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Tat-RNase H and its use in HIV gene therapy

Research Article

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Abbreviations: blood T-lymphocytes, (PBLs); $CD4^+$ T-lymphoid, (MT4); HIV-1 virion protein R, (Vpr); HIV-2 virion protein X, (Vpx); human immunodeficiency virus type-1, (HIV-1); Moloney murine leukemia virus, (MoMuLV); neomycin phosphotransferase, (*neo*); phosphate buffered saline, (PBS); reverse transcriptase, (RT)

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Summary

A targeted RNase, Tat-RNase H, was designed and tested for its activity *in vitro* and inhibition of HIV-1 replication *in vivo*. The Tat-RNase H protein consists of the TAR (*trans*-activation response) element-binding domain of the HIV-1 Tat (*trans*-activator of transcription) and the RNase H domain of the HIV-1 reverse transcriptase (RT) (Melekhovets and Joshi, 1996). The Tat protein binds specifically to the TAR element present in all HIV-1 RNA molecules, whereas HIV-1 RNase H specifically degrades RNA within RNA/DNA hybrid *in vivo* (Skalka and Goff, 1993; Telesnitsky and Goff, 1997) and to a lesser degree within RNA/RNA hybrid *in vitro* (Ben-Artzi et al, 1992; Gotte et al, 1995). Thus, there are two anticipated modes of action of the Tat-RNase H protein. It could cleave HIV-1 TAR RNA in RNA/RNA hybrid or in RNA/DNA hybrid. RNA cleavage in RNA/RNA hybrid was previously shown to be specific and to depend on interaction between the Tat domain of Tat-RNase H and the TAR element of HIV-1 RNA (Melekhovets and Joshi, 1996). We demonstrate here that the Tat-RNase H mediated cleavage of RNA in RNA/DNA hybrid is non-specific as both TAR and mutant TAR RNA/DNA hybrids could be efficiently cleaved. A retroviral vector expressing Tat-RNase H was then constructed to assess whether Tat-RNase H can inhibit HIV-1 replication. However, the Tat-RNase H protein failed to inhibit HIV-1 replication in transduced MT4 cells and in peripheral blood T lymphocytes (PBLs). The possible reasons why Tat-RNase H might have failed to inhibit HIV-1 replication in MT4 cells and PBLs are discussed.

I. Introduction

AIDS is caused by the human immunodeficiency virus type-1, HIV-1, the genome of which consists of two "+" strand RNAs. Destruction of HIV RNA molecules within the cell or within virion could prove to be a successful strategy in inhibiting HIV replication. The therapeutic potential of a number of strategies based on the ability of ribozymes and ribonucleases to cleave RNA/DNA molecules is currently being investigated.

Three RNase-based strategies may be used to inactivate HIV RNA (Singwi and Joshi, 2000). In the first strategy, "cytotoxic" RNases may be employed to specifically destroy cells that become infected by HIV (Singwi and Joshi, 2000). In the second strategy, "co-localized" RNases may be designed to be packaged within the progeny virus and cleave the virion RNA (Singwi and Joshi, 2000). This would inactivate the progeny virus by cleaving the virion RNA. The *Staphylococcal* nuclease (SN) fused with the HIV-1 virion protein R (Vpr) or HIV-2 virion protein X (Vpx) has been shown to allow its packaging within the HIV virion (Wu et al, 1995); however, this nuclease was inactivated by the HIV protease. In a third strategy, others and we have developed targeted RNases that can cleave specific RNA sequences (Melekhovets and Joshi, 1996; Singwi et al, 1999). The probability of escape mutants interfering with the function of targeted RNases is significantly lower since it would require mutations in both the viral protein from which the binding moiety of the RNase is derived and the RNA sequence it binds to.

