from each infected cell culture was removed, frozen at -70°C and replaced with fresh complete medium to a total of 3 mls. After day 30, the frozen samples were thawed and centrifuged at 250 x *g* for 5

min. The amount of HIV-1 p24 antigen present in the cell culture supernatants was determined by a p24 ELISA kit (Abbott; Chicago, USA).

RESULTS

I. DEVELOP IMPROVED $tRNA_3^{Lys}{}_{AC}$-Rz VIA SELECTION *IN VITRO*

A.Design and *in vitro* selection strategy

A population of $tRNA_{AC}$-Rzs that contained 5' and 3' linkers connecting the ribozyme to the anticodon loop was generated. Each RNA within this population contained the $tRNA_3^{Lys}$ with the ribozyme and the flanking sequence as well as the ribozyme target site. However, each RNA varied in the length and sequence of 5' and 3' linkers connecting the ribozyme and its flanking sequences to the anticodon loop within the $tRNA_3^{Lys}$. The 5' and the 3' linkers ranged in size from 0 to 20 nucleotides and possessed random sequences. These linkers varied in length with an increment of two, providing eleven different lengths of 5' linkers that could combine with any of the eleven 3' linkers. The three nucleotides on either side of the anticodon loop (5'-CUU-3' and 5'-UAA-3') were retained and not counted as part of the 5' or 3' linker (Fig. 7).

The ribozyme target site was provided *in cis* with enough nucleotides between the $tRNA_{AC}$-Rz and the target site to allow hybridization. It was hypothesized that this design would allow $tRNA_{AC}$-Rzs which contain the ideal combinations of linkers to freely recognize and cleave the target site *in cis*. As the frequency of *cis* cleavage is expected to be much greater than that of *trans* cleavage, the 5' cleavage products should mainly consist of active $tRNA_{AC}$-Rzs.

Active $tRNA_{AC}$-Rzs that allow *cis* cleavage of the downstream target site could be easily separated from the uncleaved or inactive ribozymes. These $tRNA_{AC}$-Rzs were cloned and characterized to determine whether they (i) can cleave a target RNA *in trans*, (ii) maintain catalytic activity similar to that of a linear ribozyme, (iii) are as stable as wild type $tRNA_3^{Lys}$, (iv) retain promoter elements (appropriate distance between boxes A and B) that permit RNA pol III-

driven transcription, and (v) inhibit HIV-1 replication in a CD4+ human T cell line compared to an inactive tRNA-ribozyme or the vector alone without a ribozyme.

The ribozyme used for *in vitro* selection was targeted against a highly conserved region within the *env*-coding region of HIV-1 RNA. The choice of this target site was based on previous *in vitro* comparisons of ribozymes (Ramezani and Joshi, 1996). However, once the structures which are advantageous to ribozyme cleavage are determined, $tRNA_{AC}$-Rzs could be easily designed to target other RNAs.

B. Template DNA library encoding different $tRNA_{AC}$-Rzs with 0-20 nt-long random sequence linkers

Six PCRs were performed to construct the template DNA library (Fig. 8*B*). First-round PCR products were used as overlapping PCR templates in order to obtain the final PCR products which were 988-1028 bp. Each template in this population contained a T7 promoter, a $tRNA_{AC}$-Rz with random-sequence, variable-length 5' and 3' linkers in the $tRNA_3^{Lys}$ anticodon loop, and the ribozyme target site.

C. Controls

1. To confirm greater occurrence of *cis* over *trans* cleavage

Although the $tRNA_{AC}$-Rzs and the ribozyme target site were placed *in cis* such that the ribozyme would be able to cleave its target site, the ribozyme can also hybridize and cleave *in trans* a target site located in another RNA. Because the *in vitro* selection strategy was dependent on an active $tRNA_{AC}$-Rz cleaving its target site *in cis*, it must be ensured that *trans*-cleavage was not taking place under the experimental conditions utilized. To verify that the 5' cleavage product would correspond to active $tRNA_{AC}$-Rzs cleaving *in cis*, a cleavage reaction was performed

wherein both *cis* and *trans* target sites were provided to the ribozyme. Upon *in vitro* transcription of PCR products containing Rz_{Env}-Env, Rz_{Env} should cleave the ribozyme target site located downstream. A target RNA was provided during *in vitro* transcription which lacked a *cis* acting ribozyme and could only be cleaved as a result of *trans* cleavage. This target RNA was designed such that if it were cleaved, the *trans* cleavage products would possess sizes different from the products of *cis* cleavage.

Upon analysis of the *in vitro* transcription reaction products by PAGE, 5' (73 nts) and 3' (62 nts) *cis* cleavage products were observed (Fig. 9). No 5' (163 nts) and 3' (170 nts) *trans* cleavage products could be detected. Because Rz_{Env} was equally capable of cleaving the ribozyme target site present within the same RNA or in another RNA, under the conditions used and for *in vitro* transcription (in the absence of a separate *trans* cleavage reaction) the cleavage products must be resulting from *cis* cleavage.

2. To confirm that $tRNA_{AC}$-Rzs present in the library are able to cleave a target RNA *in trans*

Although selection was based on the *cis* cleavage ability of active $tRNA_{AC}$-Rzs, the selected $tRNA_{AC}$-Rzs would be eventually used to cleave target RNAs *in trans*. Thus, before proceeding to selection, the pool of $tRNA_{AC}$-Rzs (113-153 nts) and the ribozyme target RNA (434 nts) were separately transcribed and used in a *trans*-cleavage reaction. Both the 5' (264 nts) and 3' (170 nts) *trans* cleavage products were observed (Fig. 10). This result confirmed that the $tRNA_{AC}$-Rzs present in the template DNA library could cleave the target RNA *in trans*. The extra band corresponding to the 3' cleavage product may have resulted from cleavage of premature transcription products.

FIG. 9. ***Cis vs trans*** **cleavage by $tRNA_{AC}$-Rzs transcribed from the template DNA library.** PCR DNA was used for *in vitro* transcription incubated at increasing time intervals (0.5, 1, 1.5 or 2 h). The target RNA (333 nts) containing the ribozyme target site was added to each transcription mixture. The full-length transcript (135 nts) along with the 5' (73 nts) and 3' (62 nts) *cis* cleavage products were detected, but not the products (170 nts and 163 nts) which would result from *trans* cleavage. T, target RNA alone. The uncleaved target RNA in lane "0.5h" and 5' cleavage product in lane "2h" have been excised from the gel and used for subsequent experiments.

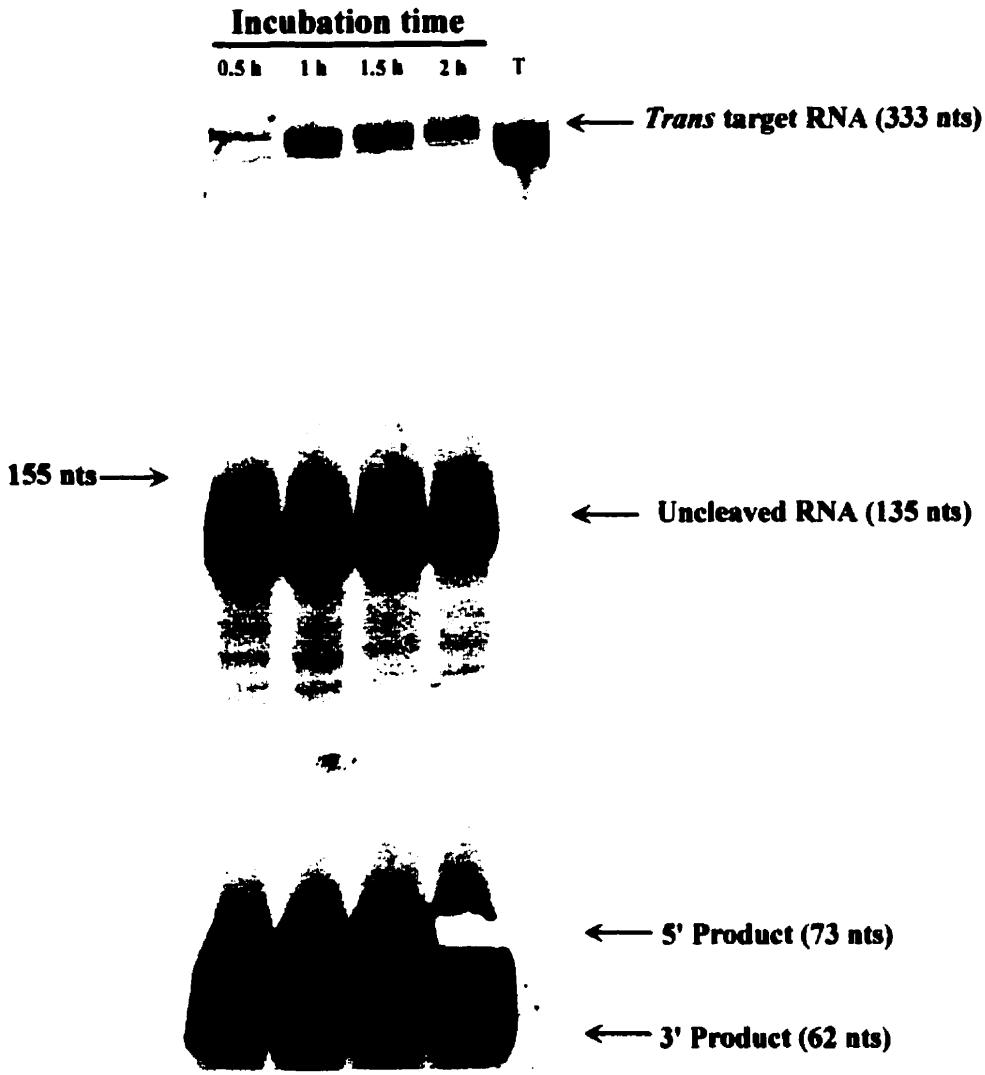

Incubation time
0.5 h
1 h
1.5 h
2 h
T
Trans target RNA (333 nts)
155 nts
Uncleaved RNA (135 nts)
5' Product (73 nts)
3' Product (62 nts)

Figure 9.

FIG. 10. **Ability of the pool of $tRNA_{AC}$-Rzs transcribed from the template DNA library to cleave the target RNA *in trans*.** The ^{32}P-labeled target RNA (434 nts) was cleaved by the $tRNA_{AC}$-Rzs and resulted in 5' (264 nts) and 3' (170 nts) cleavage products. The lower band of the two 3' cleavage products may have resulted from cleavage of premature transcription products. -, target RNA by itself; +, *trans* cleavage reaction.

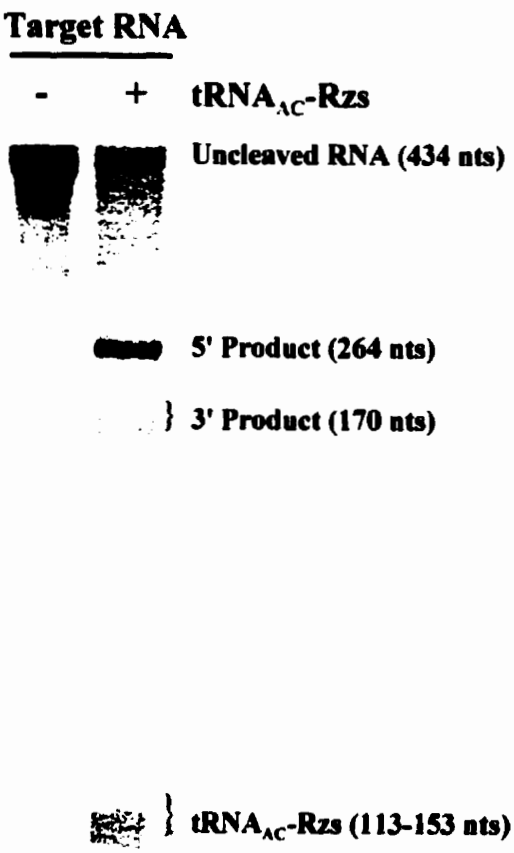

Target RNA
-
+
tRNA$_{AC}$-Rzs
Uncleaved RNA (434 nts)
5' Product (264 nts)
3' Product (170 nts)
tRNA$_{AC}$-Rzs (113-153 nts)

Figure 10.

D. *In vitro* transcription of DNA templates expressing $tRNA_{AC}$-Rzs and *cis* cleavage reaction

In order to identify the 5' *cis* cleavage products resulting from *cis* cleavage, a 5' product size marker was prepared as follows. The region in the template DNA library from the T7 promoter to the ribozyme cleavage site (Fig. 11*A*) was PCR-amplified and *in vitro* transcribed. The resulting transcript would then correspond in size to the 5' cleavage products.

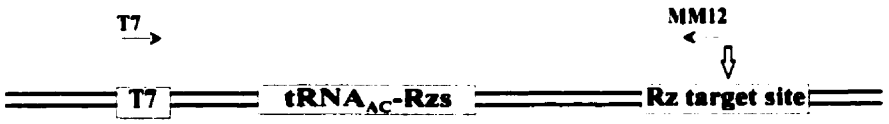
The $tRNA_{AC}$-Rz template DNA library was transcribed *in vitro* resulting in a pool of RNAs each containing a $tRNA_{AC}$-Rz and the ribozyme target site. As shown previously, active $tRNA_{AC}$-Rzs capable of cleaving the target site *in cis* were able to do so during *in vitro* transcription. Cleavage products were observed upon analysis of the *in vitro* transcripts by PAGE (Fig. 11*B*). The majority of the 5' cleavage products must have resulted from *cis* cleavage by the $tRNA_{AC}$-Rzs of the target sequence present in the same RNA molecule, and not from *trans* cleavage by $tRNA_{AC}$-Rzs present in another RNA molecule. The band corresponding to the 5' cleavage products which co-migrated with the 5' product marker was cut, and the RNA eluted and stored at -20°C (Fig. 12).

E.Cloning of selected $tRNA_{AC}$-Rzs

After elution, the 5' cleavage products were RT-PCR amplified and cloned in pGEM4Z. Clones were screened by PCR and by restriction enzyme analysis. Twenty-one independent clones containing different $tRNA_{AC}$-Rzs with variable linkers were isolated (Table 3).

FIG. 11. **Isolation of active $tRNA_{AC}$-Rzs resulting from *cis* cleavage of $tRNA_{AC}$-Rzs transcribed from the template DNA library.** *A*, A marker was prepared which would correspond in size to the 5' cleavage products upon transcription and *cis* cleavage of the template DNA library. The 5' cleavage products correspond to the region starting from the 5' end of the RNA where transcription was initiated, and ending at the cleavage site (⇩). PCR primers were therefore designed which hybridized to the T7 promoter sequence (T7 primer) and to sequences just before the cleavage site (MM12). The resulting PCR product was then transcribed *in vitro*. *B*, *In vitro cis* cleavage by the pool of $tRNA_{AC}$-Rzs. The 5' product marker (373-413 nts) corresponding to the size of the expected product was analyzed in parallel. The 3' cleavage products ran out of the gel and are therefore not visible.

A


T7
MM12
T7
tRNA$_{AC}$-Rzs
Rz target site

B

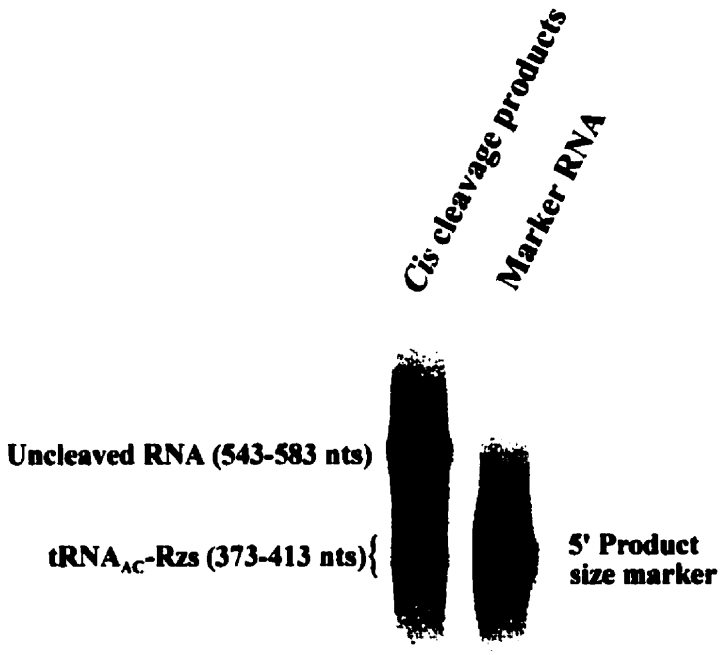

Cis cleavage products
Marker RNA
Uncleaved RNA (543-583 nts)
tRNA$_{AC}$-Rzs (373-413 nts)
5' Product size marker

Figure 11.

FIG. 12. **Conversion of the isolated $tRNA_{AC}$-Rzs into double-stranded DNA.** The pool of selected $tRNA_{AC}$-Rzs present in the 5' cleavage products was reverse-transcribed using MM20 primer. The RT-PCR product is shown. The cDNA was then PCR amplified using MM18/MM20 primer pair and cloned in the pGEM4Z plasmid.

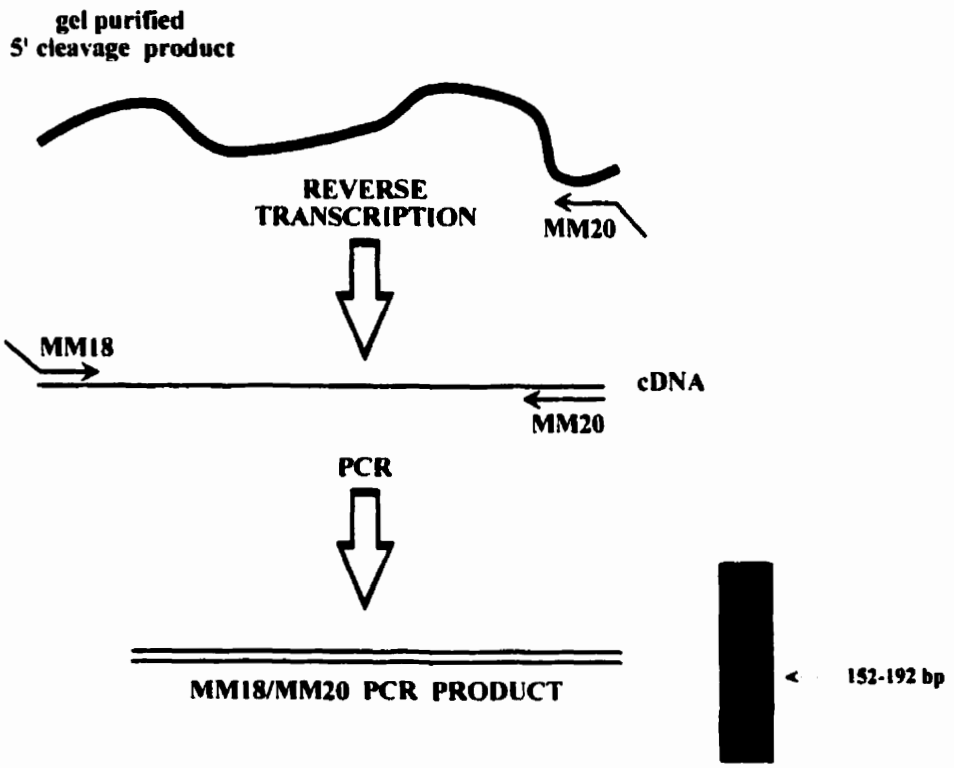

gel purified
5' cleavage product
REVERSE
TRANSCRIPTION
MM20
MM18
cDNA
MM20
PCR
MM18/MM20 PCR PRODUCT
152-192 bp

Figure 12.

TABLE 3.

Length, sequence and relative cleavage activity of various $tRNA_{AC}$-Rzs

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

*Clones selected for further characterization. For $tRNA_{AC}$-Rz (0/0), one among the six clones was used for further experiments.

***Trans* cleavage reactions were performed for 10 min at 37°C as described in the Materials and Methods. Relative cleavage activity was then determined by normalizing these values to the % target RNA cleaved by the linear ribozyme, which was 38%.

NA,Not applicable.

F.Characterization of selected $tRNA_{AC}$-Rzs

1. Electrophoretic mobility of $tRNA_{AC}$-Rzs transcribed *in vitro*

The $tRNA_{AC}$-Rzs from the selected clones were transcribed *in vitro* and analyzed by PAGE (Fig. 13). The $tRNA_{AC}$-Rzs possessed variable sizes due to the different linkers present in each $tRNA_{AC}$-Rz. The smaller transcript present in each sample could have resulted from transcription of shorter PCR products. Transcripts expressing $tRNA_3^{Lys}$, linear ribozyme and the target RNA were also produced.

2. Identification of active $tRNA_{AC}$-Rzs allowing *trans* cleavage

The $tRNA_{AC}$-Rzs, linear ribozyme and $tRNA_3^{Lys}$ were each incubated with equimolar amounts of the unlabeled target RNA to allow *trans* cleavage for 2h (Fig. 14*A*). All of the cloned $tRNA_{AC}$-Rzs were active except for two which failed to cleave. The *trans* cleavage reactions were also performed using a [γ-^{32}P]-labeled target RNA (Fig. 14*B*). All of the $tRNA_{AC}$-Rzs, except for the two inactive ones (clones 2 and 3), were also able to cleave the labeled target RNA. To compare initial rates of cleavage among all $tRNA_{AC}$-Rzs, *trans* cleavage reactions were performed for 10 min (Fig. 15, Table 3). Variable rates were observed of initial cleavage were observed.

3. Linker sequences of selected $tRNA_{AC}$-Rzs

a. 5' and 3' linkers of each $tRNA_{AC}$-Rz

Plasmid DNA from each clone was used as a template for a PCR using primers which would allow quick identification of the lengths of the 5' and 3' linkers present in each $tRNA_{AC}$-Rz. Upon amplification of the 3' linker region of $tRNA_{AC}$-Rzs present in the template DNA library, bands of identical size were observed, indicating that all possessed 3' linkers of the same length (data not shown). Upon amplification of $tRNA_{AC}$-Rzs including both 5' and 3' linker regions, bands of

FIG. 13. **Different $tRNA_{AC}$-Rzs transcribed from various clones.** The $tRNA_{AC}$-Rzs were PCR-amplified, transcribed *in vitro* and analyzed by PAGE. The $tRNA_{AC}$-Rzs possessed variable sizes due to the different linkers present in each. The numbers above each lane correspond to the clone number of the respective $tRNA_{AC}$-Rz. M, 0.16-1.77 kb RNA ladder.

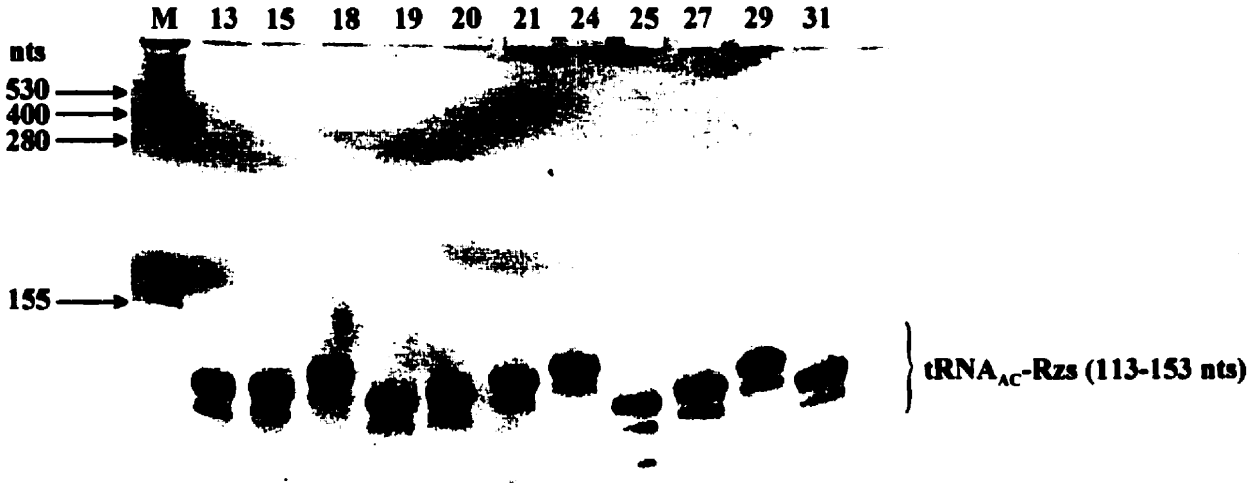

M 13 15 18 19 20 21 24 25 27 29 31
nts
530
400
280
155
tRNA$_{AC}$-Rzs (113-153 nts)

Figure 13.

FIG. 14. ***Trans*** **cleavage reactions using $tRNA_{AC}$-Rzs transcribed from the $tRNA_{AC}$-Rz clones.** *A*, Transcripts containing each cloned $tRNA_{AC}$-Rz (113-153 nts) and target RNA (333 nts) were produced by *in vitro* transcription of PCR amplified templates containing the T7 promoter. Cleavage products were analyzed by PAGE. RNA bands were visualized by staining with methylene blue. T, target RNA. *B*, *Trans* cleavage reactions were performed using a [α-^{32}P] 5'-end labeled target and unlabeled $tRNA_{AC}$-Rzs. WT, wild type $tRNA_3^{Lys}$ incubated with the target RNA. L, linear hammerhead ribozyme incubated with the target RNA. The numbers above each lane in *A* and *B* correspond to the clone number of the respective $tRNA_{AC}$-Rz.

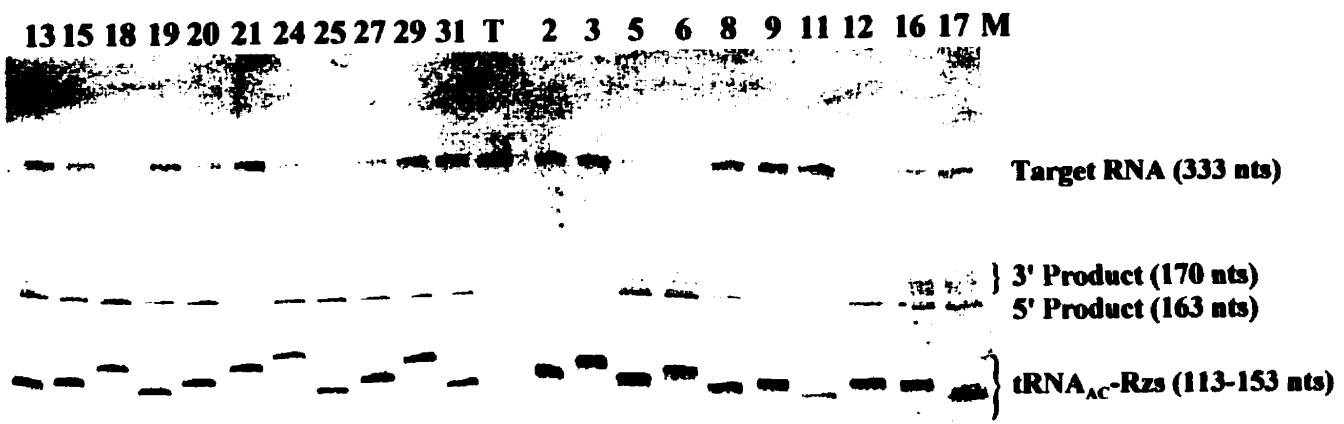


Figure 14*A*.

111


2 3 5 6 8 9 11 12 13 15 L WT
16 17 18 19 20 21 24 25 27 29 31
Target RNA (333 nts)
5' Product (163 nts)

Figure 14*B*.

FIG. 15. **Initial rates of *trans* cleavage for $tRNA_{AC}$-Rzs.** Initial rates of cleavage by various $tRNA_{AC}$-Rzs were measured by performing *trans* cleavage reactions for 10 min. The numbers above each lane correspond to the clone number of the respective $tRNA_{AC}$-Rz.


17 31 27 25 24 20 19 16 15 11 9 8
Target RNA (333 nts)
3' Product (170 nts)
5' Product (163 nts)

Figure 15.

variable sizes were observed (data not shown). Since all of the 3' linkers were of the same length, the variable size must be due to 5' linkers of different lengths.

These results were confirmed upon sequencing of the $tRNA_{AC}$-Rzs (Table 3). Sixteen $tRNA_{AC}$-Rzs were found not to possess 3' linkers. These were classified based on the length of the 5' linkers as $tRNA_{AC}$-Rz (0/0), $tRNA_{AC}$-Rz (2/0), $tRNA_{AC}$-Rz (4/0), $tRNA_{AC}$-Rz (8/0), $tRNA_{AC}$-Rz (10/0) and $tRNA_{AC}$-Rz (16/0). The $tRNA_{AC}$-Rzs with 5' linkers of a given length were not identical as these linkers varied in their sequence composition (Table 3). Two $tRNA_{AC}$-Rzs possessed 8/2 and 6/6 nt-long 5' and 3' linkers. The two inactive $tRNA_{AC}$-Rzs (clones 2 and 3) that failed to cleave contained mutations (Fig. 7) in one of the conserved residues of the ribozyme catalytic domain. One possessed a deletion in the stem loop II region of the hammerhead catalytic domain and was not analyzed further.

b. 3' linkers of $tRNA_{AC}$-Rzs present in the template DNA library

In order to determine whether the absence of 3' linkers was due to lack of variability, the 3' linker regions of the $tRNA_{AC}$-Rzs in the template DNA library were amplified. Products of different sizes were observed upon PAGE (Fig. 16). This confirmed that the absence of 3' linkers in the cloned $tRNA_{AC}$-Rzs was due to the selection procedure, and not preferential amplification of a 3' linker length during construction of the template DNA library.

4. Kinetics of *trans* cleavage by selected $tRNA_{AC}$-Rzs

$tRNA_{AC}$-Rz (0/0), $tRNA_{AC}$-Rz (2/0), $tRNA_{AC}$-Rz (4/0), $tRNA_{AC}$-Rz (8/0) and $tRNA_{AC}$-Rz (10/0) with 0, 2, 4, 8 and 10 nt-long 5' linkers (Table 3) were each used in a *trans* cleavage reaction. A linear ribozyme and $tRNA_3^{Lys}$ served as positive and negative controls, respectively.

FIG. 16. **Length of the 3' linkers of $tRNA_{AC}$-Rzs present in the template DNA library.** The 3' linker regions of the $tRNA_{AC}$-Rzs in the template DNA library were amplified using Cat^+ and MM13 primer pair. Lane 1, 10 bp ladder. Lane 2, PCR amplification.

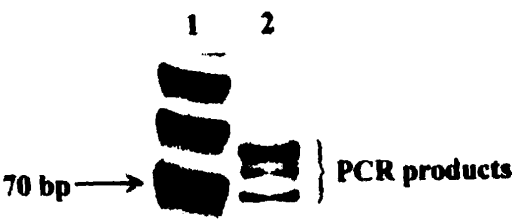


Figure 16.

Compared to the linear ribozyme, each $tRNA_{AC}$-Rz cleaved a similar amount of total target RNA by 5 h (Fig. 17*A*). The $tRNA_{AC}$-Rz (0/0) without any linkers demonstrated the slowest rate of cleavage. All the other $tRNA_{AC}$-Rzs possessed similar initial rates of cleavage. This experiment was repeated at least twice with similar results.

The $tRNA_{AC}$-Rzs with 3' linkers, $tRNA_{AC}$-Rz (8/2) and $tRNA_{AC}$-Rz (6/6) were also used in *trans* cleavage reactions. Both of these $tRNA_{AC}$-Rzs demonstrated cleavage rates lower than that observed for the linear ribozyme (Fig. 17*B*). In addition, $tRNA_{AC}$-Rz (8/2) with a 2 nt-long 3' linker possessed a cleavage rate lower than that of $tRNA_{AC}$-Rz (8/0) which does not contain a 3' linker (Fig. 17*A*).

To further compare the catalytic activities of the linear ribozyme and $tRNA_{AC}$-Rzs, *trans* cleavage reactions were performed using the same amount of either linear ribozyme or $tRNA_{AC}$-Rz (10/0) but with varying concentrations of target RNA. The kinetic constants for both the linear ribozyme and $tRNA_{AC}$-Rz (10/0) were very similar (Fig. 18).

5. Secondary structure of selected $tRNA_{AC}$-Rzs

The program mfold (Mathews et al 1999, Zuker et al 1999) was used to predict the secondary structure of $tRNA_{AC}$-Rzs. (Fig. 19). This program was capable of predicting the cloverleaf structure of $tRNA_3^{Lys}$. There was no correlation with *trans* cleavage ability and the structure of $tRNA_{AC}$-Rzs. $tRNA_{AC}$-Rz (0/0), $tRNA_{AC}$-Rz (2/0), and $tRNA_{AC}$-Rz (8/0) possessed similar structures, while $tRNA_{AC}$-Rz (4/0), $tRNA_{AC}$-Rz (10/0), $tRNA_{AC}$-Rz (8/2), and $tRNA_{AC}$-Rz (6/6) possessed structures similar to the wt $tRNA_3^{Lys}$.

FIG. 17. **Kinetics of *trans* cleavage reactions for $tRNA_{AC}$-Rzs.** *Trans* cleavage kinetics for $tRNA_{AC}$-Rzs with 5' linkers (*A*) or with 3' linkers (*B*). Aliquots were taken at the indicated time intervals and analyzed by PAGE. The intensities of the uncleaved and cleaved products were measured using a Phosphorimager. % *Trans* cleavage = cleavage product / uncleaved target RNA x 100.

