[Frontiers in Bioscience 5, d527-555, May 1, 2000]

CURRENT DEVELOMENTS AND FUTURE PROSPECTS FOR HIV GENE THERAPY USING INTERFERING RNA-BASED STRATEGIES

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1. ABSTRACT

Acquired immunodeficiency syndrome (AIDS) is a slow, progressive, degenerative disease of the human immune system, ultimately leading to premature death of the patient. This disease is primarily caused by human immunodeficiency virus type-1 (HIV-1). The major targets of HIV infection are blood cells, namely lymphocytes and macrophages. While the immune response fails to eliminate the infected cells, the virus continues to spread. The purpose of HIV gene therapy is to provide "anti-HIV" genes to cells that are susceptible to HIV infection. Anti-HIV genes may be designed to express RNAs or proteins that interfere with the function of viral or cellular RNA(s)/protein(s), thereby inhibiting virus replication. Whereas interfering proteins may be cytotoxic and/or immunogenic, interfering RNAs are not. Interfering protein-based strategies requiring inducible gene expression (under the control of HIV regulatory proteins) can only be designed to block steps subsequent to the viral regulatory protein production. In contrast, interfering RNAs can be produced in a constitutive manner, which further enhances their antiviral activity and allows one to design strategies to inhibit virus replication before viral regulatory protein production occurs. Thus, interfering RNAs are of particular interest and are the focus of this review. Genes expressing interfering RNAs were designed to inhibit syncytium formation to prevent the death of the gene-modified cells. Strategies may also be developed to prevent gene-modified cells from becoming infected by HIV or from supporting HIV replication. Genes expressing interfering RNAs have been designed to inhibit HIV-1 entry and to cleave the incoming virion RNA, thus blocking virus replication before provirus DNA synthesis can be completed. A number of genes were also designed to express interfering RNAs that inhibit HIV replication at a post-integration step, by inhibiting the function of HIV RNAs or proteins produced in the infected cell. Also in development are anti-HIV genes that produce RNAs that would not only inhibit HIV replication in the gene-modified cell, but also prevent HIV RNA packaging and/or reverse transcription such that the progeny virus produced would be non-infectious. Further refinements to these strategies may lead to the development of "self-propagating" anti-HIV genes. These genes would express interfering RNAs that not only inhibit virus replication in the cell and prevent HIV RNA packaging and/or reverse transcription in the progeny virus, but also make use of the HIV itself to deliver the anti-HIV gene(s) to other cells. Thus, more and more cells susceptible to HIV infection would become resistant. Such "self-propagation" of anti-HIV-1 genes would only occur in cells that are susceptible to HIV infection, and would continue to take place for as long as HIV exists in the body.

2. INTRODUCTION

AIDS is primarily caused by a lentivirus, HIV, which mainly infects the CD4+ T lymphocytes and macrophages (1). The continuous proliferation and differentiation of a relatively small number of pluripotent hematopoietic stem cells maintain all of the major cell types involved in the pathogenesis of AIDS. Their pluripotential, differentiative capacity and ability to self-renew make them an ideal target for gene transfer. HIV gene therapy would involve isolation of autologous or allogeneic cells, followed by their genetic modification *ex vivo*. Genetically modified cells would then be transplanted into the patient. Upon differentiation and proliferation, the gene-modified stem cells should give rise to progeny cells that are resistant to HIV infection/replication.

A successful anti-HIV gene therapy strategy would have to confer a selective advantage to the gene-modified cells, while allowing them to maintain their normal immune functions. This requires gene-modified cells to be resistant to virus replication. Thus, while HIV infections would result in the death of the unmodified cells over time, the gene-modified cells would gradually repopulate the immune system. Once the viral load begins to decrease, re-population by uninfected gene-modified and unmodified cells would result in eventual reconstitution of an HIV resistant immune system. Since gene therapy would likely be applied to patients on anti-HIV drug therapy, a low viral load (maintained by anti-HIV drugs) may not constitute enough selective pressure. Thus, the proportion of transduced cells would have to be increased by additional *in vivo* selection strategies (2).

Some degree of general immune function may be restored by transfusion of transduced peripheral blood T lymphocytes. However, gene transfer in human peripheral blood lymphocytes (PBLs) would only protect a sub-population of T-lymphocytes and monocytes/macrophages. Gene transfer into PBLs would not prevent the destruction of other cell types (like the dendritic cells and brain microglial cells), which are also susceptible to HIV infection (3, 4). Furthermore, since PBLs are not self-renewing and have a finite life span, repeated cycles of transduction and transfusion would be required to achieve a therapeutic benefit.

Several viral vectors are currently being used for human gene therapy. However, the majority of the gene therapy trials (>70%) are being performed using retroviral vectors as they allow stable, long-term gene expression. Both retroviral and lentiviral vectors are being used to deliver "anti-HIV" genes. The anti-HIV genes (5-10) may be designed to encode RNAs and/or proteins that inhibit one or multiple sites within the viral life cycle or cellular processes essential for viral replication. Interfering RNAs (10-18) and proteins (19, 20) may be designed to block the HIV-1 replication cycle by interfering with the function of cellular or HIV-1 RNA(s) or protein(s). The most effective inhibitory strategy would be one that completely blocks the viral life cycle. The step at which the viral life cycle is inhibited may not matter, however it may be better to intervene at the earliest step; *i.e.* viral entry. An alternative strategy consists of designing "suicide" genes that encode RNAs and/or proteins that would specifically disable the infected cells, causing cell death before virus production occurs.

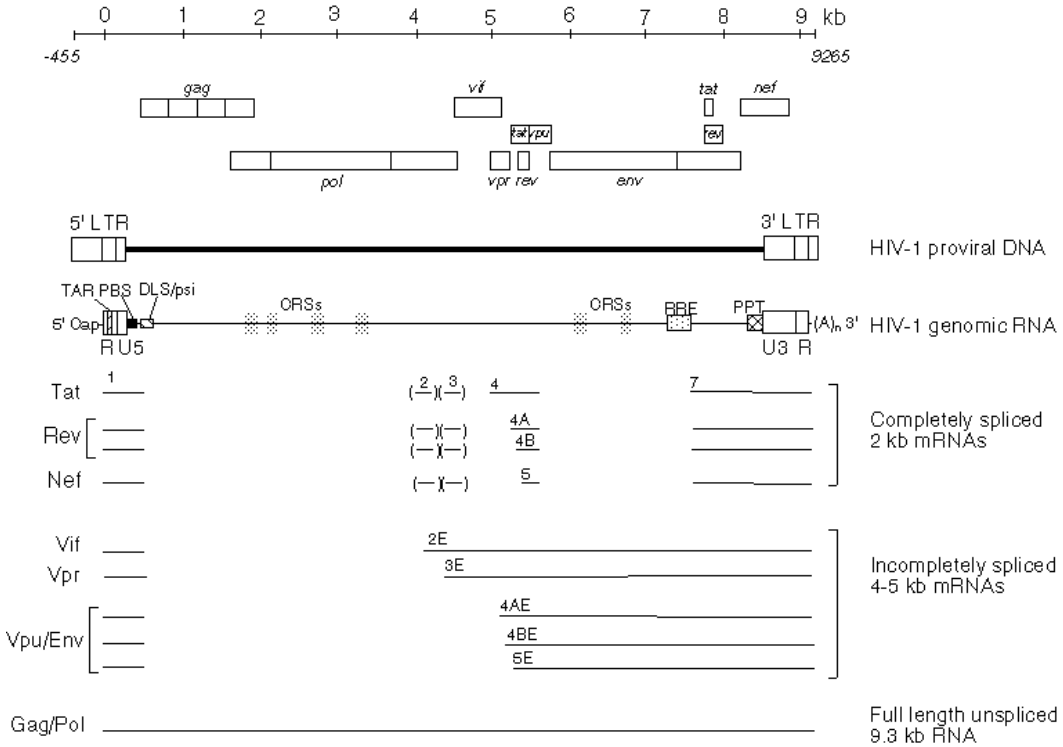

Figure 1. Structure of the HIV-1 genomic RNA, provirus DNA, and of the mRNAs produced in the infected cell. Various genes encoded by HIV-1 provirus DNA and the elements that act *in cis* at the level of viral RNA/DNA are shown. Exons are numbered 1 through 7.

Interfering RNAs include antisense RNAs (16), sense RNAs (14), and ribozymes (15, 17, 18). Antisense RNAs and ribozymes are designed to inhibit cellular or HIV RNA function, while sense RNAs are designed to disrupt HIV RNA/protein or RNA/RNA interactions. Proteins that interfere with HIV RNA function include HIV-1 RNA-specific nucleases that would specifically recognize and cleave viral mRNAs (21, 22) and packageable nucleases that would be packaged within the progeny virus and cleave the virion RNA (23). HIV protein function can be disrupted using a number of proteins, including *trans*-dominant mutants (TDMs) of viral proteins (24-47), cellular factors (48), HIV receptor (49-53), ligands of HIV coreceptors (54-56), single-chain antibodies (57-63), and interferon (64, 65). Suicide proteins, which cause the selective death of HIV-infected cells, include the herpes simplex virus (HSV) thymidine kinase (*tk*) and the attenuated diphtheria toxin A chain (66, 67). Live viruses may also be used to cause selective death of the HIV-infected cells (68, 69). A recombinant vesicular stomatitis virus (VSV) was engineered in which the gene encoding the viral glycoproteins was replaced with those encoding HIV-1 receptor (CD4) and coreceptor (CXCR4). This recombinant virus was shown to infect, propagate on, and kill the HIV-infected cells (69).

Interfering RNA- and protein-based strategies may also be combined. Several sense or antisense RNAs were combined with TDMs of viral proteins (70-74) or with a single chain antibody against an HIV-1 protein (75). All of these combination strategies were shown to confer better protection than the single anti-HIV genes.

2.1. HIV-1 molecular biology

2.1.1. Genetic map of HIV-1 RNA

The genome of HIV-1 consists of two identical copies of positive strand 9.3 kb RNA molecules (76-78). HIV-1 RNA contains nine open reading frames along with several *cis*-acting elements that act at the level of HIV RNA or DNA (figure 1).

HIV provirus DNA contains the group specific antigen (*gag*), polymerase (*pol*), and envelope (*env*) genes, common to all retroviruses. The products of these genes are packaged within the virus particles. The *gag* gene gives rise to a polyprotein precursor $Pr55^{Gag}$ which is subsequently processed into four proteins: matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p9), and p6. Frameshift gives rise to $Pr160^{Gag\text{-}Pol}$ which is processed into the Gag proteins, and the protease (Pro, p10), reverse transcriptase (RT, p66/p51), and integrase (IN, p31). The *env* gene gives rise to a glycoprotein gp160, which is processed into two proteins: surface (SU, gp120) and transmembrane (TM, gp41).

HIV-1 provirus DNA encodes six additional genes, including *trans*-activator of transcription (*tat*), regulator of expression of virion proteins (*rev*), virion infectivity factor (*vif*), viral protein u (*vpu*), viral protein r (*vpr*), and negative

effector (*nef*) (79, 80). Tat and Rev are involved in the regulation of early and late gene expression. Vif is important for the production of highly infectious mature virions, while Vpu enhances the release of virus particles. Vpr is co-packaged with Gag into the progeny virus. It regulates nuclear import of the HIV-1 pre-integration complex. Nef contributes to reduction of CD4 and MHC class I molecules on the cell.

HIV-1 RNA contains several *cis*-acting elements (figure 1) including the Repeat (R), Unique 5' (U5), Unique 3' (U3), *trans*-activation responsive element (TAR), primer binding site (PBS), packaging signal (Psi), extended packaging signal (Psi-e), dimer linkage structure (DLS), *cis*-acting repressive sequences (CRSs), Rev response element (RRE), and polypurine tract (PPT). These elements are essential for reverse transcription, integration, gene expression, nuclear export, and packaging of HIV RNA. R is a repeated region present at both 5' and 3' ends of HIV RNA and is required for HIV RNA reverse transcription. It contains the TAR element, the transcription start site, the 3' processing site, and the polyadenylation site (81). U5 and U3 are unique sequences present at the 5' and the 3' end of HIV-1 RNA, respectively. PBS is located immediately 3' to the U5 region and allows the binding of cellular $tRNA_3^{Lys}$ which is also packaged by the virus. Binding occurs through complementarity between the 18 nucleotides of the PBS and the 3' terminal 18 nucleotides of the $tRNA_3^{Lys}$. The $tRNA_3^{Lys}$ serves as a primer during reverse transcription and initiates negative strand DNA synthesis. PPT is located immediately 5' to the U3 region. It serves as a primer during reverse transcription and initiates positive strand DNA synthesis (76, 81, 82). The provirus DNA contains 5' and 3' long terminal repeats (LTRs) made of U3-R-U5 sequences. The 5' end of the U3 region within the 5' LTR and the 3' end of the U5 region within the 3' LTR allow provirus DNA integration. The U3 region contains a promoter for RNA polymerase II and enhancer sequences.

TAR is made of a 59-nt stem loop located within the 5' and 3' regions of all HIV-1 mRNAs (83, 84). A 3-nt bulge within this RNA is responsible for its interaction with viral Tat protein. Tat/TAR interaction is crucial for HIV replication and allows *trans*-activation of viral gene expression. CRS elements are located within the *gag*/*pol* and *env* coding regions of HIV RNA. These sequences act *in cis* and prevent nuclear export of the respective RNA molecules. RRE is made of 351 nucleotides with five stem-loops located within the *env* coding region of singly spliced 4-5 kb and unspliced 9.3 kb HIV mRNAs. RRE stem loop IIB interacts with the viral Rev protein. Rev/RRE interaction is required for the nuclear export of 4-5 and 9.3 kb HIV mRNAs. As these RNAs encode viral proteins involved in viral assembly and maturation, Rev regulates late gene expression. TAR and RRE also interact with other cellular factors.