Two targeted RNases were designed and constructed in our laboratory by fusing an RNase molecule with HIV RNA binding proteins, a Tat-RNase H (Melekhovets and Joshi, 1996) and a Tev-RNase T1 (Singwi et al, 1999). The Tat-RNase H protein consists of the TAR (*trans*-activation response) element-binding domain of the HIV-1 Tat and the RNase H domain of the HIV-1 reverse transcriptase (RT) (Melekhovets and Joshi, 1996). The Tat portion of the fusion protein spans the first 72 amino acids of the Tat protein and it is essential and sufficient for the transport of the protein to the nucleus and specific binding to the TAR element present in all HIV-1 RNA molecules (Hoffmann et al, 1997). The RNase portion of the HIV-1 RT specifically degrades the RNA moiety within RNA/DNA hybrid *in vivo* (Skalka and Goff, 1993; Telesnitsky and Goff, 1997) and to a lesser degree within RNA/RNA hybrid *in vitro* (Ben-Artzi et al, 1992; Gotte et al, 1995). Although the two domains (*i.e.*, the TAR binding domain of the Tat protein and the RNase H domain of HIV-1 RT) when not fused, were shown to be inactive, the chimeric Tat-RNase H protein was demonstrated to specifically recognize and cleave TAR RNA *in vitro* (Melekhovets and Joshi, 1996). The Tev-RNase T1 protein consists of the RNA binding domain of HIV-1 Tev protein and the RNase domain of the *Aspergillus oryzae* RNase T1 protein. *In vitro*, the Tev-RNase T1 protein was shown to exhibit poor specificity, however, when expressed in a $CD4^+$ human T lymphoid (MT4) cell line or human peripheral blood T-lymphocytes (PBLs) no cytotoxicity was observed (Singwi et al, 1999). In addition, HIV-1 replication was inhibited both in transduced MT4 cells and PBLs (Singwi et al, 1999).

The Tat-RNase H was previously shown to specifically recognize and cleave HIV-1 TAR RNA *in vitro* in RNA/RNA hybrid (Melekhovets and Joshi, 1996). In this paper, we demonstrate that Tat-RNase H-mediated cleavage of TAR RNA *in vitro* in RNA/DNA hybrid occurs much more efficiently. However, this activity was shown to be non-specific as both TAR RNA and mutant TAR RNA/DNA hybrids were efficiently cleaved. A retroviral vector was then engineered for the delivery and expression of *tat-RNase H* gene in the human $CD4^+$ T lymphoid cells. However, Tat-RNase H failed to inhibit HIV-1 replication in transduced MT4 cells and PBLs. The possible explanations for the inability of the Tat-RNase H to cleave HIV-RNA *in vivo* are discussed.

II. Results

A. Tat-RNase H-mediated cleavage *in vitro* of TAR RNA and mutant TAR RNA in RNA/RNA or RNA/DNA hybrid

We have previously designed and expressed a fusion Tat-RNase H protein (Melekhovets and Joshi, 1996). Tat-RNase H produced in E. coli was shown to specifically recognize and cleave RNAs containing the HIV-1 TAR element. Tat-RNase H could potentially cleave HIV-1 RNA in RNA/RNA or RNA/DNA hybrid. The RNA cleaving activity of Tat-RNase H in RNA/RNA hybrid had been previously demonstrated (Melekhovets and Joshi, 1996). The RNA cleaving activity of Tat-RNase H in RNA/DNA hybrid was studied as follows. A 75 nt-long TAR/mutant TAR RNA was transcribed and 5' end-labeled in vitro. This RNA contained the 59 nt-long stem loop structure forming the TAR/mutant TAR element at its 5' end and a 16 nt-long single stranded RNA at its 3' end. This 75 nt-long TAR/mutant TAR RNA and a 18 nt-long oligodeoxynucleotide complementary to the single stranded RNA were used to provide Tat-RNase H with a substrate containing TAR/mutant TAR element at the 5' end and RNA/DNA hybrid at the 3' end (**Figure 1A**). As the oligodeoxynucleotide is not designed to hybridize to the nucleotides forming the TAR element, the TAR stem-loop structure will form. Thus, while testing RNA cleavage in RNA/DNA hybrid, the TAR stem loop structure at the 5' end would allow Tat-RNase H/TAR element interaction as well as cleavage in an RNA/RNA hybrid. Cleavage in RNA/RNA hybrid was previously shown to yield 37, 46-49, 56-58 nt-long fragments (Melekhovets and Joshi, 1996). RNA cleavage in RNA/DNA hybrid should yield 57-75 nt-long fragments. If the cleavage was complete, only the shortest 57 nt-long fragment would be detected (**Figure 1A**); alternatively, as the two nucleotides at the 3' end of the oligodeoxynucleotide are complementary to the nts 58 and 59 forming the TAR stem structure, a 59 nt-long fragment may be detected if the formation of the TAR structure was to prevent these two nucleotides from hybridizing to the oligodeoxynucleotides.

