Published in final edited form as:

Curr HIV/AIDS Rep. 2008 February ; 5(1): 40–43.

Tat-regulated Expression of RNA Interference: Triggers for the Treatment of HIV Infection

Hoshang Unwalla, PhD and John J. Rossi, PhD

Introduction

Posttranscriptional gene silencing by RNA interference (RNAi) was first observed in the nematode, *Caenorhabditis elegans* [1] and subsequently shown to be conserved in higher eukaryotes [2,3]. RNAi mainly involves the delivery of small interfering RNA (siRNA), which are short, double-stranded RNA molecules that are processed to 21-mers by the RNase III enzyme Dicer [4,5], which is loaded into the RNA-induced silencing complex (RISC) that can recognize and cleave target RNAs that are complementary to the guide strand of siRNA [6•]. siRNA molecules can be delivered as synthetic short, double-stranded molecules [7,8] or can be expressed within the cells as individual sense and antisense strands to allow them to hybridize in vivo [9]. Another approach involves the expression of short hairpin RNA (shRNA) molecules that can be processed to 21-mer siRNA molecules [10]. Most studies have demonstrated that the expression of these molecules from constitutive RNA polymerase III xx promoters can translate to potent inhibition of HIV-1 gene expression [11]. However, there are certain drawbacks to continuous expression in that a mere seven-base complementarity to the 3' untranslated region of an unrelated mRNA molecule can silence the expression of that RNA, raising the specter of nonspecific side effects [12,13,14•]. Another cause for concern is that shRNA molecules mimic precursors of another class of small RNA molecules that use a similar biochemical pathway for export and processing and are involved in important cellular development functions [15,16]. Thus, it becomes imperative to design expression systems that can be regulated to express in a tissue-specific or conditional manner. In this study, we developed one such shRNA expression system specific for HIV-infected cells, which the promoter uses a protein made by HIV-1, Tat, for its gene expression to turn on the anti-HIV-1 shRNA molecule, thus establishing a negative feedback loop [17•,18].

HIV-1 transcription in cells is activated when the viral protein Tat binds to a stem loop structure at the 5' end of viral RNA called the transactivation response element (TAR). Tat recruits the positive transcriptional elongation factor B (PTEF-b), which is a heterodimer of two proteins CDK9 and cyclin T1 to polymerase, which is otherwise stalled on viral DNA due to an elongation block. This results in elongation block release and transcription activation.

Most studies examining gene expression in response to HIV infection have directly cloned the therapeutic genes under the control of the viral long terminal repeat (LTR). The drawback of extrapolating this approach to shRNA expression is that adding non-target-derived sequences to the shRNA 5' end can interfere with processing. In this scenario, the entire TAR sequence would be added on to the shRNA sequence. To address this issue, we looked at other promoters that are similarly regulated by an elongation block of the polymerase. One such promoter, the

Copyright © 2008 by Current Medicine Group LLC

Corresponding author John J. Rossi, PhD Division of Molecular Biology, Beckman Research Institute of the City of Hope, 1450 East Duarte Road, Duarte, CA 90101, USA. E-mail: E-mail: jrossi@coh.org.

Disclosures No potential conflicts of interest relevant to this article were reported.

heat shock protein 70 (hsp70) promoter of *Drosophila melanogaster* is similarly regulated whereas activation by heat shock recruits the same PTEF-b kinase to release the elongation block. Moreover, one can remove the heat shock elements and place other transcription factor binding sites that recruit PTEF-b and see a similar activation. Taking advantage of this similarity in activation of transcription, we designed a chimeric promoter such that the HIV-1 LTR, up to and including the TAR element, was placed immediately upstream of the core hsp70 promoter in place of the heat shock elements [17•,18] (Fig. 1). The shRNA sequence, along with a minimal polyA sequence for termination, is placed under the hsp70 promoter such that transcription of shRNA begins from +1, and no extra sequences are added on to the shRNA. Such an arrangement would result in transcription initiation from both the LTR and the hsp70 promoter with the TAR loop formed in between the two promoters. In the presence of virus, Tat would be produced, which would recruit PTEF-b and bind to the TAR element and activate transcription from both promoters. The transcription from the hsp70 promoter would synthesize shRNA molecules that can be processed to 21-mer siRNA molecules and mediate inhibition of HIV-1. In absence of the virus or when the viral transcription has been inhibited, the system would again become silent.