The Psi signal within the genomic RNA is essential for HIV RNA packaging (85, 86). It is made of four stem-loops and is located near the *gag* coding region (86). Another *cis*-element required for efficient RNA packaging (87) is located in the *env* coding region, which includes the RRE. Hence, the extended HIV-1 packaging signal, Psi-e, includes both *cis*-elements. HIV RNA is packaged in the form of a dimer. The DLS responsible for this process is contained within the Psi signal (88). The viral RNAs dimerize by a kissing loop mechanism.

2.1.2. HIV-1 life cycle

HIV infects CD4+ cells including monocytes/macrophages and CD4+ T lymphocytes. CD4 is the primary receptor used for HIV infection. HIV also utilizes chemokine receptors, CCR5 and CXCR4, as coreceptors (89, 90); several additional coreceptors have been also identified for HIV. Monocytes/macrophage (M)-tropic viruses use CCR5 (91-93), whereas the T cell (T)-tropic viruses use CXCR4 (94).

Following initial binding of HIV-1 envelope proteins to the CD4 receptor and coreceptors, fusion takes place between the viral and cellular membranes, allowing entry of the viral core into the cell (figure 2) (95). The HIV-1 genomic RNA is then reverse transcribed within the viral core to give rise to a double stranded provirus DNA (82). The preintegration complex, containing provirus DNA, RT, IN, Vpr, and possibly MA (96), is then transported to the nucleus (97). IN protein then allows proviral DNA integration within the host genome (81).

Transcription from the 5' LTR promoter gives rise to a primary 9.3 kb HIV-1 RNA, which is differentially spliced to give rise to multiply spliced 2 kb, singly spliced 4-5 kb, and unspliced 9.3 kb mRNAs (figure 1) (76, 98). During the early phase of viral life cycle, 2 kb mRNAs are translated to give rise to the Tat, Rev and Nef proteins. Upon entry within the nucleus, Tat interacts with the nascent TAR transcripts and increases the processivity of RNA pol II. This results in a 100-1000 fold increase in viral transcription (24, 99).

In the absence of Rev, only multiply spliced HIV mRNAs are found in the cytoplasm (figure 2) (100). Unspliced 9.3 kb RNA and singly spliced 4-5 kb HIV mRNAs contain CRSs, which prevent their nuclear export into the cytoplasm (101). These mRNAs also contain the RRE which interacts with Rev protein that shuttles between the nucleus and the cytoplasm. Rev also has a nuclear export signal that interacts with a nucleoporin-like protein located at the nuclear pore, through a nuclear export receptor. This then results in nuclear export of singly spliced 4-5 kb and unspliced 9.3 kb HIV-1 mRNAs.

Translation of 9.3 kb mRNA gives rise to the $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$. Singly spliced 4-5 kb RNAs give rise to Vpu, Env, Vif, and Vpr proteins. Env is translated in the endoplasmic reticulum, and processed by a cellular protease in the Golgi apparatus into SU and TM glycoproteins.

The viral core is assembled from the $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$ polyproteins, which interact with each other. The NC domain of $Pr55^{Gag}$ is involved in the selective packaging of the RNA genome. The RT domain within the $Pr160^{Gag\text{-}Pol}$ allows selective packaging of eight molecules of cellular $tRNA_3^{Lys}$ (102). Vpr is incorporated into viral particles through interactions with p6. An immature progeny virus buds from the cell surface that undergoes a morphologic change. Processing of $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$ by the

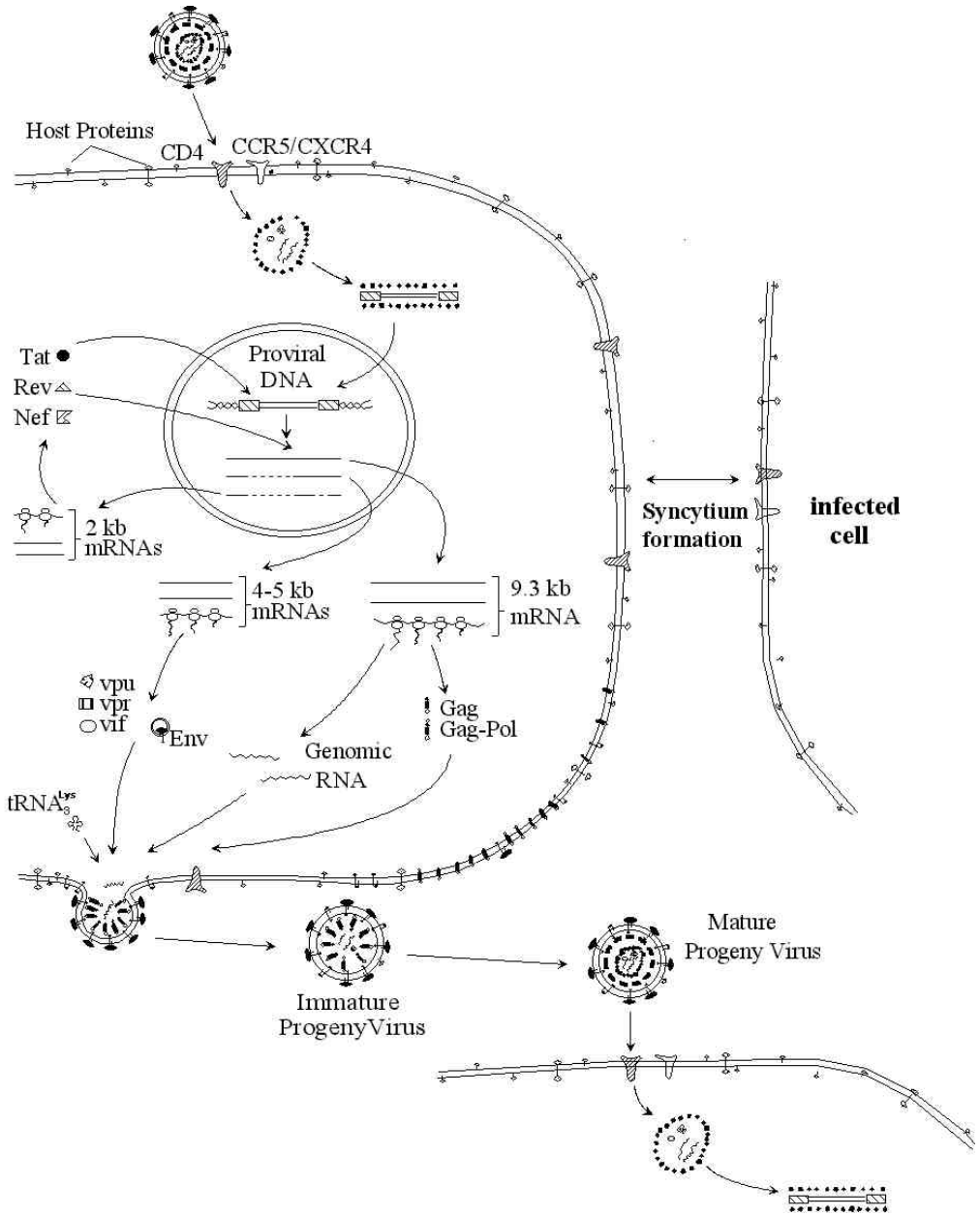

Figure 2. HIV-1 life cycle. Following entry inside the cell, HIV-1 RNA is reverse-transcribed and the provirus DNA is integrated within the cellular genome. Upon transcription, the full length 9.3 kb viral RNA is produced which is differentially spliced to give rise to various HIV-1 mRNAs. The 2 kb RNAs give rise to Tat, which enhances gene expression, and Rev, which allows nuclear export of 4-5 and 9.3 kb HIV-1 RNAs. Translation of these RNAs gives rise to various structural and maturation proteins. Virus assembly then takes place and recruits two copies of full length HIV RNA and cellular $tRNA_3^{Lys}$. Initial steps of the subsequent round of infection are also shown. In addition, an HIV-infected cell is shown as it may lead to syncytium formation, resulting in the death of the gene-modified cell (in the absence of virus replication).

viral protease results in mature, infectious progeny virus (103).

2.2. Interfering RNAs

Interfering RNAs may be designed to inhibit cellular (*i.e.* CCR5) or HIV-1 RNA/protein function (table 1, table 2). The effectiveness of an interfering RNA would depend on its interference site, antiviral potential, and ability to prevent the production of escape mutants. Furthermore, should recombination occur, inclusion of an anti-HIV gene should not be advantageous to the virus.

2.2.1. Antisense RNAs

Antisense RNAs can be designed to contain

Table 1. Interfering RNA-based strategies in HIV gene therapy.

Interfering RNAs used in HIV gene therapy		Target RNA/protein	Localization required for activity	Interference site(s)	Fate of HIV-infected gene-modified cell
Antisense RNA		Cellular CCR5 mRNA	Nuclear/Cytoplasmic	Viral entry, syncytium formation with infected cells	Protected
		HIV mRNAs	Nuclear/Cytoplasmic	RNA splicing, translation, trans-activation, nuclear export	Protected
		HIV progeny virus RNA	Cytoplasmic/Virion	RNA packaging, reverse transcription of progeny virus RNA	Subsequent rounds of infection
Sense RNA	U3-R-U5 RNA	Incoming virion RNA	Cytoplasmic	Virion RNA reverse transcription	Protected
	TAR/RRE RNA	HIV Tat/Rev proteins	Nuclear	Trans-activation/nuclear export of 4-5 kb and 9.3 kb HIV mRNAs	Protected
	Psi-e RNA	HIV Progeny virus RNA	Cytoplasmic/Virion	RNA packaging, reverse transcription of progeny virus RNA	Subsequent rounds of infection
Ribozymes		Cellular CCR5 mRNA	Nuclear/Cytoplasmic	Viral entry, syncytium formation with infected cells	Protected
		Incoming HIV virion RNA	Cytoplasmic	Reverse transcription	Protected
		HIV mRNAs	Nuclear/Cytoplasmic	Translation	Protected
		HIV progeny virus RNA	Virion	RNA packaging, reverse transcription of progeny virus RNA	Subsequent rounds of infection

Table 2. Steps within the HIV-1 life cycle that may be inhibited using various interfering RNA-based strategies.

			Syncytia formation	A										B
Target RNA/ protein	Interfering RNAs		Syncytia formation	Viral Entry	Virion RNA	RT	HIV mRNAs	Splicing	Trans-lation	Trans-activation	Nuclear export	RNA packaging	Progeny virus RNA	RT
CCR5 mRNA/ protein	Antisense RNAs		+	+										
	Ribozymes		+	+										
HIV mRNAs/ proteins	Antisense RNAs							+	+	+	+	+		+
	Sense RNAs	U3-R-U5*				+								
		TAR								+				
		RRE									+			
		Psi-e										+		+
	Ribo-zymes	Hairpin*			+		+						+	
		Hammer-head					+						+	

*: Expressed as part of a tRNA., A: 1st round of infection, B: 2nd round of infection, RT: Reverse transcription

sequences complementary to portions of cellular (*i.e.* CCR5 or CXCR4) or HIV-1 RNA. The RNA hybrids may then be cleaved by RNase 1 (104), which would result in a permanent loss of the target RNA. Antisense RNAs spanning 800 nucleotides or more were shown to inhibit HIV replication more effectively (105-108). As antisense RNAs are not likely to be toxic to the cells, they may be expressed in a constitutive manner.

Antisense RNAs could, upon hybridization with HIV RNA, disrupt viral RNA splicing, translation, *trans*-activation, nuclear export of all HIV mRNAs, RNA packaging, and/or reverse transcription of the progeny virus RNA. Lack of protein production would also result in inhibition of protein function. Inhibition of CCR5 mRNA translation would result in inhibition of viral entry and syncytium formation.

2.2.2. Sense RNAs

Sense RNAs are designed to contain HIV-1 RNA sequences which are involved in specific viral RNA/RNA or RNA/protein interactions. These RNAs act *via* competition with the HIV RNA for binding to viral RNAs or proteins. Sense RNAs may be used to prevent *trans*-activation, nuclear export, packaging, or reverse transcription of the progeny virion RNA.

Sense RNAs to HIV TAR and RRE act as decoys. Binding of these RNAs to the corresponding Tat and Rev proteins is expected to decrease the effective concentration of these proteins. And, as Tat/HIV-1 TAR and Rev/HIV-1 RRE interactions are required for *trans*-activation and nuclear export, virus replication would be inhibited.

Sense RNAs containing HIV-1 Psi signal may form dimers with HIV RNA, which would compete with HIV-1 RNA dimers for packaging into the virions. Furthermore, depending on the presence or absence of various *cis*-acting elements required for HIV-1 RNA reverse transcription, the co-packaged sense RNA may either compete with HIV RNA for reverse transcription or prevent both sense and HIV RNA reverse transcription.

Several cellular factors have been characterized, which interact with HIV TAR and RRE. Thus, in addition to inhibiting Tat or Rev function, the decoy RNAs would also inhibit the normal function of these cellular factors and may cause toxicity. Therefore, TAR and RRE may have to be produced in a Tat-inducible manner. However, Tat-inducible expression of a molecule that inhibits Tat function may not be ideal, as the amount of TAR produced in the cell may not reach excess concentration required to inhibit virus replication. To solve this problem, minimal TAR and RRE decoys (lacking the binding sites for cellular factors) are being developed that could be constitutively expressed without being cytotoxic. Sense RNAs containing HIV Psi-e (which includes RRE) may be produced in a Tat or Rev inducible manner.

