[Frontiers in Bioscience 12, 3104-3116, May 1, 2007]

Overcoming HIV-1 resistance to RNA interference

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TABLE OF CONTENTS

1. ABSTRACT

RNAi refers to the sequence-specific degradation of RNA that follows the cellular introduction of homologous short interfering (si) RNA. RNAi has emerged as a powerful tool to probe the function of genes of known sequence *in vitro* and *in vivo*. Advances in vector design permit the effective expression of siRNA in human cells. Numerous recent investigations have described the ability of RNAi to decrease the replication of human immunodeficiency virus type 1 (HIV-1) in lymphocytic cells using siRNA targeting viral (e.g. *tat*, *gag*, *rev*) and host (e.g. CCR5, CD4) proteins. Can RNAi be used as a form of genetic therapy for HIV-1 infection? Recent data indicate that the dynamic replication kinetics of HIV-1 pose a considerable barrier to achieving durable virus suppression by RNAi with the rapid emergence of HIV-1 mutants resistant to siRNA. This review summarizes recent work on HIV-1 specific RNAi with a focus on potential strategies to overcome HIV-1 resistance to RNAi.

2.INTRODUCTION

2.1. Identification of RNA interference

RNAi has revolutionized the conduct of gene knock out experiments and has the potential of emerging as a novel nucleic acid based therapeutic modality. RNAi was originally discovered in *Caenorhabditis elegans* (18). Fire and colleagues studied the *unc-22* gene that encodes a nonessential myofilament protein. Knock-out of *unc-22* leads to a severe twitching phenotype in the animals. Surprisingly, the investigators found that the injection of a mixture of sense and antisense RNA molecules homologous to a 742 nucleotide (nt) segment of *unc-22* produced more potent gene inhibition than when sense or antisense compounds were injected alone. Electrophoretic analysis of cytoplasmic extracts revealed that the mixture of injected sense/antisense compounds had annealed intracellularly to create dsRNA. Thus, the burgeoning technique of dsRNA mediated gene silencing- " RNA interference" - was born.

The cellular mechanics of RNAi have been characterized using *C. elegans* and *Drosophila* as model systems. Work by several laboratories has demonstrated that the initial substrate of RNAi is long dsRNA. These long dsRNA molecules are cleaved by cellular enzymes such as Dicer, a member of the RNase III family of double-stranded RNA-specific endonucleases (2). Enzymatic cleavage yields short RNA duplexes, 21- to 23- nt long, which have been termed short interfering (si) RNA, each with a 2-nt, 3' overhanging end (15, 21, 57). The siRNA is subsequently incorporated into an inactive protein complex (41). ATP-dependent unwinding of the double-stranded siRNA generates an active RNA-induced silencing complex (RISC) that then uses the antisense siRNA sequence to identify homologous mRNA through complementary base pairing. Degradation of target mRNA proceeds from the center of the region spanned by the guide siRNA (16, 17, 56).

2.2. RNA interference as an *in vitro* genetic tool in mammalian cells

As mentioned above, RNAi can be achieved by the cellular introduction of long (>30 bp) dsRNA in *C. elegans* and *Drosophila*. With few exceptions, similar experiments involving mammalian cells have been largely unsuccessful. The mere cytoplasmic presence of long dsRNA mimics an acute viral infection and the cell mounts a broad, non-specific interferon response that leads to global inhibition of cellular function. Specifically, cellular introduction of dsRNA of > 30 bp leads to the activation of protein kinase PKR and 2',5'-oligoadenylate synthetase (2',5'-OAS). The resulting PKR-mediated phosphorylation of the translation initiation factor eIF2α, and 2',5'-OAS-mediated activation of ribonuclease L, leads to generalized stalled translation and mRNA degradation, respectively (3, 11).

Given that the final effectors of RNAi in *Drosophila* were 21- to 23-nt long RNA duplexes, Tuschl and colleagues reasoned that direct cellular introduction of similar molecules would lead to RNAi in mammalian cells. To test this, siRNA were synthesized that targeted endogenous genes encoding the cytoskeletal proteins, lamin A/C and lamin B1, among others. Important structural parameters of siRNA included an overall length of 21-nt and the presence of 2-nt 3' overhangs. The siRNA were transfected into mammalian cells using liposomal technology. Forty-eight hours after transfection, cytoplasmic extracts were prepared and the expression levels of targeted and non-targeted proteins were determined by Western blotting. Lamin A/C expression was selectively reduced by > 90% in cells transfected with Lamin A/C but not in cells treated with an unrelated siRNA species (16).

The ability to specifically down modulate mammalian gene expression by chemically synthesized siRNA duplexes has rendered RNAi an indispensable laboratory tool. Although liposomal technology permits the efficient cellular introduction of siRNA, several limitations exist. Firstly, the repertoire of cells that can be used is largely limited to adherent cell types. Secondly, upon introduction into mammalian cells *in vitro*, siRNA remain maximally active for 2 to 4 days before intracellular destruction and loss of gene silencing occurs (40). Thus, a significant advance has been the design of DNA vectors encoding siRNA expression cassettes (8, 32, 39, 42, 43, 48). Two general approaches have been used to construct expression cassettes; both make use of RNA polymerase III (Pol III) promoters to drive the intracellular expression of siRNA. Pol III promoters are ideal in this context as their natural transcripts are small, noncoding RNA's with defined 3' ends terminated in a stretch of 4 to 5 thymidines.

Rossi and colleagues described the construction of a siRNA expression cassette that utilized the mammalian Pol III U6 promoter (32). Their system consisted of two U6 promoters, each promoter driving the expression of the sense and antisense siRNA strand, respectively. Northern blot analysis of cells transfected with these constructs verified the intracellular expression of sense and antisense RNA. The robust gene silencing activity of this system was tested using HIV-1 infection as a model system. The investigators designed siRNA expression vectors targeting HIV-1 *rev*. They demonstrated that co-transfection of HIV-1 proviral DNA along with expression vectors encoding anti-*rev* siRNA dramatically down-regulated viral replication compared to similar experiments employing sense, antisense, ribozyme, and irrelevant siRNA controls. Co-transfection with two different *rev* expression constructs in combination provided the most potent inhibition (> 4 logs). This experiment provided the first experimental data supporting the utility of using multiple siRNA's simultaneously to achieve additive gene silencing effects.