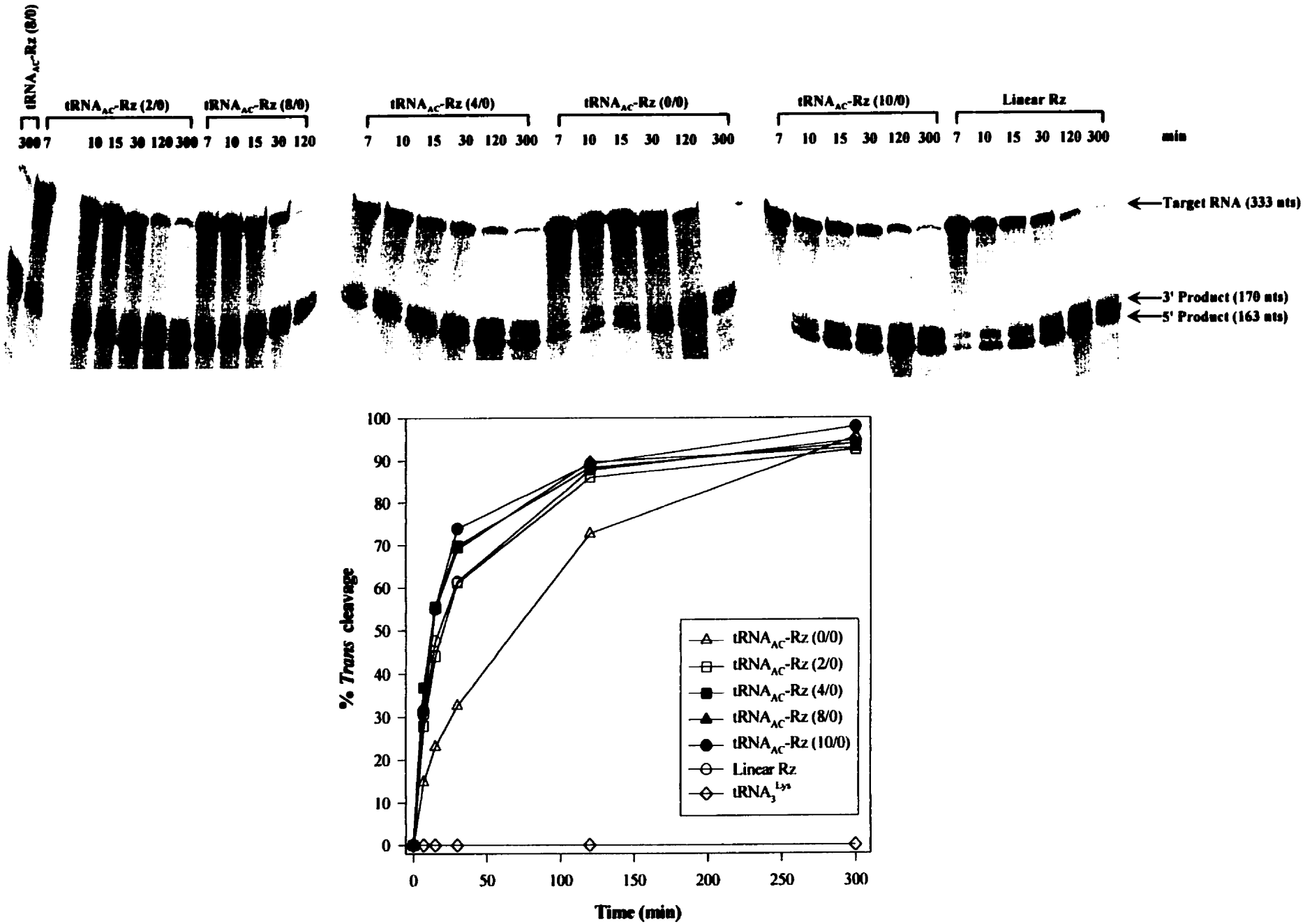
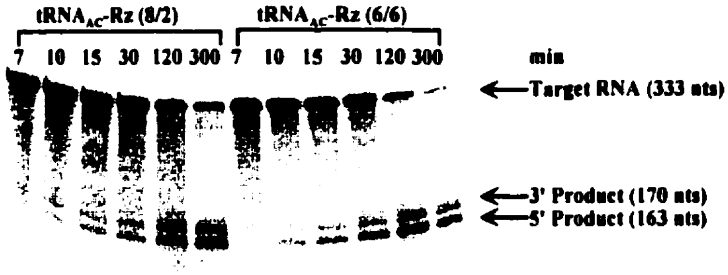

tRNA$_{AC}$-Rz (8/0)
tRNA$_{AC}$-Rz (2/0)
tRNA$_{AC}$-Rz (8/0)
tRNA$_{AC}$-Rz (4/0)
tRNA$_{AC}$-Rz (0/0)
tRNA$_{AC}$-Rz (10/0)
Linear Rz
300 7 10 15 30 120 300 7 10 15 30 120
7 10 15 30 120 300 7 10 15 30 120 300
7 10 15 30 120 300 7 10 15 30 120 300
min
Target RNA (333 nts)
3' Product (170 nts)
5' Product (163 nts)
% Trans cleavage
Time (min)
0 50 100 150 200 250 300
0 10 20 30 40 50 60 70 80 90 100
tRNA$_{AC}$-Rz (0/0)
tRNA$_{AC}$-Rz (2/0)
tRNA$_{AC}$-Rz (4/0)
tRNA$_{AC}$-Rz (8/0)
tRNA$_{AC}$-Rz (10/0)
Linear Rz
tRNA$_3^{Lys}$

Figure 17*A*.


tRNA$_{AC}$-Rz (8/2)
tRNA$_{AC}$-Rz (6/6)
7 10 15 30 120 300 7 10 15 30 120 300
min
Target RNA (333 nts)
3' Product (170 nts)
5' Product (163 nts)

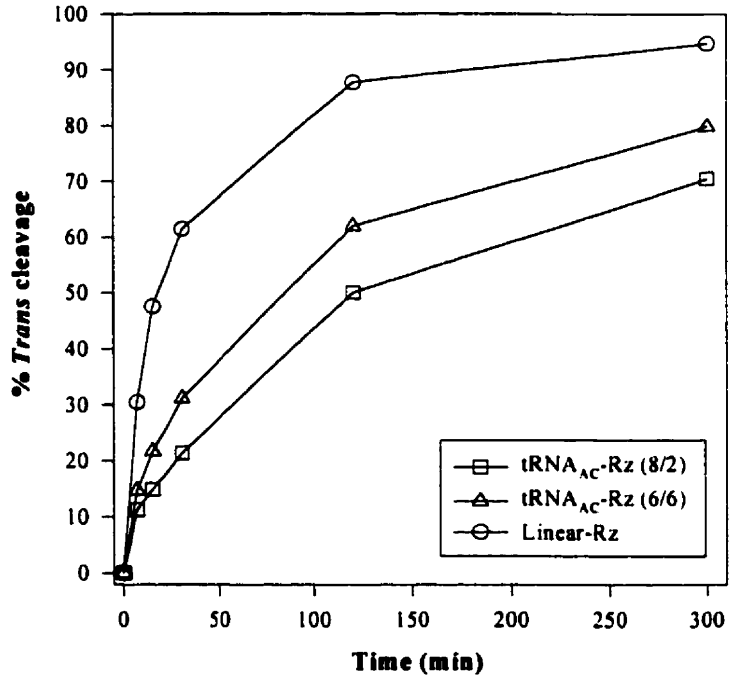
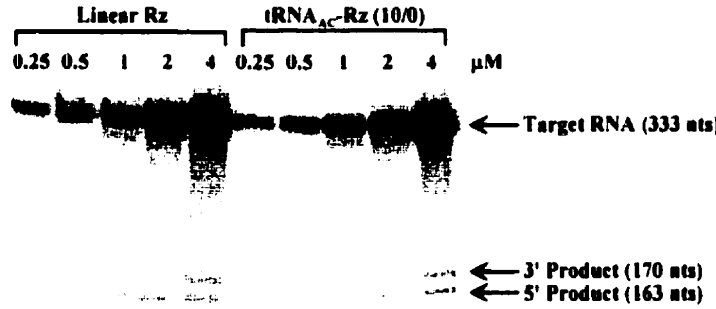

% Trans cleavage
Time (min)
tRNA$_{AC}$-Rz (8/2)
tRNA$_{AC}$-Rz (6/6)
Linear-Rz
0
10
20
30
40
50
60
70
80
90
100
0
50
100
150
200
250
300

Figure 17*B*.

Fig. 18. **Eadie-Hofstee plot for Linear Rz and $tRNA_{AC}$-Rz (10/0).** *Trans* cleavage reactions using 0.25 μM of each ribozyme and increasing amounts of [α-^{32}P]-labeled target RNA were performed for 5 min. Products were analyzed by 8 M urea-8 % PAGE followed by exposure to a phosphor screen. Band intensities of uncleaved RNA and cleavage products were measured using ImageQuant software. Vo and Vo/S were calculated and plotted.


Linear Rz
tRNA$_{AC}$-Rz (10/0)
0.25 0.5 1 2 4 0.25 0.5 1 2 4 µM
Target RNA (333 nts)
3' Product (170 nts)
5' Product (163 nts)

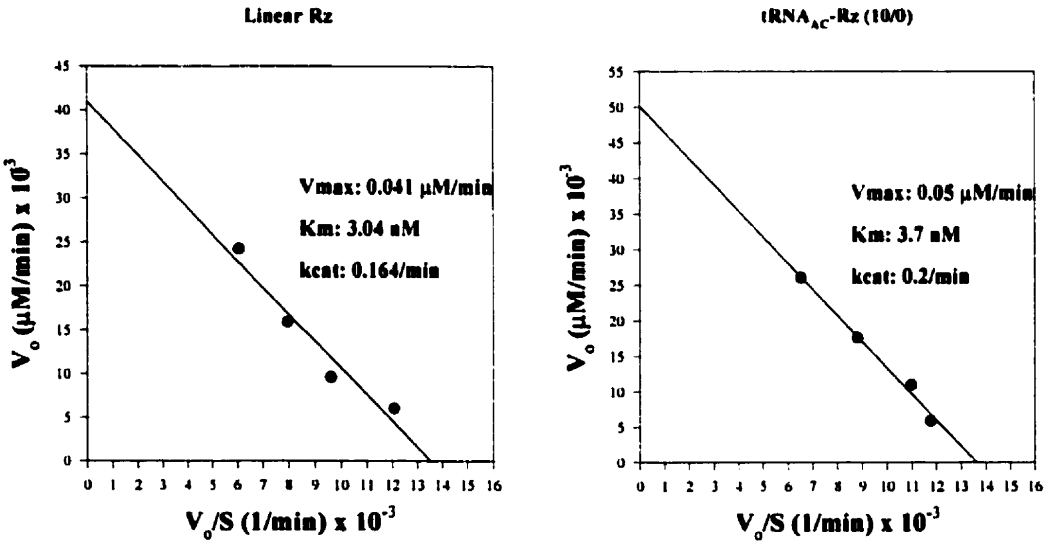
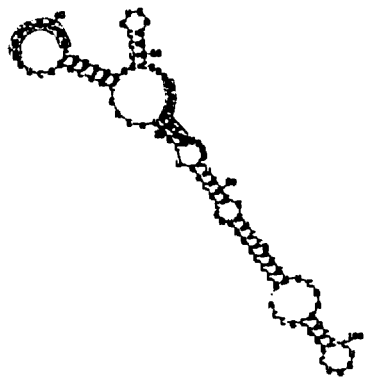
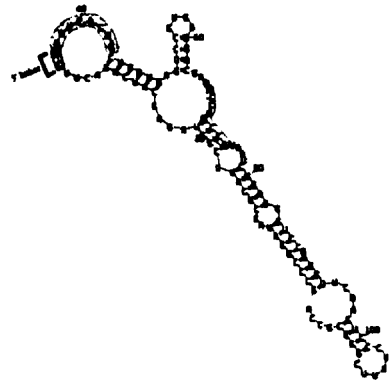

Linear Rz
Vmax: 0.041 µM/min
Km: 3.04 nM
kcat: 0.164/min
V_o (µM/min) x 10^{-3}
V_o/S (1/min) x 10^{-3}
tRNA$_{AC}$-Rz (10/0)
Vmax: 0.05 µM/min
Km: 3.7 nM
kcat: 0.2/min
V_o (µM/min) x 10^{-3}
V_o/S (1/min) x 10^{-3}

Figure 18.

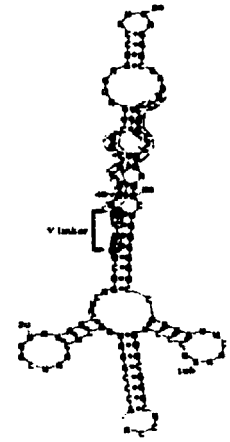
FIG. 19. **Secondary structures of selected $tRNA_{AC}$-Rzs.** The mfold program was used to render secondary structural models of $tRNA_{AC}$-Rz (0/0), $tRNA_{AC}$-Rz (2/0), $tRNA_{AC}$-Rz (4/0), $tRNA_{AC}$-Rz (8/0), $tRNA_{AC}$-Rz (10/0), $tRNA_{AC}$-InRz, $tRNA_{AC}$-Rz (8/2), and $tRNA_{AC}$-Rz (6/6). The 8-nt 5' and 8-nt 3' flanking sequences of the ribozyme are crosshatched. The 5' and 3' linker regions are also indicated by brackets. The calculated energy is shown underneath each structure.



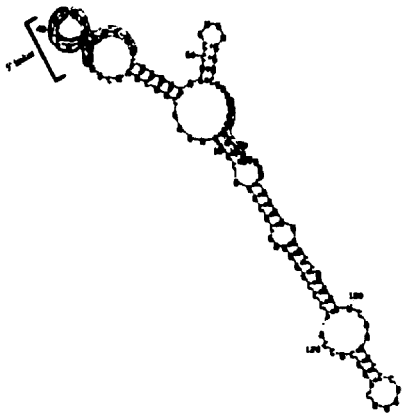
$tRNA_{AC}$-Rz (0/0)
Energy = -34.5



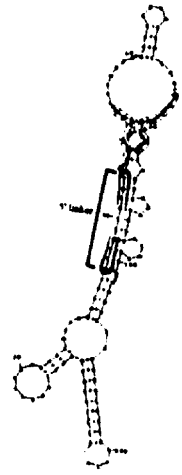
$tRNA_{AC}$-Rz (2/0)
Energy = -34.3



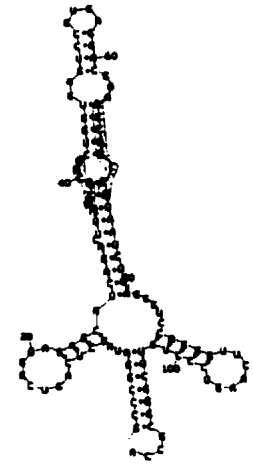
$tRNA_{AC}$-Rz (4/0)
Energy = -36.7



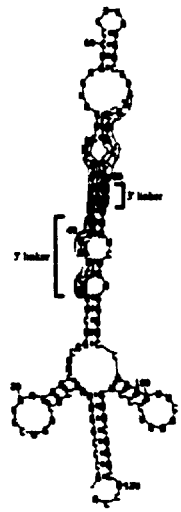
$tRNA_{AC}$-Rz (8/0)
Energy = -34.8



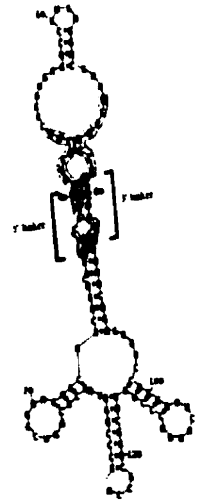
$tRNA_{AC}$-Rz (10/0)
Energy = -34.8



$tRNA_{AC}$-InRz
Energy = -46.0



$tRNA_{AC}$-Rz (8/2)
Energy = -38.1



$tRNA_{AC}$-Rz (6/6)
Energy = -34.4

Figure 19.

6. Stability of selected $tRNA_{AC}$-Rzs

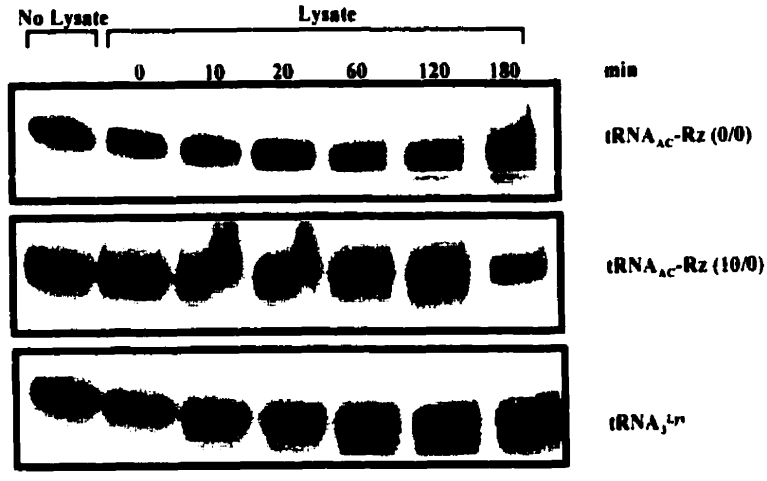
To compare the stability of ^{32}P-labeled $tRNA_{AC}$-Rzs with ^{32}P-labeled $tRNA_3^{Lys}$, $tRNA_{AC}$-Rz (0/0), $tRNA_{AC}$-Rz (10/0) and $tRNA_3^{Lys}$ were each incubated with a cell lysate (500 μg/ml) obtained from MT4 cells, a human CD4+ T cell line, and the reactions were stopped using a buffer containing unlabeled yeast tRNA followed by chilling on dry ice. Both $tRNA_{AC}$-Rz (0/0) and $tRNA_{AC}$-Rz (10/0) appeared to be very stable (Fig. 20). The half-lives of $tRNA_{AC}$-Rz (0/0), $tRNA_{AC}$-Rz (10/0) and $tRNA_3^{Lys}$ in the presence of MT4 cell lysate were 50 min, 80 min and 25 min, respectively. $tRNA_{AC}$-Rz (10/0) was also incubated in the absence of cell lysate for up to 10 min. No RNA degradation was observed. Furthermore, $tRNA_{AC}$-Rzs used for *trans* cleavage reactions for 2 hrs also remained intact when analyzed by PAGE (Fig. 14*A*).

This experiment has also been performed wherein the reactions were not stopped completely using a buffer which contained 5 mM EDTA followed by incubation on ice. Under these conditions, the RNAs were degraded rapidly (data not shown), even after the reaction was stopped. In this instance, half-lives could not be compared between different $tRNA_{AC}$-Rzs and $tRNA_3^{Lys}$. $tRNA_3^{Lys}$ may have appeared to be more stable at 10 min compared to the $tRNA_{AC}$-Rzs; however, since the degradation reactions were not performed at the same time, this may have been due to less actual incubation time in the cell lysate. Nevertheless, the result of this experiment showed that extent of degradation of all $tRNA_{AC}$-Rzs and $tRNA_3^{Lys}$ was still comparable. Thus, insertion of ribozyme sequences in the anticodon loop did not decrease the inherent stability of $tRNA_3^{Lys}$.

7. RNA pol III-driven transcription from the tRNA promoter

Plasmids containing $tRNA_{AC}$-Rzs and $tRNA_3^{Lys}$ were individually transcribed using HeLa nuclear extract. Transcripts corresponding to $tRNA_{AC}$-Rz (0/0), $tRNA_{AC}$-Rz (2/0), $tRNA_{AC}$-Rz

FIG. 20. **Stability of $tRNA_{AC}$-Rzs.** [α-^{32}P]-labeled $tRNA_{AC}$-Rz (0/0), $tRNA_{AC}$-Rz (10/0), and $tRNA_3^{Lys}$ were each incubated with MT4 cell lysate (500 μg/ml) at 30°C and aliquots were taken at various time intervals. Samples were analyzed by 8 M urea-8 % PAGE. Band intensities of intact RNA were measured using ImageQuant software and % intact RNA remaining compared to time 0 was calculated. No lysate, RNA without incubation with MT4 cell lysate.


No Lysate
Lysate
0
10
20
60
120
180
min
tRNA$_{AC}$-Rz (0/0)
tRNA$_{AC}$-Rz (10/0)
tRNA$_3^{Lys}$

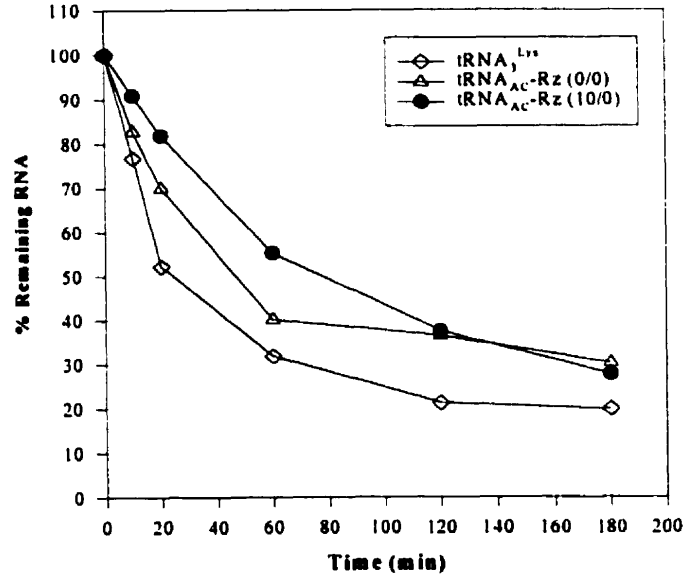

% Remaining RNA
Time (min)
tRNA$_3^{Lys}$
tRNA$_{AC}$-Rz (0/0)
tRNA$_{AC}$-Rz (10/0)

Figure 20.

(4/0), $tRNA_{AC}$-Rz (8/0) and $tRNA_{AC}$-Rz (10/0) were detected (Fig. 21*A*). A negative control consisting of HeLa nuclear extract alone without the addition of template DNA did not yield any transcript. This result shows that the intragenic tRNA promoters are intact and that the insertion of the ribozyme in the anticodon loop does not disrupt transcription by RNA pol III.

$tRNA_3^{Lys}$ transcribed from pSW201 plasmid that contains only the $tRNA_3^{Lys}$-coding region, but lacking sequences upstream and downstream of the coding region, resulted in a product (Fig. 21*B*) smaller than expected (76 nt). However, transcription of $tRNA_3^{Lys}$ from plasmid pM13-Lys,3 that contains $tRNA_3^{Lys}$ plus sequences upstream and downstream of the coding region resulted in a transcript of the expected size (Fig. 21*A*). The transcript produced from pM13-Lys,3 was of comparable length to the $tRNA_i^{Met}$ transcribed from pDTNβ (Fig. 21*B*), a plasmid that contains the $tRNA_i^{Met}$ gene. Thus, sequences other than the coding region of $tRNA_3^{Lys}$ may regulate termination of RNA pol III. The presence of the 4T residues in the anticodon of $tRNA_3^{Lys}$ may act as a termination signal in the absence of upstream and downstream sequences. But since this region was disrupted upon insertion of the ribozyme in the anticodon loop, all of the $tRNA_{AC}$-Rzs could be transcribed. As the inserted ribozyme and its flanking sequence did not contain consecutive T residues, the only terminator sequence was a run of 6T residues located downstream of the coding regions of the $tRNA_{AC}$-Rzs.

The *trans* cleavage activity of RNA pol III-transcribed $tRNA_{AC}$-Rz (10/0) was tested using an *in vitro* transcribed target RNA. A band corresponding to the 5' cleavage product was observed (Fig. 21*C*).

FIG. 21. **RNA pol III-driven expression of $tRNA_{AC}$-Rzs.** *A*, Plasmids expressing $tRNA_3^{Lys}$ (pM13-Lys,3) and the $tRNA_{AC}$-Rzs were transcribed *in vitro* using a HeLa nuclear extract and analyzed by 8 M urea-8 % PAGE to determine whether the $tRNA_3^{Lys}$ promoter of the $tRNA_{AC}$-Rzs were intact and could be recognized by RNA pol III, the polymerase which transcribes tRNAs in cells. $tRNA_{AC}$-Rz (0/0), $tRNA_{AC}$-Rz (2/0), $tRNA_{AC}$-Rz (4/0), $tRNA_{AC}$-Rz (8/0), $tRNA_{AC}$-Rz (10/0), and $tRNA_3^{Lys}$ could all be transcribed. A negative control consisting of the transcription mixture without any plasmid DNA did not yield a transcription product. *B*, Transcription of pSW201 plasmid that contains the $tRNA_3^{Lys}$-coding region, but lacking sequences upstream and downstream of the coding region, resulted in a product smaller than expected (33 nts). The transcript produced from pDTNβ (75 nts) was of comparable length to the $tRNA_i^{Met}$ transcribed from pM13-Lys,3 (76 nts). *C. Trans* cleavage reactions were performed using either the linear Rz (lane 1) or RNA pol III-transcribed $tRNA_{AC}$-Rz (10/0) (lane 2) with T7 RNA polymerase-transcribed target RNA (333 nts).

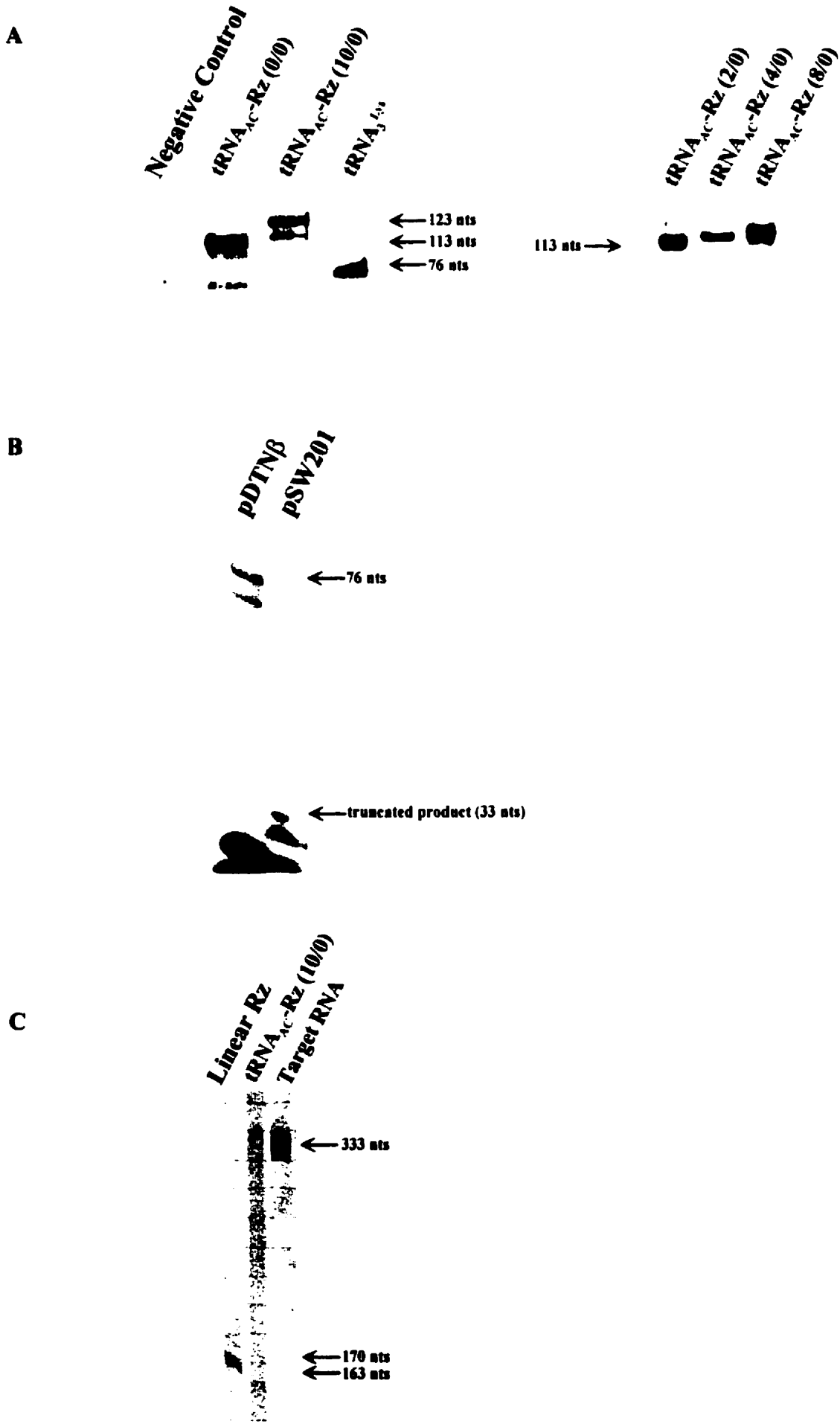

A
Negative Control
tRNA$_{AC}$-Rz (0/0)
tRNA$_{AC}$-Rz (10/0)
tRNA^{Lys}
tRNA$_{AC}$-Rz (2/0)
tRNA$_{AC}$-Rz (4/0)
tRNA$_{AC}$-Rz (8/0)
123 nts
113 nts
76 nts
113 nts
B
pDTNβ
PSW201
76 nts
truncated product (33 nts)
C
Linear Rz
tRNA$_{AC}$-Rz (10/0)
Target RNA
333 nts
170 nts
163 nts

Figure 21.

II. TEST RETROVIRAL VECTORS EXPRESSING $tRNA_{AC}$-RZS FOR INHIBITION OF HIV-1 REPLICATION IN A HUMAN CD4+ LYMPHOID MT4 CELL LINE

A. Development of retroviral vectors expressing $tRNA_{AC}$Rzs and various controls

The ribozyme in all $tRNA_{AC}$-Rzs is designed to cleave a highly conserved sequence within the *env*-coding region of HIV-1 subtype B. In order to assess the ability of various $tRNA_{AC}$-Rzs to inhibit HIV-1 replication in a human CD4+ T cell line, a MMLV-based pUCMoTiN vector was used to construct retroviral vectors expressing various $tRNA_{AC}$-Rzs (Fig. 22). A retroviral vector expressing $tRNA_{AC}$-InRz (one of the two inactive ribozymes; contains an A to G mutation in one of the conserved residues of the hammerhead catalytic domain) was also constructed to serve as a negative control. Other retroviral vectors which were constructed included a linear Rz control pUCMoTiN-Rz_{Env} which expressed the ribozyme alone, and a downstream Rz control pUCMoTiN-tRNA-Rz which expressed the ribozyme downstream of $tRNA_3^{Lys}$. RNAs from pUCMoTiN and pUCMoTiN- Rz_{Env} will be expressed from the promoter present in the 5`LTR and the *tk* promoter, while RNAs from the pUCMoTiN-tRNA-Rz, pUCMoTiN-$tRNA_{AC}$-InRz, and pUCMoTiN-$tRNA_{AC}$-Rzs would also originate from the tRNA promoter.

B. *In vitro* cleavage activities of $tRNA_{AC}$-Rzs and various controls cloned within the retroviral vector

Various $tRNA_{AC}$-Rzs, $tRNA_{AC}$-InRz, tRNA-Rz, linear Rz, and Env target RNA containing the ribozyme cleavage site were each transcribed using T7 RNA polymerase from DNA templates containing the T7 promoter, which were PCR amplified from pUCMoTiN-$tRNA_{AC}$-Rzs, pUCMoTiN-$tRNA_{AC}$-InRz, pUCMoTiN-tRNA-Rz, pUCMoTiN-Rz_{Env}, and pHEnv plasmids, respectively. The target RNA was internally labeled and subjected to *in vitro* cleavage by each $tRNA_{AC}$-Rz, $tRNA_{AC}$-InRz, tRNA-Rz, and linear Rz. As expected, all ribozyme-containing

FIG. 22. **Various retroviral vectors expressing selected $tRNA_{AC}$-Rzs and controls.** pUCMoTiN was used to clone the $tRNA_{AC}$-Rzs downstream of the *neo* gene. This vector contains the HSV *tk*-TAR fusion promoter (P) that allows *neo* gene expression. pUCMoTiN-Rz consists of a linear hammerhead ribozyme. In pUCMoTiN-tRNA-Rz, the hammerhead ribozyme was cloned downstream of $tRNA_3^{Lys}$. pUCMoTiN-$tRNA_{AC}$-InRz contains an inactive ribozyme cloned within the anticodon loop. pUCMoTiN-$tRNA_{AC}$-Rzs depicts the retroviral vector constructed for each $tRNA_{AC}$-Rz. RNAs expressed from the 5'LTR (1), *tk*-TAR (2) and tRNA (3) promoters are shown. The binding sites of primers Mo-F, Mo-R, and T7-Mo-R which flank the cloning site in pUCMoTiN, and primers MM13, MM18, MM19 and MM22 which bind to either the 5' or 3' region of $tRNA_3^{Lys}$, are shown. LTR, long terminal repeat.