Cleavage products were analyzed on a 12-15% polyacrylamide gel. As shown in **Figure 1B**, no TAR RNA (lane 1) or TAR RNA/DNA (lane 2) cleavage was observed in control samples that were incubated in the absence of Tat-RNase H. Tat-RNase H-mediated cleavage of TAR RNA is shown in lane 3. The expected 37, 46-49 and 46-48 nt-long cleavage products were observed. Tat-RNase H-mediated cleavage of TAR RNA/DNA hybrid is shown in lane 4. Almost 99% of the TAR RNA is cleaved in RNA/DNA hybrid; both 57 and 59 nt-long products were observed. Bands corresponding to RNA cleavage in the RNA/RNA hybrid are also observed (lane 4); however, the intensity of these bands is very week compared to those obtained from cleavage in RNA/DNA hybrid. Thus, the efficiency of Tat-RNase H-mediated cleavage of TAR RNA in RNA/DNA hybrid is much higher than in RNA/RNA hybrid.

To determine cleavage specificity of Tat-RNase H in cleaving TAR RNA in RNA/DNA hybrid, TAR-RNase H was used to cleave both TAR and mutant TAR RNA/DNA hybrids (**Figure 1C**). Cleavage was performed for 5, 10 and 15 min. RNA was effectively cleaved within both TAR RNA/DNA (lanes 1-3) and mutant TAR RNA/DNA (lanes 4-6) hybrids. Mutant TAR RNA/DNA hybrid cleavage by Tat-RNase H indicates that Tat-RNase H can