2.2.3. Ribozymes

Hammerhead and hairpin ribozymes are small catalytic RNAs which can be designed to specifically pair with and cleave a specific target RNA *in trans* (15). The following criteria must be fulfilled for designing a specific hammerhead ribozyme (figure 3) (109-111). The cleavage site within the target RNA must contain an NUH (N, any nucleotide; H, C/U/A) (112). The ribozyme catalytic domain must contain 11 of the 13 conserved nucleotides (113), and

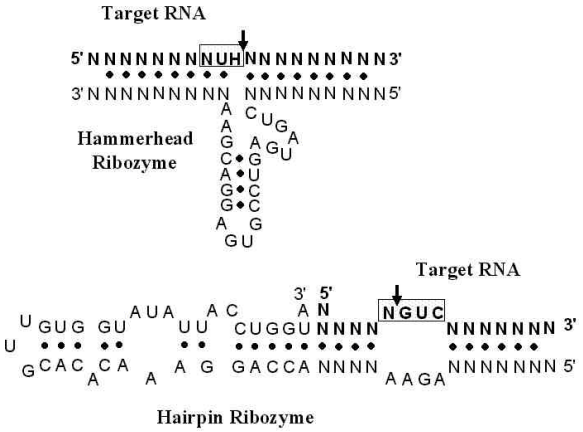

Figure 3. Secondary structure of a *trans*-cleaving hammerhead (top) and a hairpin (bottom) ribozyme. The catalytic domain is flanked by the 5' and 3' flanking complementary sequences, which are designed to be complementary to the H (hammerhead ribozymes) or NGUC (hairpin ribozyme) adjacent to the cleavage site (↓). Target RNA sequences are shown in bold. Cleavage occurs 3' to the NUH (hammerhead ribozymes) or 5' to the GUC (hairpin ribozyme). N, any nucleotide; H, C/U/A.

must be flanked by nucleotides complementary to either side of the H adjacent to the cleavage site (109, 113). Cleavage by hammerhead ribozymes occurs 3' to the H and results in a 5' product with a 2', 3' cyclic phosphate and a 3' product with a 5' hydroxyl group.

Hairpin ribozymes have been derived from the tobacco ringspot satellite virus RNA (114). The conserved nucleotides within the ribozyme catalytic domain are shown in figure 3. The substrate specificity is conferred by providing the ribozyme with nucleotides complementary to the sequences flanking the NGUC adjacent to the cleavage site within the target RNA. Cleavage occurs 5' to the GUC sequence.

Ribozymes may be designed to specifically recognize and cleave a number of sites within a specific cellular RNA (*i.e.* CCR5 mRNA) or HIV RNA. The most important criteria in designing an HIV RNA-specific ribozyme is to chose a target site that is accessible and highly conserved. Ribozymes may be designed to cleave the incoming HIV virion RNA in the cytoplasm before reverse transcription occurs, the HIV transcripts in the nucleus or cytoplasm, and/or the virion RNA in the progeny virus. The incoming RNA in the cytoplasm or the primary HIV-1 transcripts within the nucleus may be targeted anywhere within the HIV-1 RNA. However, if the cleavage occurs post-splicing within the nucleus or in the cytoplasm, it may be preferable to target regions that are shared by all spliced and unspliced HIV mRNAs. These regions include the first 289 nucleotides within the 5' untranslated region (exon 1), 69 nucleotides near the center (exon 5), and the last 1259 nucleotides near the 3' end (exon 7) of HIV-1 RNA (figure 1). While CCR5 ribozymes would have to be expressed in a constitutive manner, anti-HIV ribozymes may be expressed in a constitutive or constitutive and Tat-inducible manner (to allow overproduction in HIV-infected cells).

2.3. Steps within the viral life cycle that may be blocked using interfering RNA-based strategies

Interfering RNA-based strategies may be used to block the virus life cycle at a number of different steps. These RNAs may be designed to inactivate either the HIV RNA or a cellular mRNA (*i.e.* CCR5 mRNA) encoding a protein required for viral infection or replication. Interfering RNAs can also be designed to inhibit the function of viral proteins (*i.e.* Tat and Rev). The key considerations for designing interfering RNA-based strategies are (i) whether viral entry can be inhibited; (ii) whether reverse transcription can be inhibited, before provirus DNA integration takes place; (iii) whether post-integration steps can be inhibited in the gene-modified cells that cannot prevent provirus DNA integration (as well as in the chronically infected cells that are subject to gene transfer); and (iv) whether viral progeny can be rendered inactive by preventing viral RNA packaging and/or virion RNA reverse transcription during the subsequent round of infection.

Inhibition of virus replication prior to provirus DNA integration should be preferred, as this would avoid the constant battle between HIV willing to replicate and the interfering RNA trying to stop it. All of these strategies are aimed at conferring resistance to gene-modified cells that may be challenged by HIV. However, gene-modified cells may also die because of syncytia formation with HIV-infected cells. Thus, for the success of HIV gene therapy, it is equally important to design strategies that protect the gene-modified cells from the cytopathic effects that can occur in the absence of HIV infection or replication.

Furthermore, for gene-modified cells to maintain their immune functions, virus replication should be blocked as soon as possible. Intervention at a post-integration step may still allow the cell to retain its normal cell function, provided that it occurs at a stage within the virus life cycle where viral gene products are not harmful to the cell. However, inhibition of subsequent rounds of infection would not confer a selective advantage to the gene-modified cells that become infected.

2.3.1. Intervention at the level of viral entry

Viral entry may be blocked by designing antisense RNAs or ribozymes that inhibit coreceptor (*i.e.* CCR5, CXCR4) mRNA translation. This strategy is of particular interest, as it would also protect the gene-modified cells from being killed as a result of syncytium formation with HIV infected cells.

2.3.2. Intervention at a pre-integration step

Incoming HIV-1 virion RNA reverse transcription might be blocked using antisense RNA or ribozymes. As HIV RNA reverse transcription takes place in partially uncoated virions, strategies must be designed to allow the interfering RNA to access the incoming HIV virion RNA, before it is reverse transcribed. This is

particularly important for blocking replication of the incoming HIV virion RNA and should not be confused with reverse transcription of the progeny virus released from the gene-modified cells, which can easily be blocked by allowing the interfering RNA to be co-packaged in the virion (see below).

2.3.3. Intervention at a post-integration step

2.3.3.1. Interference with *trans*-activation of HIV gene expression

Tat/TAR interaction is required for *trans*-activation of HIV-1 gene expression. Antisense RNAs or ribozymes could be designed against the TAR and/or the *tat* coding region to inhibit *trans*-activation. Alternatively, TAR decoy RNAs may be developed to block Tat protein function.

2.3.3.2. Interference with nuclear export of singly spliced and unspliced HIV mRNAs

Rev-RRE interaction is required for nuclear export of singly-spliced and unspliced viral RNAs. Antisense RNAs and ribozymes could be designed against the RRE sequence present in these viral RNAs. Alternatively, antisense RNAs or ribozymes may be designed against the *rev* coding region to inhibit Rev function. RRE decoys may also be developed to block Rev protein function.

2.3.3.3. Interference with HIV RNA translation

Viral mRNA translation could be inhibited by designing antisense RNA and/or ribozymes against the exon 1 sequences located within the 5' untranslated region of all HIV mRNAs. Thus, translation of all (2, 4-5, and 9.3 kb) HIV mRNAs would be inhibited. Alternatively, antisense RNAs and/or ribozymes may be designed against a specific coding region to inhibit translation of a particular HIV-1 mRNA.

2.3.4. Intervention at the level of infectious progeny virus production

Several viral proteins including Pr55,^{Gag} Pr160,$^{Gag\text{-}Pol}$ Env, Vpu, and Vif are required for the assembly, release, maturation, and infectivity of virus particles. Therefore, antisense RNAs and ribozymes directed against these coding regions could interfere with the assembly and release of infectious virus particles by hybridizing to and/or cleaving the respective viral mRNAs. Sense and antisense RNAs may be designed to compete with and/or interfere with HIV RNA for packaging and reverse transcription. These interfering RNAs may also be used to allow copackaging of ribozymes that would cleave the virion RNA, rendering the progeny virus non-infectious. In addition, $tRNA_3^{Lys}$ may be used to develop packageable sense RNA, antisense RNA, and ribozymes.

2.4. Delivery and testing of anti-HIV genes expressing interfering RNAs

2.4.1. Delivery

Retroviral vectors based on the Moloney murine leukemia virus (MoMuLV) have been extensively used to introduce genes into primary hematopoietic and mature lymphoid cells with relatively high efficiencies (35, 115). Recently, Mouse stem cell virus (MSCV)-based retroviral vectors were also developed (116, 117). Since hematopoietic stem cells are quiescent (118), gene transfer into pluripotent hematopoietic stem cells *via* a retroviral vector requires that the stem cell be induced to divide in the absence of differentiation, which is difficult to achieve. Defective, non-pathogenic lentivirus (*i.e.* HIV-1) based vectors were therefore developed to allow gene delivery into non-dividing cells (119, 120). HIV-based vectors were shown to transduce activated CD34+ cells in G_0/G_1 phase (121). As the receptors for VSV-G and gibbon ape leukemia virus (GALV) Env are widespread, pseudotyping with VSV-G (122, 123) and GALV-Env (124) was shown to further increase gene transfer efficiency (125). Markers such as enhanced green fluorescence protein (*EGFP*) have been expressed to allow rapid selection of transduced cells as well as to facilitate *in vitro* and *in vivo* tracking of the progeny of transduced cells (116, 117).

Retroviral vectors are produced by replacing the viral genes with the therapeutic gene(s) and with a gene encoding a selectable marker to allow *in vitro* selection of transduced cells. Several *cis*-acting elements are required for retroviral vector gene expression (5' and 3' LTRs), vector RNA encapsidation into the progeny virus (Psi signal), vector RNA reverse transcription in the transduced cells (PBS, PPT, and R repeats), and proviral vector DNA integration into the target cell genome (5' and 3' ends of the 5' and 3' LTRs, respectively). These elements are retained in these vectors. The *gag*, *pol* and *env* genes are deleted and are instead supplied *in trans* from helper plasmids. The retroviral vector and the helper plasmids are co-transfected into a cell line to generate retroviral vector particles that can then be collected from the cell culture supernatant. The vector particles are only capable of one round of infection and are therefore replication-defective. These particles are then tested for their titre, lack of recombination, and lack of replication-competent virus (126).

Upon infection of the target cells, the vector RNA is reverse transcribed forming the proviral DNA, which then becomes integrated into the target cell genome. Based on the number of times the anti-HIV gene or the expression cassette encoding the anti-HIV gene is present in the proviral DNA, retroviral vectors are called single copy, double copy, or triple copy (figure 4). When the gene (or the expression cassette) is cloned between the 5' and the 3' LTR, its copy number is not affected by reverse transcription. However, when cloned within the U3 region of the 3' LTR, reverse transcription results in its duplication in the 5' LTR. Genes (or expression cassettes) may be cloned in a forward or reverse orientation with respect to the vector orientation. Forward orientation usually results in higher level expression. Note that when an expression cassette is cloned in a reverse orientation, complementary sequences would be present on the vector RNA. Thus unless strategies were used to inactivate the 5' LTR promoter, interfering RNA would be subject to antisense RNA-mediated inhibition.

The retroviral or lentiviral vectors can be designed to allow constitutive and/or Tat-inducible expression of anti-HIV genes. Tat-inducible expression is

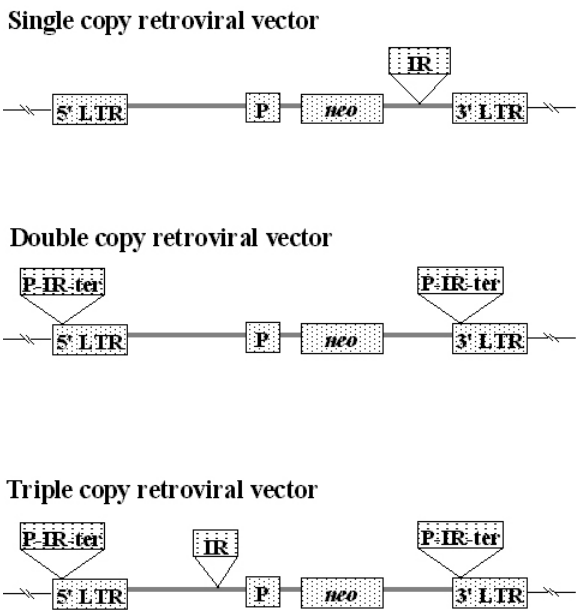

Figure 4. Retroviral vectors. Structure of the provirus DNA resulting from the single copy, double copy, and triple copy retroviral vectors is shown. An internal promoter (P) may be used for expression of a gene (*i.e.* neomycin-phosphotransferase, *neo*) conferring drug resistance. The 5' LTR promoter and the internal promoter P would allow RNA polymerase II-driven expression. In single copy retroviral vectors, the interfering RNA (IR) gene is expressed either under control of the 5' LTR promoter (cloning upstream of the *neo* gene) or under control of both the 5' LTR and the internal promoter (cloning downstream of the *neo* gene, as shown in the figure). An expression cassette containing RNA polymerase III-driven tRNA or Ad VA1 promoter, the IR gene, and a terminator (P-IR-ter) may also be used. These cassettes may be cloned in forward or reverse orientation, before or after the *neo* gene. In double copy vectors, an RNA polymerase III-driven expression cassette is commonly utilized. Cloning is performed upstream of the promoter within the 3' LTR. Thus, upon reverse transcription, the expression cassette is also duplicated in the 5' LTR promoter. In triple copy vectors, in addition to cloning an expression cassette within the 3' LTR, an IR gene is also cloned upstream or downstream of the *neo* gene.

mainly used for multi-copy TAR and RRE decoy RNA production, as sequestering of cellular factors may cause cytotoxicity. Tat-inducible gene expression can be achieved by using the HIV LTR promoter or heterochimeric promoters containing the HIV TAR element (127, 128). MoMuLV (24) and HIV (129) based vectors were designed to allow Tat-inducible expression of therapeutic gene(s). This allows the cell to evade cytotoxic effects in the absence of HIV infection. Most other interfering RNAs are expressed constitutively. In some instances, interfering RNAs may be produced in a constitutive and Tat-inducible manner. Thus, the interfering RNA would be present in the cell at all times, and should HIV infect the cell, it would be overproduced. Heterologous promoters such as herpes simplex virus thymidine kinase (*tk*) (130), cytomegalovirus (CMV) immediate-early (128), and Rouse sarcoma virus (RSV) (131) promoters were fused to the HIV-1 TAR sequence to allow constitutive and Tat-inducible gene expression.

2.4.2. Testing

To determine whether production of the interfering RNA occurs, and if it is sufficient to inhibit HIV replication, retroviral vector particles can first be used to transduce a human CD4+ T cell line.