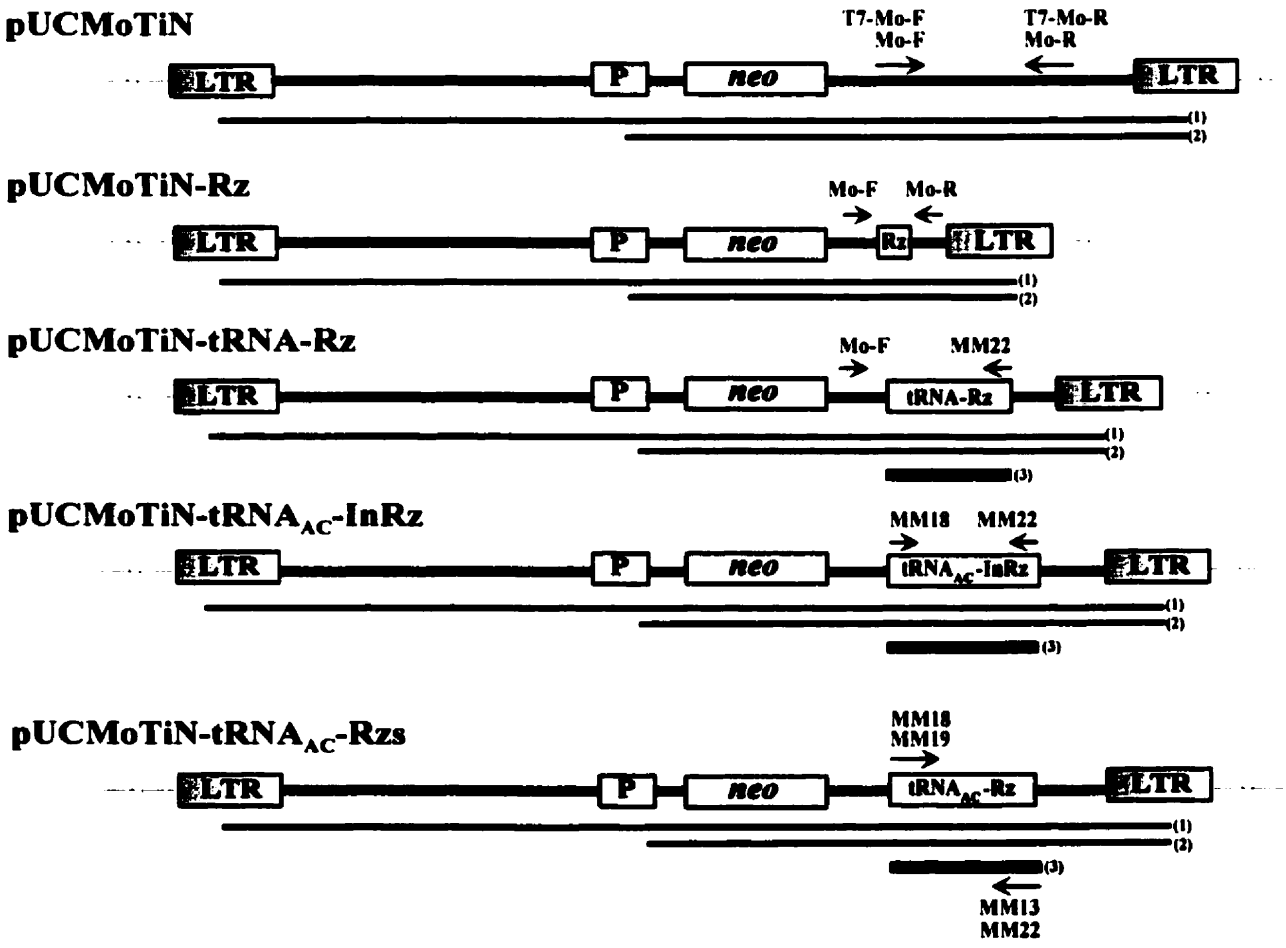

pUCMoTiN
T7-Mo-F
Mo-F
T7-Mo-R
Mo-R
LTR
P
neo
LTR
(1)
(2)
pUCMoTiN-Rz
Mo-F
Mo-R
LTR
P
neo
Rz
LTR
(1)
(2)
pUCMoTiN-tRNA-Rz
Mo-F
MM22
LTR
P
neo
tRNA-Rz
LTR
(1)
(2)
(3)
pUCMoTiN-tRNA$_{AC}$-InRz
MM18
MM22
LTR
P
neo
tRNA$_{AC}$-InRz
LTR
(1)
(2)
(3)
pUCMoTiN-tRNA$_{AC}$-Rzs
MM18
MM19
LTR
P
neo
tRNA$_{AC}$-Rz
LTR
(1)
(2)
(3)
MM13
MM22

Figure 22.

transcripts were capable of at least partially cleaving the target RNA, while tRNA-InRz failed to cleave (Fig. 23). This result shows that $tRNA_{AC}$-Rzs cloned in the retroviral vector are capable of cleaving their target RNA *in trans*.

C. Development of pools of stable MT4 transductants expressing $tRNA_{AC}$-Rzs and various controls

1. Establish stable transductants expressing $tRNA_{AC}$-Rzs and various controls

Each of the pUCMoTiN-based plasmids was used to transfect the ψ-2 packaging cell line. Ecotropic vector particles released by this cell line were then used to transduce the PA317 packaging cell line. Amphotropic MoTiN, various MoTiN-$tRNA_{AC}$-Rzs, MoTiN-$tRNA_{AC}$-InRz, MoTiN-tRNA-Rz, and MoTiN-Rz vector particles were each used to transduce MT4 cells, a human CD4+ T cell line (Fig. 24). Pools of stable MT4 transductants lacking or expressing various $tRNA_{AC}$-Rzs or controls were selected and tested without cloning.

The amphotropic vector particles were also used to transduce human PBLs. However, transduction did not take place even with the MoTiN vector particles, as there were no viable cells that remained after G418 selection. Presumably, the low titers of vector particles were not enough to transduce PBLs.

2. Confirm the presence of proviral vector DNA within the cellular genome

To confirm that retroviral sequences have integrated within the cellular genome, PCRs were performed using primers which bind to retroviral vector sequences upstream (Mo-F primer) and downstream (Mo-R) of the cloning site. Genomic DNA from transduced PA317 cells was isolated and used as a PCR template. Bands corresponding to the predicted size PCR products

FIG.23. ***In* vitro cleavage activity of $tRNA_{AC}$-Rzs amplified from pUCMoTiN $tRNA_{AC}$-Rz clones.** The $tRNA_{AC}$-Rz sequences were amplified from the respective pUCMoTiN clones to generate templates used for *in vitro* transcription. Transcripts were used in *trans* cleavage reactions with [α-^{32}P]-labeled target RNA and the products analyzed by 8 M urea-8 % PAGE. The 5' (163 nts) and 3' (170 nts) cleavage products can be detected. The two bands corresponding to the 3' cleavage product may have resulted from cleavage of premature transcription products.

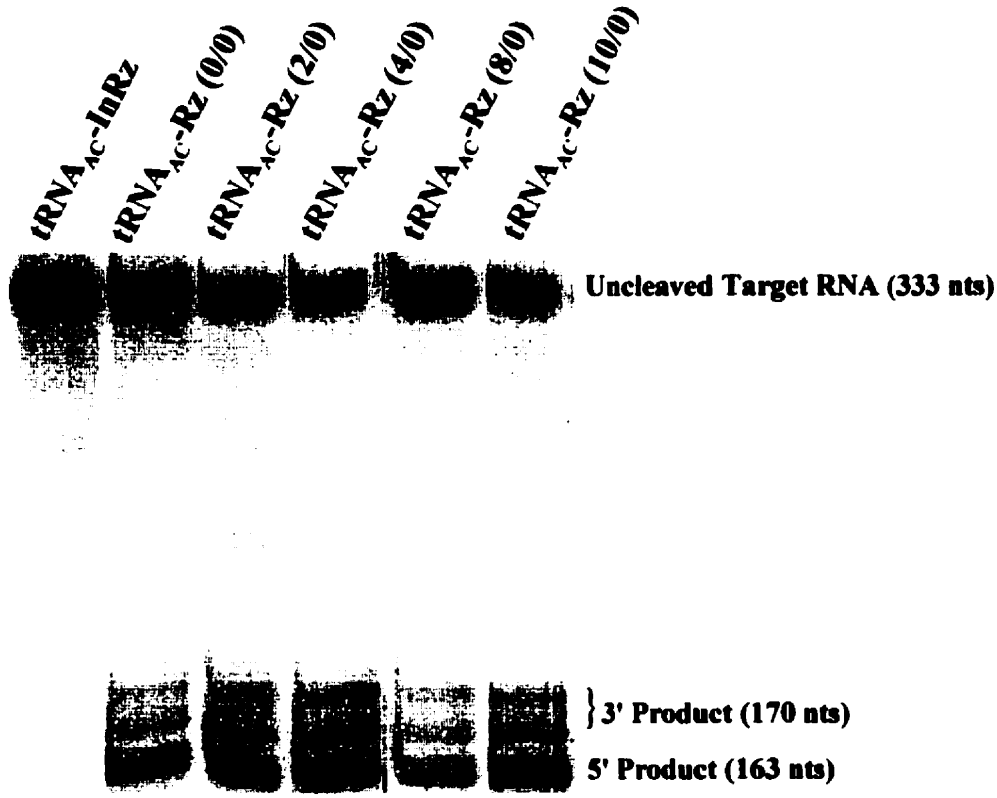

tRNA$_{AC}$-InRz
tRNA$_{AC}$-Rz (0/0)
tRNA$_{AC}$-Rz (2/0)
tRNA$_{AC}$-Rz (4/0)
tRNA$_{AC}$-Rz (8/0)
tRNA$_{AC}$-Rz (10/0)
Uncleaved Target RNA (333 nts)
3' Product (170 nts)
5' Product (163 nts)

Figure 23.

FIG. 24. **Development of stable MT4 transductants expressing $tRNA_{AC}$-Rzs and various controls.** Retroviral vectors were separately transfected into the ψ-2 packaging cell line which expresses viral structural proteins. These cells release infectious virus, but not replication-competent virus. Retroviral vector RNAs which contain the ψ signal were packaged and released as vector particles which were used to transduce the PA317 packaging cell line. Amphotropic vector particles released by these cells were used to transduce CD4+ cells such as the human MT4 lymphoid cell line. Upon reverse transcription and integration of the proviral DNA in the target cells, $tRNA_{AC}$-Rzs are produced. The presence of proviral DNA in the genome of PA317 packaging cells transduced with ecotropic ψ-2 vector particles was confirmed by PCR using the Mo-F and Mo-R primer pair. 0, $tRNA_{AC}$-Rz (0/0). 2, $tRNA_{AC}$-Rz (2/0). 4, $tRNA_{AC}$-Rz (4/0). 8, $tRNA_{AC}$-Rz (8/0). 10, $tRNA_{AC}$-Rz (10/0). In, $tRNA_{AC}$-InRz. D, tRNA-Rz. L, linear Rz. M, MoTiN.

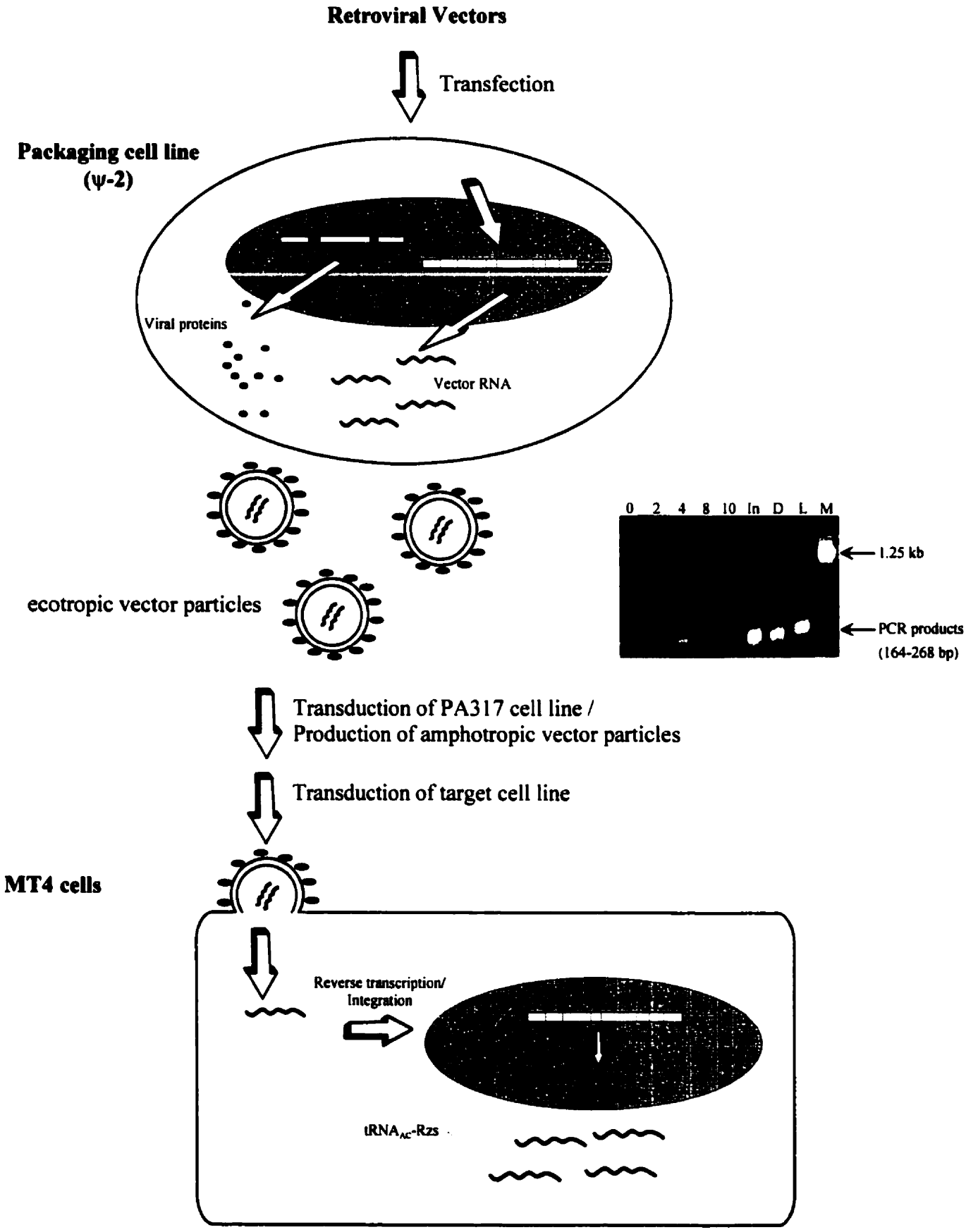

Retroviral Vectors
Transfection
Packaging cell line
(ψ-2)
Viral proteins
Vector RNA
ecotropic vector particles
0 2 4 8 10 In D L M
1.25 kb
PCR products
(164-268 bp)
Transduction of PA317 cell line /
Production of amphotropic vector particles
Transduction of target cell line
MT4 cells
Reverse transcription/
Integration
tRNA$_{AC}$-Rzs

Figure 24.

(164-268 bp) were observed (Fig. 24). Amplification of the corresponding region in the parent pUCMoTiN plasmid resulted in a 1.25 kb product.

3. Confirm the expression of $tRNA_{AC}$-Rzs and various controls

Transcription of $tRNA_{AC}$-Rzs, $tRNA_{AC}$-InRz, tRNA-Rz, and linear Rz RNA was determined by RT-PCR analysis of total cellular RNA extracted from stable MT4 transductants. The predicted size products were detected in each case (Fig. 25*A*). This confirms the presence of $tRNA_{AC}$-Rz sequences within the cellular RNAs; however, this does not distinguish from RNA pol II- or RNA pol III-transcribed RNA.

To distinguish between RNA pol II- and RNA pol III-transcribed RNAs, total RNAs were isolated from MT4 cells using a kit which allows sequential isolation of LMW RNA, HMW RNA, and genomic DNA. Contamination of HMW RNA in the LMW RNA fraction was tested by performing RT-PCRs using primers (Mo-F and T7-Mo-R) which would bind to HMW RNA sequences (Fig. 26). No products were observed. The same primers were then used for PCRs using $tRNA_{AC}$-Rz (0/0) HMW RNA as template to ensure that the primers were indeed capable of binding to and amplifying sequences within HMW RNA. A band of the expected size (284 nts) was observed (data not shown) thus confirming RNA pol II-driven expression. The LMW RNA was then used in RT-PCRs using primers (MM19 and MM22) that bound to the 5' end and 3' regions of $tRNA_3^{Lys}$. These primers should amplify both wt $tRNA_3^{Lys}$ and $tRNA_3^{Lys}$-based ribozymes. Only one band was observed for all samples, which corresponded in size to the wt $tRNA_3^{Lys}$ (Fig. 25*B*, Fig. 26). To confirm that the observed PCR product consists of $tRNA_3^{Lys}$ sequences alone, the PCR products were used for *in vitro* transcription as the T7 promoter is present in the forward primer. All of the transcripts corresponded in size to an *in vitro* transcribed $tRNA_3^{Lys}$ (Fig. 27). These transcripts were then used in *trans* cleavage reactions. None of the

FIG. 25. **$tRNA_{AC}$-Rz expression in transduced MT4 cells.** *A*, Total RNA was extracted from MT4 cells expressing $tRNA_{AC}$-Rzs, treated with DNase, and used as an RT-PCR template using primers Mo-F and T7-Mo-R. *B*, LMW RNA was used for RT-PCRs using primers MM19 and MM22. Plasmids (P) expressing $tRNA_{AC}$-Rz (10/0) and $tRNA_{AC}$-Rz (0/0) were amplified using the same primers, and the PCR products used as markers.

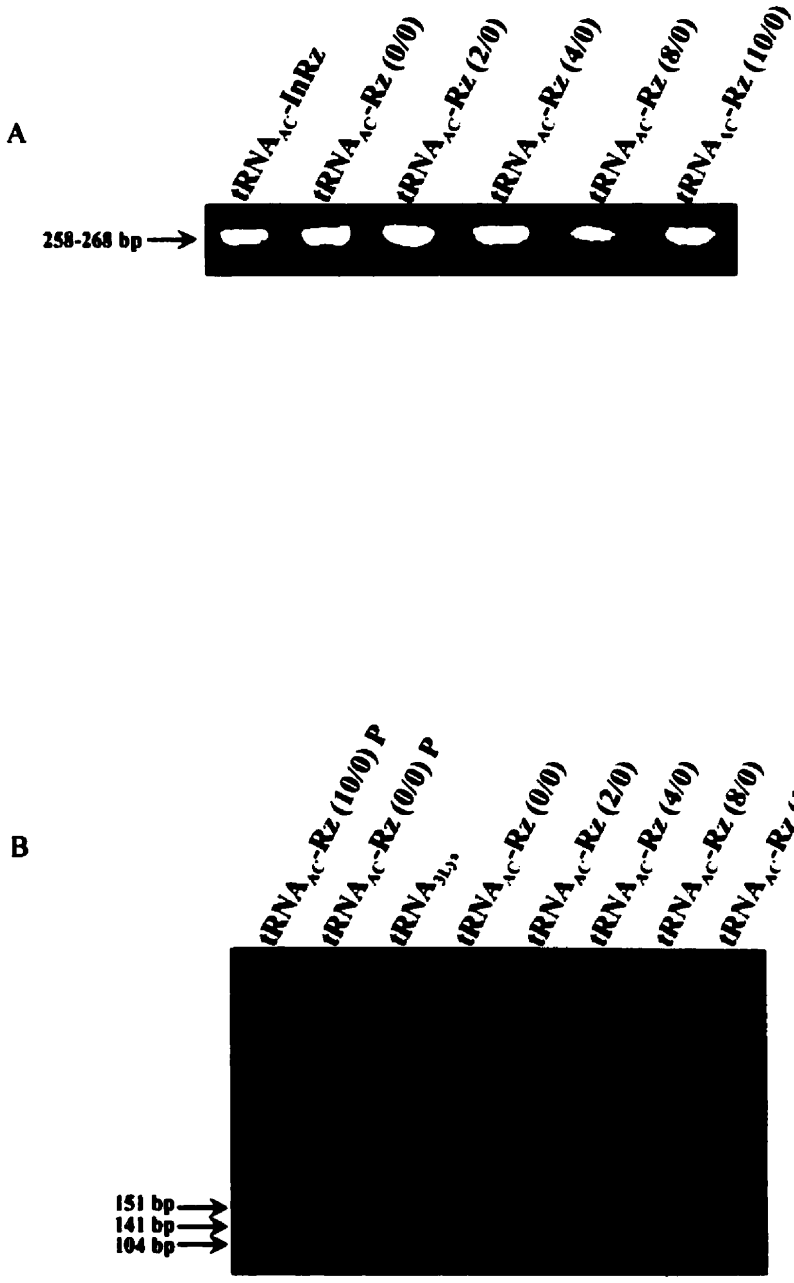
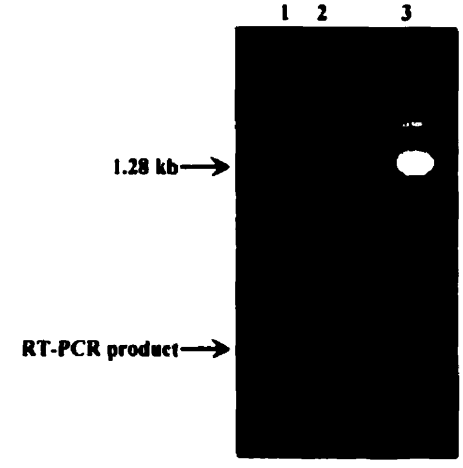
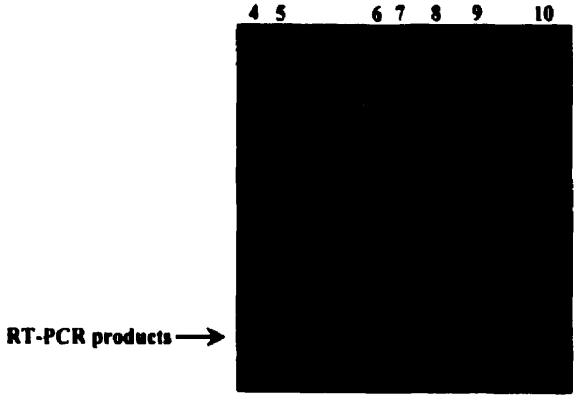

A
tRNA$_{AC}$-InRz
tRNA$_{AC}$-Rz (0/0)
tRNA$_{AC}$-Rz (2/0)
tRNA$_{AC}$-Rz (4/0)
tRNA$_{AC}$-Rz (8/0)
tRNA$_{AC}$-Rz (10/0)
258-268 bp
B
tRNA$_{AC}$-Rz (10/0) P
tRNA$_{AC}$-Rz (0/0) P
tRNA$_{AC}$-Rz (0/0)
tRNA$_{AC}$-Rz (2/0)
tRNA$_{AC}$-Rz (4/0)
tRNA$_{AC}$-Rz (8/0)
tRNA$_{AC}$-Rz (10/0)
151 bp
141 bp
104 bp

Figure 25.

FIG. 26. **RT-PCRs to confirm identity of LMW RNAs.** LMW RNAs isolated from pools of transduced cells expressing $tRNA_{AC}$-Rz (0/0) (lanes 5, 9), $tRNA_{AC}$-Rz (2/0) (lanes 11, 13), $tRNA_{AC}$-Rz (4/0) (lanes 1, 2), $tRNA_{AC}$-Rz (8/0) (lanes 12, 14) and $tRNA_{AC}$-Rz (10/0) (lanes 6, 10) were used in RT-PCRs either with primer pairs Mo-F and T7-Mo-R (lanes 1, 5, 6, 13, 14) or MM19 and MM22 (lanes 2, 9, 10, 11, 12). RT-PCR product using MoTiN RNA (lane 8) and PCR product using pUCMoTiN plasmid (lane 3) were used as markers. PCR controls without any plasmid DNA (lanes 4, 7) were also performed.

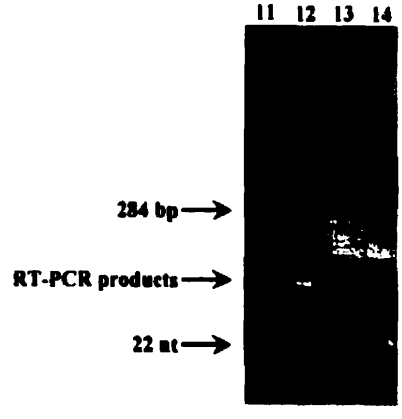


Lane 1. Mo-F/T7-Mo-R (4/0)
Lane 2. MM19/MM22 (4/0)
Lane 3. Mo-F/T7-Mo-R (pUCMoTiN)



Lane 4. Mo-F/T7-Mo-R water control
Lane 5. Mo-F/T7-Mo-R (0/0)
Lane 6. Mo-F/T7-Mo-R (10/0)

Lane 7. MM19/MM22 water control
Lane 8. MM19/MM22 (MoTiN)
Lane 9. MM19/MM22 (0/0)
Lane 10. MM19/MM22 (10/0)



Lane 11. MM19/MM22 (2/0)
Lane 12. MM19/MM22 (8/0)

Lane 13. Mo-F/T7-Mo-R (2/0)
Lane 14. Mo-F/T7-Mo-R (8/0)

Figure 26.

FIG. 27. ***Trans*** **cleavage reactions using $tRNA_{AC}$-Rzs RT-PCR-amplified and transcribed from LMW RNA.** Transcripts were obtained by *in vitro* transcription of RT-PCR products. These RT-PCR products were obtained from amplification of LMW RNA isolated from transduced MT4 cells expressing $tRNA_{AC}$-Rz (0/0), $tRNA_{AC}$-Rz (10/0) or MoTiN. Transcripts containing $tRNA_{AC}$-Rz (0/0) or $tRNA_{AC}$-Rz (10/0) were also obtained by *in vitro* transcription of PCR DNA obtained from amplification of plasmids (P) expressing these $tRNA_{AC}$-Rzs. The transcripts were used in *trans* cleavage reactions. Only the transcripts which originated from the plasmids were able to cleave the target RNA. Size of a transcript containing $tRNA_3^{Lys}$ sequence alone is indicated.

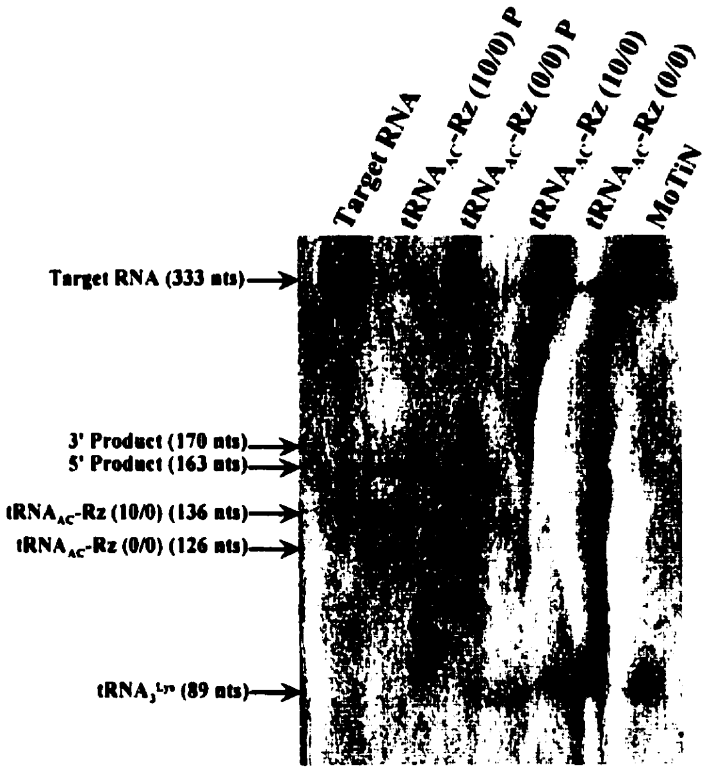

Target RNA
tRNA$_{AC}$-Rz (10/0) P
tRNA$_{AC}$-Rz (0/0) P
tRNA$_{AC}$-Rz (10/0)
tRNA$_{AC}$-Rz (0/0)
MoTiN
Target RNA (333 nts)
3' Product (170 nts)
5' Product (163 nts)
tRNA$_{AC}$-Rz (10/0) (136 nts)
tRNA$_{AC}$-Rz (0/0) (126 nts)
tRNA$_3^{Lys}$ (89 nts)

Figure 27.

transcripts demonstrated *trans* cleavage activity (Fig. 27). Thus, transcription of $tRNA_{AC}$-Rzs in transduced cells is driven mainly by the RNA pol II promoter.

Lack of RNA pol III transcription was also confirmed by primer extension. Identical sized products were obtained when total RNAs isolated from transduced MT4 cells expressing $tRNA_{AC}$-Rz (10/0) or MoTiN were extended using a 5' end labeled primer (Fig. 28). RNA from $tRNA_{AC}$-Rz (4/0) was also used in a Northern blot using a probe which hybridizes to tRNA sequences. Only the bands corresponding to the RNA pol II-transcribed RNAs were observed (data not shown).

Total RNA isolated from the transduced cells was also tested in an RNase protection assay. An antisense probe derived from pUCMoTiN-$tRNA_{AC}$-Rz (0/0) was designed, which was expected to hybridize to $tRNA_{AC}$-Rz sequences upstream and downstream of the 5' linker sequence. The unhybridized 5' linker sequence in RNA pol III-driven transcripts was expected to be cleaved by the RNases, thus resulting in two protected bands corresponding to sequences upstream (34 nts) and downstream (79 nts) of the 5' linker sequence. The same probe could also demonstrate the presence of RNA pol II-driven transcripts, resulting in protected bands (269 nts and 182 nts) that are larger in size due to the retroviral sequences in the probe that would be protected by RNA pol II-driven transcripts. The protected bands expected from using $tRNA_{AC}$-Rz (0/0) were 452 nts (pol II) and 113 nts (pol III). In addition, all of the cells express $tRNA_3^{Lys}$ which, if protected by the probe, would give rise to protected bands (34 nts and 41 nts). The presence of the 41-nt protected band in all of the samples would have served as an internal control for the probe's ability for hybridization.

The conditions for RNase protections were set up using *in vitro* transcribed $tRNA_{AC}$-Rz (10/0) and the antisense probe. Protected bands which corresponded in size to 79 nts and 34 nts were observed (Fig. 29). The full- length probe and $tRNA_{AC}$-Rz (10/0) were both degraded when each

FIG. 28. **Primer extension of total RNAs isolated from MT4 cells.** Total RNAs isolated from transduced MT4 cells expressing $tRNA_{AC}$-Rz (10/0) or MoTiN were extended using a 5' end-labeled primer d'. The arrow on the left indicates full-length product.

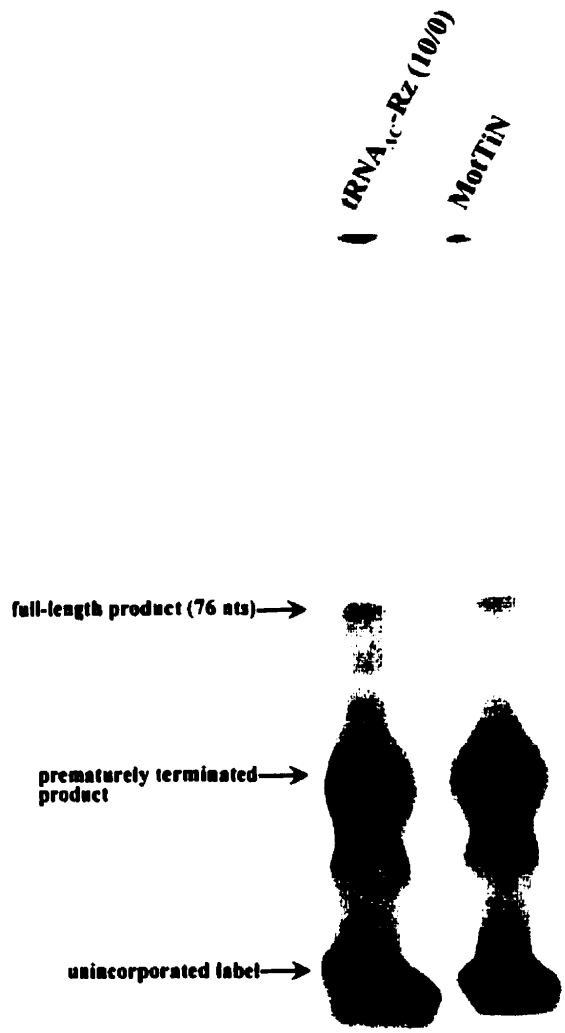

tRNA$^{Ala}_{UC}$-Rz (10/0)
MorTiN
full-length product (76 nts)
prematurely terminated product
unincorporated label

Figure 28.

FIG. 29. **RNase protections.** RNase protection was performed using *in vitro* transcribed $tRNA_{AC}$-Rz (10/0) (123 nts) and probe (451 nts). As controls, $tRNA_{AC}$-Rz (10/0) and the probe were each incubated with the RNases in the absence of the probe and $tRNA_{AC}$-Rz (10/0), respectively.

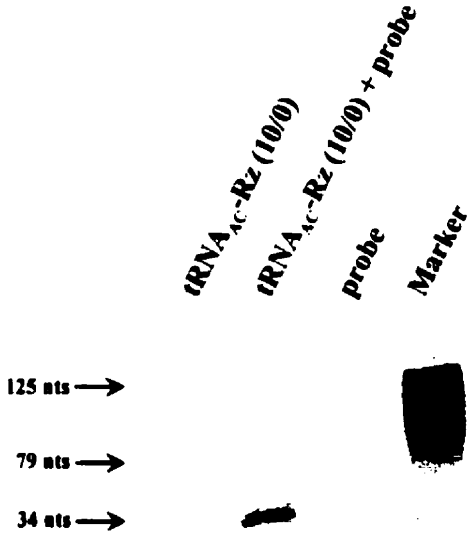

tRNA$_{AC}$-Rz (10/0)
tRNA$_{AC}$-Rz (10/0) + probe
probe
Marker
125 nts
79 nts
34 nts

Figure 29.

RNA was used alone for RNase protections. When total RNA from transduced cells expressing the $tRNA_{AC}$-Rzs was used for the RNase protections, identical-sized bands were again observed for all the samples, including $tRNA_{AC}$-Rz (0/0). Thus, a sequence common to all the samples was binding to the probe, as the same bands were also observed when using total RNA isolated from untransduced MT4 cells. The expected products for RNA pol III-driven transcription were not observed (data not shown).