Another siRNA expression cassette system was described by the Agami group. In this system, a Pol III H1 promoter was used to transcribe 19-nt sense and antisense sequences which were separated by a short spacer (9). The resulting transcript contained two-nt 3' overhangs similar to synthetic siRNA and was predicted to fold back on itself to produce a stem-loop structure (short hairpin RNA, or shRNA). The investigators demonstrated the functionality of shRNA by selectively silencing the *CDH1* protein, which activates the anaphase-promoting complex in MCF-7 cells. Transient transfection of cells with *CDH1* shRNA expressing vectors reduced protein expression by approximately 90%, similar to the levels achieved by transient transfection with CDH1-specific siRNA. Northern blot analysis verified endogenous production of shRNA. The exquisite sequence specificity of shRNA was examined by constructing vectors with a single nucleotide mutation in the shRNA sequence. Transfection with these constructs led to intracellular synthesis of shRNA, but failed to suppress *CDH1* expression. The investigators also demonstrated that the shRNA expression cassette could be incorporated into viral vectors thus allowing the generation of cells stably expressing shRNA.

2.3. HIV-1 specific RNAi

Investigators have used a variety of approaches to explore RNAi-mediated inhibition of HIV-1 replication. Recent work has demonstrated that cellular

introduction/expression of siRNA targeting HIV-1 proteins such as *tat*, *rev* and *gag* inhibits virus replication raising the possibility of using RNAi as an antiviral therapeutic (10, 12, 25, 32, 40, 49, 55). A few representative reports are outlined below.

Initial HIV-1 RNAi experiments utilized siRNA. For example, in one of the first reports, Novina et al. targeted different stages in the HIV-1 life cycle by using siRNA targeting host CD4 receptor and viral structural *gag* proteins (40). Sequential transfection of HeLa-CD4 cells with *gag* siRNA and HIV_{IIIB} led to a 25-fold reduction in supernatant p24 antigen level and 10-fold reduction in full length viral transcripts compared to control transfections. Targeting the CD4 cellular receptor with siRNA reduced receptor expression by 8-fold which, in turn, led to a 4-fold reduction in viral entry and HIV-1 replication. The investigators considered these experiments as "proof-of-principle" as CD4 silencing *in vivo* is impractical given its requirement for normal immune function, but that similar approaches could be used to target the CCR5 chemokine co-receptor.

Surabhi and Gaynor used RNAi to target both viral and host proteins (49). When MAGI cells were transfected with *tat* siRNA or reverse transcriptase siRNA and HIV-$1_{NL4.3}$, viral replication was reduced by >90% compared to control experiments, as measured by supernatant p24 level. The investigators next targeted NF-κB to better understand its role in regulating viral replication. NF-κB siRNA also decreased HIV-1 replication, but was less effective than *tat* siRNA. The efficacy of these siRNAs was tested in human $CD4^+$ Jurkat cells with a similar decrease in HIV-1 p24 antigen levels.

While most studies have assessed the antiviral activity of RNAi on post-integration steps of the viral life cycle, Jacque et al. described pre-integration inhibition of HIV-1 replication by RNAi. These results were extremely significant in that they suggested that HIV-1 infection could be prevented by RNAi (25). $CD4^+$ HeLa cells were sequentially transfected with HIV-1_{NL-GFP} and siRNA targeting HIV-1 long terminal repeat (LTR) and the accessory genes *vif* and *nef*. Transfection of siRNA led to a significant decrease in viral cDNA, indicating that siRNA directs specific degradation of genomic HIV-1 RNA, which serves as the template for cDNA synthesis. Virus production in siRNA-transfected cells was suppressed 20- to 30-fold compared to non-transfected cells. Finally, the investigators assessed the importance of sequence homology in RNAi. The antiviral activity of RNAi was compromised when the nucleotide sequence of the siRNA differed from the target by one nucleotide. This phenomenon represents a problem similar to that facing antiretroviral therapy in which drug efficacy is reduced or abrogated by sequence variability in the viral genome.

Joshi and colleagues compared the antiviral activity of aptamers and shRNA in CEMx174 cells (27). Interestingly, both classes of inhibitors were inhibitory to early events in reverse transcription when cells were challenged with low multiplicities of infection (moi). At higher moi, the aptamers proved superior to shRNA. Capodici et al. provided additional evidence that siRNA can act at pre- and post-integration steps of the viral life cycle (10). U87 CXCR4 and U87 CCR5 cells were transfected with *gag* siRNA and subsequently infected with HIV-1_{IIIB} or HIV-1_{Ba-l}. siRNA transfection was associated with a reduction in the level of HIV-1 *gag* DNA per cell. To demonstrate that RNAi can inhibit ongoing virus replication, cells were transfected with siRNA three days after HIV-1 infection. P24 Gag protein analysis showed a decrease in HIV-1 replication in cells transfected with virus-specific siRNA. Taken together, the findings of Jacque and Capodici suggest that siRNA-mediated inhibition of HIV-1 replication can occur before reverse transcription, possibly by cleaving cytoplasmic genomic RNA.

In contrast, Bushman and colleagues found no significant effect of HIV-1 specific siRNA on genomic RNA (22). Using HOS.T4.CXCR4 cells, the accumulation of reverse transcription products was quantified following transfection with *gag* siRNA and $HIV_{NL4.3}$. The investigators found no differences in viral cDNA copies between siRNA treated and non-treated cells.

The majority of HIV-1 RNAi studies have focused on the use of viral targets. Recent work has shown that the HIV-1 co-receptor, CCR5, can also be exploited as a target for RNAi (36, 45). The CCR5 chemokine receptor is an obligatory co-receptor required for CCR5-tropic HIV/SIV entry into target cells. Its congenital absence in a minority of humans with no apparent phenotypic consequences other than resistance to HIV-1 infection has rendered CCR5 an ideal drug target (14, 51, 53). Qin and colleagues described a 10-fold reduction in CCR5 expression mediated by siRNA, which led to a 3- to 7-fold decrease in HIV-1 replication. Taken together, these results suggest that RNAi can be used effectively to inhibit HIV-1 replication through inhibition of both cellular and viral gene expression, and highlight the potential for targeting multiple genes in efforts to achieve durable suppression of HIV-1 replication. Indeed, Martinez and Este have suggested that antiviral RNAi take a similar approach to highly active anti-retroviral treatment (HAART) in that siRNA constructs be engineered to simultaneously target distinct viral genes, thus inducing "highly active anti-retroviral gene silencing" (36).