The above-mentioned construct was tested for both its activation in the presence of Tat supplied in trans or infectious virus and the ability of the synthesized siRNA to inhibit the virus. The HIV-1-inducible shRNA expressing DNA vector was introduced (transfected) along with the infectious proviral DNA or the DNA encoding the viral protein Tat into human embryonic kidney cell line 293 (HEK 293). Culture supernatant was collected every 24 hours and assayed for the viral protein p24 as an index of viral inhibition. Seventy-two hours post-transfection, the HEK293 cells were harvested and total RNA from the cells was extracted and probed for expression of siRNA in response to Tat or the proviral DNA.

To test the system in a more stable, long-term gene therapy setting, the HIV-1-inducible shRNA-expressing DNA was placed in a lentiviral vector backbone and introduced (transduced) into a T-cell line (CEM T cells) or primary CD34 stem cells, which are progenitor cells for macrophages. The lentiviral vector carrying the therapeutic shRNA integrates into the genome of the cell and can then be stably maintained and is ready to respond when the virus enters the cells. CEM T cells were then infected with the IIIB strain of the virus (T-cell tropic) and CD34 cells with the JRFL strain (macrophage tropic). On designated days, the culture supernatant was collected and assayed for viral p24 protein.

Results

Expression and processing of shRNA to 21-mer siRNA products were only observed when the cells were transfected with the DNA encoding the viral protein Tat or in the presence of the infectious proviral DNA [17•,18]. The p24 assay of culture supernatants from cells co-transfected with the HIV-inducible shRNA construct and the proviral DNA demonstrated approximately 90% inhibition of HIV-1 72 hours post-transfection. In this case, viral DNA enters the cell and encodes Tat for its own replication. Instead, Tat protein also turns on shRNA expression, which inhibits the virus. Moreover, the expression of active siRNA occurs only from the hsp70 promoter and not from the LTR-derived transcripts as mutating the hsp70 promoter so as to render it inactive resulted in complete shutdown of shRNA expression and processing. By reverse transcriptase polymerase chain reaction, we were able to detect RNA from both promoters, suggesting that placing these two promoters in close proximity does not interfere with each other. However, only the hsp70-derived transcripts were processed to siRNA.

CEM T cells or CD34 cells stably expressing the inducible shRNA construct were able to inhibit viral replication up to 80% when compared with cells transduced with a dummy

lentiviral vector, suggesting that this approach can be employed for long-term gene therapy protocols.

Conclusions

RNAi is a potent inhibitor of targeted gene expression in a wide variety of organisms [1,19, 20]. The triggers for RNAi are siRNAs that are processed from long, double-stranded or hairpin RNA precursors and become part of a ribonucleoprotein complex [21,22]. In an effort to create an inducible system that would respond to HIV infection, we placed HIV-1 LTR, up to and including the TAR element, upstream of the core hsp70 promoter. Both HIV-1 LTR and hsp70 promoters are similarly regulated and require PTEF-b for release of elongation block and activation of transcription [23,24]. Our design assumed that transcription from the viral LTR would result in TAR loop formation to which Tat would bind and recruit PTEF-B. This, in turn, would result in release of the paused RNA polymerase II complex assembled at the downstream hsp70 core promoter. The resulting transcript should initiate at position +1 of the shRNA. Using this fusion promoter, efficient expression and processing of siRNA-sized products could be seen only when the cells were cotransfected with HIV-1. Cotransfection of the inducible construct with wild-type, proviral HIV-1 DNA resulted in functional expression of the shRNA transcript. This result demonstrates that Tat expressed by infectious HIV-1 is sufficient to induce shRNA expression from the chimeric promoter. Infection of CD34 stem cells or CEM cells transduced with inducible shRNA in a lentiviral vector showed approximately 85% to 90% inhibition of HIV-1 gene expression, thus establishing the potential use of this expression system in a more stable gene therapy setting.

In the context of developing RNAi-based therapies against viruses, off-target effects are a concern because the siRNAs may trigger apoptosis of nonvirally infected cells. These off-target effects could be due to partial complementarity and the ability of some siRNA molecules to induce an innate immune response in cells. Alternately, sequestration of the microRNA pathway machinery by the overexpressed siRNA could lead to deregulation of the genes that these microRNAs modulate [25••]. These nonspecific or off-target effects could lead to undesired clinical side effects. Several studies have focused on tetracycline-inducible siRNA expression systems [26,27,28••]. In these systems, addition of drugs like tetracycline can activate siRNA expression. A drug-inducible system, although good in a research setting, is not practical to use in a therapeutic setting as every cell harboring the siRNA construct would express siRNA upon administration of the drug.