D. HIV-1 susceptibility of pools of stable MT4 transductants

The HIV-1 titer was determined from infection of HeLa-CD4-LTR-β-gal cells which express high levels of CD4 and contain a β-galactosidase gene driven by the HIV-1 LTR promoter. Thus, infection of these cells by HIV-1 results in production of β-galactosidase protein. The number of infectious particles could be determined by counting the number of cells which turn blue upon addition of X-gal and IPTG.

The pools of stable MT4 transductants lacking or expressing $tRNA_{AC}$-Rzs, $tRNA_{AC}$-InRz, tRNA-Rz, linear Rz, or MoTiN were each infected with HIV-1 at an m.o.i. of 0.1 (Fig. 30*A*) and 1 (Fig. 30*B*). Virus production by the infected cells was measured by determining the amount of HIV-1 p24 antigen in the infected cell culture supernatants that were collected every three days. Cells expressing retroviral vector sequences alone (MoTiN) produced high amounts of virus. In contrast, HIV-1 production was greatly reduced in $tRNA_{AC}$-Rz-expressing cells. The inhibition conferred by the $tRNA_{AC}$-Rzs could not have been due to an antisense effect by the ribozyme flanking sequences, since cells expressing $tRNA_{AC}$-InRz also produced high amounts of virus. Similar results were obtained in challenge experiments performed at the two (0.1 and 1) m.o.i.'s. Thus, all $tRNA_{AC}$-Rzs were capable of inhibiting HIV-1 replication. By day 6 post-infection,

FIG. 30. **HIV-1 susceptibility of transduced MT4 cells.** Transduced MT4 cells were infected with HIV-1 at an m.o.i. of 0.1 (*A*) or 1 (*B*) and the amount of HIV-1 p24 antigen released in the culture supernatants was measured at various time intervals

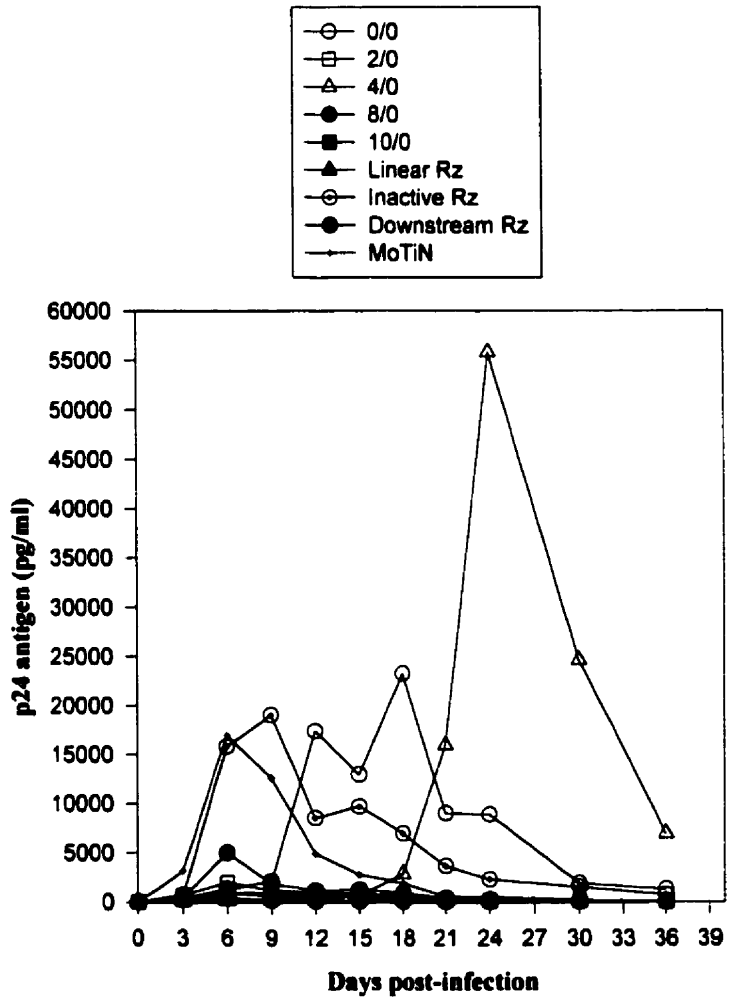

0/0
2/0
4/0
8/0
10/0
Linear Rz
Inactive Rz
Downstream Rz
MoTiN
60000
55000
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
p24 antigen (pg/ml)
0 3 6 9 12 15 18 21 24 27 30 33 36 39
Days post-infection

Figure 30*A*.

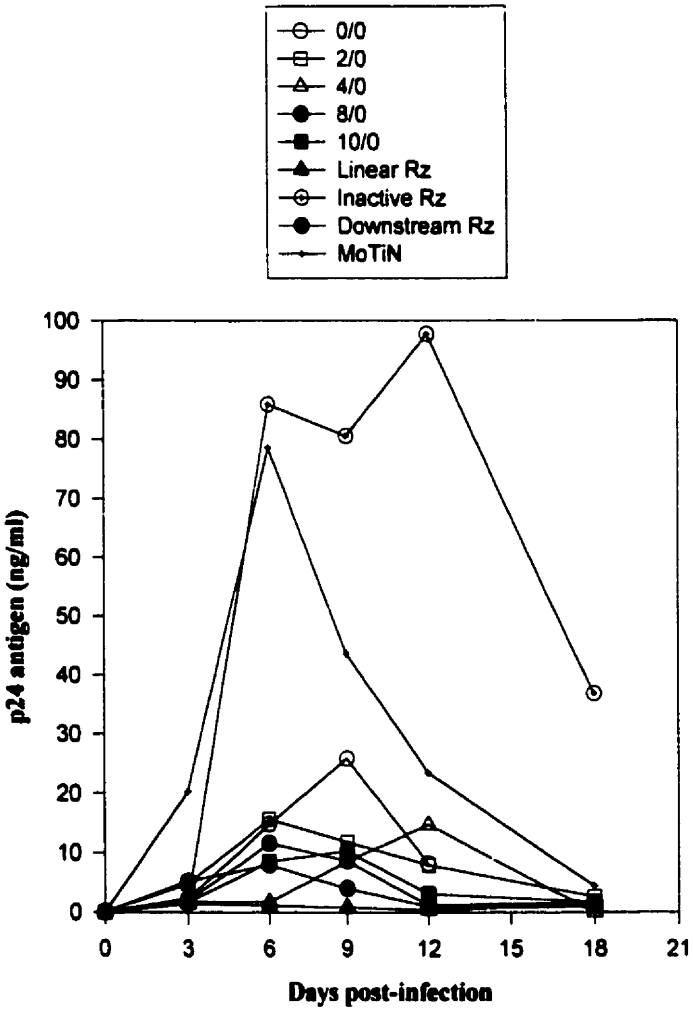

0/0
2/0
4/0
8/0
10/0
Linear Rz
Inactive Rz
Downstream Rz
MoTiN
p24 antigen (ng/ml)
100
90
80
70
60
50
40
30
20
10
0
0
3
6
9
12
15
18
21
Days post-infection

Figure 30*B*.

control cells lacking any $tRNA_{AC}$-Rzs or expressing $tRNA_{AC}$-InRz produced HIV-1 p24 antigen as high as 16-17 ng/ml p24 for the experiment performed with 0.1 m.o.i., and 78-86 ng/ml p24 for the one performed at an m.o.i. of 1. In contrast, cells expressing $tRNA_{AC}$-Rzs produced 0.3-26 ng/ml p24 until day 9 post-infection. All $tRNA_{AC}$-Rzs must have been capable of inhibiting HIV-1 replication since HIV-1 production was greatly reduced in $tRNA_{AC}$-Rz-expressing cells, compared to control cells lacking any $tRNA_{AC}$-Rz or expressing the inactive ribozyme at both m.o.i.'s.

DISCUSSION

Transfer RNAs are produced in very high concentrations and possess stable secondary structures in cells. Intragenic sequences constitute the promoter elements used for tRNA expression under the control of RNA pol III. Transfer RNA genes are therefore ideal for expression of small RNA molecules. Sequences encoding these RNAs may be cloned either within the tRNA gene between the promoter sequences, or downstream of the tRNA gene. In both cases, tRNA processing sites may be preserved such that the transcripts produced will be processed to yield two separate RNAs. Alternatively, these sites may be modified to prevent tRNA processing. In this case, the RNA of interest will remain as part of the tRNA. To develop $tRNA_3^{Lys}$-based ribozymes, cloning between the promoter elements seems the most attractive as the ribozyme is expressed within a region of highly ordered secondary structure which should confer protection against nucleases within the cell. The only drawback of this strategy is that the catalytic activity of the ribozyme may be decreased. Care should therefore be taken to design or select $tRNA_{AC}$-Rzs that retain full ribozyme activity.

In the past, linker sequences connecting the ribozyme to the rest of the tRNA were arbitrarily chosen to develop $tRNA_i^{Met}$-based $tRNA_{AC}$-Rzs. However, ribozyme activity was shown to be significantly decreased in one study where the effect of these sequences on ribozyme activity was investigated. While cloning within the anticodon loop may allow ribozymes to be expressed in high concentrations and possess stable secondary structures, structural constraint may force ribozymes to adopt a conformation detrimental to their activity. Based on the results we obtained, it is clear that sequences immediately surrounding the ribozyme influence its catalytic activity.

We have developed an *in vitro* selection strategy that led to the selection of $tRNA_{AC}$-Rzs that contained 5' and 3' linkers optimal for ribozyme cleavage (Fig. 7). Various $tRNA_{AC}$-Rzs with

different linkers were isolated (Table 3). These $tRNA_{AC}$-Rzs could be transcribed by RNA pol III (Fig. 21) and were as stable as $tRNA_3^{Lys}$ (Fig. 20). *Trans* cleavage rates of these ribozymes varied (Fig. 17*A* and *B*). $tRNA_{AC}$-Rz (0/0) without a 5' linker demonstrated the slowest rate, while $tRNA_{AC}$-Rz (4/0), $tRNA_{AC}$-Rz (8/0) and $tRNA_{AC}$-Rz (10/0) possessed rates similar to that of the linear ribozyme (Fig. 17*A* and 18). Retroviral vectors encoding $tRNA_{AC}$-Rzs were constructed (Fig. 22). Stable MT4 transductants were shown to express active $tRNA_{AC}$-Rzs (Fig. 25*A*) and inhibit HIV-1 replication (Fig. 30*A* and *B*). Virus production from the infected MT4 cells expressing $tRNA_{AC}$-Rzs was consistently lower compared to control cells.

Issues related to the *in vitro* selection strategy, *in vivo* testing and potential mode(s) of inhibition are discussed below.

I. ISSUES RELATED TO SELECTION *IN VITRO*

A. Complexity of the pool and variability within the 5' and 3' linker regions of the $tRNA_{AC}$-Rzs

Use of 5' and 3' primers containing 0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 nt-long linkers to amplify the respective linker regions of $tRNA_{AC}$-Rzs should have resulted in a total of 121 combinations of linkers, since any of the eleven 5' linkers could have combined with any of the eleven 3' linkers. The variability in the lengths of the 5' and 3' linkers present in the pool was confirmed by PCR (Fig. 16).

Because the nucleotide composition of the linkers was randomized, 10^{24} combinations with different secondary structures should have existed. Only a fraction of the library was obtained upon *in vitro* transcription of the template DNA library, due to limitations on the amount of RNA that could be potentially transcribed in one reaction. However, this did not affect the overall selection, since the goal was to isolate $tRNA_{AC}$-Rzs with similar activity as a linear ribozyme,

and this was achieved. As the selected $tRNA_{AC}$-Rzs possessed 100% activity compared to the linear ribozyme, subsequent rounds of selection were not required. Since only the linker sequences were optimized, an increase in ribozyme catalytic activity was not anticipated.

B. Choice of ribozyme target site

The ribozyme used in this study was designed to target a highly conserved sequence within the *env*-coding region, present in the unspliced and singly spliced HIV-1 mRNAs. This target site was chosen since a ribozyme targeted against the same target site demonstrated the best catalytic activity *in vitro* compared to ribozymes targeted against *gag*, *PR*, *RT*, and *tat*-coding regions (Ramezani and Joshi 1996). Other sites may also be targeted by modifying the antisense sequences flanking the ribozyme catalytic domain of the $tRNA_{AC}$-Rzs.

C. Isolation of $tRNA_{AC}$-Rzs with mutations outside the flanking sequence

Out of 20 cloned $tRNA_{AC}$-Rzs, two were found to be inactive. These may have been selected due to *trans* cleavage by the active $tRNA_{AC}$-Rzs prior to their purification. One ribozyme possessed deletions in the hammerhead ribozyme domain which were not part of the conserved region and were therefore not expected to affect ribozyme activity. This region has been shown by several groups not to be important for ribozyme cleavage (McCall et al 1992). Formation of this $tRNA_{AC}$-Rz may have arisen from mutations that occurred during PCR. Since the minimized ribozyme containing deletions retained activity, it was subsequently isolated as part of the 5' cleavage product.

D. Linkers of the $tRNA_{AC}$-Rzs

1. Proposed reasons for selection of $tRNA_{AC}$-Rzs without a 3' linker

Two clones that possessed 3' linkers had a decreased activity compared to the linear control ribozyme (Fig. 17*B*). The absence of 3' linkers in the rest of the clones must be due to the selection procedure, as variable lengths of 3' linkers were detected upon PCR analysis of the $tRNA_{AC}$-Rz template DNA library (Fig. 16). Absence of 3' linkers implies that under the conditions used, the ideal sequences to connect the ribozyme upon insertion in the anticodon loop are the anticodon loop sequences themselves. This preference may be due to potential involvement of 3' linkers in the formation of inhibitory $tRNA_{AC}$-Rz structures that prevent *cis* cleavage. Alternatively, lack of selection of clones containing $tRNA_{AC}$-Rzs with 3' linkers may be due to their toxicity to bacterial cells (see section II.C.)

2. Proposed reasons for selection of $tRNA_{AC}$-Rzs with shorter vs. longer 5' linkers

The selection procedure resulted in more $tRNA_{AC}$-Rz clones that contained shorter linkers (Table 3). However, further analysis revealed that $tRNA_{AC}$-Rzs with longer linkers are better at performing *trans* cleavage (Fig. 17*A* and *B*). As selection relied on *cis* cleavage, it is possible that $tRNA_{AC}$-Rzs with shorter linkers are better at performing *cis* cleavage, but longer linkers were better *trans* cleavers. Since the ribozyme dissociates from its target upon cleavage, it was not possible to select for ribozymes by *trans* cleavage.

Another possibility is that the position of the downstream target favoured the selection of $tRNA_{AC}$-Rzs with shorter linkers. Structural constraints on the ribozyme and target sequences could have been dictated by the length of the sequence separating them (~200 nts from the 3' end of the tRNA sequence).

E. Target RNA cleavage by $tRNA_{AC}$-Rzs

Although the $tRNA_{AC}$-Rzs were provided in excess, the target RNA was not completely cleaved when used during the *trans* cleavage reactions. This could have resulted from some $tRNA_{AC}$-Rzs, within the same sample, assuming inactive structures, such that only a proportion of the $tRNA_{AC}$-Rzs were actually involved in *trans* cleavage. Provision of the optimal linker regions could have contributed to greater numbers of $tRNA_{AC}$-Rzs assuming ideal structures suitable for *trans* cleavage, which was reflected as an increase in the amount of target RNA cleaved with increasing incubation time. Likewise, some of the target RNA could have also assumed structures that prevented binding of the $tRNA_{AC}$-Rzs, such that only a proportion of the target RNA was cleaved. For comparing the $tRNA_{AC}$-Rzs *in vitro*, the target RNA must not be completely cleaved by all $tRNA_{AC}$-Rzs tested at 10 min. Otherwise, the activity of the $tRNA_{AC}$-Rzs could not be compared with each other.

II. ISSUES RELATED TO *IN VIVO* TESTING

A. $tRNA_3^{Lys}$ promoter sequences necessary for transcription *in vivo*

The promoters in the $tRNA_{AC}$-Rzs were intact as they could be transcribed using HeLa nuclear extracts. This is in agreement with previous results that show foreign sequences can be inserted in a tRNA anticodon loop between boxes A and B without disruption of the RNA pol III promoter (Cotten and Birnstiel 1989). Although the possibility exists that these transcripts may have been generated by RNA polymerase II, this seems unlikely since the correct-sized transcripts were observed.

The parent plasmid pSW201 yielded a smaller product upon transcription using a HeLa nuclear extract. However, transcription of plasmid pM13-Lys,3 resulted in a product with a size similar to the product obtained upon transcription of plasmid pDTNβ containing $tRNA_i^{Met}$. Thus,

additional sequences other than the RNA pol III terminator may govern transcription termination in wt $tRNA_3^{Lys}$, since the anticodon loop possesses 4 T residues which could potentially act as a terminator. Sequences upstream and/or downstream of the $tRNA_3^{Lys}$-coding region may contribute to determining the site of termination, and may be necessary when expressing $tRNA_{AC}$-Rzs in cells in order to regulate, or prevent, premature termination. Transcription of other RNA pol III-driven RNAs such as adenovirus VA RNA and U6 snRNA (He and Huang 1997, Prislei et al 1997) have also demonstrated the importance of sequences other than the RNA pol III terminator for proper termination to occur.

B. Design of retroviral vectors used to express $tRNA_{AC}$-Rzs

The $tRNA_{AC}$-Rzs were cloned between the two MMLV LTRs downstream of the *neo* gene in a single copy (SC) design. Cloning may also be performed within the 3' LTR in a double copy (DC) design, which results in gene duplication upon reverse transcription. In addition to gene duplication in the 5' LTR, the DC design has the advantage of positioning the duplicated RNA pol III expression cassette upstream of the RNA pol II promoter. The DC design has been found to be more effective than the SC design (Ilves et al 1996). This could be due to interference between the 5' LTR promoter and the downstream tRNA promoter in the SC design.

Initial attempts were made to develop a DC vector containing a tRNA with a 3' processing site to allow expression of $tRNA_{AC}$-Rzs (See Appendix B). However, the tRNA cassette in this vector was found to be deleted in both 5' and 3' LTRs upon reverse transcription. Similar results have also been obtained by another group (Junker et al 1995). The deletion may have been mediated by direct repeats flanking the tRNA-based expression cassettes. We have also observed gene rearrangement in *E. coli* when using the same vector.

The lack of RNA pol III-driven expression from the SC vector in the MT4 cells may also be due to the cloning site used in the retroviral vector. The upstream 5'LTR promoter may have prevented transcription of the $tRNA_{AC}$-Rzs. When a $tRNA_i^{Met}$-hairpin ribozyme was cloned in a retroviral vector between the LTRs, no RNA pol III-driven RNA could be detected in cells (Ilves et al 1996). When cloning was performed in the 3' LTR, only RNA pol II-driven expression could be detected in transfected HeLa and PA317 cells. However, upon transduction resulting in gene duplication of the tRNA expression cassettes, RNA pol III-driven transcripts accumulated in higher levels (Ilves et al 1996).

C. Toxicity of the $tRNA_{AC}$-Rzs

The pGEM-$tRNA_{AC}$-Rzs contained a T7 promoter and the $tRNA_3^{Lys}$ promoter. Therefore, the $tRNA_{AC}$-Rzs were not transcribed by bacterial cells since they contain neither T7 RNA pol nor RNA pol III. However, even if the $tRNA_{AC}$-Rzs were transcribed, any potentially toxic ribozymes would have been eliminated, as the bacterial cells containing these $tRNA_{AC}$-Rzs would not grow.

The $tRNA_{AC}$-Rzs are not expected to be toxic in mammalian cells, since transduced MT4 cells expressing $tRNA_{AC}$-Rzs did not demonstrate any changes in viability even after 3 months of cell culture (data not shown). Cell counts were also performed every 3-4 days for 2 weeks, and the number of viable cells determined by trypan blue exclusion (data not shown). The viability of transduced cells expressing $tRNA_{AC}$-Rzs was comparable to that of untransduced and vector-transduced cells. Other groups have reported long-term expression of tRNA-chimeras for up to 6 months in cell culture with no apparent changes in cell viability (Yamada et al 1994a).

The $tRNA_{AC}$-Rzs detected in MT4 cells were expressed as part of longer, RNA pol II transcripts. The potential of a $tRNA_{AC}$-Rz to serve as $tRNA_3^{Lys}$ while part of a long RNA transcript is minimal due to the presence of surrounding sequences which could interfere with the

interaction between the mRNA and the tRNA. Furthermore, RNA pol III-transcribed $tRNA_{AC}$-Rzs should not be able to inhibit translation by serving as a $tRNA_3^{Lys}$ since the anticodon has been disrupted. In addition, these $tRNA_{AC}$-Rzs were not expected to be aminoacylated, since one of the binding sites of aminoacyl-tRNA synthetases is the anticodon.

D. Inhibition of HIV-1 replication

Use of the *env*-coding region as a target site meant that only the unspliced and singly-spliced RNAs, which possessed *env* sequences, could be cleaved in the nucleus and the cytoplasm. Cleavage of the unspliced and singly-spliced RNAs would prevent production of Gag, Gag-Pol, Env, Vif, Vpr and Vpu proteins as cleaved RNAs are more likely to be unstable and will be degraded. Cleavage of the primary HIV-1 transcripts before splicing in the nucleus should reduce the production of all viral proteins, as production of spliced mRNAs would be blocked. In both instances, whether cleavage takes place in the nucleus prior to splicing, or in the nucleus and cytoplasm after splicing, infectious virus would not be produced, since structural proteins required for virus particle production will not be produced (Hunter 1997).

The level of inhibition conferred by the $tRNA_{AC}$-Rzs was determined by measuring the amount of HIV-1 CA (p24) proteins released by cells expressing the $tRNA_{AC}$-Rzs, compared to cells expressing the inactive ribozyme control, or the vector alone. However, it is possible that the CA proteins that were detected using the p24 assay resulted from virus-like particles composed only of Gag proteins, and that the decline in CA production resulted from the death of the cells during prolonged *in vitro* culture. But if this were the case, similar amounts of CA proteins should have been produced by all the cells at the same time point. This was not the case, since cells expressing the MoTiN vector sequences, or the inactive ribozyme $tRNA_{AC}$-Rz, started producing high amounts of HIV-1 p24 antigen at day 6, compared to cells expressing the

$tRNA_{AC}$-Rzs. Most probably, the CA proteins detected using the p24 assay originated from both infectious and non-infectious virus. Cleavage of the ribozyme target site within the viral RNA would have lead to a corresponding decrease in the production of infectious virus, and ultimately to the total amount of p24 antigen present in the supernatant. Also, the virus particles detected using the p24 assay may not have contained Env proteins, and therefore would be non-infectious.

The challenge experiments were performed until days 36 (m.o.i., 0.1) and 18 (m.o.i., 1). However, conclusions could only be made regarding the data obtained until day 9 post-infection because cell death was observed for some cell cultures beyond day 12. This could have been due to the high m.o.i. (0.1 and 1) used for the challenge experiment.

Because only RNA pol II-driven transcripts were detected, the actual inhibition imparted by $tRNA_{AC}$-Rzs may be actually greater than observed. The inhibition conferred by the linear Rz was therefore greater since $tRNA_{AC}$-Rzs were also being expressed as part of the long RNA pol II transcript. Presumably the tRNA sequences in some of the $tRNA_{AC}$-Rzs (0/0 and 4/0) were involved in secondary structures which inhibited ribozyme activity.

The downstream Rz control (tRNA-Rz), also expressed as part of a RNA pol II transcript, conferred inhibition similar to that as the linear Rz and $tRNA_{AC}$-Rz (10/0). More appropriate comparisons of the antiviral activity of tRNA-Rz and the $tRNA_{AC}$-Rzs may be made upon development of retroviral vectors that allow RNA pol III-driven expression of tRNA-Rz and the $tRNA_{AC}$-Rzs.

Anticipated modes of inhibition of HIV-1 replication by $tRNA_{AC}$-Rzs (RNA pol III transcripts) are as follows.

1. Ability of the $tRNA_{AC}$-Rzs to bind to HIV-1 RNA in cells

Each Hela cell contains 6 x 10^7 cytoplasmic tRNAs, and 7 x 10^5 cytoplasmic mRNAs (Lodish et al, 1995). Thus, tRNAs are approximately 100-fold more abundant than mRNAs. HIV-1 RNAs expressed in cells should therefore be present at a 100-fold lower concentration than $tRNA_{AC}$-Rzs. However, to be effective, $tRNA_{AC}$-Rzs must be provided with the ability to localize in the same cellular compartment as HIV-1 RNA. Furthermore, since HIV-1 RNA is involved in numerous interactions with various viral proteins, $tRNA_{AC}$-Rzs should also be able to bind to viral proteins which should then bring it to the same site as HIV-1 RNA. Although the incoming viral RNA would be difficult to access as it would be part of a nucleoprotein complex, the $tRNA_{AC}$-Rzs could be assisted by NC proteins which surround the viral RNA in the nucleoprotein complex. NC proteins have been demonstrated to promote unwinding and annealing of nucleic acids, and to increase ribozyme activity *in vitro* (Tsuchihashi et al 1993, Bertrand and Rossi 1994). On the other hand, use of $tRNA_3^{Lys}$ to express ribozymes should enable ribozymes to colocalize with viral RNA both in the cytoplasm during viral assembly, and within the virion upon co-packaging.

2. Competition for co-packaging

The $tRNA_{AC}$-Rzs are expected to be co-packaged since all of the $tRNA_3^{Lys}$ sequences are present except for 1 nt in the anticodon. $tRNA_3^{Lys}$, along with $tRNA_1^{Lys}$, and $tRNA_2^{Lys}$, make up 60% of the viral tRNA population in the virion, whereas, approximately 2% of cellular tRNA is $tRNA_3^{Lys}$. The remaining 40% virion tRNA are non-$tRNA^{Lys}$, which probably result from random incorporation of small amounts of cytoplasmic tRNAs (Huang et al 1994). Assuming that $tRNA_{AC}$-Rzs are expressed from one integrated copy in the cellular genome, then the $tRNA_{AC}$-Rz would only represent a fraction of the total $tRNA_3^{Lys}$ in the cell, and would have to compete with

wt $tRNA_3^{Lys}$ for packaging. But despite this, a $tRNA_3^{Lys}$-ribozyme against HIV-1 PBS (Westaway et al 1998) and a $tRNA_3^{Lys}$-antisense RNA (Lu et al 1997) have been shown to be packaged. The presence of even 1 $tRNA_{AC}$-Rz in the virion should still be effective, as unlike antisense RNAs, a ribozyme would cause irreversible cleavage of the viral RNA. Being in such close proximity within the virion, the $tRNA_{AC}$-Rzs should easily bind to and inactivate HIV-1 genomic RNA.

3. Inhibition of reverse transcription

Once co-packaged, $tRNA_{AC}$-Rzs may still bind to the PBS since the last 18 nts of $tRNA_3^{Lys}$ were not altered. However, reverse transcription may not initiate since the interaction between HIV-1 and $tRNA_3^{Lys}$ has been mapped to other sites aside from the PBS and the last 18 nts of $tRNA_3^{Lys.}$ For example, an interaction between the anticodon and an A-rich loop upstream of the PBS was shown to be involved in the initiation of reverse transcription (Kang and Morrow 1999). Thus, it is unlikely that $tRNA_{AC}$-Rzs would allow reverse transcription of HIV-1 RNA to proceed.

E. Therapeutic value of the $tRNA_{AC}$-Rzs

Because tRNAs are expressed in all cells, tRNA promoters could be utilized for expression in different tissues and organs. Thus, tRNA promoters should also be useful for expression of RNAs in hematopoietic cells, which differentiates into different progeny cells. In contrast, other promoters may be cell or tissue-specific and prevent expression upon differentiation of the parent cell.

The $tRNA_{AC}$-Rzs characterized in this study should be of benefit for expressing ribozymes and other RNAs in cells. Further testing of the expression levels, toxicity, and efficacy of these $tRNA_{AC}$-Rzs in cells and animal models would be needed to proceed to a phase I/II clinical trial.

FUTURE WORK

I. USE OF AN IMPROVED DOUBLE COPY RETROVIRAL VECTOR FOR EXPRESSING MONOMERIC AND MULTIMERIC $tRNA_{AC}$-Rzs

The lack of RNA pol III-driven expression of $tRNA_{AC}$-Rzs in MT4 cells may be due to the absence of $tRNA_3^{Lys}$ sequences necessary for proper expression. The $tRNA_{AC}$-Rzs could be modified to contain these sequences by site directed mutagenesis *via* PCR.

A DC vector that does not contain direct repeats flanking the tRNA expression cassette could also be used to express the $tRNA_{AC}$-Rzs. At present, DC vectors based on MMLV vectors have been developed by several groups. The ability of these vectors to maintain the cloned tRNA expression cassette would have to be determined to ensure that the cloned sequences are still present upon transduction of a target cell line.

A. Monomeric $tRNA_{AC}$-Rzs

1. Ability of $tRNA_{AC}$-Rzs to interfere with cellular tRNAs

The $tRNA_{AC}$-Rzs selected during the course of this thesis may be further characterized as follows. The effect of $tRNA_{AC}$-Rzs in translation may be determined by performing an *in vitro* translation reaction using cell lysates in the presence of $tRNA_{AC}$-Rzs. The capability of $tRNA_{AC}$-Rzs to be aminoacylated may also be determined by using lysyl-tRNA synthetase and a labeled amino acid.

2. Ability of $tRNA_{AC}$-Rzs to bind to reverse transcriptase

A $tRNA_3^{Lys}$ containing a ribozyme cloned downstream of the tRNA coding sequence, which was shown to be co-packaged in virus particles, retained its ability to bind RT *in vitro* (Westaway et al 1998). Thus, to determine whether selected $tRNA_{AC}$-Rzs can be co-packaged

within virus particles, the ability of $tRNA_{AC}$-Rzs to bind to the heterodimeric form of RT, p66/51 (Richter-Cook et al 1992, Mak et al 1994), could be assessed.

3. *In vitro* selection of other $tRNA_{AC}$-Rzs

By varying the selection criteria, $tRNA_{AC}$-Rzs with a particular phenotype may be isolated from the pool. Thus, $tRNA_{AC}$-Rzs capable of selectively binding to HIV-1 RT could be selected from the pool through gel mobility assays.

B. Multimeric $tRNA_{AC}$-Rzs

In order to further improve the efficacy of the ribozyme approach, multimeric $tRNA_{AC}$-Rzs could be developed. A multimeric design is ideal for HIV-1 which has a high mutation rate. Thus, in the event that one site has mutated and is no longer recognized by a $tRNA_{AC}$-Rz, the other $tRNA_{AC}$-Rzs present can still bind and cleave their targets. Vectors expressing these $tRNA_{AC}$-Rzs should prove to be more effective than those expressing monomeric $tRNA_{AC}$-Rzs. Targeting $tRNA_{AC}$-Rzs simultaneously against sequences that are highly conserved within the HIV-1 genome should prevent formation of escape mutants of HIV-1. It is anticipated that it would be much more difficult for the virus to create mutations in all of the target sites, as this could result in a serious growth disadvantage.

A multimeric $tRNA_{AC}$-Rz could also be developed to target highly conserved RNA sites, all derived from a single HIV-1 gene. This could more severely limit the mutations that the virus undergoes, since the probability of maintaining a functional gene with multiple mutations within highly conserved sequences is minimal.

Multimeric $tRNA_{AC}$-Rzs could be constructed by using partially overlapping oligonucleotides which would be annealed and amplified by PCR. The PCR product would then be cloned in a

plasmid. Prior to testing for inhibition of HIV-1 replication in human cells, the multimeric $tRNA_{AC}$-Rzs could also be transcribed *in vitro* and used in *trans* cleavage reactions.

II. DEVELOPMENT OF STABLE TRANSDUCTANTS AND TESTING FOR EXPRESSION OF $tRNA_{AC}$-Rzs BY RNA POLYMERASE III

Retroviral vector constructs allowing RNA pol III-driven expression of monomeric or multimeric $tRNA_{AC}$-Rzs, could be used for transfection to generate amphotropic vector particles. These could then be used to transduce CD4+ cells. RNA pol III-driven expression in cells could be demonstrated by RT-PCR analysis of LMW RNA. To further confirm the presence of ribozyme sequences in the amplified products, a PCR primer may be used which contains the T7 promoter, such that the PCR products could be used as templates for *in vitro* transcription. The transcripts could then be used in *trans* cleavage reactions. Alternatively, RNA pol III-driven expression may be demonstrated by performing primer extension, RNase protection, or Northern blot analysis. Expression by RNA pol III may be confirmed *in vitro* by using α-amanitin which, at a concentration of 10 μg/ml, inhibits RNA pol II but not RNA pol III.

III. TESTING FOR ANTIVIRAL ACTIVITY OF $tRNA_{AC}$-Rzs WITHIN VIRUS PARTICLES

A. HIV-1 challenge of transduced cells expressing RNA pol III-driven monomeric or multimeric $tRNA_{AC}$-Rzs

Challenge of transduced cells with HIV-1 would determine whether the RNA pol III-driven monomeric or multimeric $tRNA_{AC}$-Rzs were able to inhibit HIV-1 replication. The amount of HIV-1 produced by the infected cells may be determined by a p24 assay.

B. Determine RNA content of HIV-1 virion released by stable transductants expressing $tRNA_{AC}$-Rzs

Once RNA pol III-driven expression of $tRNA_{AC}$-Rzs has been established, RNA from virus particles released by stable transductants expressing $tRNA_{AC}$-Rzs could be tested for presence of co-packaged ribozyme sequences. Viral RNA could be isolated using a kit (Qiagen; Santa Clarita, USA), and co-packaging of $tRNA_{AC}$-Rzs could be determined by RT-PCR using ribozyme-specific primers.