3. MATERIALS AND METHODS

3.1. Design and *in vitro* testing of DNA vectors containing short hairpin RNA (shRNA) expression cassettes

To enable the intracellular synthesis of shRNA, we designed a universal expression cassette consisted of an upstream pol III-type promoter followed by a region containing *shRNA* sense and antisense sequences, which were separated by a 6-nt spacer. Unique restriction sites were placed surrounding the promoter and shRNA region to allow easy interchange of different promoters (H1, U6, U6+1, U6+27, MTD) and shRNA sequences (targeting HIV-1 *tat*, *gag*) for downstream experiments. A control

cassette was constructed by replacing *HIV-1*-specific sequences with luciferase (*luc*)-specific sequences. Both expression cassettes were introduced into a pAAV, a DNA vector chosen for downstream applications involving adeno-associated virus as a RNAi delivery agent. 293T cells were co-transfected with either pAAV-*HIV-1 shRNA* or pAAV-*luc* and HIV-$1_{NL4.3}$. Cell-free supernatant was collected two days after transfection and HIV-1 p24 antigen level was quantified by ELISA (Beckman-Coulter, Fullerton, CA). All constructs were sequence verified.

3.2. Synthetic siRNA transfection

293T cells were used for cell culture experiments. Briefly, cells were trypsinized and plated in 6 well plates (4×10^5 cells/well) 24 h prior to siRNA transfection in DMEM containing 10% fetal bovine serum (FBS). 200 pmol of the indicated siRNA was co-transfected with 1 μg HIV-$1_{NL4.3}$ using 6 μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA) per reaction. Viral p24 antigen level in cell-free supernatant was quantified by ELISA on day 4. siRNA sequences were as follows: *tat* (sense): 5'-CUG CUU GUA CCA AUU GCU AdTdT-3'; *tat* (antisense): 5'- UAG CAA UUG GUA CAA GCA GdTdT-3'; non-HIV (sense): 5'-UGG CCC AGC GUA AGA AUG CdTdT-3'; non-HIV (antisense): 5'-GCA UUC UUA CGC UGG GCC AdTdT-3'.

3.3. Generation of shRNA expressing cell lines

An AAV Helper-Free System (Stratagene) was used to generate cells stably expressing shRNA. The AAV transduction system consists of three components: pAAV expression, pAAV-RC packaging and pAAV-helper plasmids. The AAV expression vector was modified by removing the CMV promoter and replaced with an H1-driven *tat* shRNA expression cassette followed by the neomycin selection gene. 293T cells were plated at $5x10^5$ in 2 ml DMEM containing 10% FBS in 6-well plates 24 h prior to transfection with all three AAV plasmids. Cells were harvested 72 hours post-transfection and subjected to four rounds of freeze/thaw in dry ice-ethanol and a 37^0C water bath, respectively. Cell debris was removed by centrifugation at 5000 x g for 10 min and viral particles from the supernatant were used for the transduction of H9 target cells. Cells were transduced with 0.3 MOI of rAAV at 37^0C for two hours. Two days later, cells were washed and resuspended in RPMI containing 600 μg/ml of neomycin and maintained for four weeks. Subsequently, cells were challenged with 100 $TCID_{50}$ HIV-$1_{NL4.3}$ and p24 antigen levels were assessed over a two-month period. Northern blot analysis was performed to verify shRNA expression during the entire time period.

3.4. Genotypic analysis of the *tat* siRNA target region of HIV-$1_{NL4.3}$

Viral RNA was isolated from cell-free culture supernatant using the QIAamp Viral RNA kit (Qiagen) according to the manufacturer's instructions and reverse transcribed. PCR amplification of the *tat* target region was conducted using, 20 pmol of sense primer Tat-A, 5'- ATG GAG CCA GTA GAT CCT A -3', and antisense primer Tat-B, 5'- TGC TTT GAT AGA GAA ACT TGA TG -3', 1 mM dNTPs, and 2.5 U Taq polymerase (Qiagen). Parameters of the thermal program were: 95^0C 1 min, 35 x (95^0C 15 s, 58^0C 30 s, 72^0C 30 s), 72^0C 5 min. The PCR product representing the 215 bp *tat*-exon 1 was analyzed on a 1% agarose gel and column purified by the QIAquick PCR kit (Qiagen). Cycle sequencing was performed using dye-labeled terminator chemistry.

4. RESULTS

4.1. Sequence specificity of RNAi: Implications for HIV-1

The enthusiasm surrounding RNAi as a gene knockout technique has largely derived from the exquisite sequence specificity of si/shRNA. We reasoned that the first challenge of HIV-1 specific RNAi would derive from the enormous heterogeneity of viral sequences observed among different strains of HIV-1. This issue is further complicated by the presence of a swarm of different viruses within any single chronically infected individual. To more precisely determine the extent of sequence homology required between HIV-1 siRNA and its viral target for maximal gene silencing, we designed a potent 21 nt shRNA targeting HIV-1 *gag* (4). We also designed 21 variant shRNAs, each containing a single point mutation at each position (Figure 1). Plasmids containing the various shRNA expression cassettes were co-transfected into 293 T cells along with HIV-$1_{NL4.3}$ and the antiviral effect was assessed by quantifying levels of p24 antigen in cellular supernatant. As seen in Figure 2, all mutants harbored less antiviral activity than wild-type *gag* shRNA. The antiviral activity of the individual mutant shRNAs depended on the position of the nucleotide mismatch within the shRNA. For example, single mutations at positions 9 to 11 in the middle of the 21-nt target sequence severely impaired gene silencing while mismatches in the extreme 3'-end of the shRNA sense sequence were tolerated much more so than those in the extreme 5'end region of the target sequence. These results are in agreement with current models of RNAi which indicate that the cleavage of target RNA proceeds from the middle of the complementary region to the RNA duplex (15). For example, using *D. melanogaster* lysates, Elbashir et al demonstrated that target cleavage occurs 11 or 12 nt downstream of the target position complementary to the extreme 3'-nucleotide of the sequence-complementary 21-nt guide siRNA duplex. In our *gag* shRNA experiments, mismatches in corresponding positions 10 and 11 relative to the mRNA target sequence compromised RNAi to 1 % remaining activity when compared to wild-type shRNA. Thus, perfect sequence homology is required for maximal gene silencing. On a practical level, these results indicate that enormous care must be exercised in the pre-clinical design of siRNA targeting HIV-1. For maximal utility, sequences must be chosen that target extremely conserved regions of the viral genome that, in theory, would be effective against a heterogeneous population of viruses.