Our fusion promoter system takes regulation one step further. In the absence of the ability to target therapeutic siRNA molecules only to HIV-1-infected cells, it becomes imperative to develop inducible systems that can be delivered to all HIV susceptible cells but are activated only when the virus infects the cells, thereby eluding potential toxicities that may ensue with long-term expression of sh/siRNAs in a therapeutic setting. Our fusion promoter system provides the safety of an HIV-1 inducible system without compromising on the efficacy of the antiviral shRNA.

HIV infection is a lifelong problem requiring continual medication for suppressing viral replication. Current strategies of antiretroviral drug combinations have proven effective in prolonging the time from infection to the symptoms of AIDS. Nevertheless, chemotherapy is not without its problems, which include toxicities and eventual emergence of virus mutants that are resistant to current antiretrovirals. Gene therapy refers to the introduction of effector oligonucleotides to transiently or stably alter gene expression or the delivery and expression of an exogenous gene within a specific target cell. A number of studies have demonstrated effective silencing/inhibition of HIV-1 replication by using RNA-based effector oligonucleotides for RNA interference. In this study, we have taken advantage of

lentiviral vector-mediated delivery of anti-HIV short hairpin RNA for the treatment of HIV infection in hematopoietic cells.

References and Recommended Reading

Papers of particular interest, published recently, have been highlighted as:

• Of importance

•• Of major importance

1. Fire A, Xu S, Montgomery MK, et al. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998;391:806–811. [PubMed: 9486653]
2. Hannon GJ. RNA interference. *Nature* 2002;418:244–251. [PubMed: 12110901]
3. Montgomery MK. RNA interference: historical overview and significance. *Methods Mol Biol* 2004;265:3–21. [PubMed: 15103066]
4. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 2001;409:363–366. [PubMed: 11201747]
5. Bitko V, Barik S. An endoplasmic reticulum-specific stressactivated caspase (caspase-12) is implicated in the apoptosis of A549 epithelial cells by respiratory syncytial virus. *J Cell Biochem* 2001;80:441–454. [PubMed: 11135374]
- 6•. Chendrimada TP, Gregory RI, Kumaraswamy E, et al. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* 2005;436:740–744. [PubMed: 15973356] An important discovery of a cofactor for Dicer, the TAR RNA binding protein (TRBP) is involved in binding siRNAs and handing them to the effector argonautes in RISC. Importantly, TRBP is an inhibitor of double-stranded RNA-activated protein kinase, which itself is activated by double-stranded RNAs. TRBP was first discovered as a HIV TAR element binding protein and may also play a role in preventing HIV TAR from activating double-stranded RNA-activated protein kinase.
7. Caplen NJ, Parrish S, Imani F, et al. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc Natl Acad Sci U S A* 2001;98:9742–9747. [PubMed: 11481446]
8. Elbashir SM, Harborth J, Lendeckel W, et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001;411:494–498. [PubMed: 11373684]
9. Lee NS, et al. Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat Biotechnol* 2002;20:500–505. [PubMed: 11981565]
10. Xia H, Mao Q, Paulson HL, Davidson BL. siRNA-mediated gene silencing in vitro and in vivo. *Nat Biotechnol* 2002;20:1006–1010. [PubMed: 12244328]
11. An DS, Xie Y, Mao SH, et al. Efficient lentiviral vectors for short hairpin RNA delivery into human cells. *Hum Gene Ther* 2003;14:1207–1212. [PubMed: 12908971]
12. Jackson AL, Bartz SR, Schelter J, et al. Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol* 2003;21:635–637. [PubMed: 12754523]
13. Scacheri PC, Rozenblatt-Rosen O, Caplen NJ, et al. Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proc Natl Acad Sci U S A* 2004;101:1892–1897. [PubMed: 14769924]
- 14•. Jackson AL, Burchard J, Leake D, et al. Position-specific chemical modification of siRNAs reduces “off-target” transcript silencing. *RNA* 2006;12:1197–1205. [PubMed: 16682562] This study confirms that most off-targeting mediated by siRNAs is via the first two to eight nucleotides serving as “seed sequences” for illicit binding to the 3' untranslated regions of nontargeted mRNAs. The authors show that a 2' OMe modification of the sugar on the second base can abrogate this effect. Because expressed hairpins cannot be modified at this position, caution needs to be used when expressing shRNAs ectopically in cells because the seed sequences of the sense and antisense strands can bind to non-targeted 3' UTRs and cause nontargeted RNAi.
15. Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev* 2003;17:3011–3016. [PubMed: 14681208]