C. Determine infectivity of HIV-1 virion released by stable transductants expressing $tRNA_{AC}$-Rzs

As HIV-1 RNA cleavage should result in reduced infectivity of released virions, an infectivity assay could be performed using the HeLa-CD4-LTR-β-gal cell line. Expression of β-gal in this cell line is under control of the HIV LTR; thus expression of β-galactosidase results from *trans* activation by the HIV Tat protein. Virus particles, released from the infected cells expressing $tRNA_{AC}$-Rzs, could be used to infect HeLa-CD4-LTR-β-gal cells. Infectivity could then be measured by counting the number of blue cells upon staining the cells with X-gal.

IV. TESTING FOR INHIBITION IN CHRONICALLY INFECTED CELLS

The challenge experiments demonstrated protection conferred by $tRNA_{AC}$-Rzs to uninfected cells. To demonstrate protection of cells which already harbour virus, latently infected cells, such as ACH-2 (Clouse et al 1989, Folks et al 1989), and J1.1 (Perez et al 1991), could be transduced with vector particles expressing $tRNA_{AC}$-Rzs. Induction of these cells with phorbol myristate acetate or TNF-α leads to secretion of high levels of infectious HIV. Therefore, a reduction in HIV-1 production by these cells would denote protection by the $tRNA_{AC}$-Rzs.

V. TESTING FOR INHIBITION IN PERIPHERAL BLOOD LYMPHOCYTES AND HEMATOPOIETIC STEM CELLS

Retroviral vectors expressing the $tRNA_{AC}$-Rzs could also be tested in human PBLs and hematopoietic stem cells. Use of a pluripotent hematopoietic stem cell precursor population as a target may allow expression of anti-HIV-1 genes in progeny cells without the need for multiple reinfusion of PBLs. However, retroviral vectors have limited use in hematopoietic stem cell transduction as they require cell division for their integration, and the titers of recombinant vectors commonly achieved are low. Lentiviral vectors may therefore be more useful as they have been shown to be capable of transducing quiescent cells such as neurons and hepatocytes (reviewed by Klimatcheva et al 1999). The $tRNA_{AC}$-Rzs may therefore be cloned in a lentiviral vector to allow testing of their efficacy in the progeny cells differentiated from transduced hematopoietic stem cells.

REFERENCES

Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, Martin MA: **Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone.** (1986) *J Virol* **59**:284-291.

Adeniyi-Jones S, Romeo PH, Zasloff M: **Generation of long read-through transcripts in vivo and in vitro by deletion of 3' termination and processing sequences in the human $tRNA_i^{Met}$ gene.** *Nucleic Acids Res* (1984) **12**:1101-1115.

Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA: **CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1.** *Science* (1996) **272**:1955-1958.

Amado RG, Mitsuyasu RT, Symonds G, Rosenblatt JD, Zack J, Sun LQ, Miller M, Ely J, Gerlach W: **A phase I trial of autologous CD34+ hematopoietic progenitor cells transduced with an anti-HIV ribozyme.** *Hum Gene Ther* (1999) **10**:2255-2270.

Amado R, Rosenblatt J, Zack J, Miles S, Ely J, Symonds G, Mitsuyasu R: **A phase I gene therapy clinical study of autologous CD34+ cells transduced with an anti-HIV-1 ribozyme.** *Blood Suppl* (1998) **92**:665a.

Arts GJ, Fornerod M, Mattaj IW: **Identification of a nuclear export receptor for tRNA.** *Curr Biol* (1998a) **8**:305-314.

Arts GJ, Kuersten S, Romby P, Ehresmann B, Mattaj IW: **The role of exportin-t in selective nuclear export of mature tRNAs.** *EMBO J* (1998b) **17**:7430-7441.

Bachand F, Yao XJ, Hrimech M, Rougeau N, Cohen EA: **Incorporation of Vpr into human immunodeficiency virus type 1 requires a direct interaction with the p6 domain of the p55 gag precursor.** *J Biol Chem* (1999) **274**:9083-9091.

Baier G, Coggeshall KM, Baier-Bitterlich G, Giampa L, Telford D, Herbert E, Shih W, Altman A: **Construction and Characterization of *lck*- and *fyn*-specific tRNA:Ribozyme chimeras.** *Mol Immunol* (1994) **31**:923-932.

Barcellini-Couget S, Fenard D, Bertrand E, Singer RH, Lefebvre JC, Doglio A: **3'-End modification of the adenoviral VA1 gene affects its expression in human cells: consequences for the design of chimeric VA1 RNA ribozymes.** *Antisense Nucleic Acid Drug Dev* (1998) **8**:379-390.

Bauer G, Valdez P, Kearns K, Bahner I, Wen SF, Zaia JA, Kohn DB: **Inhibition of human immunodeficiency virus-1 (HIV-1) replication after transduction of granulocyte colony-stimulating factor-mobilized CD34+ cells from HIV-1-infected donors using retroviral vectors containing anti-HIV-1 genes.** *Blood* (1997) **89**:2259-2267.

Bechtel C: **Gene Therapy for AIDS Using Retroviral Mediated Gene Transfer to Deliver HIV-1 Antisense TAR and Transdominant Rev Protein Genes to Syngeneic Lymphocytes in HIV-1 Infected Identical Twins.** NIH/00783.

Beck J, Nassal M: **Efficient hammerhead ribozyme-mediated cleavage of the structured hepatitis B virus encapsidation signal in vitro and in cell extracts, but not in intact cells.** *Nucleic Acids Res* (1995) **23**:4954-4962.

Bertrand E, Castanotto D, Zhou C, Carbonnelle C, Lee NS, Good P, Chatterjee S, Grange T, Pictet R, Kohn D, Engelke D, Rossi JJ: **The expression cassette determines the functional activity of ribozymes in mammalian cells by controlling their intracellular localization.** *RNA* (1997) **3**:75-88.

Bertrand EL, Rossi JJ: **Facilitation of hammerhead ribozyme catalysis by the nucleocapsid protein of HIV-1 and the heterogeneous nuclear ribonucleoprotein A1.** *EMBO J* (1994) **13**:2904-2912.

Biasolo MA, Radaaelli A, DelPup L, Franchin E, DeGiuli-Morghen C, Palu G: **A new antisense tRNA construct for the genetic treatment of human immunodeficiency virus type 1 infection.** *J Virol* (1996) **70**:2154-2161.

Birikh KR, Heaton PA, Eckstein F: **The structure, function and application of the hammerhead ribozyme.** *Eur J Biochem* (1997) **245**:1-16.

Bonyhadi ML, Moss K, Voytovich A, Auten J, Kalfoglou C, Plavec I, Forestell S, Su L, Bohnlein E, Kaneshima H: **RevM10-expressing T cells derived in vivo from transduced human hematopoietic stem-progenitor cells inhibit human immunodeficiency virus replication.** *J Virol* (1997) **71**:4707-4716.

Brown PO: **Integration.** In Retroviruses. Eds JM Coffin, SH Hughes, HE Varmus. Cold Spring Harbor Laboratory Press, Plainview, N.Y 161-204 (1997).

Browning CM, Cagnon L, Good PD, Rossi J, Engelke DR, Markovitz DM: **Potent inhibition of human immunodeficiency virus type 1(HIV-1) gene expression and virus production by an HIV-2 tat activation-response RNA decoy.** *J Virol* (1999) **73**:5191-5195.

Buchholz CJ, Peng KW, Morling FJ, Zhang J, Cosset FL, Russell SJ: **In vivo selection of protease cleavage sites from retrovirus display libraries.** *Nat Biotechnol* (1998) **16**:951-954.

Buchholz CJ, Stitz J, Cichutek K: **Retroviral cell targeting vectors.** *Curr Opin Mol Ther* (1999) **1**:613-621.

Cagnon L, Cucchiarini M, Lefebvre JC, Doglio A: **Protection of a T-cell line from human immunodeficiency virus replication by the stable expression of a short antisense RNA sequence carried by a shuttle RNA molecule.** *J Acquir Immune Defic Syndr Hum Retrovirol* (1995) **9**:349-358.

Cairns JS, D'Souza MP: **Chemokines and HIV-1 second receptors: the therapeutic connection.** *Nat Med* (1998) **4**:563-568.

Castanotto D, Rossi JJ, Deshler JO: **Biological and functional aspects of catalytic RNAs.** *Crit Rev Eukaryot Gene Expr* (1992) **2**:331-357.

Chadwick MP, Morling FJ, Cosset FL, Russell SJ: **Modification of retroviral tropism by display of IGF-I.** *J Mol Biol* (1999) **285**:485-494 .

Chen CC, Banerjea AC, Harmison GG, Haglund K, Schubert M: **Multitarget-Rz directed to cleave at up to nine highly conserved HIV-1 Env RNA regions inhibits HIV-1 replication-potential effectiveness against most presently sequenced HIV-1 isolates.** *Nucleic Acids Res* (1992) **20**: 4581-4589.

Chomczynski, P, Sacchi N: **Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction.** *Anal Biochem* (1987) **162**:156-159.

Chuah MKL, Vandendriessche T, Chang HK, Ensoli B, Morgan RA: **Inhibition of human immunodeficiency virus type 1 by retroviral vectors expressing antisense-TAR.** *Hum Gene Ther* (1994) **5**:1467-1475.

Clouse KA, Powell D, Washington I, Poli G, Strebel K, Farrar W, Barstad P, Kovacs J, Fauci AS, Folks TM: **Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone.** *J Immunol* (1989) **142**:431-438.

Cohli H, Fan B, Joshi RL, Ramezani A, Li X, Joshi S: **Inhibition of HIV-1 replication in a human CD4$^+$ lymphocytic cell line expressing antisense and sense RNA molecules containing HIV-1 packaging signal and Rev response element.** *Antisense Res Dev* (1994) **4**: 19-26.

Cotten M, Birnstiel ML: **Ribozyme mediated destruction of RNA *in vivo*.** *EMBO J* (1989) **8**:3861-3866.

Cooper D, Penny R, Symonds G, Carr A, Gerlach W, Sun LQ, Ely J: **A marker study of therapeutically transduced CD4+ peripheral blood lymphocytes in HIV discordant identical twins.** *Hum Gene Ther* (1999) **10**:1401-1421.

Cujec TP, Okamoto H, Fujinaga K, Meyer J, Chamberlin H, Morgan DO, Peterlin BM: **The HIV transactivator TAT binds to the CDK-activating kinase and activates the phosphorylation of the carboxy-terminal domain of RNA polymerase II.** *Genes Dev* (1997) **11**:2645-2657.

de Falco G, Giordano A: **CDK9 (PITALRE): a multifunctional cdc2-related kinase.** *J Cell Physiol* (1998) **177**:501-506.

de Polo NJ, Harkleroad CE, Bodner M, Watt AT, Anderson CG, Greengard JS, Murthy KK, Dubensky TW Jr, Jolly DJ: **The resistance of retroviral vectors produced from human cells to serum inactivation in vivo and in vitro is primate species dependent.** *J Virol* (1999) **73**:6708-6714.

Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR: **Identification of a major co-receptor for primary isolates of HIV-1.** *Nature* (1996) **381**:661-666.

Ding SF, Noronha J, Joshi S: **Co-packaging of sense and antisense RNAs: a novel strategy for blocking HIV-1 replication.** *Nucleic Acids Res* (1998) **26**:3270-3278.

Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA: **HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5.** *Nature* (1996) **381**:667-673.

Dropulic B, Lin NH, Martin MA, Jeang K: **Functional Characterization of a U5 Ribozyme: Intracellular Suppression of Human Immunodeficiency Virus Type 1 Expression.** *J Virol* (1992) **66**:1432-1441.

Du Z, Ricordi C, Inverardi L, Podack E, Pastori RL: **Efficient *Ex vivo* Inhibition of Perforin and Fas Ligand Expression by Chimeric tRNA-Hammerhead Ribozymes.** *Hum Gene Ther* (1998) **9**:1551-1560.

Duan L, Zhu M, Ozaki I, Zhang H, Wei DL, Pomerantz RJ: **Intracellular inhibition of HIV-1 replication using a dual protein- and RNA-based strategy.** *Gene Ther* (1997) **4**:533-43.

Emerman M, Malim MH: **HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology.** *Science* (1998) **280**:1880-1884.

Engel BC, Kohn DB: **Stem cell directed gene therapy.** *Front Biosci* (1999) **1**:26-33.

Feng Y, Broder CC, Kennedy PE, Berger EA: **HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor.** *Science* (1996) **272**:872-877.

Fielding AK, Maurice M, Morling FJ, Cosset FL, Russell SJ: **Inverse targeting of retroviral vectors: selective gene transfer in a mixed population of hematopoietic and nonhematopoietic cells.** *Blood* (1998) **91**:1802-1809.

Folks TM, Clouse KA, Justement J, Rabson A, Duh E, Kehrl JH, Fauci AS: **Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone.** *Proc Natl Acad Sci USA* (1989) **86**:2365-2368.

Forster AC, Symons RH: **Self-Cleavage of Plus and Minus RNAs of a Virusoid and a Structural Model for the Active Sites.** *Cell* (1987) **49**:211-220.

Frankel AD, Young JA: **HIV-1: fifteen proteins and an RNA.** *Annu Rev Biochem* (1998) **67**:1-25.

Freed EO: **HIV-1 gag proteins: diverse functions in the virus life cycle.** *Virology* (1998) **251**:1-15.

Gallay P, Stitt V, Mundy C, Oettinger M, Trono D: **Role of the karyopherin pathway in human immunodeficiency virus type 1 nuclear import.** *J Virol* (1996) **70**:1027-1032.

Gebhard JR, Perry CM, Mahadeviah S, Whitton JL: **Use of a non-viral vector to express a chimeric tRNA-ribozyme against Lymphocytic Choriomeningitis Virus:cytoplasmic accumulation of a catalytically-competent transcript, but minimal antiviral effect.** *Antisense Nucleic Acid Drug Dev* (1997) **7**:3-11.

Geiduschek EP, Tocchini-Valentini GP: **Transcription by RNA polymerase III.** *Ann Rev Biochem* (1988) **57**:873-914.

Gervaix A, Li X, Kraus G, Wong-Staal F: **Multigene antiviral vectors inhibit diverse human immunodeficiency virus type 1 clades.** *J Virol* (1997a) **71**:3048-3053.

Gervaix A, Schwarz L, Law P, Ho AD, Looney D, Lane T, Wong-Staal F: **Gene therapy targeting peripheral blood CD34+ hematopoietic stem cells of HIV-infected individuals.** *Hum Gene Ther* (1997b) **8**:2229-2238.

Goila R, Banerjea AC: **Sequence specific cleavage of the HIV-1 coreceptor CCR5 gene by a hammer-head ribozyme and a DNA-enzyme: inhibition of the coreceptor function by DNA-enzyme.** *FEBS Lett* (1998) **436**:233-238.

Gonzalez MA, Serrano F, Llorente M, Abad JL, Garcia-Ortiz MJ, Bernad A: **A hammerhead ribozyme targeted to the human chemokine receptor CCR5.** *Biochem Biophys Res Commun* (1998) **251**:592-596.

Good PD, Krikos AJ, Li SX, Bertrand E, Lee NS, Giver L, Ellington A, Zaia JA, Rossi JJ, Engelke DR: **Expression of small, therapeutic RNAs in human cell nuclei.** *Gene Ther* (1997) **4**:45-54.

Hampel A: **The hairpin ribozyme: discovery, two-dimensional model, and development for gene therapy.** *Prog Nucleic Acid Res Mol Biol* (1998) **58**:1-39.

Haseloff J, Gerlach W: **Simple RNA enzymes with new and highly specific endoribonuclease activities.** *Nature* (1988) **334**:585-591.

He Y, Huang L: **Growth inhibition of human papillomavirus 16 DNA-positive mouse tumor by antisense RNA transcribed from U6 promoter.** *Cancer Res* (1997) **57**:3993-3999.

He Y, Zeng Q, Drenning SD, Melhem MF, Tweardy DJ, Huang L, Grandis JR: **Inhibition of human squamous cell carcinoma growth in vivo by epidermal growth factor receptor antisense RNA transcribed from the U6 promoter.** *J Natl Cancer Inst* (1998) **90**:1080-1087.

Heidenreich O, Eckstein F: **Hammerhead Ribozyme-mediated Cleavage of the Long Terminal Repeat RNA of Human Immunodeficiency Virus Type 1.** *J Biol Chem* (1992) **267**:1904-1909.

Heusch M, Kraus G, Johnson P, Wong-Staal F: **Intracellular immunization against SIVmac utilizing a hairpin ribozyme.** *Virology* (1996) **216**:241-244.

Homann M, Tzortzakaki S, Rittner K, Sczakiel G, Tabler M: **Incorporation of the catalytic domain of a hammerhead ribozyme into antisense RNA enhances its inhibitory effect on the replication of human immunodeficiency virus type 1.** *Nucleic Acids Res* (1993).**21**:2809-2814.

Hope TJ: **The ins and outs of HIV Rev.** *Arch Biochem Biophys* (1999) **365**:186-191.

Huang Y, Mak J, Cao Q, Li Z, Wainberg MA, Kleiman L: **Incorporation of excess wild-type and mutant tRNA(3Lys) into human immunodeficiency virus type 1.** *J Virol* (1994) **68**:7676-7683.

Huang Y, Shalom A, Li Z, Wang J, Mak J, Wainberg MA, Kleiman L:**Effects of modifying the tRNA(3Lys) anticodon on the initiation of human immunodeficiency virus type 1 reverse transcription.** *J Virol* (1996) **70**:4700-4706.

Hunter E: **Viral Entry and Receptors.** In Retroviruses. Eds JM Coffin, SH Hughes, HE Varmus. Cold Spring Harbor Laboratory Press, Plainview, N.Y 71-120 (1997).

Ilves H, Barske C, Junker U, Bohnlein E, Veres G: **Retroviral vectors designed for targeted expression of RNA polymerase III-driven transcripts: a comparative study.** *Gene* (1996) **171**:203-208.

Jackson WH Jr, Moscoso H, Nechtman JF, Galileo DS, Garver FA, Lanclos KD:. **Inhibition of HIV-1 replication by an anti-tat hammerhead ribozyme.** *Biochem Biophys Res Commun* (1998) **245**:81-84.

James W: **The use of ribozymes in gene therapy approaches to AIDS.** *Recent Results Cancer Res* (1998) **144**:139-146.

Jennings PA, Molloy PL:**Inhibition of SV40 replication function by engineered antisense RNA transcribed by RNA polymerase III** *EMBO J* (1987) **6**:3043-3047.

Jiang M, Mak J, Ladha A, Cohen E, Klein M, Rovinski B, Kleiman L: **Identification of tRNAs Incorporated into Wild-Type and Mutant Human Immunodeficiency Virus Type 1.** *J Virol* (1993).**67**:3246-3253.

Jones KA: **Taking a new TAK on tat transactivation.** *Genes Dev* (1997) **11**:2593-2599.

Joshi S, Davison L, Van der Elst I, Van Brunschot A: **A retroviral vector to allow constitutive and tat-inducible gene expression for anti-HIV-1 gene therapy..** *Mol Biol Life Sci Adv* (1993) **12**:135-189.

Joshi S, Joshi RL: **Molecular biology of human immunodeficiency virus type-1.** *Transfus Sci* (1996) **17**:351-378.

Joshi S, Van Brunschot A, Asad S, van der Elst I, Read SE, Bernstein A: **Inhibition of human immunodeficiency virus type 1 multiplication by antisense and sense RNA expression.** *J Virol* (1991) **65**:5524-5230.

Junker U, Bohnlein E, Veres G: **Genetic instability of a MoMLV-based antisense double-copy retroviral vector designed for HIV-1 gene therapy.** *Gene Ther* (1995) **2**:639-646.

Junker U, Rittner K, Homann M, Bevec D, Bohnlein E, Sczakiel G: **Reduction in replication of the of human immunodeficiency virus type 1 in human T cell lines by polymerase III-driven transcription of chimeric tRNA-antisense RNA genes.** *Antisense Res Dev* (1994) **4**:165-172.

Kang SM, Morrow CD: **Genetic analysis of a unique human immunodeficiency virus type 1 (HIV-1) with a primer binding site complementary to tRNAMet supports a role for U5-PBS stem-loop RNA structures in initiation of HIV-1 reverse transcription.** *J Virol* (1999) **73**:1818-1827.

Kawasaki H, Eckner R, Yao T, Taira K, Chiu R, Livingston DM, Yokoyama KK: **Distinct roles of the co-activators p300 and CBP in retinoic-acid-induced F9-cell differentiation** *Nature* (1998) **393**:284-289.

Kawasaki H, Ohkawa J, Tanishige N, Yoshinari K, Murata T, Yokoyama KK, Taira K: **Selection of the best target site for ribozyme-mediated cleavage within a fusion gene for adenovirus E1A-associated 300kDa protein (p300) and luciferase.** *Nucleic Acids Res* (1996) **24**:3010-3016.

Kimpton J, Emerman M: **Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene.** *J Virol* (1992) **66**:2232-2239.

Kleiman L, Caudry S, Boulerice F, Wainberg MA, Parniak MA: **Incorporation of tRNA into normal and mutant HIV-1.** *Biochem Biophys Res Commun* (1991) **174**:1272-1280.

Klimatcheva E, Rosenblatt JD, Planelles V: **Lentiviral vectors and gene therapy.** *Front Biosci* (1999) **4**:D481-496.

Kohn DB, Bauer G, Rice CR, Rothschild JC, Carbonaro DA, Valdez P, Hao Ql, Zhou C, Bahner I, Kearns K, Brody K, Fox S, Haden E, Wilson K, Salata C, Dolan C, Wetter C, Aguilar-Cordova E, Church J: **A clinical trial of retroviral-mediated transfer of a rev-responsive element decoy gene into CD34(+) cells from the bone marrow of human immunodeficiency virus-1-infected children.** *Blood* (1999) **1**:368-371.

Koseki S, Ohkawa J, Yamamoto R, Takebe Y, Taira K: **A simple assay system for examination of the inhibitory potential *in vivo* of decoy RNAs, ribozymes and other drugs by measuring the Tat-mediated transcription of a fusion gene composed of the long terminal repeat of HIV-1 and a gene for luciferase.** *J Controlled Release* (1998) **53**:159-173.

Koseki S, Tanabe T, Tani K, Asano S, Shioda T, Nagai Y, Shimada T, Ohkawa J, Taira K: **Factors governing the activity *in vivo* of ribozymes transcribed by RNA polymerase III.** *J Virol* (1999) **73**:1868-1877.

Kozu T, Sueoka E, Okabe S, Sueoka N, Komori A, Fujiki H: ***In vitro*** **catalytic activities of DNA/RNA chimeric hammerhead ribozymes against AML1-MTG8 mRNA, a fused gene transcript in acute myeloid leukemia with t(8;21).** *Biochimie* (1996) **78**:1067-1073.

Kunkel GR, Pederson T: **Transcription of a human U6 small nuclear RNA gene in vivo withstands deletion of intragenic sequences but not of an upstream TATATA box.** *Nucleic Acids Res* (1989) **17**:7371-9.

Kutay U, Lipowsky G, Izaurralde E, Bischoff FR, Schwarzmaier P, Hartmann E, Gorlich D: **Identification of a tRNA-specific nuclear export receptor.** *Mol Cell* (1998) **1**:359-369.

Kuwabara T, Warashina M, Nakayama A, Ohkawa J, Taira K: **Activities of tRNA-embedded dimeric minizymes.** *Nucleic Acids Symp Ser* (1997a) **37**:307-308.

Kuwabara T, Warashina M, Nakayama A, Ohkawa J, Taira K : **tRNAVal-heterodimeric maxizymes with high potential as gene inactivating agents: simultaneous cleavage at two sites in HIV-1 tat mRNA in cultured cells.** *Proc Natl Acad Sci USA* (1999) **96**:1886-1891.

Kuwabara T, Warashina M, Tanabe T, Tani K, Asano S, Taira K: **Comparison of the specificities and catalytic activities of hammerhead ribozymes and DNA enzymes with respect to the cleavage of BCR-ABL chimeric L6 (*b2a2*) mRNA.** *Nucleic Acids Res* (1997b) **25**:3074-3081.

Kuwabara T, Warashina M, Tanabe T, Tani K, Asano S, Taira K: **A novel allosterically trans-activated ribozyme, the maxizyme, with exceptional specificity *in vitro* and *in vivo*.** *Mol Cell* (1998b) **2**:617-627.

Kuwabara T, Warashina M, Orita M, Koseki S, Ohkawa J, Taira K: **Formation of a catalytically active dimer by tRNA(Val)-driven short ribozymes.** *Nat Biotechnol* (1998a) **16**:961-965.

Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA: **Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody.** *Nature* (1998) **393**:648-59.

Larder BA, Darby G, Richman DD: **HIV with reduced sensitivity to AZT isolated during prolonged therapy.** *Science* (1989) **243**:1731-1734.

Larsson S, Hotchkiss G, Su J, Kebede T, Andang M, Nyholm T, Johansson B, Sonnerborg A, Vahlne A, Britton S, Ahrlund-Richter L: **A novel ribozyme target site located in the HIV-1 nef open reading frame.** *Virology* (1996) **219**:161-169.

Lazarev VN, Shmarov MM, Zakhartchouk AN, Yurov GK, Misurina OU, Akopian TA, Grinenko NF, Grodnitskaya NG, Kaverin NV, Naroditsky BS: **Inhibition of influenza A virus reproduction by a ribozyme targeted against PB1 mRNA.** *Antiviral Res* (1999) **42**:47-57.

Leavitt MC, Yu M, Yamada O, Kraus G, Looney D, Poeschla E, Wong-Staal F: **Transfer of an anti-HIV-1 ribozyme gene into primary human lymphocytes.** *Hum Gene Ther* (1994) **5**:1115-1120.

Lee SW, Gallardo HF, Gaspar O, Smith C, Gilboa E: **Inhibition of HIV-1 in CEM cells by a potent TAR decoy.** *Gene Ther* (1995) **2**:377-384.

Lee SW, Gallardo HF, Gilboa E, Smith C: **Inhibition of human immunodeficiency virus type 1 in human T cells by a potent Rev response element decoy consisting of the 13-nucleotide minimal Rev-binding domain.** *J Virol* (1994) **68**:8254-8264.

Lee TC, Sullenger BA, Gallardo HF, Ungers GE, Gilboa E: **Overexpression of RRE-derived sequences inhibits HIV-1 replication in CEM cells.** *New Biol* (1992) **4**:66-74.

Li X, Gervaix A, Kang D, Law P, Spector SA, Ho AD, Wong-Staal F: **Gene therapy targeting cord blood-derived CD34+ cells from HIV-exposed infants: preclinical studies.** *Gene Ther* (1998) **5**:233-239.

Liem SE, Ramezani A, Li X, Joshi S: **The development and testing of retroviral vectors expressing trans-dominant mutants of HIV-1 proteins to confer anti-HIV-1 resistance.** *Hum Gene Ther* (1993) **4**:625-634.

Lodish H, Baltimore D, Berk A, Zipursky SL, Matsudaira P, Darnell (1995) in *Molecular Cell Biology* (Scientific American Books, New York) pp. 142-147.

Lorentzen EU, Wieland U, Kuhn JE, Braun RW: **In vitro cleavage of HIV-1 vif RNA by a synthetic ribozyme.** *Virus Genes* (1991) **5**:17-23.

Lu Y, Planelles V, Li X, Palaniappan C, Day B, Challita-Eid P, Amado R, Stephens D, Kohn DB, Bakker A, Fay P, Bambara RA, Rosenblatt JD: **Inhibition of HIV-1 replication using a mutated tRNALys-3 primer.** *J Biol Chem* (1997) **272**:14523-14531.

Macpherson JL, Ely JA, Sun LQ, Symonds GP: **Ribozymes in gene therapy of HIV-1.** *Front Biosci* (1999) **1**;D497-505.

Mak J, Jiang M, Wainberg MA, Hammarskjold H, Rekosh D, Kleiman L: **Role of $Pr160^{gag\text{-}pol}$ in Mediating the Selective Incorporation of $tRNA^{Lys}$ into Human Immunodeficiency Virus Type 1 Particles.** *J Virol* (1994).**68**:2065-2072.

Mak J, Kleiman L: **Primer tRNAs for reverse transcription.** *J Virol* (1997) **11**:8087-8095.

Mancebo HS, Lee G, Flygare J, Tomassini J, Luu P, Zhu Y, Peng J, Blau C, Hazuda D, Price D, Flores O: **P-TEFb kinase is required for HIV Tat transcriptional activation in vivo and in vitro.** *Genes Dev* (1997) **11**:2633-2644.

Mann R, Mulligan RC, Baltimore D: **Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus.** *Cell* (1983) **33**:153-159.

Marcello A, Giaretta I: **Inducible expression of herpes simplex virus thymidine kinase from a bicistronic HIV1 vector.** *Res Virol* (1998) **149**:419-431.

Marsh JW: **The numerous effector functions of Nef.** *Arch Biochem Biophys* (1999) **365**:192-198.

Mathews DH, Sabina J, Zuker M, Turner DH: **Expanded sequence dependence of thermodynamic parameters provides robust prediction of RNA secondary structure.** *J Mol Biol* (1999) **288**:911-940.

Mathews MB, Shenk T: **Adenovirus virus-associated RNA and translation control.** *J Virol* (1991) **65**:5657-5662.

McCall MJ, Hendry P, Jennings PA: **Minimal sequence requirements for ribozyme activity.** *Proc Natl Acad Sci USA* (1992) **89**:5710-5714.

Melekhovets YF, Joshi S: **Fusion with an RNA binding domain to confer target RNA specificity to an RNase: design and engineering of Tat-RNase H that specifically recognizes and cleaves HIV-1 RNA in vitro.** *Nucleic Acids Res* (1996) **24**:1908-1912.

Mhashilkar AM, LaVecchio J, Eberhardt B, Porter-Brooks J, Boisot S, Dove JH, Pumphrey C, Li X, Weissmahr RN, Ring DB, Ramstedt U, Marasco WA: **Inhibition of human immunodeficiency virus type 1 replication in vitro in acutely and persistently infected human CD4+ mononuclear cells expressing murine and humanized anti-human immunodeficiency virus type 1 Tat single-chain variable fragment intrabodies.** *Hum Gene Ther* (1999) **10**:1453-1467.

Michienzi A, Conti L, Varano B, Prislei S, Gessani S, Bozzoni: **Inhibition of human immunodeficiency virus type 1 replication by nuclear chimeric anti-HIV ribozymes in a human T lymphoblastoid cell line.** *Hum Gene Ther* (1998) **9**:621-628.

Michienzi A, Prislei S, Bozzoni I X: **U1 small nuclear RNA chimeric ribozymes with substrate specificity for the Rev pre-mRNA of human immunodeficiency virus.** *Proc Natl Acad Sci USA* (1996) **93**:7219-7224.

Miller AD, Buttimore C: **Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production.** *Mol Cell Biol* (1986) **6**:2895-2902.

Morgan RA, Walker R: **Gene therapy for AIDS using retroviral mediated gene transfer to deliver HIV-1 antisense TAR and transdominant Rev protein genes to syngeneic lymphocytes in HIV-1 infected identical twins.** *Hum Gene Ther* (1996) 7:1281-1306.

Ohkawa J, Yuyama N, Takebe Y, Nishikawa S, Taira K: **Importance of independence in ribozyme reactions:Kinetic behavior of trimmed and of simply connected multiple ribozymes with potential activity against human immunodeficiency virus.** *Proc Natl Acad Sci USA* (1993b) **90**:11302-11306.

Ohkawa J, Yuyama N, Takebe Y, Nishikawa S, Homann M, Sczakiel G, Taira K: **Multiple site-specific cleavage of HIV RNA by transcribed ribozymes from shotgun-type trimming plasmid.** *Nucleic Acids Symp Ser* (1993a) **29**:121-122.

Orkin SH, Motulsky AG: **Report and Recommendations of the Panel to Assess the NIH investment in research on gene therapy.** (1995) http://www.nih.gov/news/panelrep.html

Ozaki I, Zern MA, Liu S, Wei DL, Pomerantz RJ, Duan L: **Ribozyme-mediated specific gene replacement of the alpha1-antitrypsin gene in human hepatoma cells.** *J Hepatol* (1999) **31**:53-60

Pace U, Bockman JM, MacKay BJ, Miller WH Jr, Dmitrovsky E, Goldberg AR: **A ribozyme which discriminates in vitro between PML/RAR alpha, the t(15;17)-associated fusion RNA of acute promyelocytic leukemia, and PML and RAR alpha, the transcripts from the nonrearranged alleles.** *Cancer Res* (1994) **54**:6365-6369.

Paik SY, Banerjea A, Chen CJ, Ye Z, Harmison GG, Schubert M: **Defective HIV-1 provirus encoding a multitarget-ribozyme inhibits accumulation of spliced and unspliced HIV-1 mRNAs, reduces infectivity of viral progeny, and protects the cells from pathogenesis.** *Hum Gene Ther* (1997) **8**:1115-1124.

Paillart JC, Marquet R, Skripkin E, Ehresmann C, Ehresmann B: **Dimerization of retroviral genomic RNAs: structural and functional implications.** *Biochimie* (1996) **78**:639-653.

Pauwels R, De Clerq E, Desmyter J, Balzarini J, Goubau P, Herdewijn P, Banderhaeghe H, Vandeputte M: **Sensitive and rapid assay on MT4 cells for detection of antiviral compounds against the AIDS virus.** *J Virol* (1987)**16**:171-185.

Peng H, Callison D, Li P, Burrell,C: **Long-term protection against HIV infection conferred by tat or rev Antisense RNA was affected by the design of the retroviral vector.** *Virology* (1996) **220**:377-389.

Peng H, Callison DE, Li P, Burrell CJ: **Enhancement or inhibition of HIV-1 replication by intracellular expression of sense or antisense RNA targeted at different intermediates of reverse transcription.** *AIDS* (1997) **11**:587-595.