4.2. HIV-1 escape from RNAi

While the studies outlined above prove the principle that RNAi can be harnessed to transiently decrease HIV-1 replication, they offer no information on the durability of the antiviral response. We reasoned that

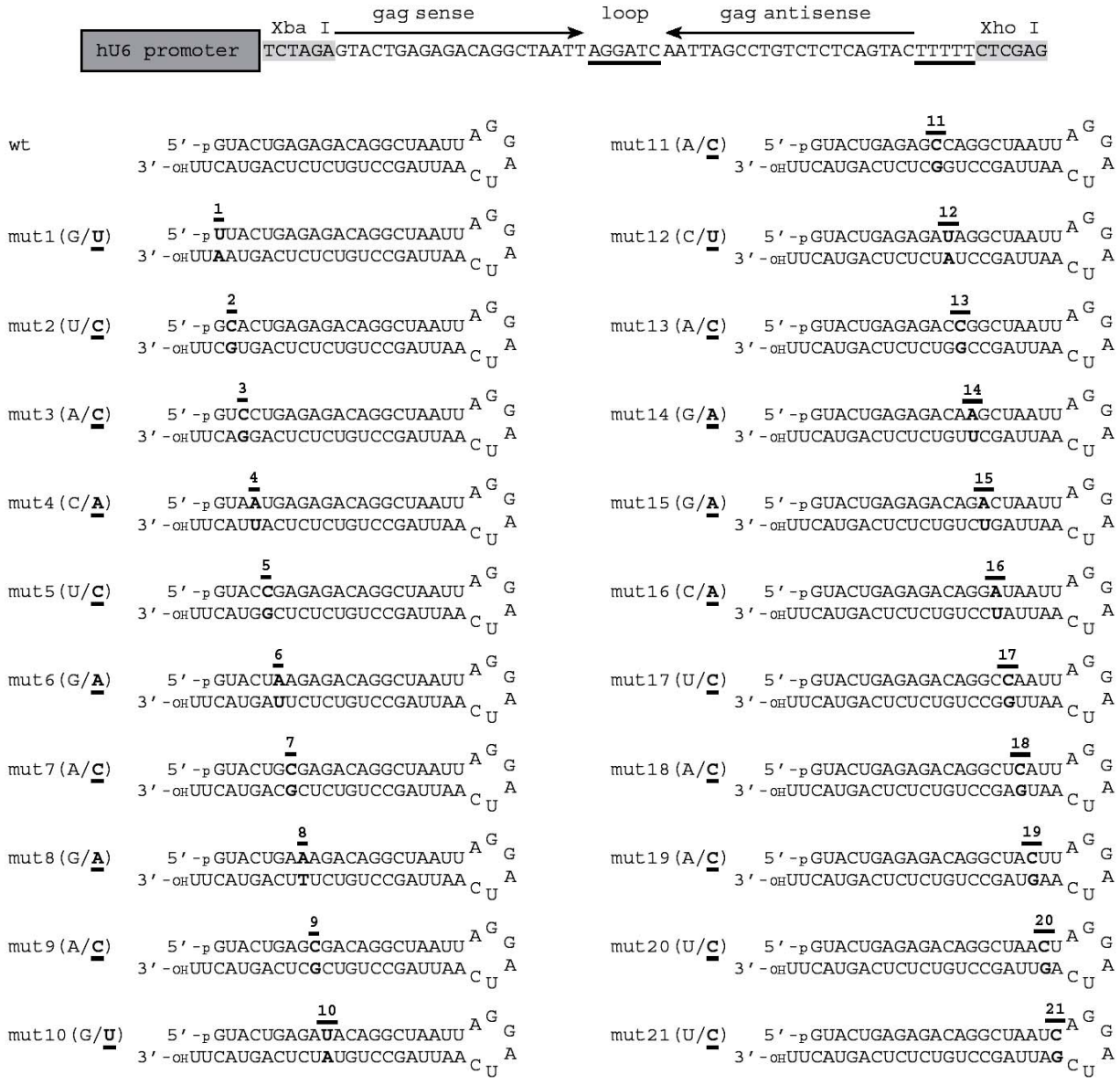

Figure 1. Schematic of mutated and wild-type versions of *gag* shRNA. Each nucleotide position in the shRNA was mutated as indicated in parenthesis. The mutants are numbered according to the position of the mutation with respect to the 5' end of the sense strand.

the highly dynamic nature of HIV-1 replication with an attendant proclivity for sequence variation and mutation, would pose a critical barrier to prolonged RNAi-mediated inhibition (5). To more formally define the durability of RNAi, we first identified an siRNA molecule targeting HIV-1 *tat*. Mutation or truncation of *tat* decreases viral replication by > 1000-fold, underscoring the critical role of *tat* in the viral life cycle (26). After verifying the antiviral activity of *tat* siRNA in cell culture, we created a *tat* shRNA expression cassette. An expression cassette directing the synthesis of shRNA targeting luciferase served as a control. Both cassettes also contained a drug resistance gene and were introduced into adeno-associated virus thereby allowing the generation of recombinant viral particles. Permanent cell lines were produced under drug selection expressing either *tat* or *luc* shRNA. H9 cells were used given that this cell line is permissive for HIV-1 replication, thereby facilitating determination of the magnitude and durability of antiviral RNAi. Prior to viral challenge, genomic integration of rAAV-2 in transduced H9 cells was verified by Southern blot, while precursor *tat* shRNA expression was quantified by Northern blot. Cells were infected with 100 $TCID_{50}$ of HIV_{NL4-3}. P24 antigen level was quantified in cell-free supernatant at weekly intervals over a two-month period. As seen in Figure 3a, the antiviral activity of *tat* shRNA was transient. On day

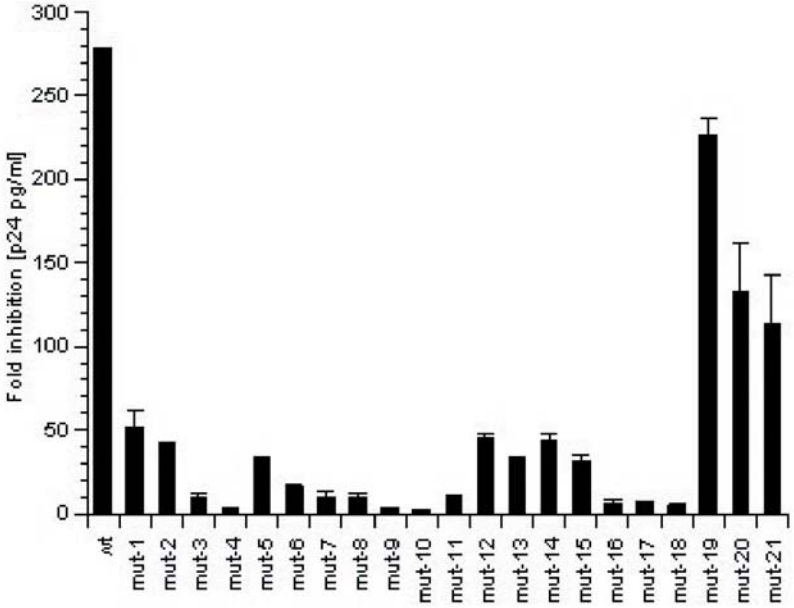

Figure 2. The antiviral activity of mutant *gag* shRNA was assessed by quantifying HIV-1 p24 antigen level in culture supernatant after co-transfection of 293 T cells with HIV-1 and DNA vectors containing the shRNA expression cassette. The inhibitory effect of *gag* shRNAs on HIV-1 replication is expressed as fold inhibition of p24 production compared to cells transfected with a DNA vector expressing luciferase shRNA. Wild-type shRNA G-WT reduced p24 production by 278-fold. All experiments were performed in duplicates with the range indicated.