T cells are transduced with amphotropic or VSV-G pseudotyped retroviral vectors. Stable transductants are isolated. The presence of anti-HIV gene(s) is confirmed, and the level of expression of interfering RNA(s) is determined. Viability of cells is observed to determine whether transgene expression has any toxic effects. Initial HIV challenge experiments are then performed using a laboratory strain of HIV-1. Virus replication is measured by a number of techniques, including HIV-1 p24 antigen production and RT activity.

If partial inhibition is observed, this may be due to (i) isolation of cells that only allowed transient expression of the transgene, (ii) cell-to-cell variability in anti-HIV gene expression, (iii) inability of interfering RNAs to confer complete protection, and (iv) escape virus production. For anti-HIV genes conferring complete inhibition of HIV replication, the interference site(s) within the virus life cycle may be elucidated. This would demonstrate how early the virus life cycle could be inhibited. Further analyses may be performed to determine the mechanism underlying resistance and to determine if any escape mutants have evolved. Next, challenge experiments may be performed using clinical isolates of HIV-1, including various subtypes and drug resistant isolates of HIV. If promising results are obtained, co-challenge experiments may be performed by simultaneously exposing cells to the different HIV-1 subtypes. As various subtypes may co-exist in the patients, this would determine if recombination between subtypes can result in an escape virus. These experiments would rank the different interfering RNAs based on the degree of resistance conferred and the absence of escape virus production.

Next, the efficacy of interfering RNA approaches may be tested in transduced PBLs from healthy or HIV-infected individuals. HIV resistance may then be tested in myeloid and lymphoid progeny cells of transduced CD34+ hematopoietic stem/progenitor cells. These experiments would reveal whether anti-HIV gene expression is maintained during differentiation, and whether it is sufficient to confer HIV resistance to both myeloid and lymphoid progeny cells. If resistance is observed, this would confirm the feasibility of an interfering RNA approach for HIV gene therapy using CD34+ cells. Retroviral vectors expressing the best of the interfering RNA molecules could then proceed to a clinical trial.

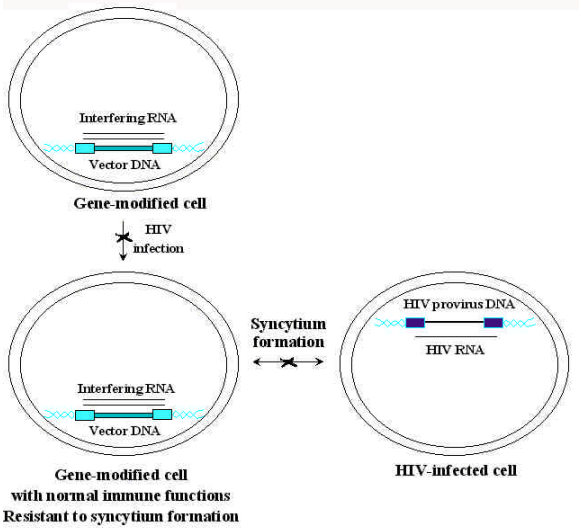

Figure 5A. Strategy to block viral entry. This strategy would result in inhibition of virus infection and syncytium formation. In the absence of viral DNA, RNA or protein, the cell is likely to retain its immune functions.

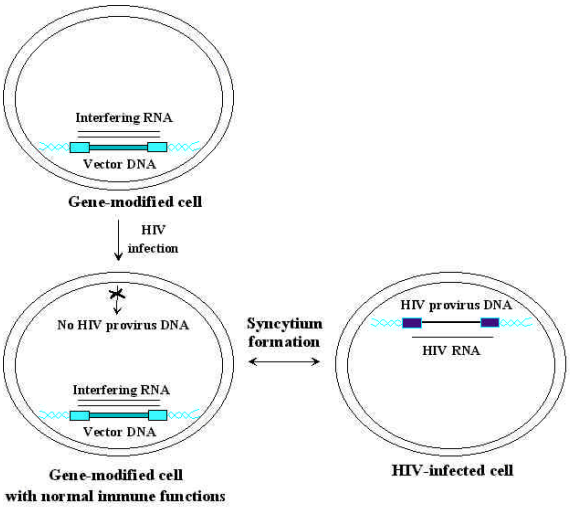

Figure 5B. Strategy to block reverse transcription of the incoming virion RNA. This strategy would result in inhibition of virion RNA reverse transcription. As no viral DNA, RNA or protein will be produced, the cell is likely to retain its immune functions. However, it will be subject to syncytium formation with other HIV-infected cells.

3. INTERFERING RNA-BASED STRATEGIES USED IN HIV GENE THERAPY

3.1. Strategies to block viral entry

3.1.1. Ribozymes

CCR5 serves as a coreceptor for HIV-1 infection in monocytes/macrophages. Downregulation of CCR5 expression is believed to inhibit HIV-1 replication and to prevent the death of gene-modified cells due to the cytopathic effects caused by the untransduced HIV-infected cells (figure 5A). As individuals with a mutation in this gene are known to lead a normal life, down-regulation of CCR5 coreceptor should not alter normal cell functions. Ribozymes have therefore been developed against the mRNA encoding the CCR5 coreceptor (132).

A hammerhead ribozyme was developed and was shown to cleave CCR5 mRNA in vitro (132). In another study, a plasmid was designed to express a hammerhead ribozyme targeted against a different site within the CCR5 mRNA (133). In transient co-transfection experiments, this ribozyme was shown to reduce CCR5 expression by up to 60% (133). No results were reported for inhibition of HIV-1 replication.

We have developed a retroviral vector expressing a multimeric ribozyme targeted against seven different sites within the CCR5 mRNA. Sites that are unique to CCR5 were chosen to avoid cytotoxicity. All individual ribozymes were shown to cleave their respective target sites in vitro (134). This vector is currently being tested in stable transductants for CCR5 coreceptor downregulation, inhibition of replication of an M-tropic HIV at the level of viral entry, and inhibition of syncytium formation.

3.2. Strategies to block incoming virion RNA reverse transcription

Sense RNA- and ribozyme-based strategies have been designed to block HIV-1 reverse transcription (figure 5B). Indirect evidence suggests that the ribozymes described below can cleave the incoming virion RNA, before it is reverse transcribed. In case the virion RNA cleavage is missed, these ribozymes might also be able to inhibit virus replication at a post-integration step. However, as the results obtained with these ribozymes are discussed here, they will not be repeated later.

3.2.1. Sense RNAs

A sense RNA-based strategy was designed to inhibit incoming virion RNA reverse transcription. Double copy retroviral vectors were designed to express $tRNA_i^{Met}$-R-U5 (201-nts) or $tRNA_i^{Met}$-U3-R-U5 (684-nts) RNAs. Stable transductants expressing the $tRNA_i^{Met}$-U3-R-U5 RNA inhibited HIV replication until 20 weeks post-infection (135). Virus replication was not inhibited in cells expressing the $tRNA_i^{Met}$-R-U5 RNA. The $tRNA_i^{Met}$-U3-R-U5 RNA lacks the PBS, and therefore, it would not serve as a template during reverse transcription. However, it may lead to abortive virion RNA reverse transcription by competing with HIV RNA during the first strand switching reaction. In contrast, $tRNA_i^{Met}$-R-U5 seems to have facilitated reverse transcription by serving as a primer during positive strand DNA synthesis.

3.2.2. Ribozymes

A plasmid expressing a hammerhead ribozyme targeted against the HIV-1 *gag* coding region was shown to inhibit HIV replication (>97%) in stable transductants until day 7 post-infection (136). Ribozyme-expressing cells were shown to contain significantly less HIV RNA. The cells also contained up to 100 times less HIV-1 provirus DNA than the infected untransduced control cells, suggesting that the incoming virion RNA may have been cleaved before reverse transcription was completed.

A hairpin ribozyme was designed to cleave a conserved site within the U5 region of HIV-1 RNA (137).

A plasmid expressing this ribozyme was shown to suppress virus replication in transient co-transfection experiments (137). Several single copy retroviral vectors were also designed to express this ribozyme under control of the MoMuLV 5' LTR promoter or both the 5' LTR promoter and an internal beta-actin promoter (138). Other single copy retroviral vectors were also designed. In these vectors, a $tRNA^{Val}$-U5 ribozyme or an Ad VA1-U5 ribozyme expression cassette was cloned in a reverse orientation with respect to the vector. In transient co-transfection experiments using the retroviral vector constructs, the best results were obtained using cells expressing the $tRNA^{Val}$-U5 ribozyme (138). This vector was then shown to inhibit HIV-1 replication in stable transductants until day 35 post-infection (139). Proviral DNA synthesis in ribozyme-expressing cells was decreased 50-100 fold compared to control cells, suggesting incoming virion RNA cleavage (139). Transduced human PBLs were also shown to be protected for 10-25 days against challenge with a clinical isolate of HIV-1 (139, 140). Retroviral vectors expressing $tRNA^{Val}$-U5 ribozyme and Ad VA1-U5 ribozyme were also tested in the progeny of transduced CD34+ cells obtained from the human fetal cord blood (141). Differentiated macrophage-like progeny cells were challenged with an M-tropic HIV-1. VA1-U5 ribozyme inhibited HIV-1 replication slightly better (>95% inhibition for 10 days post-infection), compared to $tRNA^{Val}$-U5 ribozyme (>90% inhibition for 6 days post-infection) (141). The retroviral vector expressing the $tRNA^{Val}$-U5 ribozyme was also tested in the progeny of transduced umbilical cord blood CD34+ cells from HIV-exposed infants (142). Differentiated macrophage-like progeny cells, challenged with an M-tropic HIV-1, were protected until day 20-35 post infection (142). An improved retroviral vector expressing this $tRNA^{Val}$-U5 ribozyme is currently being evaluated in a clinical trial (143, 144).

A single copy retroviral vector was also designed to allow Ad VA1 promoter-driven expression of a hairpin ribozyme with an improved catalytic activity. This ribozyme was targeted against the HIV-1 *pol* coding region. In stable transductants, this ribozyme inhibited HIV-1 replication until day 25 post-infection (145). Inhibition of HIV-1 replication with this ribozyme was slightly better (145) than with the $tRNA^{Val}$-U5 ribozyme (139).

A $tRNA^{Val}$-hairpin ribozyme was also designed against a site within the 3' untranslated region that is conserved within the SIVmac and HIV-2 RNAs. A single copy retroviral vector expressing this ribozyme was shown to inhibit SIVmac239 (an SIV strain that replicates in T cells) and HIV-2 replication in stable transductants until day 43 post-infection (146). A significant decrease in the provirus DNA synthesis was noted at day 2 post-infection in the ribozyme-expressing cells, suggesting incoming virion RNA cleavage. This vector was also tested in the lymphoid and myeloid progeny of transduced rhesus CD34+ cells. Differentiated CD4+ T lymphoid progeny cells were resistant to SIVmac239 infection until day 30 post-infection, and macrophage-like progeny cells were resistant to SIVmac316 (an SIV strain which replicates in macrophages) infection until day 20 post-infection (147).

3.2.3. Combined interfering RNAs

3.2.3.1. Ribozymes combined with a sense RNA

A single copy retroviral vector was designed to co-express 66-nt RRE stem loop II (SLII, which contains the Rev binding site) and the U5 hairpin ribozyme. Another vector was designed to co-express RRE SLII and a hairpin ribozyme targeted against the rev coding region of HIV-1 RNA. The $tRNA^{Val}$-RRE-SLII-ribozymes were found to be more efficient at inhibiting HIV replication than either one of the two ribozymes alone (148). Seven hours post-infection, the amount of HIV provirus DNA in cells expressing the $tRNA^{Val}$-RRE-SLII-U5 ribozyme was 14 and 33% of the provirus DNA in transduced cells expressing the $tRNA^{Val}$-RRE-SLII-inactive U5 ribozyme or the $tRNA^{Val}$-U5 ribozyme, respectively (148). Thus, the $tRNA^{Val}$-RRE-SLII-U5 ribozyme seems to cleave the incoming virion RNA better than RRESLII or the U5 ribozyme alone.

A retroviral vector expressing a $tRNA^{Val}$-hairpin ribozyme against an HIV-1 sequence overlapping the rev/env coding region was also designed (149). This ribozyme was shown to inhibit (>99%) virus production until day 36 post-infection (149).

A double copy retroviral vector was then designed to express $tRNA^{Val}$-RRE-SLII-U5 ribozyme (150). A triple copy retroviral vector was also designed, which contained the $tRNA^{Val}$-RRE-SLII-U5 ribozyme expression cassette in the 3' LTR and the $tRNA^{Val}$-RRE-SLII-rev/env ribozyme expression cassette between the two LTRs (150). The ribozyme transcripts produced in cells transduced with the single copy vector expressing $tRNA^{Val}$-RRE-SLII-U5 ribozyme, double copy vector expressing $tRNA^{Val}$-RRE-SLII-U5 ribozyme, or triple copy vector expressing $tRNA^{Val}$-RRE-SLII-U5 and $tRNA^{Val}$-RRE-SLII-rev/env ribozymes were $1.5x10^3$, $1.2x10^4$, and $1.2x10^5$, respectively (150). Also, the triple copy vector conferred the best protection and inhibited virus replication in stable transductants against challenge with HIV-1 from 5 clades (A, B, C, D and E) until day 27 post-infection (150). The double copy vector conferred the next best protection, followed by the single copy vector. The activity of this triple copy vector was also demonstrated in monocyte/macrophage-like cells derived from transduced CD34+ cells (151). Infection of these progeny cells with an M-tropic HIV-1 resulted in decreased virus production (70-95% inhibition) until day 28 post-infection (151).

3.3. Strategies to block post-integration steps

Antisense RNA, sense RNA, and ribozyme-based strategies have been developed to inhibit virus replication post-integration (**figure 5C**).

3.3.1. Antisense RNAs

Antisense RNAs may be designed to inhibit a number of sites within the HIV-1 life cycle. Several antisense RNAs targeted against both coding and non-coding regions of HIV-1 RNA were shown to inhibit virus replication.