Peng H, Callison D, Li P, Burrell CJ: **Interference between effector RNAs expressed from conventional dual-function anti-HIV retroviral vectors can be circumvented using dual-effector-cassette retroviral vectors.** *Hum Gene Ther* (1999) **10**:449-462.

Perez VL, Rowe T, Justement JS, Butera ST, June CH, Folks TM: **An HIV-1-infected T cell clone defective in IL-2 production and Ca2+ mobilization after CD3 stimulation.** *J Immunol* (1991) **147**:3145-3148.

Perriman R, Bruening G, Dennis ES, Peacock WJ: **Effective ribozyme delivery in plant cells.** *Proc Natl Acad Sci USA* (1995) **92**:6175-6179.

Perriman R, de Feyter R: **tRNA delivery systems for ribozymes.** *Methods Mol Biol* (1997) **74**:393-402.

Ping YH, Rana TM: **Tat-associated kinase (P-TEFb): a component of transcription preinitiation and elongation complexes.** *J Biol Chem* (1999) **274**:7399-7404.

Popov S, Rexach M, Zybarth G, Reiling N, Lee MA, Ratner L, Lane CM, Moore MS, Blobel G, Bukrinsky M: **Viral protein R regulates nuclear import of the HIV-1 pre-integration complex.** *EMBO J* (1998) **17**:909-917.

Prislei S, Buonomo SBC, Michienzi A, Bozzoni I: **Use of adenoviral VAI small RNA as a carrier for cytoplasmic delivery of ribozymes.** *RNA* (1997) **3**:677-687.

Proudfoot AE, Wells TN, Clapham PR: **Chemokine receptors--future therapeutic targets for HIV?.** *Biochem Pharmacol* (1999) **57**:451-463.

Pruzan R, Flint SJ: **Transcription of adenovirus RNA polymerase III genes.** *Curr Top Microbiol Immunol* (1995) **199**:201-226.

Rabson AB, Graves BJ: **Synthesis and Processing of Viral RNA.** In Retroviruses. Eds JM Coffin, SH Hughes, HE Varmus. Cold Spring Harbor Laboratory Press, Plainview, N.Y 205-262 (1997).

Ramezani A, Ding SF, Joshi S: **Inhibition of HIV-1 replication by retroviral vectors expressing monomeric and multimeric hammerhead ribozymes.** *Gene Ther* (1997) **4**:861-867.

Ramezani A, Joshi S: **Comparative analysis of five highly conserved target sites within the HIV-1 RNA for their susceptibility to hammerhead ribozyme-mediated cleavage in vitro and in vivo.** *Antisense Nucleic Acid Drug Dev* (1996) **6**:229-235.

Richter-Cook NJ, Howard KJ, Cirino NM, Wohrl BM, Le Grice SFJ: **Interaction of $tRNA^{Lys-3}$ with Multiple Forms of Human Immunodeficiency Virus Reverse Transcriptase.** *J Biol Chem* (1992) **267**: 15952-15957.

Rosenzweig M, Marks DF, Hempel D, Heusch M, Kraus G, Wong-Staal F, Johnson RP: **Intracellular immunization of rhesus CD34+ hematopoietic progenitor cells with a hairpin ribozyme protects T cells and macrophages from simian immunodeficiency virus infection.** *Blood* (1997) **90**:4822-4831

Rossi JJ, Cantin EM, Zaia JA, Ladne PA, Chen J, Stephens DA, Sarver N, Chang PS: **Ribozymes as Therapies for AIDS.** *Annals NY Acad Sci* (1990) **616**:184-200.

Sambrook J, Fritch EF, Maniatis T (1989) in *Molecular Cloning: A Laboratory Manual*, 2nd ed, (Cold Spring Harbor Laboratory, Cold Spring Harbor).

Sarver NE, Cantin EM, Chang PS, Zaia JA, Ladne PA, Stephens DA, Rossi JJ: **Ribozymes as Potential Anti-HIV-1 Therapeutic Agents.** *Science* (1990) **247**:1222-1225.

Shore SK, Nabissa PM, Reddy EP: **Ribozyme-mediated cleavage of the BCRABL oncogene transcript: *in vitro* cleavage of RNA and *in vivo* loss of P210 protein-kinase activity.** *Oncogene* (1993) **8**:3183-8.

Simões S, Pires P, Düzgunes N, Pedroso de Lima MC: **Cationic liposomes as gene transfer vectors: Barriers to successful application in gene therapy.** *Curr Opin Mol Ther* (1999) **1**:147-157.

Singwi S, Ramezani A, Ding SF, Joshi S: **Targeted RNases: a feasibility study for use in HIV gene therapy.** *Gene Ther* (1999) **6**:913-921.

Sioud M, Drlica K: **Prevention of human immunodeficiency virus type 1 integrase expression in *Escherichia coli* by a ribozyme.** *Proc Natl Acad Sci USA* (1991) **88**:7303-7307.

Sorrentino S, Libonati M: **Structure-function relationships in human ribonucleases: main distinctive features of the major RNase types.** *FEBS Lett* (1997) **404**:1-5.

Sprinzl M, Hartmann T, Weber J, Blank J, Zeidler R: **Compilation of tRNA sequences and sequences of tRNA genes.** *Nucleic Acids Res* (1989) **17 Suppl**:1-172.

Sullenger BA, Cech TR: **Tethering ribozymes to a retroviral packaging signal for destruction of viral RNA.** *Science* (1993) **262**:1566-1569.

Sullenger BA, Gallardo HF, Ungers GE, Gilboa E: **Overexpression of TAR sequences renders cells resistant to human immunodeficiency virus replication.** *Cell* (1990a) **63**:601-608.

Sullenger BA, Lee TC, Smith CA, Ungers GE, Gilboa E: **Expression of chimeric tRNA-driven antisense transcripts renders NIH 3t3 cells highly resistant to Moloney murine leukemia virus replication.** *Mol Cell Biol* (1990b) **10**:6512-6523.

Swanstrom R, Wills JW: **Synthesis, Assembly and Processing of Viral Proteins.** In Retroviruses. Eds JM Coffin, SH Hughes, HE Varmus. Cold Spring Harbor Laboratory Press, Plainview, N.Y 263-334 (1997).

Tanner NK: **Ribozymes: the characteristics and properties of catalytic RNAs.** *FEMS Microbiol Rev* (1999) **23**:257-275.

Telesnitsky A, Goff SP: **Reverse Transcriptase and the Generation of Retroviral DNA.** In Retroviruses. Eds JM Coffin, SH Hughes, HE Varmus. Cold Spring Harbor Laboratory Press, Plainview, N.Y 121-160 (1997).

Thompson JD, Ayers DF, Malmstrom TA, McKenzie TL, Ganousis L, Chowrira BM, Couture L, Stinchcomb DT: **Improved accumulation and activity of ribozymes expressed from a tRNA-based RNA polymerase III promoter.** *Nucleic Acids Res* (1995) **23**:2259-2268.

Tiganos E, Yao XJ, Friborg J, Daniel N, Cohen EA: **Putative alpha-helical structures in the human immunodeficiency virus type 1 Vpu protein and CD4 are involved in binding and degradation of the CD4 molecule.** *J Virol* (1997) **71**:4452-4460.

Trono D: **HIV accessory proteins: leading roles for the supporting cast.** *Cell* (1995) **82**:189-192.

Tsuchihashi Z, Khosla M, Herschlag D: **Protein enhancement of hammerhead ribozyme catalysis.** *Science* (1993) **262**: 99-102.

Uhlenbeck OC: **A small catalytic oligoribonucleotide.** *Nature* (1987) **328**:596-600.

Ventura M, Wang P, Franck N, Saragosti S: **Ribozyme targeting of HIV-1 LTR.** *Biochem Biophys Res Commun* (1994) **203**:889-898.

Ventura M, Wang P, Ragot T, Perricaudet M, Saragosti S: **Activation of HIV-specific ribozyme activity by self-cleavage.** *Nucleic Acids Res* (1993) **21**:3249-3255.

Wang L, Witherington C, King A, Gerlach WL, Carr A, Penny R, Cooper D, Symonds G, Sun LQ: **Preclinical characterization of an anti-tat ribozyme for therapeutic application.** *Hum Gene Ther* (1998) **9**:1283-1291.

Weerasinghe M, Liem SE, Asad S, Read SE, Joshi S: **Resistance to Human Immunodeficiency Virus Type 1 (HIV-1) Infection in Human CD4+ Lymphocyte-Derived Cell Lines Conferred by Using Retroviral Vectors Expressing an HIV-1 RNA-Specific Ribozyme.** *J Virol* (1991) **65**:5531-5534.

Weil PA, Segall J, Harris B, Ng SY, Roeder RG: **Faithful transcription of eukaryotic genes by RNA polymerase III in systems reconstituted with purified DNA templates.** *J Biol Chem* (1979) **254**:6163-6173.

Westaway SK, Cagnon L, Chang Z, Li S, Li H, Larson GP, Zaia JA, Rossi JJ: **Virion encapsidation of $tRNA_3^{Lys}$-Ribozyme Chimeric RNAs Inhibits HIV infection.** *Antisense Nucleic Acid Drug Dev* (1998) **8**:185-197.

Westaway SK, Larson GP, Li S, Zaia JA, Rossi JJ: **A chimeric $tRNA_3^{Lys}$-ribozyme inhibits HIV replication infection.** *Nucleic Acids Symp Ser* (1995) **33**:194-199.

Wiechen K, Zimmer C, Dietel M: **Selection of a high activity *c-erbB-2* ribozyme using a fusion gene of *c-erbB-2* and the enhanced green fluorescent protein.** *Cancer Gene Ther* (1998) **5**:45-51.

Willis IM : **RNA polymerase III. Genes, factors and transcriptional specificity.** *Eur J Biochem* (1993) **212**:1-11.

Wolin SL, Matera AG: **The trials and travels of tRNA.** *Genes Dev* (1999) **13**:1-10.

Wong-Staal F, Poeschla EM, Looney DJ: **A controlled, Phase 1 clinical trial to evaluate the safety and effects in HIV-1 infected humans of autologous lymphocytes transduced with a ribozyme that cleaves HIV-1 RNA.** *Hum Gene Ther* (1998) **9**:2407-2425.

Wyatt R, Sodroski J: **The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens.** *Science* (1998) **280**:1884-1888.

Xu Z, Oey L, Mohan S, Kawachi MH, Lee N, Rossi JJ, Fujita-Yamaguchi Y: **Hammerhead ribozyme-mediated cleavage of the human insulin-like growth factor-II ribonucleic acid *in vitro* and in prostate cancer cells.** *Endocrinology* (1999) **140**:2134-2144.

Yamada O, Kraus G, Leavitt MC, Yu M, Wong-Staal F: **Activity and cleavage site specificity of an anti-HIV-1 hairpin ribozyme in human T cells.** *Virology* (1994a) **205**:121-126.

Yamada O, Kraus G, Luznik L, Yu M, Wong-Staal F: **A chimeric human immunodeficiency virus type 1 (HIV-1) minimal Rev response element-ribozyme molecule exhibits dual antiviral function and inhibits cell-cell transmission of HIV-1.** *J Virol* (1996) **70**:1596-1601.

Yamada O, Yu M, Yee JK, Kraus G, Looney D, Wong-Staal F: **Intracellular immunization of human T cells with a hairpin ribozyme against human immunodeficiency virus type1.** *Gene Ther* (1994b) **1**:38-45.

Yu M, Leavitt MC, Maruyama M, Yamada O, Young D, Ho AD, Wong-Staal F: **Intracellular immunization of human fetal cord blood stem/progenitor cells with a ribozyme against HIV-1.** *Proc Natl Acad Sci USA* (1995a) **92**:699-703.

Yu M, Ojwang J, Yamada O, Hampel A, Rappaport J, Looney D, Wong-Staal F: **A hairpin ribozyme inhibits expression of diverse strains of human immunodeficiency virus type 1.** *Proc Natl Acad Sci USA* (1993) **90**:6340-6344.

Yu M, Poeschla E, Yamada O, Degrandis P, Leavitt MC, Heusch M, Yees JK, Wong-Staal F, Hampel A: **In vitro and in vivo characterization of a second functional hairpin ribozyme against HIV-1.** *Virology* (1995b) **206**:381-386.

Yuyama N, Ohkawa J, Koguma T, Shirai M, Taira K: **A multifunctional expression vector for an anti-HIV-1 ribozyme that produces a 5' and 3'-trimmed *trans*-acting ribozyme, targeted against HIV-1 RNA, and *cis*-acting ribozymes that are designed to bind to and thereby sequester *trans*-activator proteins such as Tat and Rev.** *Nucleic Acids Res* (1994) **22**:5060-5067.

Yuyama N, Ohkawa J, Inokuchi Y, Shirai M, Sato A, Nishikawa S, Taira K: **Construction of a tRNA-embedded-ribozyme trimming plasmid.** *Biochem Biophys Res Commun* (1992) **186**:1271-1279.

Zaia JA: **High Dose Chemotherapy and Autologous Peripheral Stem Cell Transplantation for HIV Lymphomas: A Phase IIA Study of Comparative Marking Using a Ribozyme Gene and a Neutral Gene.** FDA STUDY 2 .

Zaia JA, Rossi JJ, Ito JI, Castanotto D, Li S, Yam P, Bauer G, Valdez P, Rice CR, Rothchild JC, Li H, Banh VN, Sauter S, Peters G, Parker V, Jolly DJ, Sniecinski I, Forman SJ, Kohn DB: **One year results after autologous stem cell transplantation using retrovirus-transduced peripheral blood progenitor cells in HIV-infected subjects.** *Blood Suppl* (1998) **92**:665a.

Zakharchuk AN, Doronin KK, Karpov VA, Krougliak VA, Naroditsky BS: **The fowl adenovirus type 1 (CELO) virus-associated RNA-encoding gene: a new ribozyme-expression vector.** *Gene* (1995) **161**:189-193.

Zhao JJ, Lemke G: **Selective disruption of neuregulin-1 function in vertebrate embryos using ribozyme-tRNA transgenes.** *Development* (1999) **125**:1899-1907.

Zhou C, Bahner I, Rossi JJ, Kohn DB: **Expression of hammerhead ribozymes by retroviral vectors to inhibit HIV-1 replication: comparison of RNA levels and viral inhibition.** *Antisense Nucleic Acid Drug Dev* (1996) **6**:17-24.

Zuker M, Mathews DH, Turner D: **Algorithms and thermodynamics for RNA secondary structure prediction: a practical guide.** In RNA Biochemistry and Biotechnology. Eds J Barciszewski, BFC Clark. NATO Asi series, Kluwer Academic Publishers (1999).

APPENDIX A

Ribozyme-dependent inactivation of *lacZ* mRNA in *E. coli*: a feasibility study to set up a rapid *in vivo* system for screening HIV-1 RNA-specific ribozymes*

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ABSTRACT

Ribozymes are potentially useful tools with widespread applications in gene therapy of several diseases. In order to assess the *in vivo* cleavage efficiency of human immunodeficiency virus (HIV)-1 RNA-specific ribozymes, a bacterial indicator cell system could be developed in which the degree of inhibition of β-galactosidase activity would correlate with ribozyme activity. The suitability of this indicator cell system was assessed using a ribozyme targeted against the *env*-coding region within the HIV-1 RNA. To this end, a pGEM4Z-based plasmid was engineered wherein oligodeoxynucleotides containing a hammerhead ribozyme and its target site were cloned in frame within the *lac*Z-coding region that encodes for the α fragment of β-galactosidase. Extra nucleotides were included in the insert to ensure that the *lac*Z open reading frame was not interrupted due to a frameshift or nonsense mutation. In *E. coli* indicator cells harbouring this plasmid, ribozyme-mediated cleavage of the target site provided *in cis* and the subsequent loss of β-galactosidase activity should correlate with ribozyme activity. However, frameshift mutations were observed upon sequence analysis of plasmid DNA isolated from the selected light blue to white colonies. Because these mutations affected the production of the β-galactosidase α fragment, a direct correlation between β-galactosidase and ribozyme activities could not be established *in vivo*. Thus, in clones which demonstrated visibly lower β-galactosidase activities than the control, the effect of the frameshift mutations on *lac*Z mRNA translation can not be discounted. In clones expressing ribozymes but displaying dark blue colour, it is possible that *lac*Z mRNAs were cleaved but that the β-galactosidase substrates used were sensitive enough to allow detection of proteins translated from residual *lac*Z mRNA transcripts. The use of alternative β-galactosidase substrates with less sensitivity may enable the use of the proposed indicator cell system.

INTRODUCTION

Hammerhead ribozymes are small, catalytic RNA molecules first identified in the avocado sunblotch viroid as well as in the satellite RNAs of lucerne transient streak and tobacco ringspot viruses (reviewed in 1). The hammerhead ribozyme catalytic and substrate binding domains have been well characterized (2, 3). Hammerhead ribozymes may be targeted against any given RNA (reviewed in 4) provided that the ribozyme catalytic domain is flanked by antisense sequences to allow ribozyme binding to the target RNA. The cleavage site within the target RNA must be immediately preceded by NUH (5), with N being any nucleotide and H being any nucleotide except G. Cleavage results in a 5' product with a 5' hydroxyl group and a 3' product with a 2', 3' cyclic phosphate.

AIDS is caused by HIV, a retrovirus with an RNA genome. During its life cycle, HIV produces numerous mRNAs which are all potential targets for designing ribozymes (reviewed in 6). Monomeric hammerhead ribozymes have been developed and tested against several sites within the HIV-1 RNA (reviewed in 7); however, virus breakthrough was eventually observed in each case (reviewed in 8).

Ribozymes with increased catalytic activity have been selected *via in vitro* selection/evolution (reviewed in 9). However, the *in vivo* cleavage activity of these ribozymes may be less than what is anticipated from results *in vitro*. The *in vitro* cleavage activity of HIV-1 RNA-specific ribozymes has been shown not to correlate with their *in vivo* cleavage activity in human cell lines (10-15). A ribozyme targeted against the HIV-1 5' leader sequence, although active *in vitro*, was less active upon testing in HeLa and H9 cells (10). A ribozyme against the first coding exon of the HIV-1 *tat* which possessed short flanking sequences performed better *in vitro* than ribozymes with longer flanking sequences (11). However, upon testing in Jurkat cells,

the opposite was the case. Similarly, a ribozyme targeted against the HIV-1 *env*-coding region cleaved poorly *in vitro*, but demonstrated the highest inhibition against viral replication in the MT4 cell line (12). On the other hand, ribozymes targeted against the HIV-1 R region (13) or 5' leader sequence (14) were catalytically inactive *in vitro* but were found to be active in a cellular environment. A dimeric maxizyme possessing a 2-bp common stem loop II demonstrated weak activity *in vitro* against the HIV-1 *tat*-coding region, but in transiently transfected HeLa cells expressing a chimeric HIV-1 LTR and luciferase gene, luciferase activity was inhibited by up to 90% (15). Thus, selection of ribozymes on the basis of their *in vitro* activity alone may eliminate molecules with increased therapeutic potential *in vivo*. *In vivo* systems are therefore required for screening ribozymes with increased/altered catalytic activities. The development of such screening systems should greatly accelerate ribozyme applications, for example in gene therapy.

Ribozymes have been shown to be active in bacterial cells. A ribozyme targeted against the *A2*-coding region of RNA coliphage SP was tested in *E. coli*. Cells expressing this ribozyme produced less progeny phage than those expressing the inactive ribozyme (16). Ribozyme cleavage of HIV-1 RNA target sites have also been demonstrated in bacterial cells. RNA containing the *IN*-coding region of HIV-1 and a ribozyme targeted against it were expressed under control of the T7 promoter in bacteria producing T7 RNA polymerase (17). Upon induction, integrase mRNA could not be detected by analyzing RNA extracted from bacteria expressing the active ribozyme. However, it was present when an inactive ribozyme was expressed. Induction of target RNA synthesis prior to ribozyme induction led to the detection of one of the cleavage products. The amount of integrase protein produced *in vivo* was also shown to be decreased by Western blot analysis. Ribozymes targeted against the *RT* and *PR*- coding regions within the HIV-1 RNA were also tested in *E. coli* expressing an RNA containing HIV-1

PR and *RT*-coding regions (18). *Trans* cleavage of HIV-1 RNA was demonstrated by semi-quantitative RT-PCR and HIV-1 RT activity assay. However, although ribozyme activity against HIV-1 RNA could be demonstrated in both of these studies (17, 18), the assays used were rather time consuming, and thus would not allow the fastest possible screening of ribozyme activity *in vivo*.

We were interested in designing an *E. coli* based indicator cell system for rapid initial screening of active ribozymes without performing extensive biochemical characterizations. In the proposed bacterial indicator cell system (FIGS. 1, 2), a ribozyme and its target site were cloned in frame within the *lacZ* open reading frame (ORF) present in the plasmid pGEM4Z, which gives rise to the α fragment of β-galactosidase. Accordingly, the *lacZ* transcript would contain the ribozyme and its target site *in cis*. Ribozyme-mediated cleavage of the target RNA would prevent its translation and thus production of the α fragment of β-galactosidase. Complementation between the α fragment and the ω fragment (expressed in certain *E. coli* strains) of β-galactosidase would not occur. In the presence of a chromogenic substrate such as 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), β-galactosidase would catalyze the formation of 5-bromo-4-chloro-indigo, a blue-coloured product. If the enzyme is absent, the substrate would not break down and remain colourless. Thus, an effective ribozyme should lead to the formation of white, in contrast to blue, colonies on agar plates containing X-gal and isopropylthio-β-D-galactoside (IPTG), an inducer of the *lac* operon.

A yeast splicing protein was found to interact *in vivo* with a ribozyme and block its intracellular activity (19), whereas the nucleocapsid protein of HIV-1 (20-26), the heterogeneous nuclear ribonucleoprotein A1 (21, 22) and glyceraldehyde-3-phosphate dehydrogenase (27) were found to enhance ribozyme activity. Since hammerhead ribozymes are found in plant pathogens

(viroid and satellite RNAs of viruses), plant proteins may also be found which could enhance ribozyme cleavage. Lack of complete cleavage both *in vitro* and *in vivo* in bacterial and mammalian cells may reflect the absence of proteins which enhance ribozyme activity. Thus, aside from the assessment of ribozyme cleavage activity *in vivo*, a bacterial system may also be used for cloning protein co-factors which could affect ribozyme activity *in vivo*.

MATERIALS AND METHODS

Oligonucleotide design and cloning of Rz_{Env}-Env into pGEM4Z

Cloning of sequences encoding Rz_{Env} (28) and its HIV-1 *env* target site was performed using two sets of oligonucleotides. The first set consisted of partially overlapping oligonucleotides (53-54-nt) which were first extended *in vitro* and then cloned. The second set consisted of two complementary oligonucleotides that contained ribozyme and target sequences flanked by restriction sites, which were cloned directly into the plasmid pGEM4Z. Both ligations yielded >100 colonies upon transformation into *E. coli*.

Rz_{Env} was designed to cleave after a highly conserved GUU (nt 665 to nt 667) sequence within the *env*-coding region of HIV-1 HXB2 RNA (29). Partially overlapping oligonucleotides (5'-CCC-CCC-*AAG-CTT-GGA-TCC*-aat-cgc-aa**C-TGA-TGA-GTC-CGT-GAG-GAC-GAA**-acc-agc-3' and 5'-GGG-*GAA-TTC*-C**aa-tcg-caa-aac-cag-ccg**-*att-cga-a*cg-gct-ggt-**TTC-GTC-CTC-AC**-3') were synthesized using the Expedite™ Nucleic Acid Synthesis System (Millipore; Etobicoke, Canada). Before cloning, these oligonucleotides were extended to full-length complementary oligonucleotides for 1 h at 37°C in a 40 µl reaction containing 50 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, 8µM of each dNTP, and 5 units of Klenow (Life Technologies; Burlington, Canada). The fill-in products were ethanol-precipitated and resuspended in water and digested with *Hin*d III and *Eco*R I. In a second set of experiment, complementary oligonucleotides with 5' overhangs to allow cloning (5'-AGC-TT*G-GAT-CC*a-atc-gca-a**CT-GAT-GAG-TCC-GTG-AGG-ACG-AA**a-cca-gcc-g*tt-cga-at*c-**ggc-tgg-ttt-tgc-gat-**tCG-3' and 5'-AAT-TCG-**aat-cgc-aaa-acc-agc-cg***a-ttc-gaa*-cgg-ctg-gt**T-TCG-TCC-TCA-CGG-ACT-CAT-CAG**-ttg-cga-tt*G-GAT-CC*A-3') were synthesized. Ribozyme catalytic domain is in uppercase bold, 8-nt flanking sequences complementary to either side of the cleavage site are in lowercase, target sequence is in lowercase bold, loop sequence is in lowercase italics, restriction enzyme sites are in uppercase italics, and 5' overhangs are underlined. The full-length oligonucleotides (75 nts) are of comparable length to the *Hin*d III-*Eco*R I fragment (54 nts) being removed from pGEM4Z.

Plasmid pGEM4Z (Promega Corp.; Madison, USA) was transformed into *E. coli* strain DH5α, isolated by a miniprep procedure and digested with *Hin*d III and *Eco*R I. The DNA band corresponding to the *Eco*R I-*Hin*d III fragment was eluted using the GeneClean kit (BIO 101; Vista, USA) following 1% agarose gel electrophoresis. Full-length oligonucleotides were then cloned as described in (30) at the *Hin*d III and *Eco*R I sites within the *lac*Z gene of pGEM4Z. Ligation reactions (10 μl) containing 50 mM Tris-Cl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% (w/v) polyethylene glycol-8000, double stranded insert (300-5000 ng), vector (10 ng) and 1 unit T4 DNA ligase (Life Technologies; Burlington, Canada) were performed at 23°C for 1h. DH5α competent cells were transformed with the ligation mix and plated on Luria-Bertani (LB) agar plates containing ampicillin (50 μg/ml), X-gal (800 μg) and IPTG (0.4 μmol). A positive transformation control consisting of pGEM4Z DNA yielded over 300 colonies, a negative transformation control without DNA yielded no colonies, and the ligation mixtures each yielded ~50 colonies. Colonies which ranged in size and colour from white to light shades of blue were picked and screened by *Csp*45 I, *Dra* I, *Sma* I, *Bam*H I, *Eco*R I and *Hin*d III restriction enzyme analyses. DNA sequencing was performed using the T7 Sequencing Kit (Pharmacia Biotech Inc.; Baie d'Urfé, Canada) using instructions provided by the supplier.

β-galactosidase activity of individual pGEM-Rz_{Env}-Env clones

Individual colonies were picked and grown overnight in LB medium containing ampicillin (50 μg/ml). The next day, β-galactosidase activity was assayed using cultures at an optical density at wavelength of 600 (OD_{600}) equivalent to 1.00. The cultures were incubated for another 4 h after adding IPTG (0.1 μmol) and X-gal (200 μg). Cells were pelleted by spinning for 3 min at 8000*g*. OD_{550} and OD_{420} of the supernatants were measured to quickly assess β-galactosidase activity using culture medium containing IPTG and X-gal as a blank.

For clones selected for further characterization, β-galactosidase activity was assayed using *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) as substrate (30) and LB cultures at the logarithmic growth

phase with OD_{600} values between 0.28-0.70. After cooling on ice for 20 minutes, cell cultures (100 µl) were mixed with 50 µl 0.1% SDS, 100 µl chloroform and 900 µl Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol), vortexed for 10 seconds, and incubated at 28°C for 5 minutes. ONPG (4 mg/ml, 200 µl) was added to each tube, and the incubation continued for 80 to 220 min at 37°C. Reactions were stopped by adding 500 µl of 1M Na_2CO_3. OD_{550} and OD_{420} were then measured.

Cis and _trans_ cleavage activity of Rz_{Env}-Env

To detect *cis* cleavage activity, ribozyme and HIV-1 *env* target site were PCR amplified from pGEM-Rz_{Env}-Env #21 using a forward primer (5-CGA-AAT-TAA-TAC-GAC-TCA-CTA-TA-3') which binds to the T7 promoter and a reverse primer (5'-GTA-AAA-CGA-CGG-CCA-GT-3') which binds downstream of the ribozyme target site. PCRs were performed for 30 cycles (1 min, 95°C; 1 min, 56°C; 1 min, 72°C each). PCR DNA (2-50 µl) containing the T7 promoter sequence was transcribed *in vitro* at 37°C in a reaction mixture (100 µl) containing 40 mM Tris-Cl (pH 8.0), 25 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 1 mM of each NTP, and 200 units of T7 RNA polymerase (Life Technologies; Burlington, Canada). The reaction was stopped after 0.5-2 h by digesting the template DNA with 5 units of RQI RNase-free DNase (Promega Corp.; Madison, USA) for 10 min. *Cis* cleavage at the HIV-1 *env* target site by Rz_{Env} occurred under the condition used for transcription, without further incubation or addition of reagents. To compare *cis* and *trans* cleavage activities, the *env* target sequence was PCR amplified from the plasmid pHEnv using a primer (5'-ATA-TCA-TAT-GTA-ATA-CGA-CTC-ACT-ATA-GGG-CGA-GTG-CAG-AAA-GAA-TAT-GC-3') which binds upstream of the ribozyme target site and contains the T7 promoter sequence and a primer (5'-GTC-CGT-GAA-ATT-GAC-AG-3') which binds downstream of the ribozyme target site. PCR DNA was transcribed *in vitro* for 2 h at 37°C as described above. After phenol extraction and ethanol precipitation, the target RNA was resuspended in water and then added to the *in vitro* transcription mixture. The cleavage products were analyzed by 8 M

urea-8 % polyacrylamide gel electrophoresis (PAGE) followed by methylene blue staining (30).

Trans cleavage activity of Rz_{Env}

Target RNA was transcribed in the presence of [α-^{32}P] UTP (3000 Ci/mmol; Amersham Canada Ltd.; Oakville, Canada). Rz_{Env} was transcribed from PCR DNA which was amplified from pGEM-Rz_{Env}-Env #21 using a forward primer (5-CGA-AAT-TAA-TAC-GAC-TCA-CTA-TA-3') which binds to the T7 promoter and a reverse primer (5'-ATA-TAT-ATC-GAT-AAA-AAA-CGG-CTG-GTT-TCG-TCC-TC-3') which binds near the 3' end of the ribozyme. It was then used in a *trans* cleavage reaction with [α-^{32}P]-labelled target RNA. Essentially, Rz_{Env} and target RNA were combined in a reaction mixture containing 40 mM Tris-Cl (pH 8.0) and 10 mM NaCl. The sample was heated to 65°C for 5 min, cooled to 37°C, and the reaction initiated by adding 20 mM $MgCl_2$. Aliquots were taken after 7, 15, 30, 120, 300, 600 and 900 min incubation at 37°C, and the reaction stopped by addition of loading buffer containing 5 mM EDTA. Cleavage products were analyzed by 8 M urea-8 % PAGE followed by exposure to a phosphor screen and scanning by Storm phosphorimager (Molecular Dynamics; Sunnyvale, USA).

RESULTS

Bacterial indicator system for identification of ribozymes capable of *in vivo* cleavage

E. coli DH5α cells contain a portion of the *lac*Z gene which encodes for the ω fragment of β-galactosidase. Transformation of these cells with plasmids expressing the α fragment of β-galactosidase leads to complementation between the α and ω fragments and the consequent assembly of an active enzyme, whose activity can be detected by chromogenic substrates (FIG. 1*A*). A ribozyme (Rz_{Env}) was therefore designed to cleave the *lac*Z mRNA coding for the α fragment of β-galactosidase. This was achieved by cloning Rz_{Env} and the *env* target sequence in frame within the *lac*Z gene of plasmid pGEM4Z. Upon transcription of this modified *lac*Z gene, *lac*Z mRNA would be produced which contains Rz_{Env} and its *env* target sequence. If this mRNA remains intact, then the ribozyme must have been incapable of *in vivo* cleavage. This should lead to the formation of blue colonies on agar plates containing X-gal and IPTG. In contrast, if the conditions *in vivo* are suitable for cleavage, then the ribozyme should hybridize to its target located downstream and cleave it, effectively cutting the *lac*Z mRNA into two. Bacteria harbouring ribozymes capable of *in vivo* cleavage would not produce the α fragment of β-galactosidase and, as a result, would give rise to white colonies on plates containing X-gal and IPTG (FIG. 1*B*). The colour of the colonies should thus correlate with *in vivo* cleavage of the ribozyme target site present in the *lac*Z mRNA. A ribozyme's ability to cleave *in vivo* may therefore be easily and quickly assessed by monitoring the colour of the colonies which result after transformation in *E. coli* cells.

Ribozyme cloning, *in vivo* screening and characterization

Oligonucleotides containing the ribozyme and its target sequence were synthesized. A 7-nt loop was placed between the ribozyme and its target sequence to allow folding and consequent hybridization of the two sequences. This loop (UUCGAAU) was designed to closely resemble a naturally occurring loop, such as the tRNA anticodon loop (U/CUNNNG/AN; 31). Additional nucleotides were added so that insertion by itself of the oligonucleotide would not affect the reading frame of the *lac*Z gene present in pGEM4Z. After cloning, ligated plasmids were used to transform *E.coli* cells. Cells were then plated on X-gal/IPTG plates.

Twenty-four colonies which ranged in colour from white to light blue were screened by restriction enzyme analysis and quickly assayed for β-galactosidase activity. Clones #4, #18 and #21 demonstrated correct restriction enzyme patterns and lower β-galactosidase activities compared to cells expressing the plasmid pGEM4Z. Colonies #4 and #21 were light blue, while #18 was white on LB agar plates containing X-gal and IPTG. β-galactosidase activities of extracts from all three colonies were consistently lower, compared to extracts from cells expressing pGEM4Z (Table 1).