16. Tang G. siRNA and miRNA: an insight into RISCs. *Trends Biochem Sci* 2005;30:106–114. [PubMed: 15691656]
- 17••. Unwalla HJ, Li HT, Bahner I, et al. Novel Pol II fusion promoter directs human immunodeficiency virus type 1-inducible coexpression of a short hairpin RNA and protein. *J Virol* 2006;80:1863–1873. [PubMed: 16439542] This study demonstrates that Tat-inducible expression from an LTR-minimal hsp70 promoter expression can read through a minimal polyA sequence. By fusing an open reading frame of the Rev M10 inhibitory protein downstream of the minimal polyA signal, a readthrough transcript that expresses both shRNA and M10 protein occurs but only in the presence of Tat. This study has implications for novel anti-HIV therapeutic approaches using combinations of shRNAs and dominant negative proteins.
18. Unwalla HJ, Li MJ, Kim JD, et al. Negative feedback inhibition of HIV-1 by TAT-inducible expression of siRNA. *Nat Biotechnol* 2004;22:1573–1578. [PubMed: 15568018]
19. Wianny F, Zernicka-Goetz M. Specific interference with gene function by double-stranded RNA in early mouse development. *Nat Cell Biol* 2000;2:70–75. [PubMed: 10655585]
20. Kennerdell JR, Carthew RW. Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the wingless pathway. *Cell* 1998;95:1017–1026. [PubMed: 9875855]
21. Lipardi C, Wei Q, Paterson BM. RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. *Cell* 2001;107:297–307. [PubMed: 11701121]
22. Sijen T, Fleenor J, Simmer F, et al. On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* 2001;107:465–476. [PubMed: 11719187]
23. Krumm A, Meulia T, Groudine M. Common mechanisms for the control of eukaryotic transcriptional elongation. *Bioessays* 1993;15:659–665. [PubMed: 8274141]
24. Garriga J, Grana X. Cellular control of gene expression by T-type cyclin/CDK9 complexes. *Gene* 2004;337:15–23. [PubMed: 15276198]
- 25••. Grimm D, Streetz KL, Jopling CL, et al. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* 2006;441:537–541. [PubMed: 16724069] This study describes fatalities in mice due to high levels of expression of shRNAs in murine livers. The authors show that the endogenous microRNAs are reduced, a consequence of competition with the ectopically expressed shRNAs for exportin 5, and perhaps with other factors downstream of Drosha processing.
26. Wiznerowicz M, Trono D. Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. *J Virol* 2003;77:8957–8961. [PubMed: 12885912]
27. Matsukura S, Jones PA, Takai D. Establishment of conditional vectors for hairpin siRNA knockdowns. *Nucleic Acids Res* 2003;31:e77. [PubMed: 12888529]
- 28••. Aagaard L, Rossi JJ. RNAi therapeutics: principles, prospects and challenges. *Adv Drug Deliv Rev* 2007;59:75–86. [PubMed: 17449137] A tet-inducible shRNA expression system is described in which the shRNA is targeting Drosha, the RNase enzyme that processes pre-miRNAs. This vector has the inducible shRNA and the tet repressor on the same lentiviral vector construct, making it a fully portable, inducible system.

Figure 1.

Design of the HIV-1 long terminal repeat (LTR)-heat shock protein 70 (hsp70). HIV-1 LTR along with the transactivation response element (TAR) loop region was cloned upstream of the minimal hsp70 promoter. In absence of the viral transactivator Tat, polymerase (Pol) from the LTR initiated and hsp70-initiated transcript pauses due to an elongation block. When the virus infects the cell, Tat is produced, which then recruits a cellular protein complex called positive transcriptional elongation factor B (PTEF-b) which phosphorylates the C-terminal domain of the stalled polymerase and makes it elongation competent. Short hairpin RNA (shRNA) is made only from the hsp70-initiated transcript. mpolyA—minimal polyadenylation recognition and cleavage element; TBP—TATA-binding protein.