A non-retroviral vector was used to express several antisense RNAs to the 5' untranslated region (180-nts), 5' untranslated region-*gag* coding region (406-nts), *gag*/*pol*

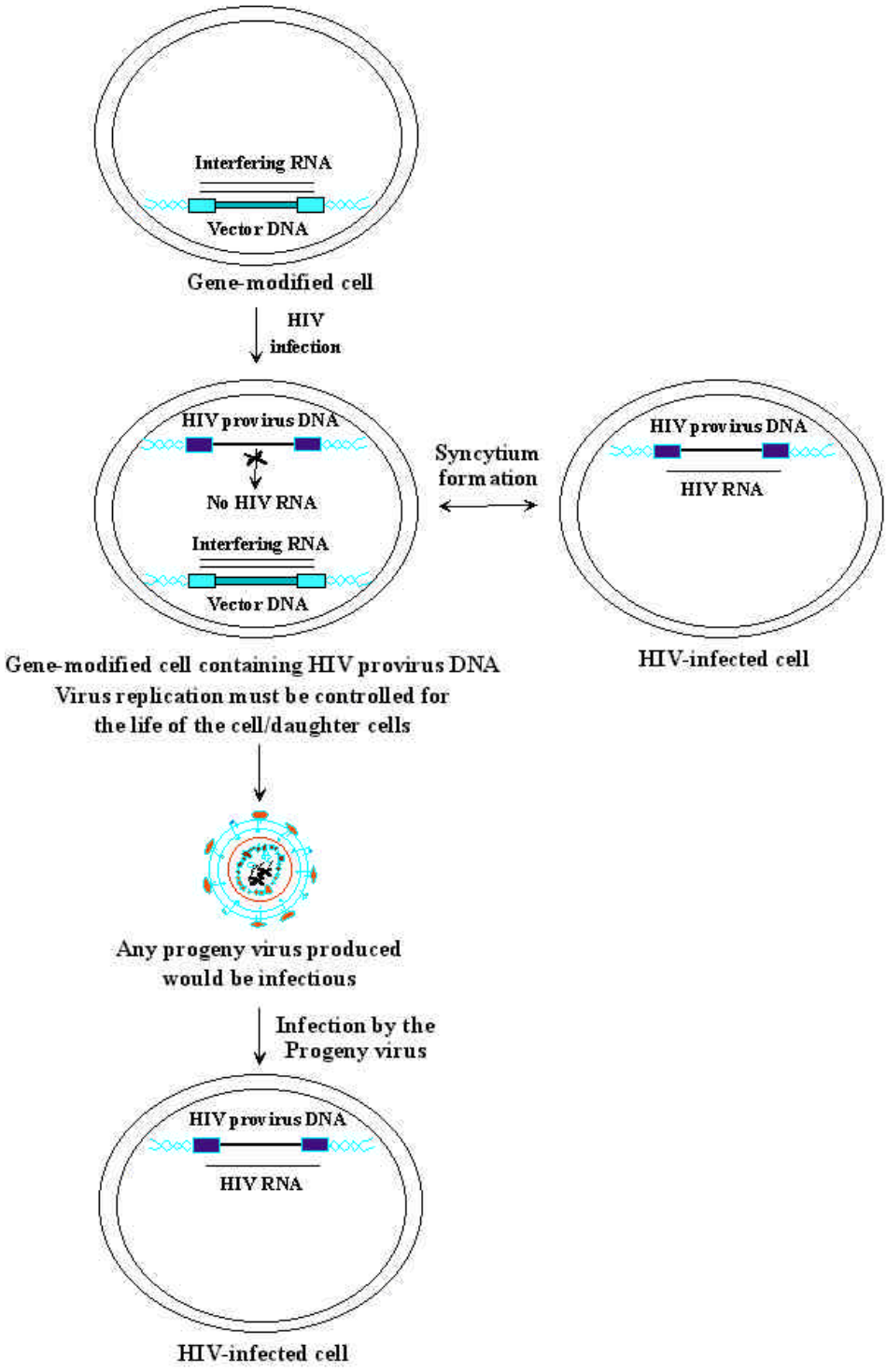

Figure 5C. Strategy to block post-integration steps in the viral life cycle. Since HIV-1 provirus DNA integration is allowed, viral RNAs and proteins will be produced. In order to protect these cells, viral RNAs and proteins must be inactivated (or their function must be inhibited) for as long as the cell or daughter cells live. Maintenance of immune functions would depend on the interference site used (early *vs* late) as well as on the level of inhibition achieved. If inhibition is not complete, infectious HIV progeny virus may be produced that will infect other cells.

frameshift site (91-nts), and the *gag* (735-nts, 1000-nts), *vif*/*vpr* (662-nts), *tat*/*rev*/*vpr* (562-nts), *vpu*/*env*/*tat*/*rev* (3109-nts), *env* (521-nts), *tat*/*rev* (330-nts), and *nef* (120-nts) coding regions (152) These vectors were tested in stably transfected T cell clones (153). The best results were obtained using the 562-nt *tat*/*rev*/*vpr* antisense RNA, followed by the 1000-nt *gag* antisense RNA, the 406-nt 5'untranslated region-*gag* antisense RNA, and then the 3109-nt *env* antisense RNA. Compared to controls, inhibition of HIV replication by these antisense RNAs was >90%, 69%, 65%, and 60%, respectively (153). The 562-nt *tat*/*rev*/*vpr* antisense RNA was further shown to inhibit virus replication for 5 weeks (154). A non-retroviral vector expressing the 406-nt 5' untranslated region-*gag* antisense RNA was also shown to inhibit virus replication in stably transduced T cell clones until day 21 post-infection (155).

A high copy number plasmid was also designed to express a 1236-nt antisense RNA against the *RT* coding region within the HIV-1 RNA (156). In transduced T cell clones, over 98% inhibition of HIV-1 replication was observed until day 28 post-infection.

Retroviral vectors were designed to express antisense RNAs to the PBS (18-nts), to the U5 region (18-nts) immediately upstream of the PBS, and to the 5' untranslated region of HIV-1 *tat* mRNA (343-nts) of which 289 nucleotides are common to all HIV-1 mRNAs (157). The two 18 nt-long antisense RNAs were expressed as part of a loop structure. Cells expressing these ribozymes were shown to delay virus replication by 4 days compared to the control. Antisense RNA (526-nts) to HIV-1 *env* coding region encompassing the RRE was also expressed from this vector (105). However, compared to control cells virus replication in cells expressing this antisense RNA was only delayed by 2-3 days.

Retroviral vectors expressing antisense RNAs to HIV-1 R-U5-PBS region (187-nts), and *vpr* (14-nts), *vpr*/*tat* (71-nts, 114-nts), *tat*/*rev* (145-nts), and *vpr*/*tat*/*rev*/*vpu*/*env* (602-nts) coding regions were also designed (158). Best results were obtained using the 602-nt antisense RNA spanning multiple coding regions, which conferred up to 70% inhibition of HIV replication for 10-12 days. This antisense RNA was also shown to confer protection against several strains of HIV-1 (159).

A retroviral vector was also designed to express a 550-nt antisense RNA to HIV-1 5' untranslated region-*gag* coding region, and was shown to inhibit HIV-1 replication in transduced PBLs for 8-12 days post-infection (160).

We have developed a retroviral vector expressing a 1.44 kb antisense RNA to the HIV-1 Psi-*gag* coding region (105). This vector was shown to confer significant inhibition of HIV replication in stably transduced cells as no virus could be detected until day 30 post-infection (105). Analysis of the little progeny virus produced in some of the experiments revealed that the antisense RNA was co-packaged with HIV RNA (108). The infectivity of the progeny virus was also shown to be significantly reduced (108). Similar results were obtained using a 5 kb antisense RNA spanning the U3-5'untranslated region-*gag*-*env* coding regions of HIV-1 RNA (108). This antisense RNA was shown to inhibit HIV replication until day 78 post-infection. It was also shown to be co-packaged with HIV-1 RNA into progeny virus, which was shown to be less infectious (108). Furthermore, both antisense RNAs (1.44 kb Psi-*gag* and 5 kb U3-5' untranslated region-*gag*-*env*) were shown to inhibit HIV replication in transduced PBLs for 21 days post-infection (108). These results demonstrate for the first time that antisense RNAs can also be used to inhibit HIV replication at the level of virion RNA encapsidation and progeny virion RNA reverse transcription. We are currently investigating the mechanism of antisense RNA packaging within the virus particles.

Based on the results obtained with the Psi-*gag* antisense RNA (105), and after comparing it to several other available interfering RNA- and protein-based strategies, Systemix (California) has developed many other 225-1425 nt-long antisense RNAs targeted against various sites within the Psi-*gag* coding region of HIV-1 RNA (106). All of these antisense RNAs were expressed using a retroviral vector. The best inhibition of HIV replication was obtained by the Psi-*gag* antisense RNA, reinforcing previous results (105). Virus replication was also shown to be inhibited in transduced PBLs expressing the Psi-*gag* antisense RNA (106). Several other similar-length antisense RNAs were then developed and compared with each other (107). These antisense RNAs were targeted against the *gag* (1.4 kb), *pol* (1.4 kb, 1.2 kb), *vif* (1.1 kb), *env* (1.4 kb), and *env*-*nef*-3'LTR (1.2 kb) coding/non coding regions (107). Retroviral vectors expressing these antisense RNAs were tested in stable transductants. HIV-1 replication was best inhibited by the *env* antisense RNA, followed by the two *pol* antisense RNAs, *gag* antisense RNA, and then the *vif* antisense RNA. A 5, 3, 2, 1 and 0 $\log_{10}$ reduction was observed at day 12 post-infection in transduced cells expressing the *env*, *pol*, *gag*, *vif*, and *env*-*vif*-3' LTR antisense RNAs, respectively. In the *env*, *pol*, and *vif* antisense RNA-expressing cells, the Tat protein production was also shown to be decreased (107). As the antisense RNAs were not targeted against this coding region, they must have acted at a pre-splicing step (107). The *env*, *pol*, *vif*, and *gag* antisense RNAs were shown to inhibit HIV replication much better than the Rev TDM (161, 162), which is being investigated in several clinical trials (39, 144). The antisense Psi-*gag* RNA was also tested in conjunction with the anti-HIV RT drug (AZT) (163). Compared to the amount of HIV produced in the presence of AZT from the control vector-transduced cells, virus production at day 16 post-infection in Psi-*gag* antisense RNA-expressing cells in the presence or absence of AZT was shown to be inhibited by 99% and 65%, respectively (163). The Psi-*gag* antisense RNA was also shown to inhibit replication of HIV isolates resistant to AZT or to AZT, ddI, and non-nucleoside RT inhibitors (163). These results demonstrate the feasibility of a combined gene–drug therapy approach.

Several single and double copy retroviral vectors were also designed to express antisense RNAs targeted against the HIV-1 *tat* coding region (~300-nts), *rev* coding region (~350-nts), 5' untranslated region-*tat* coding region

(~590-nts), and the 5' untranslated region-*rev* coding region (~640-nts) (164). As *tat* and *rev* coding regions overlap with each other, these antisense RNAs are targeted against *tat* and *rev* coding regions. Both *tat* and *rev* antisense RNAs were expressed from double copy retroviral vectors under control of HIV-1 LTR promoter or the $tRNA_i^{Met}$ promoter. The 5' untranslated region-*tat* coding region and 5' untranslated region-*rev* coding region specific antisense RNAs were expressed from a double copy retroviral vector under control of $tRNA_i^{Met}$ promoter, from a single copy retroviral vector under control of Moloney murine sarcoma virus (MSV) 5' LTR promoter, and from a single copy retroviral vector under control of $tRNA_i^{Met}$ and MSV 5' LTR promoters (164). Antisense RNA expression could not be demonstrated for many of the double copy vectors. However, all double copy retroviral vectors expressing either one of the two antisense RNAs inhibited HIV-1 replication in stably transduced cells for 20 weeks post-infection. The single copy retroviral vector expressing 5' untranslated region-*tat* antisense RNA under control of MSV 5' LTR promoter inhibited virus replication for 5 weeks, whereas no inhibition was observed for the single copy retroviral vector expressing this antisense RNA under control of MSV 5' LTR and $tRNA_i^{Met}$ promoters. The single copy retroviral vectors expressing 5' untranslated region-*rev* antisense RNA under control of MSV 5' LTR promoter or MSV 5' LTR and $tRNA_i^{Met}$ promoters inhibited virus replication for 20 and 9 weeks, respectively (164).

Double copy retroviral vectors expressing $tRNA_i^{Met}$-antisense RNAs targeted against the HIV-1 U3-R-U5 (684-nts) and U3-R-U5-Psi (800-nts) regions were also developed (135). Stable transductants expressing either one of the two antisense RNAs were shown to inhibit virus replication for 20 weeks post-infection (135).

Double copy retroviral vectors expressing $tRNA_i^{Met}$-*tat* (68-nts) antisense or $tRNA_i^{Met}$-*rev* (69-nts) antisense RNAs inhibited HIV-1 replication for 10 days (60-70% inhibition, compared to control) (165). A 20-nt antisense RNA to HIV-1 *tat* coding region was also expressed as part of the anticodon loop of $tRNA^{Pro}$. This $tRNA^{Pro}$-*tat* antisense RNA was shown to inhibit HIV replication until day 22 post-infection (166). A 28-nt antisense RNA to *rev* was also expressed as part of Ad VA1 RNA and was shown to inhibit HIV replication for 3 months post-infection (167).

A double copy retroviral vector expressing a $tRNA_i^{Met}$-71-nt TAR antisense RNA was also developed. This vector was shown to inhibit HIV replication better than a single copy retroviral vector allowing MoMuLV 5' LTR promoter-driven expression of a 258-nt *tat*/*rev* antisense RNA targeted against the entire *tat* coding region and the 3' half of the *rev* coding region of HIV-1 RNA (168). In stable transductants, $tRNA_i^{Met}$-TAR antisense RNA conferred 97% inhibition of HIV replication for 28 days post-infection. These antisense RNAs were also tested in peripheral blood CD4+ T lymphocytes against challenge with clinical isolates of HIV-1 (73). $tRNA_i^{Met}$-TAR antisense RNA inhibited HIV replication better (50-61% of control) than the *tat*/*rev* antisense RNA (30-57% of control). Similar results were obtained using a AZT^R isolate of HIV-1 (74). A retroviral vector co-expressing this $tRNA_i^{Met}$-TAR antisense RNA (double copy design) and Rev TDM (constitutively expressed from MoMuLV 5' LTR) (73) is currently being tested in a clinical trial (169).