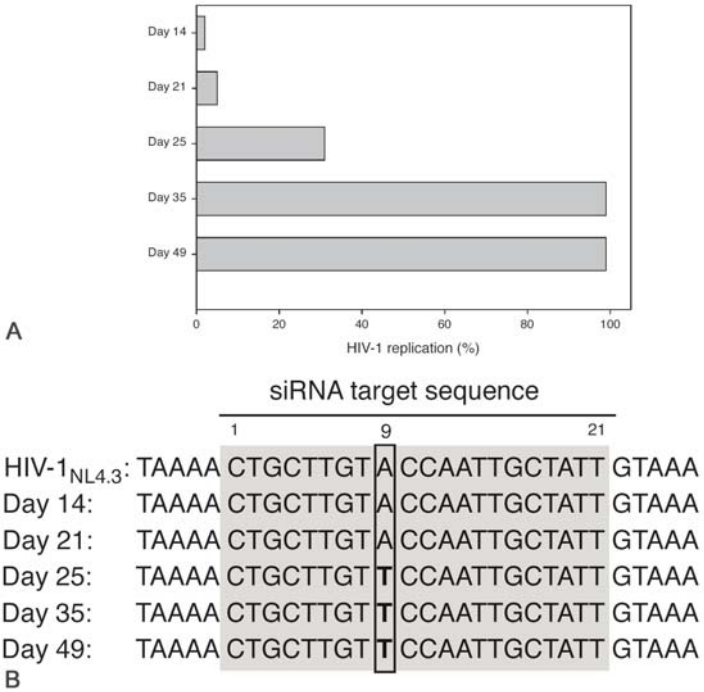

Figure 3.A. Inhibition of HIV-1 replication in H9 cells stably expressing *tat* shRNA. Cells were challenged with 100 $TCID_{50}$ HIV-$1_{NL4.3}$ and p24 antigen levels were assessed over a two-month period. Viral replication was suppressed by 95% until day 21 compared to control cells. Viral escape started to emerge on day 25 and viral replication was no longer suppressed by day 35 with observed p24 antigen levels similar to those in control cells. B. Sequence analysis of HIV-$1_{NL4.3}$ in culture supernatant at indicated time points. A mutation in position 9 of the *tat* target sequence emerged at day 25, coincident with the loss of *tat* shRNA antiviral activity.

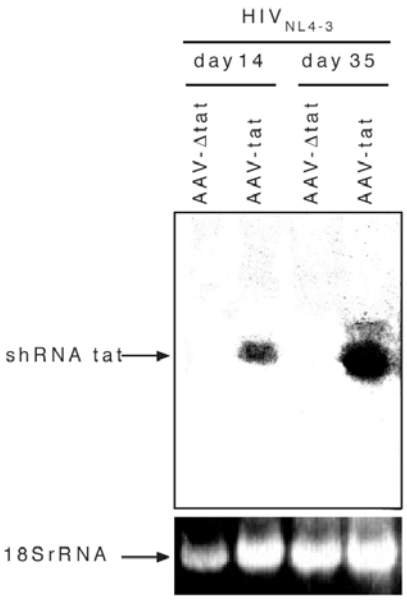

Figure 4. Northern blot analysis of *tat* shRNA expression in HIV-$1_{NL4.3}$ infected H9 cells transduced with AAV-*tat* or AAV-*Δtat*. Continuous expression of *tat* shRNA was observed on days 14 and 35 after HIV-$1_{NL4.3}$ infection. rRNA expression was used as a loading control.

21, HIV-1 replication was suppressed by 1200-fold in cultures of H9 cells expressing *tat* shRNA, compared to cultures of cells expressing *luc* shRNA. By day 25, however, HIV-1 p24 levels had increased and by day 35 there was complete loss of *tat* shRNA-mediated antiviral activity. Viral RNA was extracted from sequential samples of culture supernatants and the nucleotide sequence of the *tat* target region was determined. Interestingly, the sequence of the *tat* target region was identical to the expressed shRNA during the 21 days of infection. By day 25, however, a viral species emerged with a non-synonymous mutation at nucleotide position 9 of the targeted sequence resulting in an amino acid change from threonine (ACC) to serine (TCC) (Figure 3b). Continued intracellular production of shRNA was verified by northern blot analysis (Figure 4). Thus, low level HIV-1 replication in the presence of a single shRNA species was sufficient to generate a viral species with sequence mutations in the siRNA target region. Since the publication of these findings, other groups have also reported on viral escape from RNAi. Das *et al* found that HIV-1 escape mutants emerged in cell culture after the stable expression of siRNA targeting *nef*. The RNAi mutants emerged in a matter of weeks and consisted of viral strains that contained either deletions of the *nef* gene or point mutations in the target sequence (13). RNAi escape mutants have also been demonstrated in other systems such as polio virus (19).

4.3. Potential strategies to overcome HIV-1 resistance to RNAi

Virus escape poses a critical obstacle to using RNAi as an antiretroviral agent. What strategies can be employed to prevent escape? As with current treatment paradigms for antiretroviral therapy, it follows that residual replication in the presence of an antiviral agent, whether it is a conventional drug or a novel molecule, ensures the eventual generation of escape mutants. Can the antiviral potency of siRNA be increased to eliminate residual replication?

The overall efficiency of RNAi depends upon the gene-silencing efficacy of the chosen siRNA sequence. When RNAi was first identified, little data was present on the biophysical determinants of siRNA activity; the most important attribute of a given siRNA was its complete homology to the chosen target sequence. Thus, the actual generation of an effective molecule was largely an empiric process with dozens of siRNA sequences needing to be tested in order to identify a few active molecules. Within the past year, rules have emerged that guide siRNA design. Major determinants for the selection process of siRNAs include low internal stability at the sense strand 3'-terminus, G/C at the 5'end of the sense strand, lack of inverted repeats, and a GC percentage between 30 to 60%. Putative siRNA sequences can be "scored" according to these criteria and those sequences that satisfy all criteria are more likely to harbor potent gene silencing activity (46). Thus, the first step in preventing viral escape is the design of maximally effective HIV-1 specific siRNA following these guidelines.