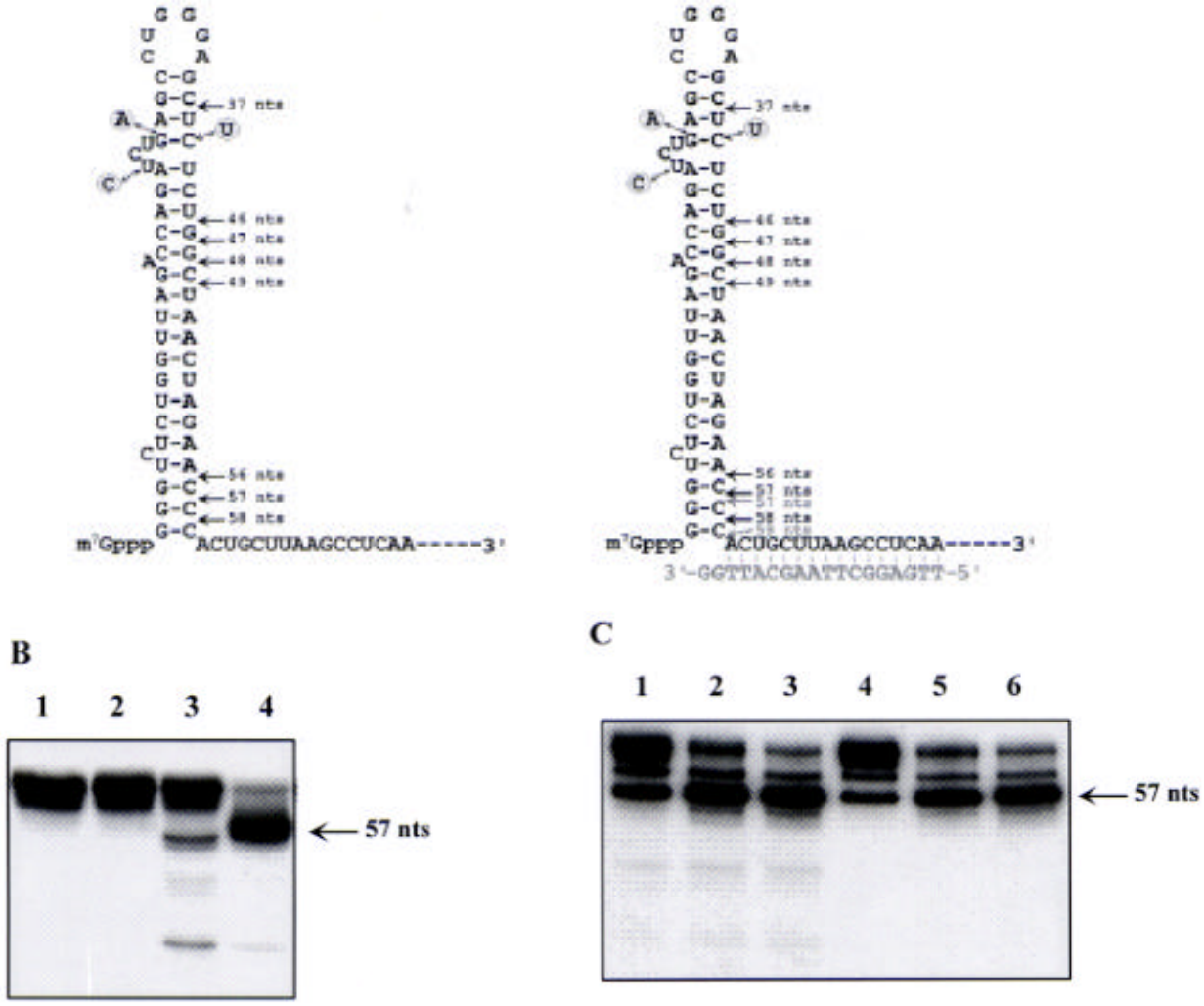

Figure 1A. Structure of TAR/mutant TAR RNA in RNA/RNA (left) and RNA/DNA (right) hybrids. Nucleotides mutated within the mutant TAR RNA are shown with an arrow and are circled. Oligodeoxynucleotide is shown in Grey. RNA cleavage sites and the size of the resulting cleavage products from cleavage within the RNA/RNA (black arrows) and RNA/DNA (Grey arrows) hybrids are shown. **B.** TAR RNA cleavage in a RNA/RNA or RNA/DNA hybrid. 5'-End-labeled TAR RNA cleavage in RNA/RNA (lanes 1 and 3) or RNA/DNA (lanes 2 and 4) hybrid in the absence (lanes 1 and 2) or presence of Tat-RNase H (lanes 3 and 4). Results are shown after 1-hour incubation at 37ºC. **C.** TAR and mutant TAR RNA cleavage in a RNA/DNA hybrid. 5'-End-labeled TAR (lanes 1-3) and mutant TAR (lanes 4-6) RNA cleavage in RNA/DNA hybrid following 5 (lanes 1 and 4), 10 (lanes 2 and 5) and 15 (lanes 3 and 6) min incubation with Tat-RNase H.

cleave RNA in RNA/DNA hybrid in the absence of Tat/TAR interaction and thus demonstrates the non-specificity of this reaction. Cleavage in RNA/RNA hybrid requires Tat-RNase H/TAR element interaction (Melekhovets and Joshi, 1996); as expected, this cleavage was only observed when the TAR RNA/DNA was used (lanes 1-3) and not when the mutant TAR RNA/DNA was used (lanes 4-6).

B. Construction of a retroviral vector expressing tat-RNase H gene

In order to test whether Tat-RNase H can inhibit HIV replication, the *tat-RNase H* gene was cloned in a Moloney murine leukemia virus (MoMuLV) based retroviral vector MoTN (Magli et al, 1987). This vector contains the MoMuLV 5' LTR promoter driving vector expression and the HSV tk promoter driving the expression of the neomycin phosphotransferase (*neo*) gene. Using a two step PCR procedure, a 1-kb cassette

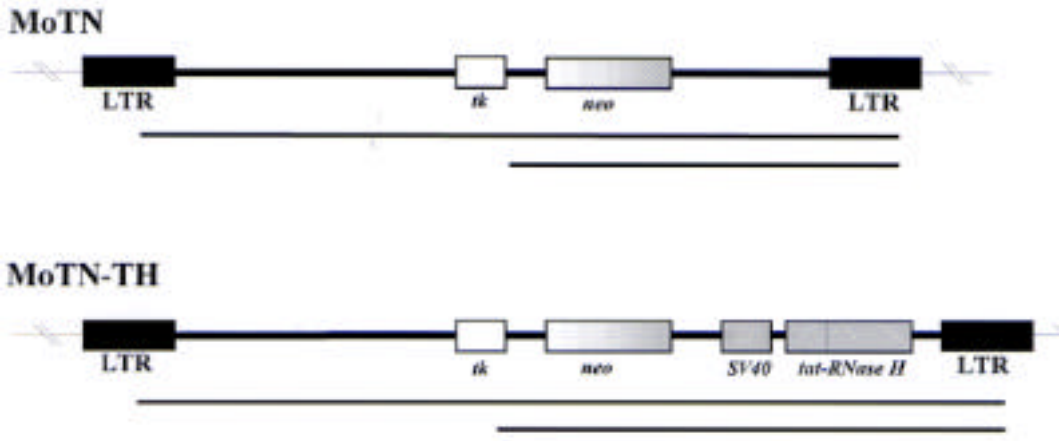

Figure 2. **Schematic representation of MoTN and MoTN-TH vectors.** Only those sequences that are part of the retroviral vector DNA are shown. The *neo* gene is expressed under control of the HSV *tk* promoter and the *tat-RNase H* gene is expressed under control of the SV40 promoter.

containing the SV40 promoter that allows the *tat-RNase H* gene expression was generated. The restriction enzyme sites *BamH* I and *Cla* I were used to clone the cassette at the *BamH* I and *Cla* I sites of the MoTN vector. The resulting vector was referred to as MoTN-TH (**Figure 2**).