3.3.2. Sense RNAs

TAR and RRE RNAs act by competing with HIV RNA for binding to Tat and Rev proteins, respectively. Psi-e RNA acts by competing with HIV-1 RNA for packaging within the virion. Depending on the way this RNA is designed, the co-packaged Psi-e RNA may inhibit HIV-1 RNA reverse transcription. Several vectors expressing TAR decoy, RRE decoy, and Psi-e RNAs were developed and were shown to confer varying degrees of inhibition of HIV-1 replication.

A single copy retroviral vector designed to express HIV-1 *tat* mRNA 5' untranslated region (which includes the TAR element) was shown to delay virus replication by 7 days, compared to the control (157). Virus replication was only inhibited when this sense RNA was expressed as part of the 5' untranslated region of the *neo* mRNA, and not when it was expressed as part of the 3' untranslated region.

A double copy retroviral vector was designed to express a $tRNA_i^{Met}$-60-nt TAR decoy. Transduced T cell clones infected with HIV-1 or SIVmac251 were shown to delay virus replication by 7 days, compared to the control cells (170). A processing site was included 3' to the $tRNA_i^{Met}$ to yield processed decoy RNAs. Addition of hairpin sequences upstream and downstream of a processed RNA conferred increased RNA stability and led to a 10-15 fold increase in RNA accumulation (171). Therefore, double copy retroviral vectors were designed to express $tRNA_i^{Met}$-TAR2 (yielding a processed TAR2 with a 5' and a 3' stem loop) or $tRNA_i^{Met}$-TAR3 (yielding a processed TAR3 with a 3' stem loop) (171). In stable transductants, TAR2 inhibited virus replication better than TAR3. However, TAR2 failed to inhibit HIV replication until day 17 post-infection, and only inhibited virus replication at later time points (171).

A single copy retroviral vector was designed to express a 20xTAR decoy under control of MoMuLV 5' LTR promoter (160). This vector was compared with two other vectors expressing an antisense RNA or a hammerhead ribozyme. The three vectors were tested for inhibition of replication of laboratory and clinical isolates of HIV-1 in transduced PBLs. The 20xTAR conferred the worse protection (50% reduction in virus production at day 8 post-infection). Multi-copy 25-50xTAR decoys were also tested in combination with antisense RNAs or ribozymes. These results will be discussed later (see section 3.3.4).

A small circular RNA containing a 27-nt TAR decoy (nts +18 to +44 of TAR RNA) was also designed (172). It was shown to specifically inhibit HIV-1 LTR-driven gene expression (172). This TAR decoy was shown to be extremely stable (172). Furthermore, as it only contained 27 of the 59 nucleotides, overproduction of this TAR decoy may be less cytotoxic as it would not interact with the cellular TAR RNA binding proteins that recognize

the lower stem of the TAR RNA. However, this TAR RNA has not been tested for inhibition of HIV-1 replication.

Plasmids expressing 1xRRE, 3xRRE, or 6xRRE decoys were also engineered (173). In transient co-transfection experiments with an infectious HIV provirus DNA, 60-70% inhibition of HIV replication was observed at day 3 post-transfection (173). Retroviral vectors expressing 2xRRE, 3xRRE, or 6xRRE were then developed (174). Stable transductants expressing these decoy RNAs were not protected (by immunofluorescence) at 2 weeks post-infection. However, at 7 weeks post-infection the percentage of 2xRRE and 3xRRE decoy RNA-expressing cells was significantly increased, whereas that of 6xRRE expressing cells remained unchanged (174). It is possible that HIV infection lead to the selection of cells expressing high levels of 2xRRE and 3xRRE, which were able to better inhibit HIV replication at later time points. The reason why 6xRRE failed to inhibit HIV replication was not investigated. In another study, single copy retroviral vectors were designed to express 1xRRE or 2xRRE decoys (105). However, virus replication was only delayed by 2-3 days, compared to control cells.

In order to reduce potential cytotoxicity (as RRE also interacts with cellular factors), single copy retroviral vectors were designed to express 41-nt RRE SLIIAB RNA, which contains the major Rev binding site. This RNA was expressed under control of the 5' LTR promoter or both the 5' LTR promoter and an internal CMV promoter (175). These vectors were compared to a double copy retroviral vector expressing $tRNA_i^{Met}$-RRE. In stable transductants, inhibition of HIV replication was best observed by the vector expressing RRE SLIIAB RNA under control of a single 5'LTR promoter; virus production in these cells was delayed by 12 days, compared to the control cells. Long-term bone marrow cultures (containing myelo-monocytotropic progeny cells) of CD34+ cells transduced with this vector, were also shown to inhibit HIV replication until day 24-36 post-infection (175). This vector is currently being evaluated in a clinical trial (176, 177).

A double copy retroviral vector was also designed to express $tRNA_i^{Met}$- (43-nt) RRE-SLIIAB (178). In two stably transduced T cell clones, virus replication was shown to be delayed by 7 days compared to control cells. A double copy retroviral vector expressing $tRNA_i^{Met}$-TAR was also tested in parallel, and was shown to confer better protection (178). Virus replication in these cells was delayed by 11 days compared to the control cells.

Double copy retroviral vectors were also designed to express processed $tRNA_i^{Met}$-minimal (13-nt)-RRE4 and RRE5. In order to increase the stability of processed minimal RREs, RRE4 was designed to contain a stem loop at both 5' and 3' ends, whereas RRE5 contained a stem loop only at its 3' end. Only RRE5 was predicted to allow proper folding of the Rev binding site. In stable transductants, HIV replication was better inhibited with RRE5 (80% inhibition at day 20 post-infection) than by RRE4 (50% inhibition at day 20 post-infection) (179).

In order to compare TAR and RRE decoys, Adeno associated virus (AAV)-based vectors were also developed to express the $tRNA_i^{Met}$-RRE4 (179) and $tRNA_i^{Met}$-TAR3 (a processed TAR stabilized with a stem loop structure at the 5' end) (180). Inhibition of HIV replication was tested in stably transduced T cell clones. Virus production was better inhibited by TAR3 (>95% inhibition until day 24 post-infection) than by RRE4 (>90% inhibition until day 20 post-infection).

We have developed a single copy retroviral vector expressing a sense RNA containing 1.44 kb Psi-*gag* coding region, which failed to inhibit virus replication (105, 106). Lack of inhibition was likely due to the inability of this RNA to efficiently compete with HIV-1 RNA for packaging within the progeny virus; inclusion of a 1 kb region within the *env* coding region (encompassing RRE) has been shown to be required for efficient viral RNA packaging (181). A single copy retroviral vector was then designed to express a 1.8 kb sense RNA containing the 5' untranslated region (which includes TAR) and the Psi-e signal (which includes RRE) (108). HIV replication in transduced cells was significantly inhibited and remained low until day 78 post-infection (108). This sense RNA was shown to be packaged into the progeny virus (108). Infectivity of the progeny virus was shown to be greatly reduced, suggesting sense RNA co-packaging with the HIV-1 RNA (108). The fact that co-packaging of non-viral RNAs can be used as an efficient means to confer resistance against retroviruses has also been demonstrated using an MoMuLV-based system (181-183).

3.3.3. Ribozymes

Hammerhead ribozymes have been designed against the 5' (R, U5) and 3' (R, U3) untranslated regions, the Psi region, and the *gag*, *pro*, *RT*, *vif*, *tat*, *rev*, *tat*/*rev*, *env*, and *nef* coding regions of HIV-1 RNA. These ribozymes were shown to confer varying degrees of inhibition of HIV replication.

Our laboratory was among the first to design and demonstrate the feasibility of the ribozyme-mediated approach in HIV gene therapy (127). This study was performed using retroviral vectors expressing a hammerhead ribozyme targeted against a highly conserved sequence within the U5 region of HIV-1 RNA; this site differs from the one used in other studies (137, 184). Compared to control cells, stably transduced cells expressing this ribozyme under control of an internal simian virus (SV) 40 or CMV promoter delayed virus production by 4 days (127). Cells expressing this ribozyme in a constitutive and Tat-inducible manner under control of the HSV *tk*-TAR fusion promoter inhibited virus replication for 22 days (127). We then developed retroviral vectors allowing constitutive and Tat-inducible expression of five other hammerhead ribozymes targeted against highly conserved sequences within the *gag*, *pro*, *RT*, *tat*, or *env* coding region of HIV-1 RNA (185). Of these, the ribozyme targeted against the *env* coding region conferred the best protection, followed by the one against the

pro coding region (185). Compared to control vector-transduced cells, these ribozymes delayed virus production in stably transduced cells for 15 and 12 days, respectively (185). Virus production was shown to occur despite the lack of an escape mutation within HIV-1 RNA target site and despite active ribozyme production (185).

A hammerhead ribozyme targeted against the U5 region of HIV-1 RNA was also expressed as part of $tRNA.^{Val}$ Three $tRNA^{Val}$-ribozymes were designed, which varied in the nucleotides connecting the tRNA to the ribozyme (186). The $tRNA^{Val}$-ribozyme which had only one flanking arm available for hybridization to the target RNA displayed increased stability in cells. Despite poor catalytic activity *in vitro*, of the three $tRNA^{Val}$-ribozymes tested, this $tRNA^{Val}$-ribozyme conferred the best (99%) inhibition of HIV replication at day 11 post-infection (186).

An *in vitro* selection strategy was used to isolate $tRNA_3^{Lys}$ molecules containing a hammerhead ribozyme (targeted against the *env coding* region) as part of the anticodon loop of the tRNA. Active $tRNA_3^{Lys}$-ribozymes, which contained the same activity as the linear ribozyme, were shown to contain 4-10 nt-long 5' linkers and lacked a 3' linker (187). These $tRNA_3^{Lys}$-ribozymes were also shown to be very stable *in vitro* (187). Single copy retroviral vectors were designed to express these $tRNA_3^{Lys}$-ribozymes. Compared to control cells expressing an inactive $tRNA_3^{Lys}$-ribozyme, HIV replication was shown to be delayed by 9 days in stable transductants expressing these $tRNA_3^{Lys}$-ribozymes (187).

A plasmid was designed to allow SV40 promoter-driven expression of a hammerhead ribozyme targeted against the HIV-1 Psi signal sequence (188). Compared to control cells, stably transduced T cell clones were shown to inhibit virus replication by >95%, until day 12 post-infection. A plasmid expressing a hammerhead ribozyme targeted against the *nef* coding region was also developed (189), and was shown to inhibit virus replication in stably transduced T cell clones until day 14 post-infection.

A hammerhead ribozyme targeted against the *tat* coding region was also designed to contain 26/22 nt-long 5'/3' flanking sequences complementary to the target site. A 48-nt antisense RNA complementary to the target site (without the ribozyme catalytic domain) was also designed (190). Compared to the control, virus replication was delayed by 8 days in stable T cell clones expressing the ribozyme, whereas it was delayed by 12 days in clones expressing the antisense RNA. Another study was performed using vectors designed to express a hammerhead ribozyme, also targeted against the *tat* coding region, with 9/9, 12/12, 15/15, 18/18, 21/21, 24/24, 27/27, 30/30, 33/33, 45/70, and 45/564 nt-long 5'/3' flanking sequences complementary to the target site (191). These ribozymes were compared for their cleavage activity *in vitro* and *in vivo*. The ribozyme activity decreased *in vitro* as the length of the flanking antisense regions increased. However, inhibition of HIV-1 replication in stable transductants was best observed with the ribozymes containing 33/33 or 45/70 nt-long 5'/3' flanking sequences (191).

A retroviral vector was designed to express a hammerhead ribozyme against the *tat* coding region of HIV-1 RNA (160). This ribozyme was targeted against a different site than the one used by Lo *et al.* (190) and was expressed under control of MoMuLV 5' LTR promoter. In transduced PBLs, replication of both laboratory and clinical isolates of HIV was shown to be inhibited until day 12 post-infection (160). At day 9 post-infection with laboratory and primary AZT^R and $Nevirapine^R$ isolates of HIV-1, this vector was shown to confer >80% inhibition of HIV-1 replication in stable transductants (192). Transduced PBLs from HIV-infected patients were shown to possess greater viability, compared to control cells (192). A single copy retroviral vector expressing a hammerhead ribozyme targeted against the same site was also developed by another group. CD4+ PBLs transduced with this vector were shown to inhibit HIV replication for 21 days post-infection (193).

Hammerhead ribozymes targeted against the R region (194), U5 region (184, 194), *pol* coding region (194), RRE (194), and another site within the *env* coding region (194) were also cloned within the infectious HIV-1 provirus itself (184, 194). However, all of these ribozymes failed to completely inactivate HIV, demonstrating the need for improved ribozyme strategies.

Minizymes contain a short oligonucleotide linker in place of the stem loop region within the hammerhead catalytic domain. Dimeric minizymes are composed of two monomers which combine to form the conventional hammerhead ribozyme structure (195, 196). $tRNA^{Val}$-dimeric minizymes have been designed to target two sites in the HIV-1 *tat* coding region and were shown to inhibit (>90%) Tat-mediated *trans*-activation. The rate of *tat* mRNA depletion in cells expressing the dimeric minizymes was shown to be faster than in those expressing the conventional ribozyme (197). However, the advantage of this strategy in inhibition of HIV replication has not been demonstrated.