4.4. Does promoter choice affect the potency of HIV-1 specific RNAi?

RNAi as a potential form of gene therapy relies upon the efficient delivery of si/shRNA to target cells. Vector mediated transfer of RNAi involves the construction of si/shRNA expression cassettes. Pol III promoters have been used to drive the expression of RNAi cassettes given that Pol III transcripts are abundant in human cells. Pol III promoters also have a proven track record in expressing high levels of RNA molecules such as ribozymes and antisense compounds (1, 9, 50). Several Pol III promoters exist and their sub-classification into 3 different categories (Type I, II, and III) is based upon the composition of the promoter elements and their position relative to the transcriptional start site (20). For example, type III promoters such as U6 and H1 are classified have a compact and relatively simple organization and are located upstream of the transcribed region. Transcription is terminated within a stretch of four to six uridines yielding small RNA transcripts with defined 3'ends. This structural feature was shown to be critical for the function of siRNA synthesized *in vitro* (15). Does the type of Pol III promoter impact the potency of anti-HIV-1 RNAi? To rigorously determine this, we created a series of *tat* shRNA expression cassettes, each driven by a unique promoter (H1, U6, U6+1, U6+27, MTD and CMV) (Figure 5). To test the efficacy of each promoter in mediating *tat* shRNA expression and antiviral activity, 293T cells were co-transfected with HIV-$1_{NL4.3}$ and DNA constructs containing different shRNA expression cassettes. *Tat* shRNA expressed by the MTD promoter resulted in 93% inhibition of HIV-1 replication compared to controls. The MTD promoter also consistently gave 30 to 55% greater inhibition of HIV-1 replication when compared to cassettes

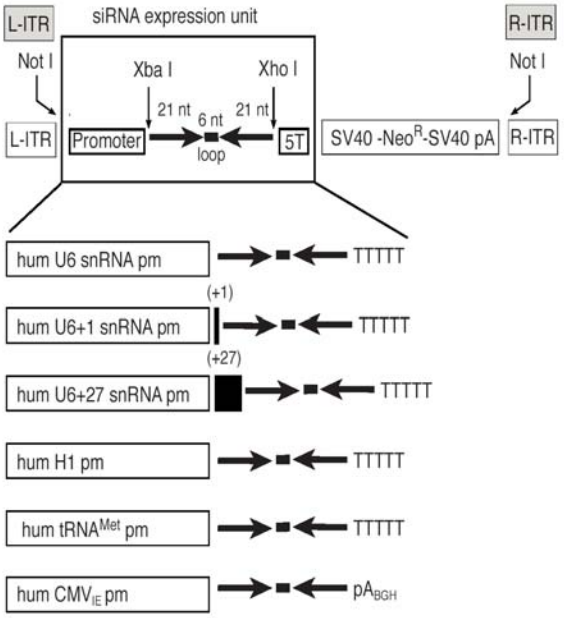

Figure 5. Schematic representation of the various shRNA expression units. Unique restriction sites in the AAV-DNA vector allowed the exchange of different promoters. *Tat* siRNA is expressed in the form of a hairpin transcript consisting of a 21-nt sense and 21-nt antisense sequence separated by a hexaloop. The five thymidine stretches serve as a RNA pol III termination signal whereas the pA_{BGH} polyadenylation sequence terminates RNA pol II transcription. Additional components of the recombinant AAV-DNA vector include inverted terminal repeats (ITRs) and a neomycin selection cassette.

driven by the U6, U6+1, U6+27 or H1 promoters (Figure 6). The CMV promoter did not yield a discernible inhibitory effect on HIV-1 replication, in agreement with previous reports that an unmodified CMV promoter is not suitable for siRNA expression (54). We also evaluated each promoter cassette for expression of precursor hairpin *tat* siRNA by northern blot. The strongest signals were obtained with the MTD and U6+27 promoters, followed by the U6+1, U6, and H1 promoters. However, the magnitude of the antiviral effect did not correlate with the expression level of the precursor transcripts. For example, the H1 promoter had the lowest expression of precursor siRNA yet showed strong inhibition of HIV-1 replication. Conversely the U6+27 promoter did not yield high suppression of HIV-1 replication yet was associated with strong expression of precursor siRNA. Currently underway is a more detailed analysis of promoter-specific effects. We have started comparing the effects of different promoters on the antiviral activity of *tat* shRNA using stable cell lines. Using our AAV-2 transduction system, we have thus far created H9 cells with H1 and MTD-promoter driven *tat* shRNA synthesis. These cells were challenged with 100 $TCID_{50}$ of HIV_{NL4-3} and experiments are still underway. At day 21, we do see a clear difference in HIV-1 replication, with viral replication being 10-fold lower in cells harboring MTD-driven expression of *tat* shRNA compared to cells with H1-driven expression. Stable cell lines employing other promoters are being prepared and virus challenge experiments will follow to determine the relevance of promoter choice vis a vis the anti-viral durability of RNAi.

4.5. Simultaneous expression of multiple shRNA leads to a synergistic antiviral effect

Pharmacologic treatment of HIV-1 infection involves at least two drugs with a preference for including a third, especially in chronically infected individuals with high HIV-1 RNA levels. Can a similar strategy be invoked for RNAi? To assess whether siRNA mediated inhibition of viral replication can be enhanced by simultaneously targeting three separate regions of the HIV-1 genome, we designed a DNA vector co-expressing three siRNAs simultaneously. This vector consisted of a MTD promoter driving the expression of a *tat* shRNA, a human U6 promoter controlling the transcription of a *gag* shRNA, and the H1 promoter expressing shRNA targeting viral protease (prot shRNA). The three shRNA expression units were separated by 400 nt long spacer elements including several thymidine termination signals to reduce potential transcriptional interference between the various shRNA expression units. Three additional DNA vectors were generated, MTD-*tat*, U6-*gag*, and H1-prot which drive the single expression of *tat*, *gag*, and prot shRNA, respectively. These shRNA expression vectors were co-transfected with $HIV\text{-}1_{NL4.3}$ into 293 cells and p24 antigen determined from culture supernatant 48 h post infection. The *tat*-*gag*-prot co-expressing vector M3 reduced viral p24 production by 165-fold. The single shRNA expressing constructs MTD-*tat*, U6-*gag*, and H1-prot reduced viral replication by 21-fold, 42-fold, and 19-fold, respectively. These results demonstrate a synergistic antiviral effect of the triple expression vector compared to the combined effect of each single shRNA expression vector. The antiviral durability of this multi-shRNA expression system is currently being tested in cell culture employing cells permanently expressing either all three shRNAs or a single shRNA species.