Upon sequencing, all three clones were found to contain mutations (FIG. 3). Clone #4 contained an insertion (G) in the ribozyme flanking sequence. Clone #18 contained a substitution (G → T) in stem loop II of the ribozyme catalytic domain and a deletion (C) in the ribozyme flanking sequence. Clone #21 contained an insertion (C) in the 7-nt loop connecting the ribozyme and the target sequence. Three additional clones that were picked and sequenced also contained mutations (data not shown). In a second set of experiment, twenty-four colonies picked from the ligation using the partially overlapping oligonucleotides were also characterized. Three colonies from this set were sequenced. Instead of single point mutations, tracts of mutated sequences were

observed (data not shown). These could have resulted from mis-alignment of the partial overlap during the extension reaction performed prior to cloning.

Cis and *trans* cleavage activity *in vitro* of a cloned ribozyme

Of the three clones selected, clone #21 contained a mutation in the loop region between the ribozyme and the target site. This mutation was not expected to affect ribozyme cleavage *per se*. The ribozyme and target site from pGEM-Rz_{Env}-Env #21 were PCR amplified and the PCR products transcribed *in vitro*. The PCR product was then used in an *in vitro* transcription and cleavage reaction (FIGS. 4*A*, 4*B*,). *Cis* cleavage occurred during the *in vitro* transcription reaction itself. This demonstrates that the ribozyme cloned in pGEM-Rz_{Env}-Env #21 was functional *in vitro*.

Relative occurrence of *cis* and *trans* cleavage *in vitro* of RNA containing Rz_{Env}-Env sequences was determined as follows. The RNA containing the Rz_{Env} target site was transcribed separately and added to the *in vitro* transcription mixture of pGEM-Rz_{Env}-Env #21, and the *cis* and *trans* cleavage products were analyzed by PAGE (FIG. 4*C*). *Trans* cleavage did not occur for up to 2 h incubation. Thus, only *cis* cleavage occurred under the conditions used for *in vitro* transcription.

To determine whether the ribozyme possesses *trans* cleavage ability, ribozyme (without the *cis* target site) was PCR amplified from clone #21 and the PCR product transcribed *in vitro*. This RNA was then used in an *in vitro trans* cleavage reaction using a target RNA which was PCR amplified and transcribed separately. The ribozyme was able to cleave the target RNA *in trans* (FIG. 5). Thus, lack of *trans* cleavage in the presence of a *cis* target site (FIG. 4*C*) is due to the higher efficiency of *cis* cleavage.

DISCUSSION

Although *in vitro* selection techniques may allow the identification of ribozymes with improved catalytic activity, the *in vivo* performance of these ribozymes may not correlate with their activity *in vivo*. *In vivo* ribozyme activity may be rapidly assessed using a bacterial indicator system, provided that a strategy is designed which allows correlation of *in vivo* ribozyme activity with a bacterial phenotype.

We attempted to test activity of the enzyme β-galactosidase produced by *lacZ* mRNA to monitor ribozyme activity *in vivo* (FIG. 1). Sequences encoding Rz_{Env} and its target site were cloned *in cis* within the N-terminal region of the *lacZ* gene in pGEM-4Z. Bacterial cells were then transformed with pGEM-Rz_{Env}-Env plasmids. Upon transcription, Rz_{Env} should have bound to and cleaved its target site, thereby inactivating the *lacZ* transcript coding for the α fragment of β-galactosidase. Absence of the α fragment should have prevented formation of a functional enzyme *via* complementation. White colonies likely to contain active Rz_{Env} were identified on agar plates containing X-gal and IPTG.

Ribozyme's ability to cleave *in cis* was demonstrated during *in vitro* transcription (FIGS. 4*A* and *B*). Upon addition of a target RNA containing Rz_{Env} target site, only the products corresponding to *cis* cleavage were detected (FIG. 4*C*). However, this does not rule out the ability of Rz_{Env} to cleave *in trans*. The ribozyme was indeed able to cleave the target RNA under *trans* cleavage conditions (FIG. 5). Thus, *cis* cleavage occurs with higher efficiency than *trans* cleavage. The use of a *cis* cleaving ribozyme is therefore a logical choice in establishing a bacterial indicator cell system.

pGEM-Rz_{Env}-Env plasmid designed to contain the ribozyme and its target sequence was used to transform *E. coli* cells. Colonies which had reduced β-galactosidase activity based on their

colour on LB agar plates containing X-gal and IPTG were identified. Lack of β-galactosidase activity within the bacterial cell extracts was confirmed by performing an assay using ONPG as a substrate (Table 1). Plasmid DNA from the clones was isolated and analyzed by restriction enzyme analysis. However, sequencing results revealed that mutations were present in the insert (FIG. 3).

The mutations present in the clones may have caused formation of white colonies by disruption of the *lac*Z ORF. In addition, Rz_{Env} could have cleaved its target site *in vivo* which could have further decreased the number of *lac*Z mRNAs available for translation of the α fragment of β-galactosidase. Therefore, the observed reduction in β-galactosidase activity in these clones could be due to an additive effect between the mutations and ribozyme activity. However, because clones containing both an active ribozyme and a frameshift mutation were the only ones which reduced β-galactosidase activity to a detectable level, only these clones were selected. Clones containing the correct ribozyme and target sequence may have been missed, as these may have appeared blue on agar plates with X-gal/IPTG and therefore not selected for further analysis. As seen during the *in vitro* transcription and cleavage reaction using pGEM-Rz_{Env}-Env #21, some of the RNA may have remained uncleaved in *E. coli*, which could then be used in translation.

Using a similar blue/white colour selection, Chuah & Galibert (32) could successfully demonstrate the activity of a *cis* cleaving ribozyme but not of a *trans* cleaving ribozyme. In this study, a ribozyme targeted to *lac*Z mRNA was cloned within the *lac*Z-coding region of plasmid $M_{13}mp_8$ to allow co-expression of the ribozyme and its target site within the same RNA molecule *in vivo*. Upon transcription, the ribozyme was expected to cleave the *lac*Z mRNA *in cis*. Out of 18 white plaques tested, 15 contained the correct ribozyme sequence, while 3 were due to cloning

of aberrant sequences leading to the loss of the ORF. When the ribozyme was designed and expressed to *trans* cleave the *lac*Z RNA encoding ω fragment of β-galactosidase transcribed in *E. coli* from the episome, all of the isolated white plaques were due to the presence of incorrect sequences (32).

In our study, all of the isolated white colonies were due to mutations. The discrepancy between our results and those by Chuah and Galibert (32) could be due to a number of reasons. The ribozyme that we designed cleaved the 5' end of the *lac*Z mRNA coding for the α fragment. This may have been less effective in reducing the amount of protein produced than if the target chosen was further downstream as is the case in Chuah and Galibert's study (32). The ribozyme used in our study may have been less active than the ribozyme used by Chuah and Galibert (32). However, Rz_{Env} was shown to cleave the *lac*Z mRNA *in vitro* (FIG. 4*A*, 4*B*); the majority of the RNA was cleaved *in cis*, suggesting that the design of the construct was appropriate. Since the ribozyme was in very close proximity to its target site, it is also unlikely that the ribozyme was bound to sequences other than its downstream target, forming an inactive complex.

Another possibility is that the white plaques obtained by Chuah and Galibert (32) may have been due to mutations which occurred elsewhere in the cloning vector and not in the insert. Also, the substrate (X-gal) concentration we have used in our system (800 μg X-gal/plate) was higher than the amount used by Chuah and Galibert (4 μg X-gal/plate; 32). Thus, it is conceivable that our system is too sensitive, allowing small amounts of β-galactosidase to produce a detectable blue-coloured product.

Ribozymes tested against HIV-1 *PR* (18), *RT* (18) and *IN* (17)-coding regions were shown to be active in *E. coli*. However, in these studies ribozyme activities were demonstrated by assays that relied on the presence of cleaved RNA and their translation products. On the other hand, the

system we and Chuah and Galibert (32) have utilized detected the presence of uncleaved products. Thus, although the majority of the *lac*Z RNA may have been cleaved *in vivo*, protein translated from the remaining uncleaved transcripts catalyzed the breakdown of the substrate to a blue coloured product, which could still be detected by the assays used. As such, the blue/white colour selection may not accurately report the *in vivo* cleavage activity of a ribozyme, since colonies containing mutations were the only ones that could be isolated in our study. For successful development of a ribozyme screening system, the amount of substrate used may have to be titrated for each ribozyme. However, this may be time-consuming. Alternatively, substrates may have to be used which have a higher cut-off limit of detection, requiring a higher amount of β-galactosidase before a colour change is observed. Thus, only those cells which are producing high amounts of β-galactosidase may turn blue.

REFERENCES

1. Vaish, N.K., Kore, A.R., and Eckstein, F. (**1998**). Recent developments in the hammerhead ribozyme field. **Nucleic Acids Res** 26, 5237-5242.
2. Haseloff, J., and Gerlach, W.L. (**1988**). Simple RNA enzymes with new and highly specific endoribonuclease activities. **Nature** 334, 585-591.
3. Uhlenbeck, O.C. (**1987**). A small catalytic oligoribonucleotide. **Nature** 328, 596-600.
4. Birikh, K.R., Heaton, P.A., and Eckstein, F. (**1997**). The structure, function and application of the hammerhead ribozyme. **Eur J Biochem** 245, 1-16.
5. Ruffner, D.E., Stormo, G.D., and Uhlenbeck, O.C. (**1990**). Sequence requirements of the hammerhead RNA self-cleavage reaction. **Biochemistry** 29, 10695-10702.
6. Joshi, S., and Joshi, R.L. (**1996**). Molecular biology of human immunodeficiency virus type-1. **Transfus Sci** 17, 351-378.
7. Macpherson, J.L., Ely, J.A., Sun, L.Q., and Symonds, G.P. (**1999**). Ribozymes in gene therapy of HIV-1. **Frontiers Biosci** 4, D497-505.
8. Ramezani, A., and Joshi, S. (**1999**). Development of hammerhead ribozymes for HIV-1 gene therapy: principles and progress. **Gene Ther Mol Biol** 3, 1-10.
9. Pan, T. (**1997**). Novel and variant ribozymes obtained through in vitro selection. **Curr Opin Chem Biol** 1, 17-25.
10. Koseki, S., Tanabe, T., Tani, K., Asano, S., Shioda, T., Nagai, Y., Shimada, T., Ohkawa, J., and Taira, K. (**1999**). Factors governing the activity *in vivo* of ribozymes transcribed by RNA polymerase III. **J Virol** 73, 1868-1877.
11. Crisell, P., Thompson, S., and James, W. (**1993**). Inhibition of HIV-1 replication by ribozymes that show poor activity in vitro. **Nucleic Acids Res** 21, 5251-5255.
12. Ramezani, A., and Joshi, S. (**1996**). Comparative analysis of five highly conserved target sites within the HIV-1 RNA for their susceptibility to hammerhead ribozyme-mediated cleavage in vitro and in vivo. **Antisense Nucleic Acid Drug Dev** 6, 229-235.
13. Ventura, M., Wang, P., Franck, N., and Saragosti, S. (**1994**). Ribozyme targeting of HIV-1 LTR. **Biochem Biophys Res Commun** 203, 889-898.
14. Domi, A.., Beaud, G., and Favre, A.. (**1996**). Transcripts containing a small anti-HIV hammerhead ribozyme that are active in the cell cytoplasm but inactive in vitro as free RNAs. **Biochimie** 78, 654-662.
15. Kuwabara, T., Warashina, M., Nakayama, A., Ohkawa, J., and Taira, K. (**1999**). tRNAVal-heterodimeric maxizymes with high potential as gene inactivating agents: simultaneous cleavage at two sites in HIV-1 tat mRNA in cultured cells. **Proc Natl Acad Sci USA** 96, 1886-1891.
16. Inokuchi, Y., Yuyama, N., Hirashima, A., Nishikawa, S., Ohkawa, J., and Taira, K. (**1994**). A hammerhead ribozyme inhibits the proliferation of an RNA coliphage SP in Escherichia coli. **J Biol Chem** 269, 11361-11366.
17. Sioud, M.., and Drlica, K. (**1991**). Prevention of human immunodeficiency virus type 1 integrase expression in *Escherichia coli* by a ribozyme. **Proc Natl Acad Sci USA** 88, 7303-7309.
18. Ramezani, A., Marhin, W., Weerasinghe, M., and Joshi, S. (**1997**). A rapid and efficient system for screening HIV-1 Pol mRNA-specific ribozymes. **Can J Microbiol** 43, 92-96.
19. Castanotto, D., Li, H., Chow, W., Rossi, J.J., and Deshler, J.O. (**1998**). Structural similarities between hammerhead ribozymes and the spliceosomal RNAs could be responsible for lack of ribozyme cleavage in yeast. **Antisense Nucleic Acid Drug Dev** 8, 1-13.
20. Tsuchihashi, Z., Khosla, M., and Herschlag, D. (**1993**). Protein enhancement of hammerhead ribozyme catalysis. **Science** 262, 99-102.
21. Bertrand, E.L., and Rossi, J.J. (**1994**). Facilitation of hammerhead ribozyme catalysis by the nucleocapsid protein of HIV-1 and the heterogeneous nuclear ribonucleoprotein A1. **EMBO J** 13, 2904-2912.

22. Herschlag, D., Khosla, M., Tsuchihashi, Z., and Karpel, R.L. (**1994**). An RNA chaperone activity of non-specific RNA binding proteins in hammerhead ribozyme catalysis. **EMBO J** 13, 2913-2924.
23. Hertel, K.J., Herschlag, D., and Uhlenbeck, O.C. (**1996**). Specificity of hammerhead ribozyme cleavage. **EMBO J** 15, 3751-3757.
24. Muller, G., Strack, B., Dannull, J., Sproat, B.S., Surovoy, A., Jung, G., and Moelling, K. (**1994**). Amino acid requirements of the nucleocapsid protein of HIV-1 for increasing catalytic activity of a Ki-ras ribozyme in vitro. **J Mol Biol** 242, 422-429.
25. Moelling, K., Mueller, G., Dannull, J., Reuss, C., Beimling, P., Bartz, C., Wiedenmann, B., Yoon, K., Surovoy, A., and Jung, G (**1994**). Stimulation of Ki-ras ribozyme activity by RNA binding protein, NCp7, in vitro and in pancreatic tumor cell line, capan 1. **Ann NY Acad Sci** 733, 113-121.
26. Mahieu, M., Hendrix, C., Ooms, J., Herdewijn, P., and Content, J. (**1995**). In vitro NCp7 enhancement of ribozyme-mediated cleavage of full-length human IL-6 mRNA. **Biochem Biophys Res Comm** 214, 36-43.
27. Sioud, M., and Jespersen, L. (**1996**). Enhancement of hammerhead ribozyme catalysis by glyceraldehyde-3-phosphate dehydrogenase. **J Mol Biol** 257, 775-789.
28 Medina, M.F.C., and Joshi, S. (**1999**). Design, characterization and testing of tRNA3Lys-based hammerhead ribozymes. **Nucleic Acids Res** 27, 1698-1708.
29. Myers, G., Hahn, B.H., Mellors, J.W., Henderson, L.E., Korber, B., Jeang, K., McCutchan, F.E., and Pavlakis, G.N. (**1995**) **Human Retroviruses and AIDS.** Los Alamos National Laboratory, Los Alamos, New Mexico.
30. Sambrook, J., Fritsch, E.F. and Maniatis, T. (**1989**) **Molecular Cloning: A Laboratory Manual.** 2nd Ed. Cold Spring Harbor University Press, Cold Spring Harbor, NY.
31. Stryer, L. (**1988**) **Biochemistry**, 3rd Ed. W. H. Freeman and Company, New York, NY.
32. Chuat, J., and Galibert, F. (**1989**). Can ribozymes be used to regulate procaryote gene expression? **Biochem Biophys Res Comm** 162, 1025-1029.

TABLE 1.

*β-galactosidase activity of pGEM4Z clones**

clone #	colour of colony	β-gal activity*
4	light blue	1.42
18	white	9.60
21	light blue	<0
pGEM4Z	dark blue	23.14

*The values listed are the average of two experiments.
Unit of β-galactosidase activity = $1000 \times [A_{420} - (1.75 \times A_{550})] / (t \times 0.1 \times A_{600})$, where t = time in minutes
N.A., not applicable

FIGURE LEGENDS

FIG. 1. **Indicator cell system for monitoring ribozyme cleavage activity *in vivo*.** *A*, α-complementation between the α-peptide produced from plasmids containing the N-terminal portion of the *lac*Z gene and bacteria which express the ω-peptide leads to formation of blue colonies in agar plates with X-gal and IPTG. *B*, An oligonucleotide was designed which contained the ribozyme and its target sequence downstream. A 7-nt loop was placed between the ribozyme and its target sequence to allow folding and consequent hybridization of the two sequences. This loop (UUCGAAU) was designed so that it closely resembles a naturally occurring loop such as the tRNA anticodon loop (U/CUNNNG/AN; 31). *Hin*d III and *Eco*R I sites were added on either side of the oligonucleotides to allow cloning between the *Hin*d III-*Eco*R I sites of the plasmid pGEM4Z located at the *lac*Z gene. The oligonucleotide was thus *Hin*d III-Rz_{Env}-loop-Env-*Eco*R I. Additional nucleotides were added such that insertion by itself of the oligonucleotide would not affect the reading frame of the *lac*Z gene present in pGEM4Z. Upon *in vitro* transcription, the ribozyme cleaves its target site, thereby inactivating the *lac*Z mRNA. Because the α-peptide is not produced, α-complementation does not occur, which leads to formation of white colonies in agar plates with X-gal and IPTG. Ribozyme catalytic domain and 7-nt loop are shown in large case. Ribozyme flanking sequences and the target sequences to which they bind are shown in small case. ⇩ denotes cleavage site.

FIG. 2. **Overview of the selection procedure for colonies with reduced β-galactosidase activity.** Selection of clones able to cleave *in vivo* was mediated by the chromogenic substrate X-gal which was added to agar plates. Lighter coloured clones expressing ribozymes were picked and grown in liquid culture, and used for an initial assay. Plasmid DNA isolated was subjected to restriction enzyme analysis. The cultures were re-streaked on agar plates. Isolated colonies were used in a β-galactosidase assay to confirm lack of *lac*Z mRNA expression.

FIG. 3. **Sequences of the Rz_{Env} clones**. Mutations in the sequences of clones 4, 18 and 21 are indicated as → for substitution, ▲ for insertion and ⇨ for deletion. The numbers in parentheses correspond to the clone # in which the mutation was found. Ribozyme catalytic domain and 7-nt loop are shown in large case. Ribozyme flanking sequences and the target sequences to which they bind are shown in small case. ⇩ denotes cleavage site. The sequence of the 75-nt insert is shown at the bottom. Locations of the different mutations within the flanking sequences, ribozyme catalytic domain and the loop region are indicated by arrows.

FIG. 4. ***Cis* and *trans* cleavage activity of Rz_{Env}-Env**. *A*, Increasing amounts of PCR DNA (2, 5, 10, 25 or 50 µl) were used for *in vitro* transcription for 2 h. *B*, PCR DNA (30 µl) was used for *in vitro* transcription and incubated at increasing time intervals (0.5, 1, 1.5 or 2 h). The full-length transcript (135 nts) along with the 5' (73 nts) and 3' (62 nts) *cis* cleavage products were detected. *C*, Same as *B*, except that RNA (333 nts) containing the ribozyme target site was added to each transcription mixture. Products (170 nts and 163 nts) which would result from *trans* cleavage were not detected. Only the *cis* cleavage products (73 nts and 62 nts) and the full-length transcript (135 nts) were detected. T, target RNA alone. The uncleaved target RNA in lane "0.5" and 5' cleavage product in lane "2" have been excised from the gel and used for subsequent experiments.

FIG. 5. ***Trans* cleavage activity of pGEM-Rz_{Env}**. Rz_{Env} and [α-^{32}P]-labelled target RNA were used in a *trans* cleavage reaction. Aliquots were taken at the indicated time intervals and analyzed by 8 M - 8% polyacrylamide gel electrophoresis followed by exposure to a phosphor screen and scanning by Storm phosphorimager (Molecular Dynamics; Sunnyvale, USA).

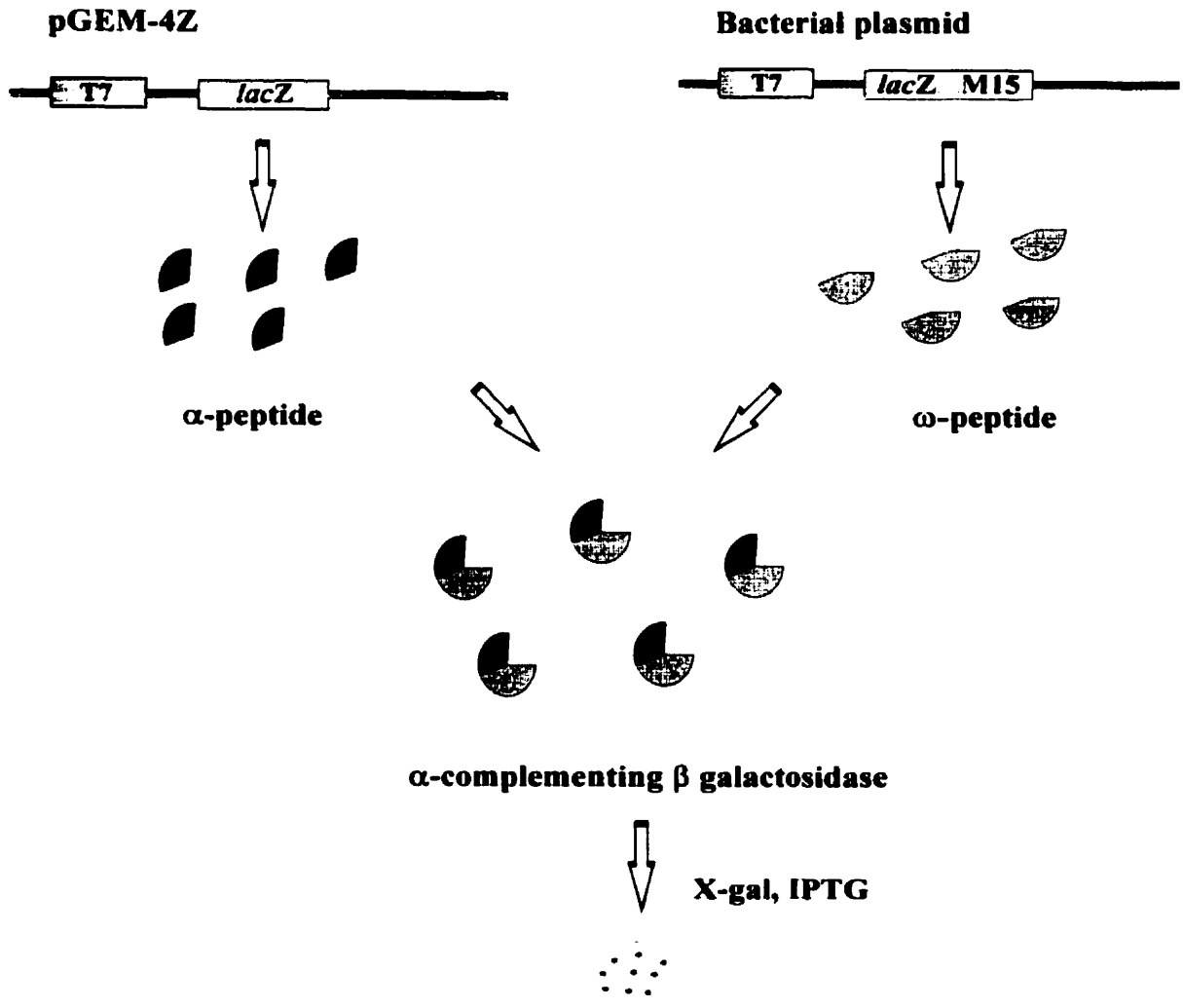

pGEM-4Z
T7
lacZ
Bacterial plasmid
T7
lacZ M15
α-peptide
ω-peptide
α-complementing β galactosidase
X-gal, IPTG

Figure 1*A*.

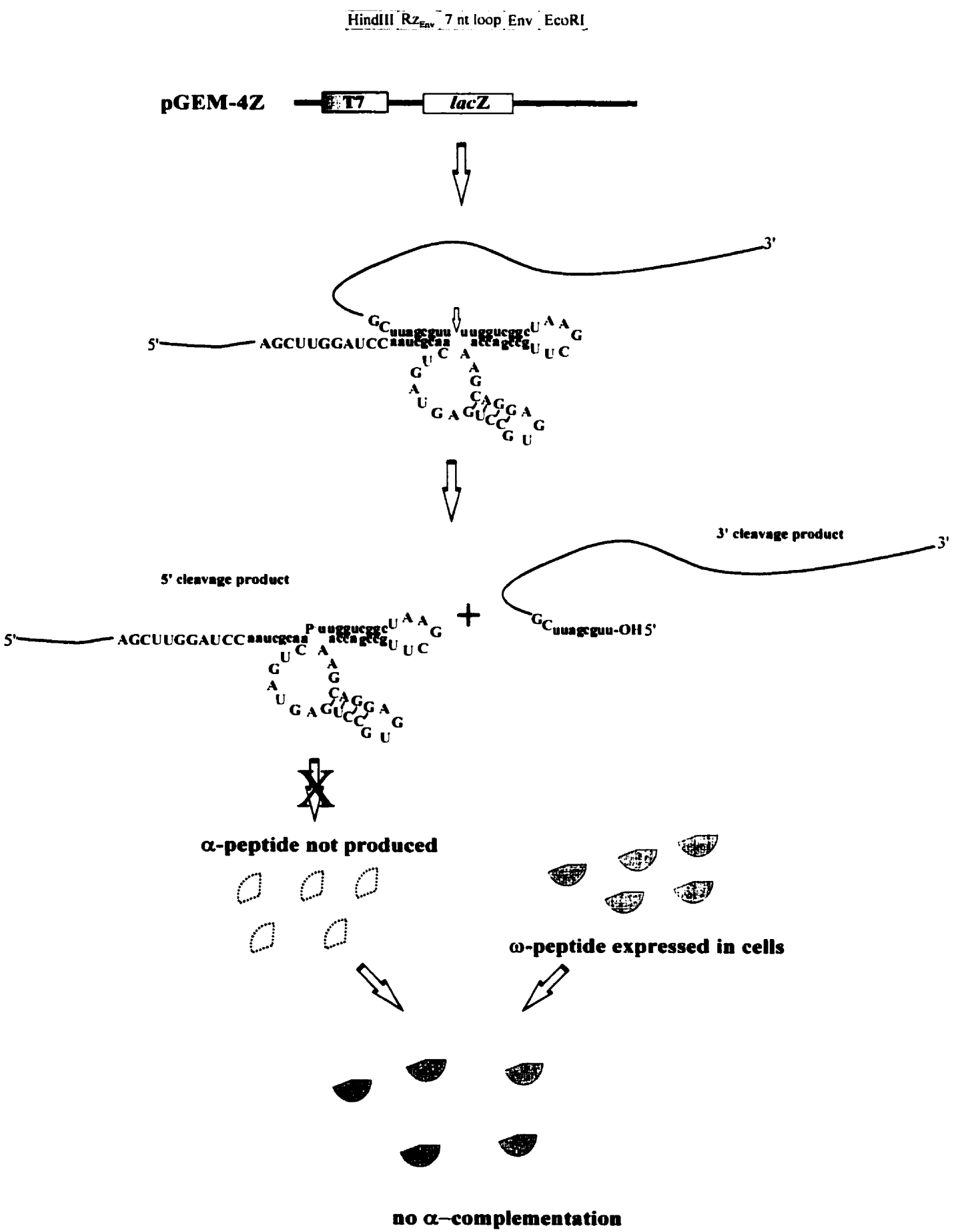

HindIII Rz$_{Env}$ 7 nt loop Env EcoRI
pGEM-4Z
T7
lacZ
3'
5'
AGCUUGGAUCC
5' cleavage product
3' cleavage product
3'
5'
AGCUUGGAUCC
GCuuagcguu-OH 5'
α-peptide not produced
ω-peptide expressed in cells
no α–complementation

Figure 1*B*.

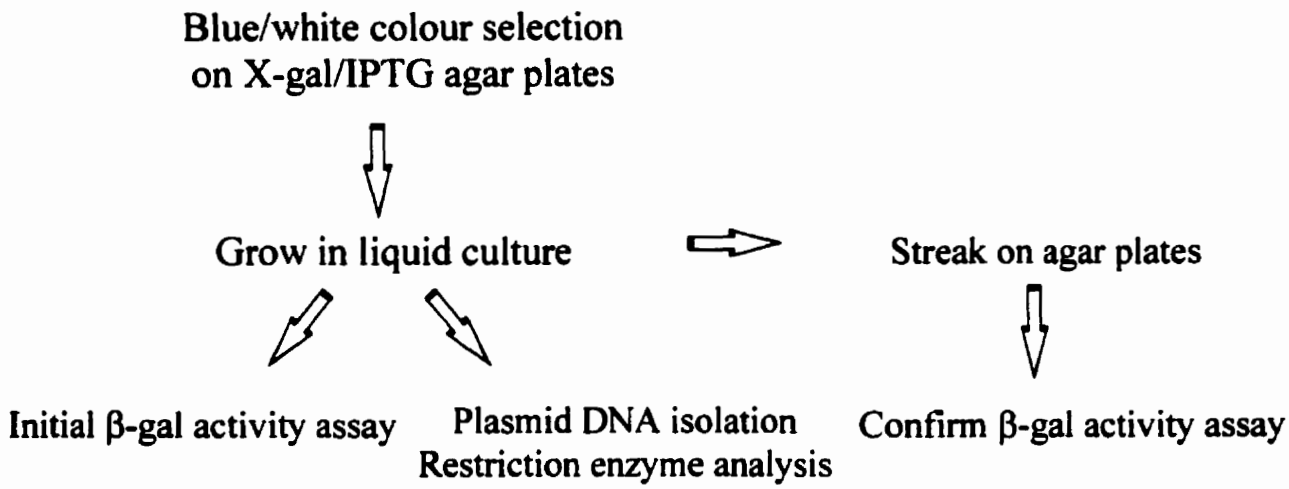
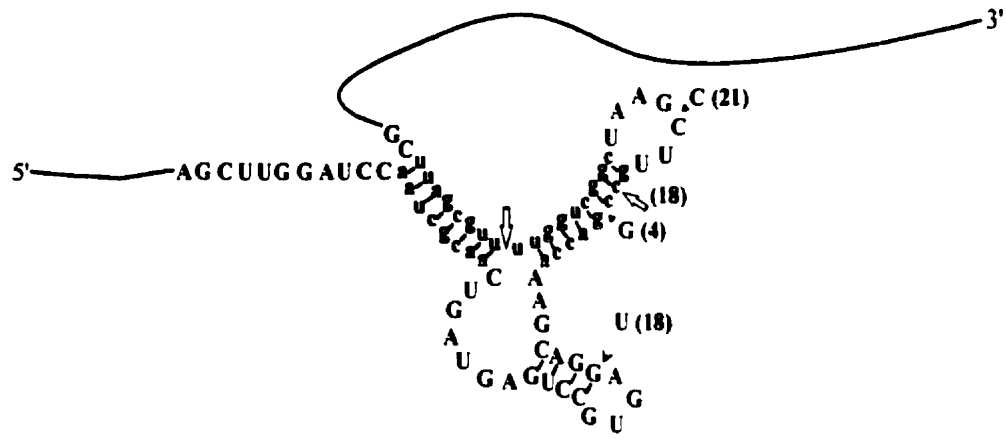
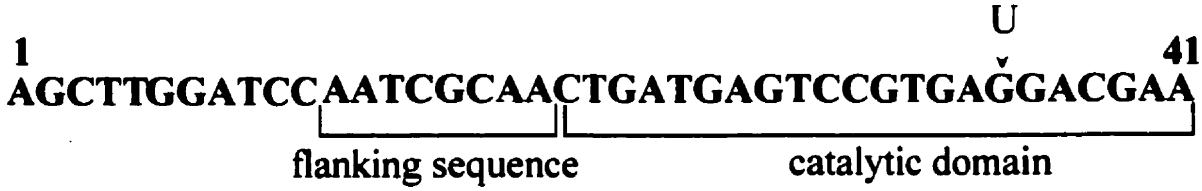

Blue/white colour selection
on X-gal/IPTG agar plates
Grow in liquid culture
Streak on agar plates
Initial β-gal activity assay
Plasmid DNA isolation
Restriction enzyme analysis
Confirm β-gal activity assay

Figure 2.


3'
5'
AGCUUGGAUCCA
A G C (21)
U
C
U U
(18)
G (4)
U
C A
G
A
A
G
U (18)
U G A G U C C G A G
G U


U
1
41
AGCTTGGATCCAATCGCAACTGATGAGTCCGTGAGGACGAA
flanking sequence
catalytic domain


42
G
C
75
ACCAGCCGTTCGAATCGGCTGGTTTTGCGATTCG
flanking sequence
loop
target sequence

Figure 3.