C. Transduction of human CD4$^+$ T-lymphoid (MT4) cells and PBLs with MoTN and MoTN-TH retroviral vectors

Amphotropic MoTN and MoTN-TH vector particles, generated from the PA317 packaging cell line, were used to transduce the human CD4$^+$ T-lymphoid (MT4) cells and PBLs. Resistance to the G418 antibiotic conferred by the *neo* gene was used to select for transduced MT4 cells and PBLs. To confirm the presence of the MoTN and MoTN-TH vector DNA in the MT4 transductants and PBLs, PCR analysis was carried out using the Tat-RNase H-5' and 3' primers. Shown in **Figure 3A** are the results obtained from the PCR analysis of genomic DNA extracted from MT4 transductants. PCR analysis of genomic DNA from MoTN vector-transduced MT4 cells served as a negative control (**Figure 3A**, lane 1) and of MoTN-TH plasmid served as a positive control (**Figure 3A**, lane 3). A 0.6-kb PCR product is observed when DNA from MoTN-TH transduced MT4 cells (**Figure 3A**, lane 2) or when MoTN-TH vector DNA only were used. Similar results (data not shown) were obtained from PBLs transduced with the MoTN or MoTN-TH vectors.

To establish the expression of the *tat-RNase H* gene, RT-PCR analyses were performed. No RT-PCR products were observed when total RNA from MT4 cells transduced with the MoTN vector was analyzed (**Figure 3B**, lane 1). A 0.6-kb RT-PCR product was observed in RNA from MT4 cells transduced with the MoTN-TH vector (**Figure 3B**, lane 3). This product was not detected when the RNA was analyzed by PCR without reverse transcription (lane 2), confirming the lack of DNA contamination in the RNA samples. Expression of the *tat-RNase* gene was also detected in PBLs transduced with the MoTN-TH vector but not with the MoTN (control) vector (data not shown).

D. Susceptibility of MoTN and MoTN-TH-transduced MT4 cells and PBLs to HIV-1 infection

After initial testing for the presence of *tat-RNase-H* gene and its expression, pools of MT4 and PBL transductants were tested for their susceptibility to HIV-1 infection.

Stable MT4 transductants were challenged with the HIV-1 strain NL4-3 and transduced PBLs were challenged with a clinical isolate of HIV-1. HIV-1 production was monitored by assaying for the presence of HIV-1 p24 antigen in culture supernatants at various time points. Similar results were obtained from both MoTN or MoTN-TH vector transduced cells. Shown in **Figure 4** are the results obtained from MT4 and PBL transductants infected with the HIV-1. Following an initial lag period of three days, an increase in the virus production, as reflected by the p24 antigen values, was observed in MT4 cells that had been transduced with the MoTN-TH vector. The increase of the viral production from MoTN-TH vector-transduced MT4 cells paralleled the viral increase observed from MT4 cells transduced with the MoTN vector. Similar results were observed with PBLs transduced with the MoTN-TH vector. The values obtained from PBLs transduced with the MoTN-TH vector were similar, although not identical, to the p24 antigen values obtained from PBLs that had been transduced with the MoTN vector. These results indicate that expression of

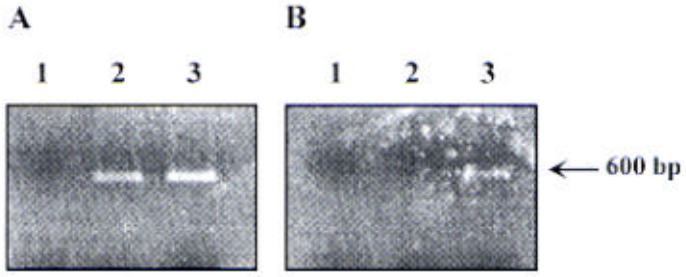

Figure 3A. PCR analysis of the genomic DNA from MT4 transductants. PCR amplification using the Tat-RNase H-5' and 3' primers (described in Materials and Methods) of total DNA isolated from MT4 cells transduced with MoTN vector (lane 1), total DNA isolated from MT4 cells transduced with the MoTN-TH vector (lane 2), or MoTN plasmid DNA (lane 3). **B.** RT-PCR analysis of total RNA **from MT4 transductants.** RT-PCR amplification using the above primers and total RNA isolated from MT4 cells transduced with the MoTN vector (lane 1), PCR amplification of total RNA isolated from MT4 cells transduced with the MoTN-TH vector (lane 2), or RT-PCR amplification of total RNA isolated from MT4 cells transduced with the MoTN-TH vector (lane 3).