4.6. Use of microRNA backbones to increase the activity of siRNA

Thus far RNAi mediated intracellular immunization against HIV-1 has been attained by the cellular introduction or expression of siRNAs or short hairpin RNAs (5, 6, 10, 25, 32, 40, 44). We hypothesized that another potential approach for delivering antiviral siRNA could utilize the structure of microRNAs (miRNA). MicroRNAs are ~ 22 nt single stranded molecules that control post-transcriptional gene expression in eukaryotes. Several hundred distinct miRNAs exist in animals and plants (29-31, 33, 47) but little is known about their precise function and mechanism of biogenesis. The maturation of miRNAs occurs through sequential processing events, the nuclear processing of long primary transcripts (pri-miRNA) into stem-loop precursors of ~70 nucleotides (pre-miRNAs), and the cytoplasmic processing of pre-miRNAs into miRNAs (35). The nuclear RNase III, human Drosha has been recently identified to execute the initiation step of human miRNA biogenesis (34) followed by cytoplasmic processing of pre-miRNA into mature miRNAs by the RNase III nuclease Dicer (2, 23, 28, 52).

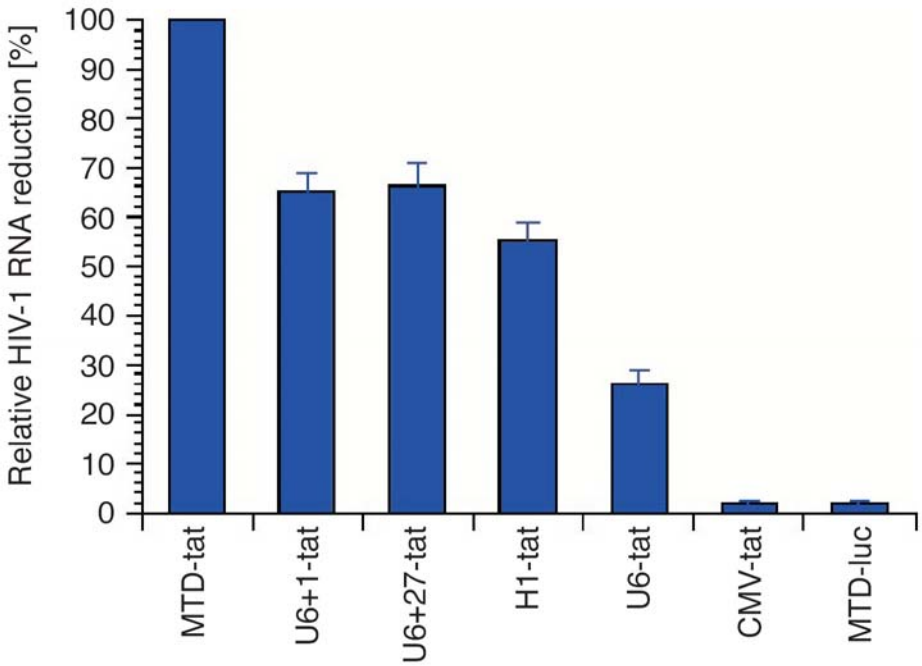

Figure 6. Inhibition of HIV-1 virus production by various *tat* shRNA expression constructs. 293T cells were co-transfected with HIV-$1_{NL4.3}$ and the indicated DNA vectors. A vector expressing luciferase specific shRNA (MTD-luc) served as a negative control. HIV-1 p24 antigen level in culture supernatant was quantified by ELISA 48 h after transfection. The antiviral effect of the MTD promoter was adjusted to 100 % and the potency of the remaining pol III promoters is presented relative to the antiviral effect of the MTD promoter. Values represent the average of duplicate independent experiments, with the range indicated.

One example of a miRNA is the miR-30 species that has been identified in the human HeLa cell line (29). Investigators have demonstrated that the miR-30 miRNA backbone can be altered to include sequences encoding siRNA. This approach has been used to effectively degrade mRNA of endogenous human genes such as the polypyrimidine tract binding protein (59). McManus et al have similarly modified the stem of mir-26a to include siRNA sequences (38). In both reports, the nucleotide sequence introduced into the stem was 100% complementary to the targeted gene, a feature thought to be critical for commencing the RNAi cascade (24).

Is the efficiency of ensuing HIV-1 specific RNAi any different when conventional shRNAs are used compared to siRNAs incorporated into a miRNA backbone? We replaced a part of the miR-30 pre-miRNA stem sequence with a siRNA duplex sequence targeting the HIV-1 *tat* (7). Two different constructs were created: miA-*tat* and miB-*tat* (Figure 7). The miA construct followed the structural motif of Zeng and Cullen who used a loop structure (miA-*tat*) that differed from the endogenously produced *mir*30 by 4 nucleotides (58). The miB construct was identical to that of endogenously produced *mir30*. The miA/B expression vectors were tested along with the previously described conventional *tat* shRNA expression vector in mammalian cell culture (5). The human U6 pol III promoter was used for all expression cassettes. Interestingly, we observed that HIV-1 production was most effectively suppressed by miB-*tat*. Quantification of HIV-1 p24 antigen by immunoassay in culture supernatant revealed that miA-*tat* and miB-*tat* reduced viral activity by 25-fold and 45-fold respectively. Both miR-*tat* constructs were up to 82% more effective in inhibiting HIV-1 replication than conventional *tat* shRNA as measured by Real Time PCR of HIV-1 RNA transcripts (Figure 8). These results were confirmed by Western blot analysis of HIV-1 p24 antigen isolated from culture supernatant and cell lysate. The production of p24 antigen was reduced in both compartments and mirrored the p24 immunoassay results. miB-*tat* RNA showed the most effective reduction of p24 antigen in both compartments followed by miA-tat RNA. Northern blot experiments were carried out to quantify the production of siRNA effector molecules. Most efficient processing of siRNA from *tat* short hairpin or pre-microRNAs was obtained with miB-*tat* RNA. The lowest level of siRNA production resulted from the conventional *tat* shRNA expression cassette.