A dimeric hammerhead ribozyme was targeted against the HIV-1 *tat* coding region (same site as the one used by Lo *et al.*) (190) and against a common site within the *tat*/*rev* coding regions of HIV-1 RNA (198). This dimeric ribozyme was expressed under the control of MoMuLV 5' LTR promoter, MoMuLV 5' LTR and an internal CMV promoter, and the $tRNA_i^{Met}$ promoter (cloned within the 3' LTR; double copy retroviral vector) (199). Ribozyme production was highest under control of the MoMuLV 5' LTR promoter. However, cells transduced with different vectors displayed similar level of resistance and delayed virus production by 10 days, compared to control. The retroviral vector allowing this dimeric ribozyme expression under control of the MoMuLV 5' LTR promoter was then tested in the long-term bone marrow cultures of $G418^R$ transduced CD34+ mobilized peripheral blood stem cells from HIV-infected volunteers (200). Replication of both laboratory and clinical isolates of HIV was shown to be inhibited in these cells until day 45-50 post-infection (200). A similar retroviral vector expressing a 41-nt RRE SLIIAB decoy was also tested in these experiments. Inhibition of HIV-1 laboratory strain was similar with both

interfering RNAs. However, inhibition of a clinical isolate of HIV was not as good with the RRE decoy. The dimeric ribozyme is being tested in two clinical trials (177, 200, 201).

A nonameric hammerhead ribozyme, Rz_{1-9}, was designed to contain ribozymes targeted against 9 different sites within the *env* coding region of HIV-1 RNA (202). We have developed and tested a retroviral vector allowing constitutive and Tat-inducible expression of this ribozyme (203). Rz_{1-9} was shown to confer excellent inhibition of HIV replication as no viral RNA or protein could be detected in the transduced cells or in the culture supernatants for the length of the experiment, 60 days (203). Rz_{1-9} was also shown to inhibit replication of laboratory and clinical isolates of HIV-1 in transduced PBLs (204). As HIV-1 provirus DNA could still be detected, our studies suggest that the incoming virion RNA was not cleaved (203). A recent study using this ribozyme also demonstrated that the newly synthesized HIV-1 mRNAs are cleaved in the nucleus, and that the incoming viral RNA is not cleaved (205). Rz_{1-9} is only targeted against the HIV-1 B subtype. To overcome the problem of variability between HIV-1 subtypes, we have developed a pentameric ribozyme, Rz_{10-14}, targeted against 5 sites that are highly conserved among most subtypes of HIV-1 (206). One of these sites is located within the 5' untranslated region, three are located within the *pol* coding region, and one is located within the *vif* coding region of HIV-1 RNA. We have also combined Rz_{1-9} and Rz_{10-14} to generate Rz_{1-14}, containing all 14 of the ribozymes. These ribozymes were cloned in MGIN vector (116) to yield MGIN-Rz_{1-9}, MGIN-Rz_{10-14}, and MGIN-Rz_{1-14} vectors, which are currently being tested.

3.3.4. Combined interfering RNAs

Combination strategies have been used to achieve increased HIV resistance and to decrease the evolution of escape mutants. The following strategies are being pursued: (i) sense RNAs combined with antisense RNAs, (ii) ribozymes combined with antisense RNAs, and (iii) ribozymes combined with decoy RNAs.

3.3.4.1. Sense RNAs combined with antisense RNAs

Antisense RNA to HIV-1 *tat* coding region (the first 107-nts) or *tat*/*rev* coding regions (258-nts, spanning the entire *tat* coding region and the 3' half of the *rev* coding region) was expressed using non-retroviral vectors (70). A plasmid expressing 5xTAR was also constructed. These vectors were tested for inhibition of Tat-mediated *trans*-activation of *CAT* gene expression in transient transfection experiments. Tat *trans*-activation was best inhibited by 5xTAR decoy RNA (~80%), followed by *tat*/*rev* antisense RNA (60%). Co-transfection with plasmids expressing both the 5xTAR and antisense *tat* or antisense *tat*/*rev* RNA conferred even better inhibition (~85% and 95%, respectively).

Inhibition of HIV replication was also demonstrated using two separate plasmids, each expressing one anti-HIV gene (70). Using a high efficiency gene transfer technique, cells were transduced with a non-retroviral plasmid allowing Tat-inducible expression of 50xTAR and a plasmid allowing constitutive expression of an antisense *gag* (1.28 kb) RNA (70). The combination strategy led to very little HIV production until day 28 post-infection, compared to single gene transduction experiments using plasmids expressing 50xTAR decoy (~90% inhibition until day 23 post-infection) or antisense *gag* RNA (~60% inhibition at day 13 post-infection).

A double copy retroviral vector was also designed to allow Tat-inducible expression of 25xTAR-*tat* antisense RNA (207). Tat-inducible expression of a TAR decoy was designed to prevent cytotoxicity in the absence of HIV infection. Transduced cells were shown to inhibit virus replication until day 40 post-infection. At a higher multiplicity of infection, a peak of virus production was observed around day 20-25. However, by day 50 post-infection, virus production was shown to be significantly decreased and remained low until day 200 post-infection. Virus replication was also inhibited in transduced PBLs until day 25 post-infection (207). Retroviral vector transduction was shown to inhibit HIV replication, syncytia formation, and T cell killing in peripheral blood mononuclear cells from late-stage AIDS patients (208).

3.3.4.2. Ribozymes combined with antisense RNAs

Double copy retroviral vectors were designed to express $tRNA_i^{Met}$-hammerhead ribozyme targeted against the U5 region or $tRNA_i^{Met}$-antisense (*tat* or *rev*) RNA (209). Vectors were also designed to co-express these RNAs as part of a single transcript ($tRNA_i^{Met}$-U5 ribozyme-*tat* or *rev* antisense RNA) or as two separate transcripts ($tRNA_i^{Met}$-U5 ribozyme and $tRNA_i^{Met}$-*tat* or *rev* antisense RNA). Stable transductants expressing either the ribozyme or the antisense RNA were shown to inhibit virus production the best (by >95% for 4 weeks). Cells expressing the ribozyme and the antisense (*tat* or *rev*) RNA as part of two separated transcripts inhibited (80-85%) virus replication for ~10 days. No inhibition of virus replication was observed when the two RNAs were co-expressed as part of a single transcript. Similar results were obtained using *tat* or *rev* antisense RNA. Thus, this study suggests that combination strategies must be carefully designed as the interfering RNAs present on a single or even a different transcript could interfere with each other's activity.

A hammerhead ribozyme targeted against the *gag* coding region was flanked with a 413 nt-long antisense RNA targeted to the 5' untranslated region-*gag* coding region (210). In co-transfection experiments with the interfering RNA (transcribed *in vitro*) and infectious HIV-1 provirus DNA, the combination approach resulted in a better (95%) inhibition of virus replication than the antisense RNA (210).

We have combined the Psi-*gag* (1.44 kb) antisense RNA (105) with the multimeric ribozymes, Rz_{1-9}, Rz_{10-14}, and Rz_{1-14}. Retroviral vectors expressing these combined interfering RNAs are currently being tested for inhibition of HIV replication.

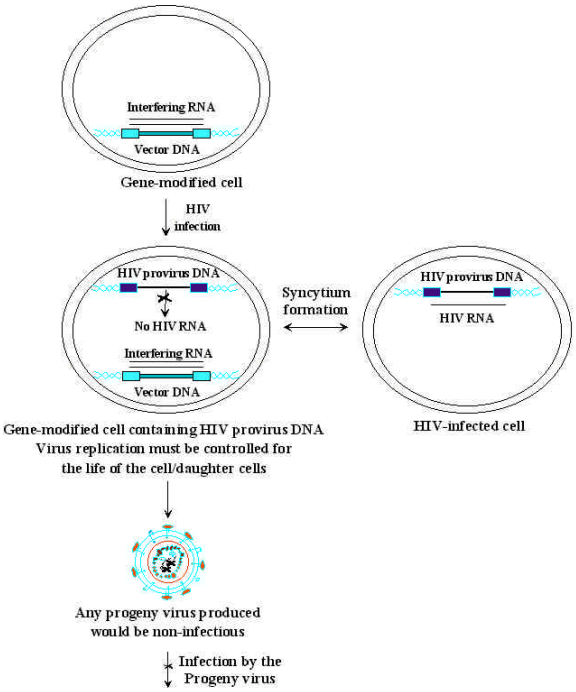

Figure 5D. Strategy to block post-integration steps in the viral life cycle and to inhibit/inactivate progeny virus RNA packaging and/or reverse transcription. This strategy differs from the one described in the figure 5C with respect to progeny virus infectivity. In this case, the interfering RNA, upon co-packaging into the progeny virus, would cleave the virion RNA and/or inhibit reverse transcription. Any progeny virus produced from these cells would be non-infectious, such that no subsequent round of infection would take place.

3.3.4.3. Ribozymes combined with sense RNAs

Hammerhead ribozymes were expressed as part of the anticodon loop of the $tRNA_i^{Met}$ (211, 212). Inclusion of *cis*-acting ribozymes was shown to enhance ribozyme activity *in vitro*. 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 sequences were added to the *cis*-acting ribozymes (162). The *cis*-acting ribozyme-decoy RNAs were shown to interact with HIV-1 Tat or Rev proteins *in vitro*. However, the usefulness of this strategy in inhibiting HIV replication has not been described.

A double copy retroviral vector was designed to allow Tat-inducible expression of 50xTAR-RRE-ribozyme (213). This ribozyme was targeted against the *gag* coding region (136). HIV replication was shown to be inhibited for 20 days post-infection (213). At day 30-40 post-infection, the amount of virus produced was ~50% of the control. However, 3 to 6 months later, very little virus production was detected. Interestingly, this combination strategy was also shown to inhibit SIV replication for 30-40 days post-infection.

3.4. Strategies to block post-integration steps and inhibit/inactivate progeny virus RNA packaging and/or reverse transcription to block subsequent rounds of infection

Packageable interfering RNAs may be developed that would not only inhibit virus replication inside the cell but also compete with HIV RNA/$tRNA_3^{Lys}$ packaging within the progeny virus, and/or inhibit HIV RNA reverse transcription during the subsequent round of infection (figure 5D). Packageable interfering RNAs may be expressed as part of Psi-e sense RNA, $tRNA_3^{Lys}$ or antisense RNA. Psi-e RNA would act by competing with HIV-1 RNA for packaging and interfere with HIV-1 RNA reverse transcription. $tRNA_3^{Lys}$ would act by competing with host $tRNA_3^{Lys}$ for packaging and initiation of reverse transcription. Antisense RNA would, upon hybridization with HIV RNA, be packaged by HIV RNA; antisense RNA may interfere with HIV genomic RNA dimerization, packaging, and/or reverse transcription.

3.4.1. Antisense RNAs

We have shown that the antisense RNAs targeted against the Psi-*gag* region (1.44 kb) and the U3-5' untranslated region-*gag-env* coding region (5 kb) can inhibit virus replication for the length of the experiment, 30 and 78 days, respectively (105, 108). This RNA was shown to be packaged within the progeny virus (108). As the antisense RNA is unlikely to be packaged on its own, it must have been co-packaged with HIV RNA. Infectivity of the progeny virus was significantly decreased, suggesting antisense RNA copackaging and its ability to abort HIV RNA reverse transcription. We have also shown that PBLs transduced with retroviral vectors expressing these antisense RNAs inhibit replication of both the laboratory and the clinical isolates of HIV-1 (108).

3.4.2. Sense RNAs

We have shown that sense RNAs containing the (1.8 kb) 5' untranslated region-Psi-e signal sequence suppress HIV-1 production for 78 days post-infection (108). This RNA was also shown to be packaged within the progeny virus. The RNA must have been co-packaged with HIV-1 RNA as the infectivity of the progeny virus was significantly decreased (108). A decrease in the progeny virus infectivity also suggests that this RNA is capable of inhibiting HIV-1 RNA reverse transcription. This RNA may also be combined with other interfering RNAs to develop packageable ribozymes and/or antisense RNAs.

A mutant $tRNA_3^{Lys}$ was also designed to be packaged into the progeny virus and lead to the formation of defective, incomplete provirus DNA by false priming at the TAR region (214). In this tRNA, the eleven nucleotides at the 3' end were complimentary to the HIV-1 TAR RNA. The mutant tRNA was shown to compete with $tRNA_3^{Lys}$ for binding to RT, to prime reverse transcription at the TAR region, and to delay HIV-1 replication for 8-10 days, compared to control (214). It was also shown to inhibit Tat-mediated *trans*-activation, suggesting that it may inhibit Tat/TAR interaction and/or translation of TAR-containing mRNAs.

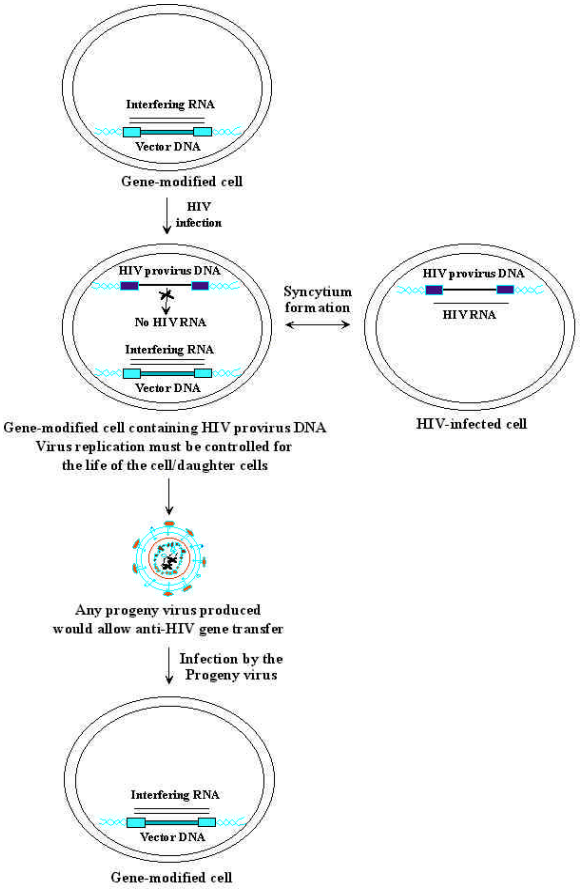

Figure 5E. Strategy to block post-integration steps in the viral life cycle, to inhibit/inactivate HIV-1 RNA packaging and/or reverse transcription, and to use progeny virus for allowing anti-HIV gene transfer into other susceptible cells. This strategy differs from the one described in the figure 5D. In this case, the interfering RNA would become packaged into the progeny virus and would cleave the co-packaged HIV virion RNA. During the next round of infection, the interfering RNA (and not the HIV-1 RNA) would be reverse transcribed, and the provirus DNA containing the anti-HIV gene would be integrated in the host cell genome. This strategy would thus allow anti-HIV gene propagation *in vivo*.