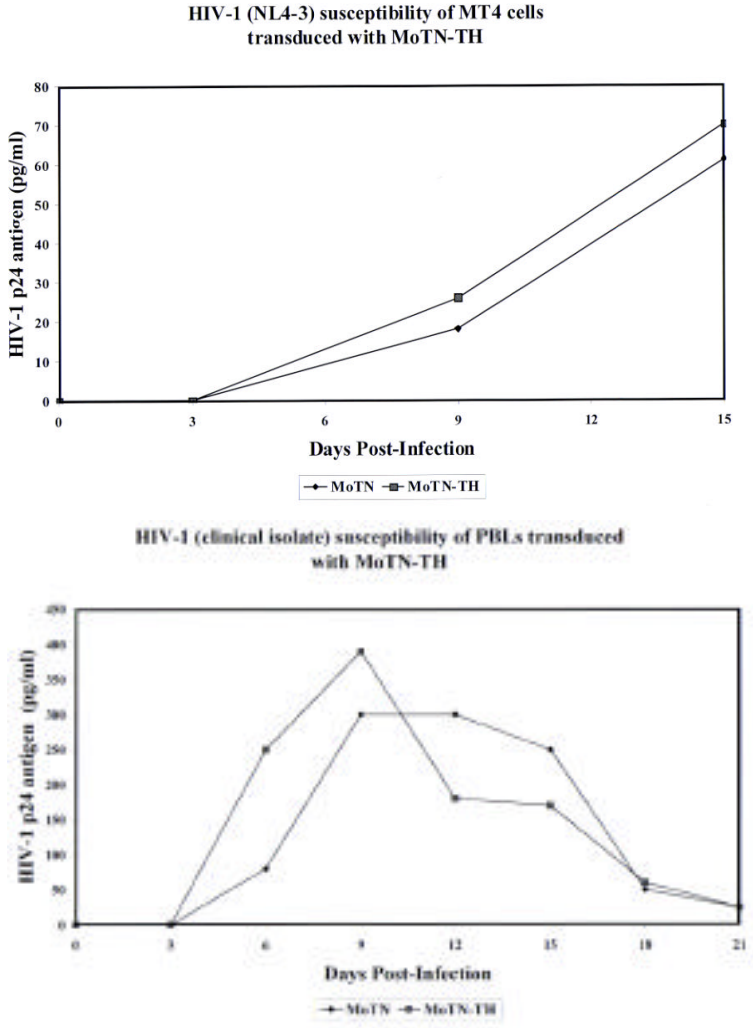

Figure 4A. HIV-1 challenge of MT4 transductants. The amount of HIV-1 p24 antigen present in the infected cell culture supernatants at various time intervals. Challenge of MT4 transductants expressing MoTN or MoTN-TH, with the laboratory HIV-1 strain NL4-3. **B.** HIV-1 challenge of transduced PBLs. The amount of HIV-1 p24 antigen present in the infected cell culture supernatants at various time intervals. Challenge of transduced PBLs with a clinical isolate of HIV-1. Results are expressed as pg/ml of the HIV-1 core antigen p24 at days post-infection.

the Tat-RNase H fusion protein does not inhibit HIV-1 replication in transduced MT4 cells and PBLs.