These results confirm the utility of using the pre-miR30 backbone as a delivery system for siRNA. The enhanced potency of gene silencing achieved with the miA/B constructs compared to conventional shRNA deserves comment. The effectiveness of RNAi mediated gene silencing is likely multifactorial. Key determinants of RNAi efficiency likely include the efficiency of siRNA generation, its nucleotide homology with the chosen target, the accessibility of the target RNA, the structural stability of the siRNA delivery molecule (miRNA, shRNA) and its ability to be processed by Dicer to enter the RNAi silencing

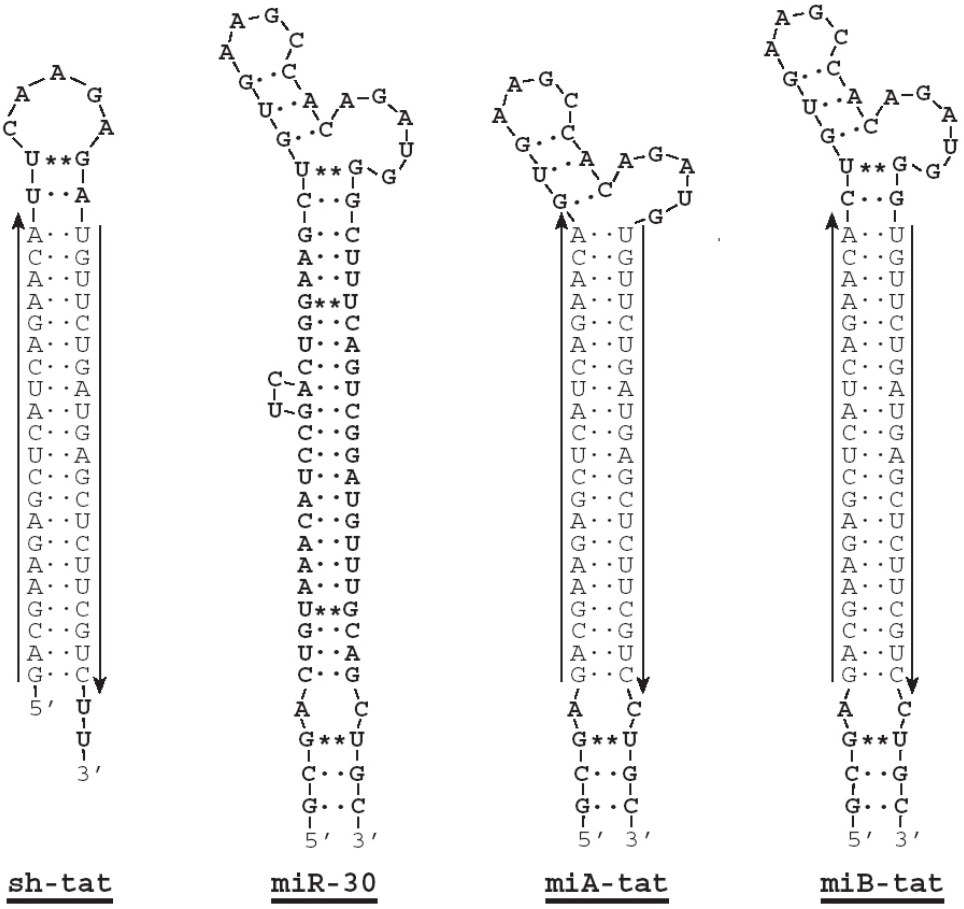

Figure 7. Schematic representation of predicted short hairpin *tat* and pre-micro mir-30 / *tat* RNA sequences. Underlined arrows indicate sense and antisense *tat* sequences embedded in a shRNA or a mir30 precursor RNA backbone. Bold letters in miA-*tat* and miB-*tat* represent stem-loop sequences derived from pre-mir-30. G:U wobbles are indicated with a star.

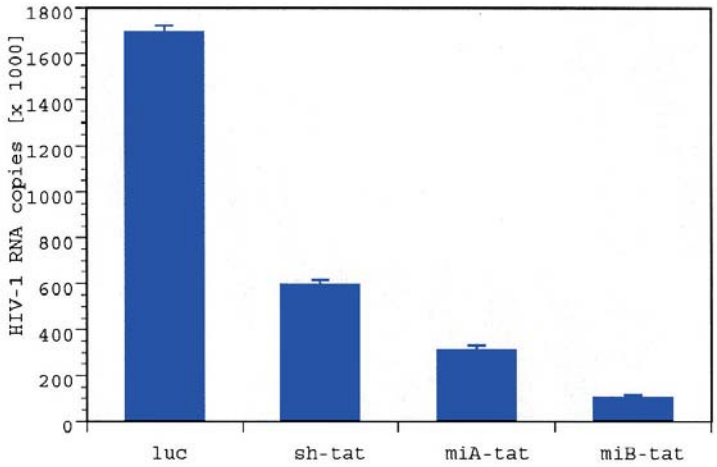

Figure 8. Reduction of total intracellular HIV-1 RNA determined by real-time PCR by conventional *tat* shRNA and the miRA/B constructs. Variation of RNA input was normalized by quantifying GAPDH expression by real-time PCR. To convert threshold cycles to copy number an external standard curve was created with known copy numbers of HIV-$1_{NL4.3}$. Values represent averages of three independent experiments, with the range indicated.

complex (RISC) / miRNA ribonucleoprotein particle (miRNP). When the secondary structure of the three different RNAs was modeled (MFold version 3.0), we note that the most potent construct (miB-tat) contained the highest local folding potential (lowest ΔG) (sh-*tat* ΔG: -38.5, *miA-tat* ΔG: -41.6, miB-*tat* ΔG: -45.9). Thus, the enhanced processing of miB-*tat* RNA may be related to the higher structural stability of the stem-loop structure.

We are currently using the miR-35-miR-41 cluster identified in C. elegans (30) to engineer a "multiference" cassette expressing multiple siRNAs against HIV-1 and CCR5. The miRNA genes included in the miR-35-miR-41 cluster show high and coordinate expression of each miRNA from the nascent precursor RNA (30). We believe that co-expression of multiple siRNAs by the miR-35-miR-41 cluster multiference vector will enhance the overall antiviral response and reduce the emergence of single siRNA-resistant virus with a comparable effect to that observed with conventional HAART regimens.

4.7. Conclusions: The HIV-1 RNAi future

Antiviral potency is the major determinant of the durability of any HIV-1 therapy. On a molecular level, the anti-HIV-1 activity of RNAi is directly related to the efficiency of si/shRNA mediated destruction of targeted RNA. Thus, the further pre-clinical development of HIV-1 specific RNAi will depend upon the identification of optimal target sequences as directed by recently elucidated guidelines. The siRNA molecules must be incorporated in muti-expression vectors either as conventional shRNA or within microRNA stems. Optimized promoters must drive their synthesis. Permanent cell lines expressing these constructs will need to be challenged not only by laboratory adapted strains but also by primary patient isolates. Extension of these approaches to animal models of HIV-1 will be required to validate antiviral potency in an *in vivo* setting prior to their translation into gene therapy approaches suitable for humans. The challenges are many but the potential clinical utility endless for such an approach.

5. ACKNOWLEDGEMENT

BR acknowledges grant support from the Doris Duke Charitable Foundation (Clinical Scientist Development Award) and the NIH (R01AI58697, P20RR018757).

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Key Words: RNA interference, HIV, AIDS, Immune deficiency, Virus, Infection, Gene Therapy, Viral Mutation, Review

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