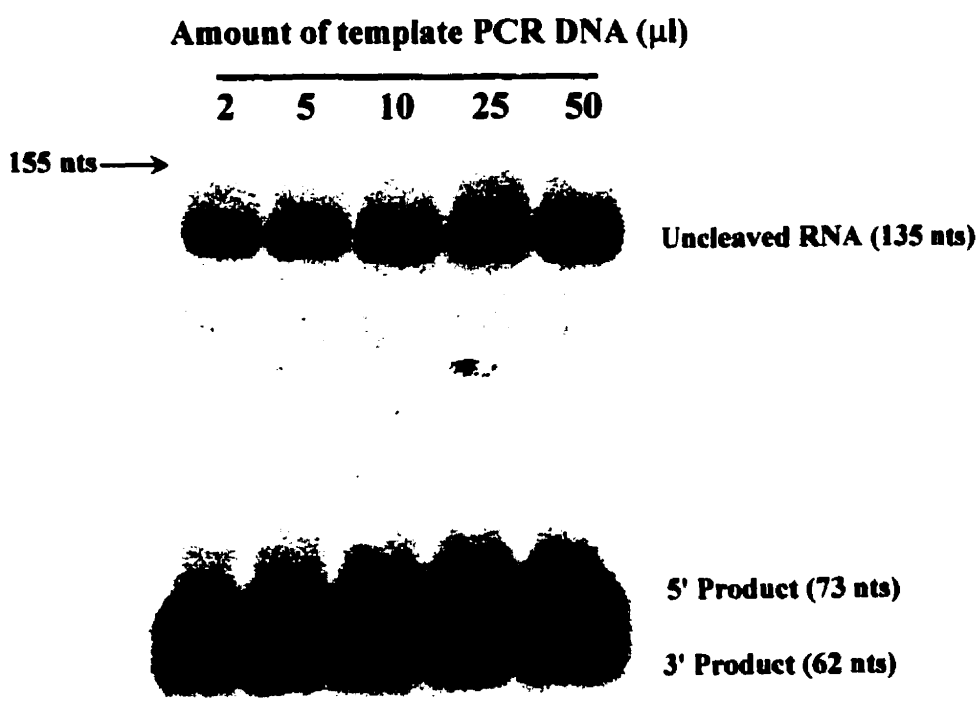

Amount of template PCR DNA (µl)
2
5
10
25
50
155 nts
Uncleaved RNA (135 nts)
5' Product (73 nts)
3' Product (62 nts)

Figure 4A.

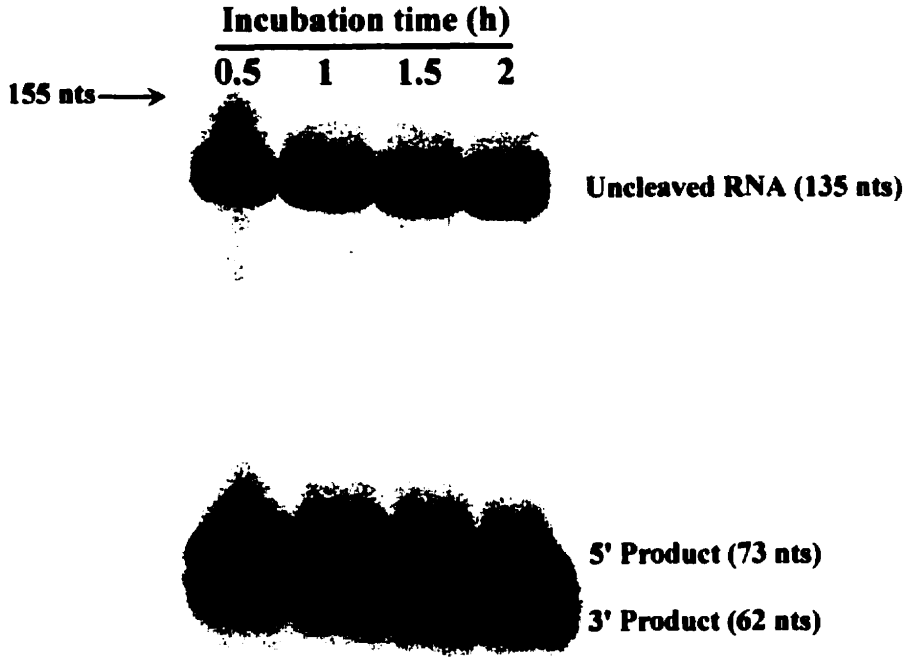

Incubation time (h)
0.5
1
1.5
2
155 nts
Uncleaved RNA (135 nts)
5' Product (73 nts)
3' Product (62 nts)

Figure 4*B*.

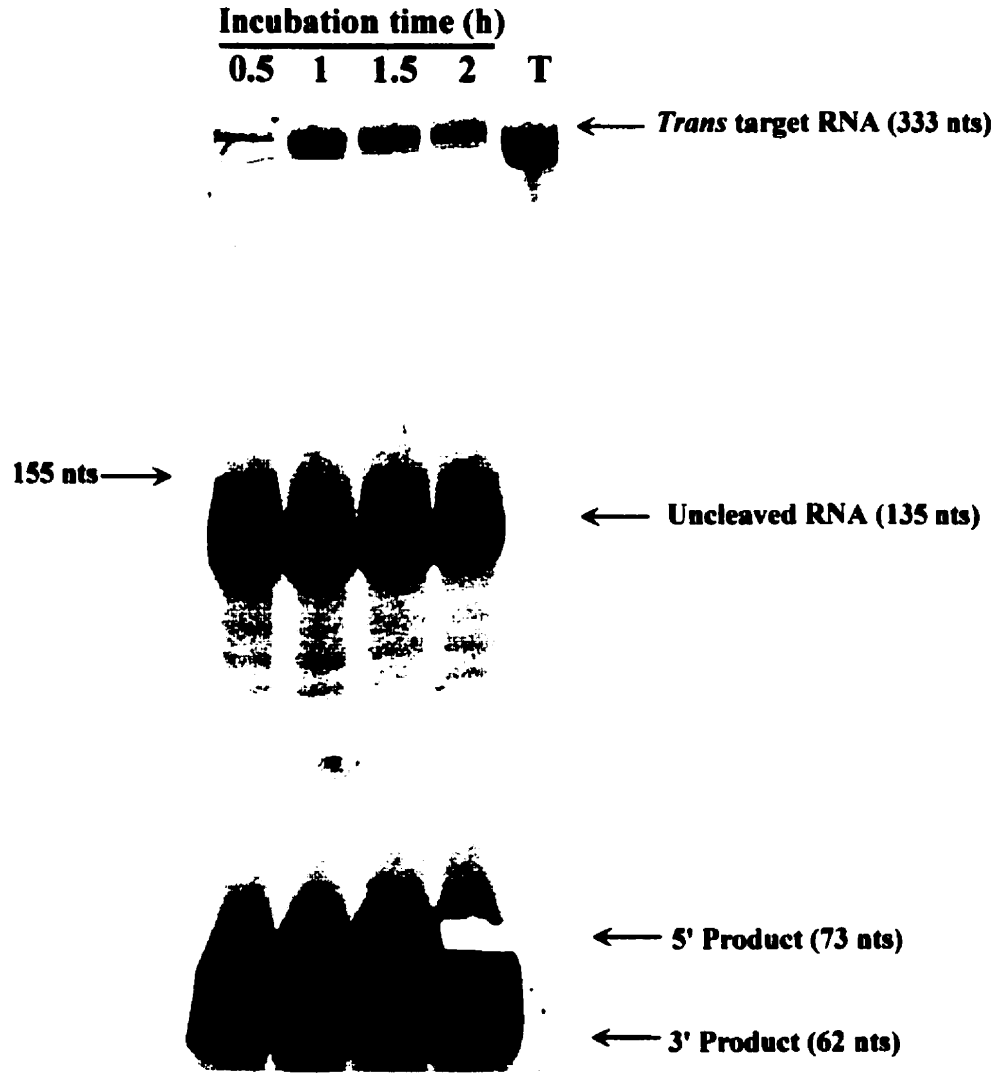

Incubation time (h)
0.5 1 1.5 2 T
Trans target RNA (333 nts)
155 nts
Uncleaved RNA (135 nts)
5' Product (73 nts)
3' Product (62 nts)

Figure 4C.

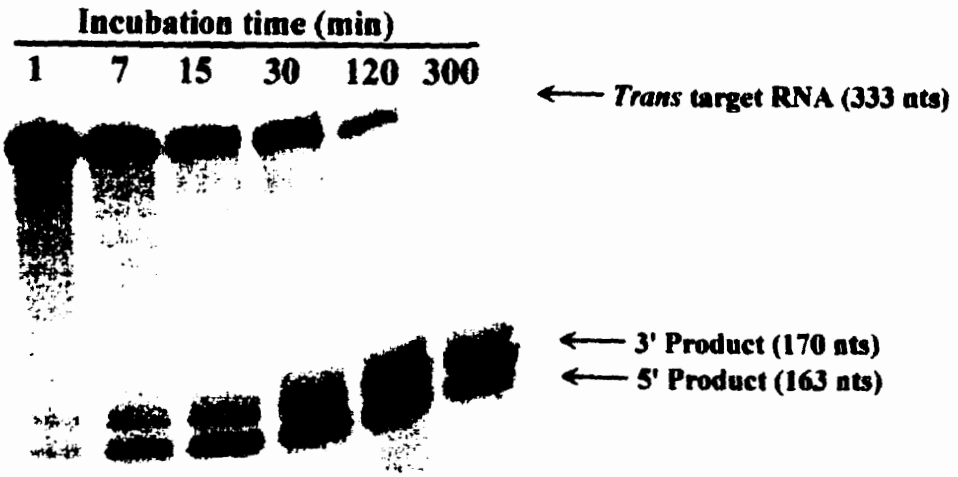

Incubation time (min)
1
7
15
30
120
300
Trans target RNA (333 nts)
3' Product (170 nts)
5' Product (163 nts)

Figure 5.

APPENDIX B

PCR Mutagenesis of the $tRNA_i^{Met}$ gene in the DC-t5t vector

ABSTRACT

A double copy vector (DC-t5t) was developed (Sullenger et al 1990b) which contains a $tRNA_i^{Met}$ gene lacking a 3' processing site, thereby allowing high levels of expression of chimeric tRNAs. In order for this vector to be used for expressing $tRNA_i^{Met}$-ribozymes, the 3' processing site would have to be restored. This is because a 3' trailer RNA may interfere with the hybridization of the ribozyme with its target through binding to the ribozyme flanking sequences, or to the target RNA. The 3' processing site was restored by site-directed mutagenesis by PCR. Clones were obtained which contained the 3' processing site; however, sequencing analysis showed that the rest of the $tRNA_i^{Met}$ sequence has been deleted, most probably mediated by a direct repeat flanking the $tRNA_i^{Met}$ sequence in DC-t5t. Excision of the tRNA cassette was observed when the DC-t5t vector was used to express multimeric ribozymes in CD4+ cells, and has also been reported by another group (Junker et al 1995).

INTRODUCTION

Moloney murine leukemia virus-based retroviral vectors are commonly used for RNA pol III-driven gene expression (Fig. 1). These vectors contain an RNA pol II promoter located in the 5' LTR. An additional promoter may be present in between the LTRs to allow expression of a selectable marker. RNA pol III-driven expression cassettes may be cloned either in a forward or reverse orientation (Fig. 1). Cloning may be performed in between the two LTRs upstream or downstream of the selectable marker (single copy design) or within the 3' LTR that results in gene duplication upon reverse transcription (double copy design). In addition to gene duplication in the 5' LTR, the latter design has the advantage of positioning the duplicated RNA pol III expression cassette upstream of the RNA pol II promoter. In either forward or reverse orientation, the double copy design was found to be more effective than the single copy design (Ilves et al 1996). This could be due to interference between the 5' LTR promoter and the downstream RNA pol III promoter in the single copy design.

DC-t5t is a DC retroviral vector, which contains a truncated $tRNA_i^{Met}$ gene inserted upstream of the promoter in the U3 region of the 3'LTR. This vector allowed expression of high levels of chimeric RNAs such as antisense RNAs (Sullenger et al 1990b) and decoy RNAs (Lee et al 1992, Sullenger et al 1990a). In order to express ribozymes that are part of a processed transcript, the deleted sequences of the $tRNA_i^{Met}$ gene, which contained the processing site, would have to be restored. By doing so, the ribozymes could be expressed as part of a processed tRNA molecule, without a 3' trailer RNA that may interfere with the hybridization of the ribozyme with its target.

MATERIALS AND METHODS

Four primers (Table 1) were designed in order to restore the deleted sequences of $tRNA_i^{Met}$ in DC-t5t. MM2 and MM3 are complementary primers, which possess the 11-nt deletion. KL1 and

MM1 bind to sequences immediately upstream and downstream, respectively, of $tRNA_i^{Met}$ in DC-t5t.

PCRs were performed using 400 ng of DC-t5t template, 1.5 mM $MgCl_2$, 200 μM of dNTPs, 0.2 μM of each forward and reverse primer and 5 units of *Taq* polymerase in a final volume of 100 μL. To generate the KL1/MM3 product (590 bp), the sample was subjected to 30 cycles of denaturation (1 min, 94°C), annealing (2 min, 56°C), and extension (3 min, 72°C). The MM2/MM1 product (181 bp) was generated using 40 cycles of denaturation (20 sec, 95°C), annealing (20 sec, 56°C), and extension (20 sec, 72°C). The KL1/MM3 and MM2/MM1 PCR products were purified to eliminate contaminating PCR products and DC-t5t template DNA. The products were size-fractionated by agarose gel electrophoresis. The DNA bands were cut and purified using theGeneClean kit (BIO 101: Vista, USA) and the MERmaid kit (BIO 101: Vista, USA). The products were then combined in a PCR using primers KL1 and MM1 for 30 cycles (1 min, 94°C; 2 min 56°C; 3 min, 72°C). The DNA product generated was approximately 717 bp.

The 717-bp fragment was cloned within the *Bgl* II and *Sac* II sites of DC-t5t. Correct clones were screened by restriction enzyme analysis using *Mro* I. Four clones demonstrated the correct bands. Sequencing was also performed using a 3. 8 kb *Sac* I fragment which was purified by GeneClean.

RESULTS AND DISCUSSION

PCR Mutagenesis was performed in two steps (Ho et al 1989, Smith et al 1993). First, PCR products were generated that contained overlapping ends. Alterations in the DNA sequence were made by changing the nucleotide composition of the overlapping PCR primers. The DNA products were combined in an extension reaction wherein the overlapping ends annealed, and allowed the 3' overlap of each strand to serve as the primer for the extension of the

complementary strand. The extension product was further amplified by PCR (Fig. 1). This product was then cloned within the DC-t5t vector.

Four correct clones were identified by restriction enzyme analysis. However, sequencing results showed that although the 11-nt deletion of $tRNA_i^{Met}$ has been restored, the rest of the tRNA sequence has been deleted. Furthermore, use of this vector to express a multimeric ribozyme resulted in excision of the tRNA cassette in the transduced cells (our unpublished results). Similar results were also reported by another group (Junker et al 1995). The excision of the $tRNA_i^{Met}$ cassette in transduced cells was thought to occur during reverse transcription because of the presence of direct repeats flanking the $tRNA_i^{Met}$ gene. The same repeats may have also caused recombination in bacterial cells.

REFERENCES

Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR: **Site-directed mutagenesis by overlap extension using the polymerase chain reaction.** *Gene* (1989) **77**:51-59.

Ilves H, Barske C, Junker U, Bohnlein E, Veres G: **Retroviral vectors designed for targeted expression of RNA polymerase III-driven transcripts: a comparative study.** *Gene* (1996) **171**:203-208.

Junker U, Bohnlein E, Veres G: **Genetic instability of a MoMuLV-based antisense double-copy retroviral vector designed for HIV-1 gene therapy.** *Gene Ther.* (1995) 2:639-646.

Lee TC, Sullenger BA, Gallardo HF, Ungers GE, Gilboa E: **Overexpression of RRE-derived sequences inhibits HIV-1 replication in CEM cells.** *New Biol.* (1992) **4**:66-74.

Smith KD, Valenzuela A, Vigna JL, Aalbers K, Lutz CT: **Unwanted Mutations in PCR Mutagenesis: Avoiding the Predictable.** *PCR Methods and Applications* (1993) **2**:253-257.

Sullenger BA, Gallardo HF, Ungers GE, Gilboa E: **Overexpression of TAR sequences renders cells resistant to human immunodeficiency virus replication.** *Cell* (1990a) **63**:601-608.

Sullenger BA, Lee TC, Smith CA, Ungers GE, Gilboa E: **Expression of chimeric tRNA-driven antisense transcripts renders NIH 3t3 cells highly resistant to Moloney murine leukemia virus replication.** *Mol. Cell. Biol.* (1990b) **10**:6512-6523.

TABLE 1.

List of primers used to restore the deleted sequences of $tRNA_i^{Met}$ in DC-t5t

KL1	17	CCA-CCT-GTA-GGT-TTG-GC	forward primer; binds upstream of $tRNA_i^{Met}$ in DCt5t
MM1	17	GGT-CAG-GAA-CAG-ATG-GA	reverse primer; binds downstream of $tRNA_i^{Met}$ in DCt5t
MM2	35	TGG-ATC-GAA-ACC-ATC-CTC-TGC-TAG-TCC-GGA-TCG-TA	forward primer; contains eleven nucleotides missing in $tRNA_i^{Met}$ in DCt5t
MM3	35	TAC-GAT-CCG-GAC-TAG-CAG-AGG-ATG-GTT-TCG-ATC-CA	forward primer; contains eleven nucleotides missing in $tRNA_i^{Met}$ in DCt5t
MM4	17	CGA-TCC-GGA-CTA-GCA-GA	reverse primer) derived from bases 3-19 of MM3 which was used as a sequencing primer

FIGURE LEGENDS

FIG. 1. **Cloning sites in a retroviral vector.** A retroviral vector is shown which contains a neomycin phosphotransferase gene (*neo*) driven by an internal promoter (P). RNA pol III-driven expression cassettes may be cloned either in a forward (black arrow) or reverse (gray arrow) orientation. Cloning may be performed in between the two LTRs upstream or downstream of the selectable marker (A and B) in the single copy (SC) design. Alternatively, expression cassettes may be cloned within the U3 region of the 3' LTR (C) in the double copy (DC) design. Upon transfection, RNAs are expressed from the 5'LTR, the internal promoter P, and each of the expression cassettes. Upon reverse transcription in transduced cells, the expression cassette cloned within the 3' LTR (C) is duplicated upstream of the RNA pol II promoter and is now placed upstream of the RNA pol II transcript. Thin and thick lines correspond to RNA pol II- and pol III-driven transcripts, respectively.

FIG. 2 **Overlap PCRs to restore the processing site of $tRNA_i^{Met}$.** Primers KL1 and MM3 were used in a PCR resulting in a 590-bp PCR product, while primers MM2 and MM1 were used in a PCR resulting in a 181-bp PCR product. The gray box on the primers corresponds to the missing sequences in $tRNA_i^{Met}$ which contains the 3' processing site. The two PCR products (590 bp and 181 bp) were used as overlapping templates to generate the 717-bp KL1/MM1 PCR product.

FIG. 3. **PCR products.** The PCR products were analyzed by 2% agarose gel electrophoresis and the DNA bands were visualized by staining with ethidium bromide. The image shown is a scanned photograph that has been inverted using Adobe PhotoShop. Lane 1, KL1/MM3 product. Lane 2, MM2/ MM1 product. M, ϕx174-DNA *Hae* III marker.

FIG. 4. **Enlarged view of the U3 region in the 3' LTR of DC-t5t.** The 717-bp KL1/MM1 PCR product was cloned in the unique *Bgl* II and *Sac* II sites flanking the mutated $tRNA_i^{Met}$ in the DC-t5t vector. The parent vector contains a unique *Mro*I site (not shown), whereas clones containing the insert would possess a second *Mro*I site.

FIG. 5. **Restriction enzyme analysis of DC-t5t clones.** The DC-t5t vector contains a single *Mro* I site, resulting in a single fragment (10.2 kb) upon *Mro* I digestion. Correct clones contain two *Mro* I sites, resulting in two fragments (7.7 kb and 2.5 kb) upon *Mro* I digestion. M, λ/*Hin*d III marker. D, DC-t5t. Bracketed lanes are the different clones. Arrows show the linearized DC-t5t plasmid (10.2 kb) and the restriction fragments (7.7 kb and 2.5 kb).

FIG. 6. **Sequencing results for the DC-t5t clones.** Plasmid DNA was isolated from three clones (19, 20 and 22), and used for *Sac* I digestion. The 3.8 kb *Sac* I fragment was separated from undigested DNA by agarose gel electrophoresis, and purified by GeneClean. It was then used for sequencing using the ^{T7}Sequencing Kit (Pharmacia Biotech Inc.; Baie d'Urfé, Canada) following instructions provided by the supplier. The brackets correspond to the 11-nt sequence that was restored to the $tRNA_i^{Met}$.

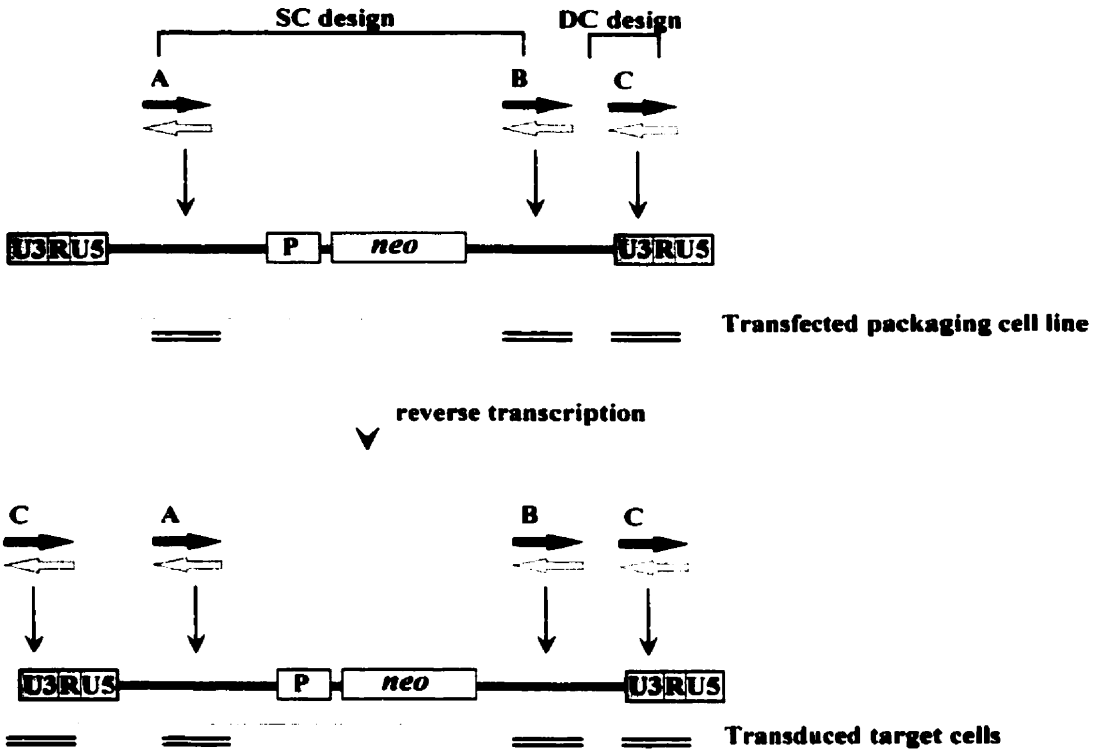

SC design
DC design
A
B
C
U3RU5
P
neo
U3RU5
Transfected packaging cell line
reverse transcription
C
A
B
C
U3RU5
P
neo
U3RU5
Transduced target cells

Figure 1.

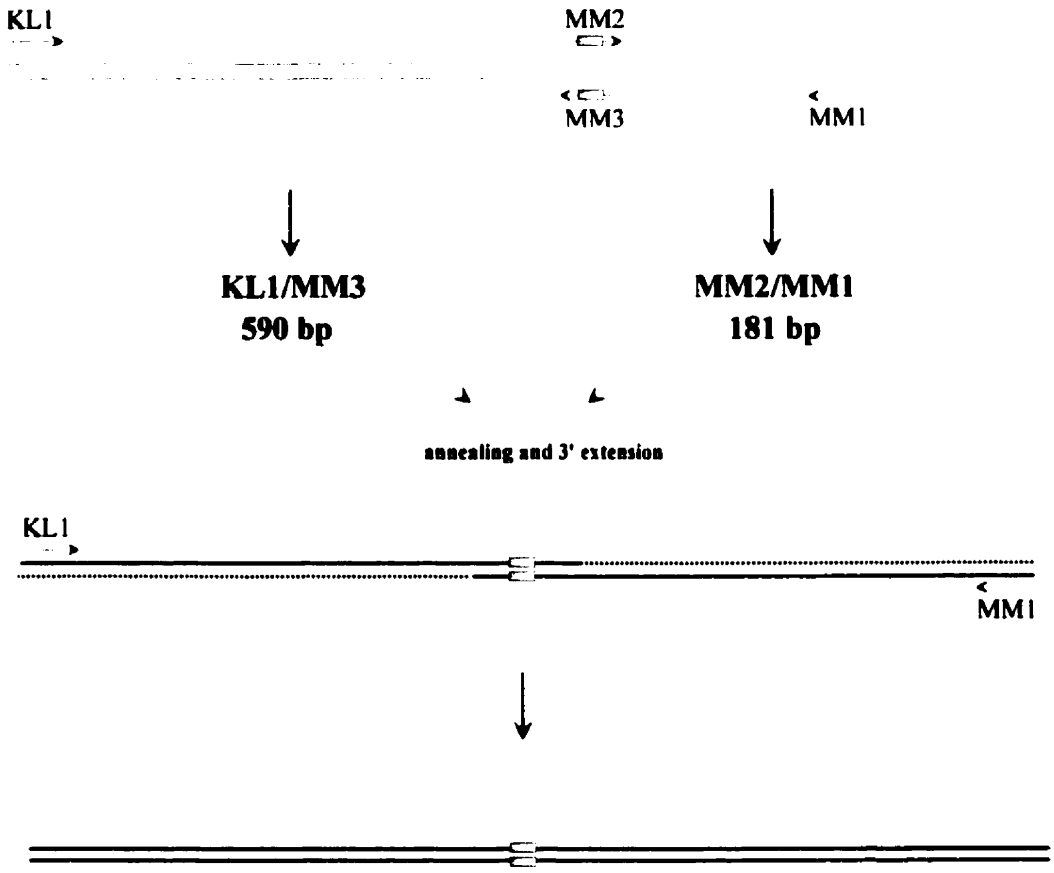

KL1
MM2
MM3
MM1
KL1/MM3
590 bp
MM2/MM1
181 bp
annealing and 3' extension
KL1
MM1
FINAL PCR PRODUCT (717 bp)

Figure 2.

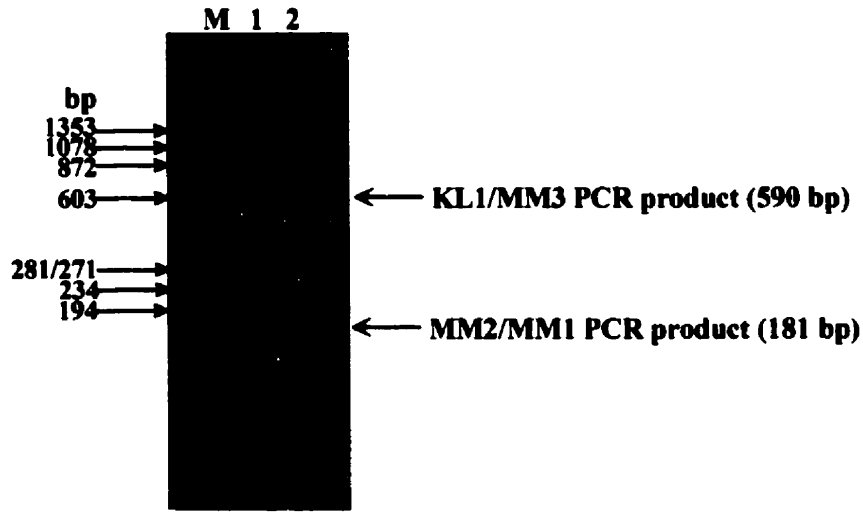

M 1 2
bp
1353
1078
872
603
281/271
234
194
KL1/MM3 PCR product (590 bp)
MM2/MM1 PCR product (181 bp)

Figure 3.

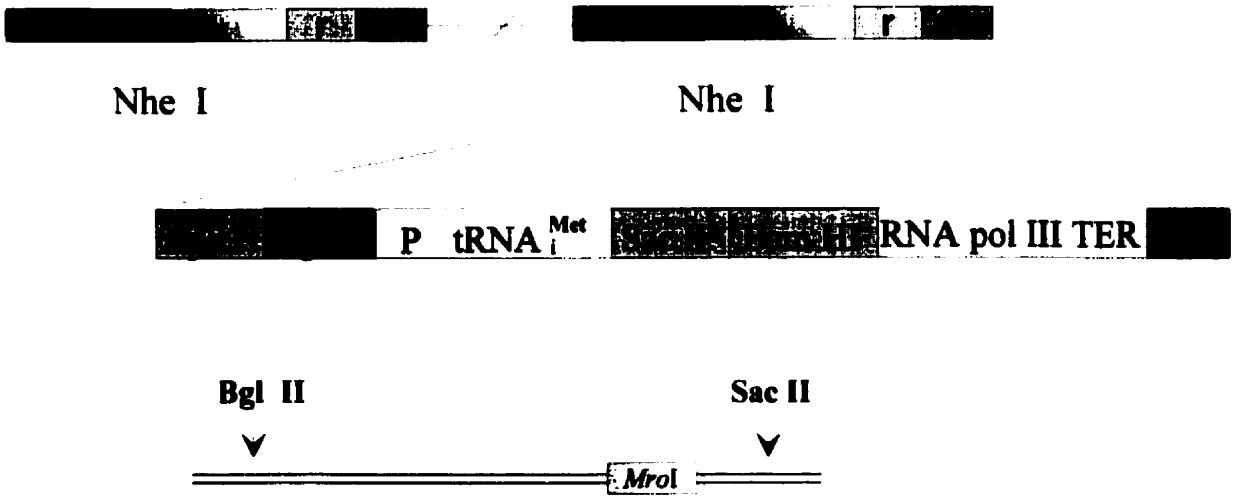

Nhe I
Nhe I
P
tRNA i Met
RNA pol III TER
Bgl II
Sac II
MroI
PCR product (717 bp)

Figure 4.

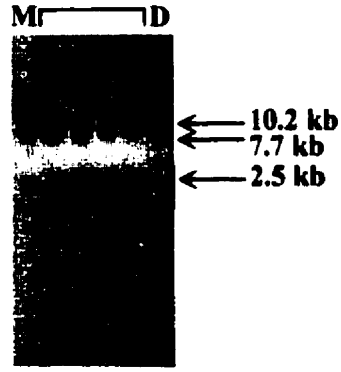


Figure 5.

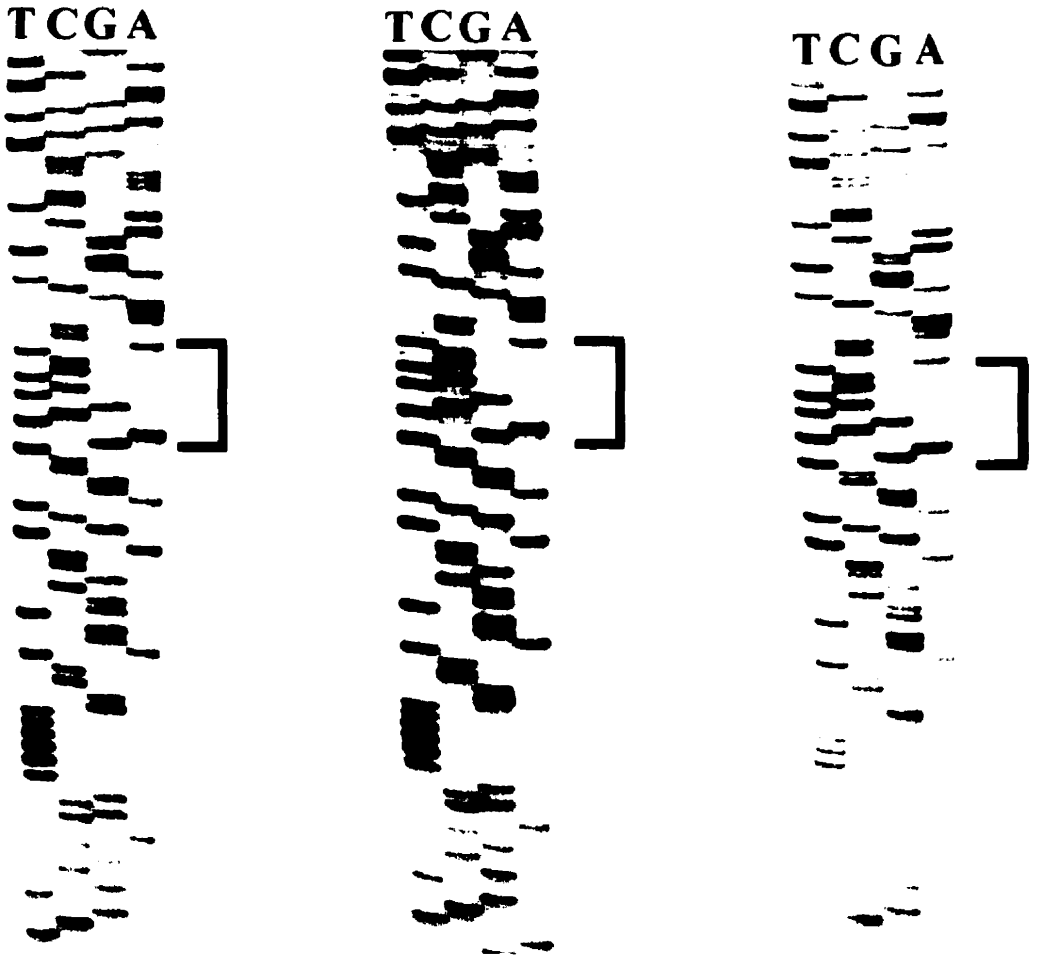


Figure 6.