III. Discussion

A number of functions have been attributed to the RNase H domain of the HIV-1 RT (Hostomsky et al, 1992, 1994). RNase H activity is required for strand transfer and strand displacement both of which are essential functions for the viral replication (Pop, 1996; Gabbara et al, 1999). RNase H shows a strong affinity for RNA/DNA hybrids *in vivo* (Skalka and Goff, 1993; Telesnitsky and Goff, 1997) and to a lesser degree for RNA/RNA hybrids *in vitro* (Ben-Artzi et al, 1992; Gotte et al, 1995). The HIV-1 RNase H has been shown to exhibit both endonuclease and exonuclease activities. The exonucleolytic activity on RNA/DNA complex is also referred to as "directional processing" (Zhan et al, 1994). It has been proposed that the dual function of RNase H might be the result of two distinct conformations (Zhan et al, 1994; Cirino et al, 1995). The two conformations are regulated by the presence and relative amounts of divalent ions (Davis et al, 1991; Cirino et al, 1995). HIV-1 RNase H binds two divalent ions, namely Mn^{2+} or Mg^{2+}. The presence of Mn^{2+} has been implicated in the exonucleolytic activity of the enzyme in RNA/DNA and RNA/RNA hybrids, while Mg^{2+} is required for both the exonucleolytic and endonucleotic activities (Ben-Artzi et al, 1993). The intimate association of the RNase H function with viral replication and its ability to recognize and cleave both RNA/RNA and RNA/DNA hybrids, renders it an attractive candidate for anti-HIV-1 gene therapy.

We have previously reported the design and expression of the fusion gene *tat-RNase H*, composed of the sequence encoding a portion of the Tat protein of HIV-1 and of the sequence encoding the RNase H domain of the HIV-1 RT (Melekhovets and Joshi, 1996). *In vitro* studies using Tat-RNase H fusion protein expressed in *E. coli* and an HIV-1 TAR RNA had previously shown that the fusion protein specifically recognizes the TAR element and cleaves RNA in RNA/RNA hybrid (Melekhovets and Joshi, 1996). Tat-RNase H could cleave RNA in RNA/RNA and also in RNA/DNA hybrid (Ben-Artzi et al, 1992; Skalka and Goff, 1993; Gotte et al, 1995; Telesnitsky and Goff, 1997). The RNA cleaving activity of Tat-RNase H in RNA/DNA hybrid was therefore studied. The efficiency of Tat-RNase H-mediated cleavage of TAR RNA in RNA/DNA hybrid was found to be much higher than in RNA/RNA hybrid. However, the RNA cleaving activity of Tat-RNase H in RNA/DNA hybrid was non-specific and did not require Tat-RNase H/TAR element interaction as both TAR and mutant TAR RNAs were efficiently cleaved in RNA/DNA hybrid. How the mutant TAR RNA/DNA hybrid is recognized by Tat-RNase H was not investigated.

Tat-RNase H-mediated cleavage of HIV RNAs, which all contain the TAR element at their 5' end, should result in inhibition of HIV-1 replication. Thus, a retroviral vector MoTN-TH was designed to allow constitutive expression of *tat-RNase H* gene under the control of the SV 40 promoter. Note that constitutive expression is the only effective way to express Tat-RNase H. Tat-inducible expression would require Tat-RNase H mRNA to contain the TAR element and therefore would be suicidal for Tat-RNase H's own production. Rev-inducible expression would make it too late for Tat-RNase-H to inhibit virus replication.

Tat-RNase H mediated inhibition of HIV-1 replication was tested in both transduced human $CD4^+$ T-lymphoid cell line (MT4) and in PBLs. Amphotropic MoTN and MoTN-TH vector particles were used to transduce MT4 cells and PBLs. PCR and RT-PCR analyses confirmed the presence and expression of MoTN and MoTN-TH vectors. Also the viability of MoTN or MoTN-TH transduced packaging cell lines, MT4 cells and PBLs was similar, suggesting lack of cytotoxicity. This result indicates that the RNA/DNA cleaving activity of Tat-RNase H is not predominant *in vivo*. As this activity is not dependent on Tat-RNase H/TAR element interaction, if present it was expected to cause cytotoxicity.

However, Tat-RNase H failed to inhibit HIV-1 replication in both MT4 transductants challenged with a laboratory strain of HIV-1 and PBL transductants challenged with a clinical isolate of HIV-1. As virus production in MoTN-TH transduced cells was not delayed as compared to cells transduced with the MoTN vector, it seems unlikely, that the lack of inhibition of HIV-1 replication by the Tat-RNase H protein would be due to escape virus production. These results indicate that, the Tat-RNase H fusion protein cannot cleave HIV-1 RNA in MT4 cells or PBLs.