3.4.3. Ribozymes

Since host $tRNA_3^{Lys}$ is packaged by the virus, this tRNA was used to develop packageable ribozymes (187, 215, 216). The $tRNA_3^{Lys}$-ribozymes are expected to allow HIV-1 RNA cleavage both within the cell and, upon co-packaging, within the virions.

A plasmid was designed to express $tRNA_3^{Lys}$-hammerhead ribozymes (215). This ribozyme is targeted against the PBS. The progeny virus released from transient co-transfection experiments using this plasmid and the HIV proviral DNA was analyzed (215). The infectivity of the progeny virus was shown to be reduced 6 fold, suggesting co-packaging of $tRNA_3^{Lys}$-ribozyme (215). In addition to allowing HIV-1 RNA cleavage, $tRNA_3^{Lys}$-ribozyme may also inhibit HIV RNA reverse transcription by competing with the host $tRNA_3^{Lys}$ for binding to the RT and/or to the PBS within the HIV RNA. Also, upon hybridization with the PBS, the $tRNA_3^{Lys}$-ribozyme may not be able to prime reverse transcription since the ribozyme was expressed 3' to the tRNA. Thus, a $tRNA_3^{Lys}$-inactive ribozyme is also likely to inhibit progeny virion RNA reverse transcription.

We have used the *in vitro* selection strategy to isolate $tRNA_3^{Lys}$ chimeras containing a hammerhead ribozyme targeted against the *env* coding region of HIV-1 RNA (187). This ribozyme was expressed as part of the anticodon loop of the tRNA. The *in vitro trans* cleavage activity of $tRNA_3^{Lys}$-ribozymes was found to be affected by the length of linkers connecting the ribozyme to the $tRNA_3^{Lys}$. The presence of 4-10 nt-long 5' linkers, and the absence of a 3' linker, was found to be optimal for *in vitro trans* cleavage activity (187). These ribozymes were shown to be as active as a linear ribozyme and as stable as $tRNA_3^{Lys}$. Single copy retroviral vectors expressing these tRNA-ribozymes were then developed and used to transduce T cells. Virus replication in stable transductants was delayed by 9 days, compared to control cells expressing an inactive ribozyme (187).

3.4.4. Combined interfering RNAs

3.4.4.1. Ribozymes combined with interfering RNAs

The Psi-*gag* antisense RNA was used to develop packageable multimeric ribozymes. We have developed retroviral vectors expressing the 1.44 kb Psi-*gag* antisense RNA, multimeric hammerhead ribozymes (Rz_{1-9}, Rz_{10-14}, and Rz_{1-14}) (210, 211), as well as both the antisense RNA and the multimeric ribozymes (Psi^--Rz_{1-9}, Psi^--Rz_{10-14}, and Psi^--Rz_{1-14}). These vectors are currently being tested (134). In addition to providing a combined antiviral effect, we expect the antisense RNA to (i) enhance ribozyme activity within the cell through hybridization with the HIV RNA (217), (ii) permit HIV-1 RNA cleavage in the progeny virus, and (iii) enhance ribozyme activity as a result of co-localization within the progeny virus (218). Any viral progeny released from cells expressing these interfering RNAs is expected to be non-infectious, thus preventing subsequent rounds of infection.

3.5. Strategies to block post-integration steps, inhibit/inactivate progeny virus RNA packaging and/or reverse transcription, and use HIV for anti-HIV gene transfer during the subsequent round of infection

HIV based vectors may be used to express ribozymes that would not only cleave HIV RNA in the cell and in the progeny virus, but would also use HIV to allow anti-HIV gene transfer upon subsequent round of infection (figure 5E).

3.5.1. Ribozymes

As reported previously (206), interfering RNAs are being developed in our laboratory that could be packaged by the HIV progeny, inactivate virion RNA, and be transduced during subsequent round of infection. This strategy is designed to allow ribozymes to not only cleave HIV-1 RNA within the cell and viral progeny, but also use HIV to allow "self-propagation" of interfering RNAs.

An HIV-based vector (120) was used in this study. This vector allows Tat-inducible expression of vector RNA from the HIV-1 5' LTR promoter. We have modified this vector to allow *EGFP* expression under control of an internal CMV promoter. A multimeric ribozyme, $Rz_{1\text{-}10}$ containing nine ribozymes targeted against the *env* coding region (B subtype) and one ribozyme targeted against the *vif* coding region (all major subtypes) of HIV-1 RNA. Ribozymes targeted against regions of HIV RNA that are also present in the packaging constructs were excluded since they would prevent vector particle production. In HIV-infected cells, virus replication should be inhibited by the multimeric ribozyme. However, should any progeny virus be produced, the full length vector RNA encoding $Rz_{1\text{-}10}$ is expected to be packaged by the progeny virus, cleave HIV-1 virion RNA, and allow anti-HIV gene transfer *in vivo*. This vector is currently being tested in stable transductants for inhibition of HIV replication, interfering RNA packaging within the viral progeny, cleavage of HIV-1 RNA within the cell and the viral progeny, virion infectivity, and self-propagation of the interfering RNA. In addition, we will determine if self-propagation allows cells to become resistant to HIV infection.

RRE and Psi-e sequences within the HIV-based vector RNA should bring ribozymes in close proximity to HIV RNAs during nuclear export and during/after virion assembly. Since the HIV-1 NC protein binds to the Psi signal (219) and has been shown to enhance ribozyme activity (220), we expect enhanced intracellular and virion RNA cleavage. We also expect TAR, RRE, and Psi-e sequences to inhibit virus replication by interfering with HIV-1 RNA/protein and RNA/RNA interactions. Note that the HIV-based vector expresses RNAs with only a single copy of TAR and RRE. As multiple TAR and RRE decoys are not being used, the degree of sequestration of cellular factors is not likely to be cytotoxic. Furthermore, an internal CMV promoter is used to express multimeric ribozymes. This would allow constitutive expression of ribozymes as part of RNAs that would lack TAR and RRE. HIV-1 based vector RNAs (spliced and unspliced) would only be produced in HIV-infected cells and would contain ribozymes. This strategy is therefore designed to allow upregulated expression of packageable multimeric ribozymes capable of self-propagation during subsequent rounds of replication. In addition to allowing HIV RNA cleavage in the cell and in the viral progeny, this strategy takes advantage of the ability of HIV to deliver anti-HIV genes to the next cell that becomes infected. Self-propagation would only occur in cells susceptible to HIV infection and only for as long as HIV would exist in the system. Since not all cells can be modified during *ex vivo* gene transfer, self-propagation of ribozymes to cells susceptible to HIV infection should significantly enhance the overall efficacy of anti-HIV gene therapy.

4. CURRENT ANTI-HIV GENE THERAPY CLINICAL TRIALS USING INTERFERING RNA-BASED STRATEGIES

Phase 1 clinical trials address the safety and feasibility of an anti-HIV gene, while phase II trials address whether an anti-HIV gene can provide the desired therapeutic benefit to the patients. Several interfering RNAs and proteins are now being evaluated in phase I and phase II clinical trials (144, 221-223). Efficacy of anti-HIV genes is being assessed in CD4+ T lymphocytes (144), as this may provide some short term benefit to the patients. Studies are also underway to assess the rate of reconstitution of transduced CD34+ stem cells *in vivo* (224) and to test the efficacy of anti-HIV-1 genes in transduced CD34+ cells (144). Therapeutic effects resulting from gene transfer into human hematopoietic stem cells, which are the ideal targets of HIV-1 gene therapy, have not yet been achieved. Since only a fraction of stem cells are used for *ex vivo* gene therapy, the problem of untransduced stem cells giving rise to cells susceptible to HIV infection remains to be solved. Our self-propagation strategy is designed to specifically address this issue.

4.1. Antisense RNAs

A phase I clinical trial is underway using syngeneic CD4+ lymphocytes from the uninfected identical twins of HIV-infected patients (169, 225). This clinical trial is performed using a retroviral vector lacking or expressing $tRNA_i^{Met}$-antisense TAR (from a double copy design) and TDM Rev (expressed constitutively from the 5' LTR promoter).

4.2. Sense RNAs

A phase I clinical trial was performed to evaluate the safety, feasibility and efficacy of using retrovirus-mediated transduction of an RRE decoy gene (175) into CD34+ cells from the bone marrow of HIV-1 infected children (176, 177). The retroviral vector used in this study is designed to allow 5' LTR promoter-driven expression of RRE (41-nt) SLIIAB decoy RNA (175). Cells transduced with a vector lacking or expressing RRE SLIIAB were transfused to the patients. No adverse effects were observed. Transduction efficiency of clonogenic progenitors in the marrow was between 7%-30%. However, the gene-modified cells could only be seen in the peripheral blood on the first day following transfusion. The number of gene-modified cells in peripheral blood was very low during the 1-year period post-treatment.

4.3. Ribozymes

A phase I clinical trial is underway using a single copy retroviral vector expressing a $tRNA^{Val}$-U5 hairpin ribozyme to determine the safety, feasibility, and potential efficacy of this monomeric ribozyme in HIV gene therapy (143). Autologous CD4+ peripheral blood mononuclear cells of asymptomatic HIV-1 seropositive individuals were used in this study. Preliminary results show that infusion of gene-modified, activated T-cells into HIV infected patients is safe. Transduced cells were shown to persist until 24 weeks, suggesting that ribozyme-transduced cells may possess a survival advantage *in vivo*.

Another phase I clinical trial was set up using CD4+ cells from uninfected identical twins (226, 227). Healthy CD4+ PBLs from the uninfected twins were transduced with a single copy retroviral vector lacking or expressing a hammerhead ribozyme targeted against the HIV-1 *tat* coding region (under control of 5' LTR

promoter) (160, 192). The gene-modified cells were then infused into the corresponding HIV-positive twins. Results have not yet been reported. This vector is also being tested in another clinical trial involving the use of mobilized peripheral blood CD34+ stem cells from HIV-1 infected individuals (160, 192, 223, 228). No adverse effects were observed at 3 months post-treatment. In four patients, transduced cells were detected in bone marrow at 4 weeks, and in peripheral blood T cells and monocytes at 12 weeks (228). Whether transduced cells expressing ribozyme possess a selective advantage has not been reported.

Another clinical trial is being conducted using CD34+ peripheral blood stem cells from HIV-1 seropositive adults. A single copy retroviral vector lacking or expressing a dimeric hammerhead ribozyme targeted against the HIV-1 *tat* and *tat*/*rev* coding regions (under control of the 5' LTR promoter) is being used in this study (177, 207). No adverse effects were observed. Gene-modified cells could be detected in peripheral blood mononuclear cells and/or bone marrow at 6 months post-transfusion, but the frequency of these cells was low. Transduced cells could not be detected at 6 or 12 months post-transfusion. Thus, transduction and engraftment of long-lived stem cells did not take place. This vector is also being used in a phase II clinical trial using CD34+ peripheral blood stem cells from HIV^+ patients undergoing chemotherapy and stem cell transplantation for non-Hodgkin's lymphoma (177). Initial results in the few months following transplantation showed detectable level of ribozyme-expression in peripheral blood mononuclear cells and granulocytes. Enduring engraftment of transduced CD34+ cells and production of ribozyme-containing cells remains to be determined.

5. CONCLUSIONS AND FUTURE PROSPECTS

A one time *ex vivo* gene transfer into the hematopoietic stem cells may theoretically be sufficient to control virus replication for the life of the patient, provided that, upon transplantation and differentiation, the gene-modified progeny cells that reconstitute the immune system maintain their functions. This necessitates that the gene-modified cells have a selective advantage over the untransduced cells; a negative selection would not allow immune reconstitution. Most interfering RNA-based strategies are likely to confer a selective survival advantage to the gene-modified cells. Conferring a selective advantage would not be an issue in HIV gene therapy, if all susceptible cells within a patient were transduced. This may require *in vivo* gene transfer. Safe, replication-competent, targeted viral vectors are being developed for this purpose. Replication-competent recombinant viruses were developed for specific destruction of HIV-infected cells.

Several HIV gene therapy trials are underway using retroviral vectors expressing anti-HIV genes. While clinical protocols are being established for the *ex vivo* gene transfer into CD4+ and CD34+ cells, retroviral vectors and anti-HIV gene therapy strategies are continually being improved. These vectors/anti-HIV genes are likely to confer even better protection to the patients than those previously developed.

Gene therapy may be used in combination with drug therapy. Anti-viral drugs have extended the lives of many HIV-infected individuals (229). However, there are problems associated with patient compliance, toxic side effects, and viral resistance to all classes of anti-HIV drugs. Interestingly, several anti-HIV genes were shown to confer resistance against drug-resistant isolates of HIV-1, and escape mutants of HIV have not been reported for anti-HIV genes. The feasibility of a combined drug-gene therapy approach has already been demonstrated for several anti-HIV genes.

Over 33 million people have been infected world-wide. The high cost of anti-viral drugs makes it impossible for developing countries, where >95% of all HIV-infected people live, to have access to the treatment. Therefore, despite tremendous progress in the field of drug therapy, the only hope for these millions of individuals is in a one-time treatment without an expensive hospitalization cost. Thus, the eventual development of a safe, replication-competent recombinant virus, which allows *in vivo* delivery of anti-HIV genes conferring resistance or selective death of infected cells, may be an ideal choice while awaiting a vaccine.

6. ACKNOWLEDGEMENTS

This research was funded from a grant from the Medical Research Council of Canada. We thank Reza Nazari for assisting us with bibliographic searches.

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Key words: AIDS, Gene therapy, HIV, Antisense RNA, Decoy RNA, Ribozymes, Review

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