There are three stages in the virus life cycle that could be affected by the Tat-RNase H activity: during reverse transcription in the cytoplasm, during transcription in the nucleus, or post-transcription in the nucleus or in the cytoplasm. However, in order for the Tat-RNase H to cleave HIV-1 RNA in RNA/RNA or RNA/DNA hybrid during reverse transcription, it would have to enter the partially uncoated virion which is rather unlikely. Next, Tat-RNase H could cleave RNA in RNA/RNA or RNA/DNA hybrid during transcription in the nucleus. Note that Tat-RNase H contains the nuclear localization signal of Tat and therefore it should have been localized in the nucleus (Endo et al, 1989; Hoffmann et al, 1997). Lastly, HIV RNA in RNA/RNA hybrid could have been cleaved post-transcription in the nucleus or in the cytoplasm. However, as Tat is predominantly localized in the nucleus, chances of Tat-RNase H localization and cleaving HIV RNA in the nucleus are much greater than in the cytoplasm. Failure of the Tat-RNase H to inhibit HIV-1 replication, by virtue of destroying HIV-1 RNA, could be attributed to a number of reasons discussed below.

As Tat-RNase H-mediated cleavage of RNA in RNA/DNA hybrid is non-specific *in vitro* and does not require Tat/TAR interaction, this activity could not have resulted in specific inhibition of HIV replication. If present, this activity would have caused cytotoxicity, which was not observed. The RNA cleaving activity of Tat-RNase H in RNA/RNA hybrid observed *in vitro* (Melekhovets and Joshi, 1996) is the one that was expected to specifically inhibit virus replication. Thus, it seems that this activity was poorly exhibited *in vivo*.

Tat-RNase H was designed to contain the nuclear localization signal of Tat for transport of the fusion protein to the nucleus to be localized in the nucleus. This should have enabled the Tat-RNase H to recognize and cleave nascent HIV-1 transcripts. However, the last few amino acids present in the C-terminus of the Tat protein have not been included in the Tat-RNase H protein. Although the significance of this sequence is not clear, some evidence exists to implicate it in the nuclear localization of the protein (Hoffmann et al, 1997). Therefore, it is possible that inadequate localization of the Tat-RNase H protein in the nucleus might have resulted in poor inhibition of HIV-1 replication. Alternatively, it is also possible that Tat-RNase H does enter the nucleus but that TAR RNA recognition and/or cleavage by the fusion protein is inhibited *in vivo*. This could be due to steric interference caused by other TAR RNA binding proteins, or those typically interacting with the Tat protein (Kato et al, 1992; Kaczmarski and Khan, 1993). High levels of viral Tat protein could also out-compete the Tat-RNase H protein for TAR RNA binding, rendering the HIV-1 RNA inaccessible to the Tat-RNase H activity.

A poor Tat-RNase H-mediated cleavage of TAR RNA in RNA/RNA hybrid *in vivo* is consistent with the fact that RNase H activity of RT/RNase H during viral reverse transcription only shows RNase activity within RNA/DNA hybrids and not within RNA/RNA hybrids. Our *in vitro* studies have shown that the Tat-RNase H protein can cleave TAR RNA in an RNA/RNA or RNA/DNA hybrid in the presence of Mn^{2+} but not in the presence of Mg^{2+}. It is possible that the physiological concentrations of Mn^{2+} in MT4 cells or PBLs are sub-optimal for RNA cleaving activity of Tat-RNase H. As this activity (RNA cleavage in RNA/RNA hybrid) would have been detrimental for virus evolution, it seems that the reason why this targeted RNase failed to inhibit HIV-1 replication is because of the choice of the RNase used. Indeed, Tev-RNase T1 was shown to inhibit HIV-1 replication in both MT4 cells and PBLs (Singwi et al, 1999).

IV. Materials and Methods

A. *In vitro* cleavage of TAR RNA and mutant TAR RNA in RNA/RNA and RNA/DNA hybrids by Tat-RNase H

Tat-RNase H was produced and purified from *E. coli* (Melekhovets and Joshi, 1996). TAR RNA and mutant TAR RNA (75 nt-long) were transcribed *in vitro* and 5' end-labeled as described previously (Melekhovets and Joshi, 1996). These RNAs (30,000 cpm) were then subjected to Tat-RNase H cleavage in the presence of 10 mM Mn^{2+} as described previously (Melekhovets and Joshi, 1996). Control samples lacked Tat-RNase H. In order to study the RNA/DNA cleaving activity of Tat-RNase H, an oligodeoxynucleotide (5'-TTG-AGG-CTT-AAG-CAG-TGG-3') complementary to the last 18 nucleotides of the TAR/mutant TAR RNA was added. All cleavage reactions were performed for 1 hour at 37ºC, unless specified otherwise. The cleaved and uncleaved RNA was then ethanol precipitated and analyzed by electrophoresis on a 12-15% polyacrylamide gel.

B. MoTN-TH vector construction

The *tat-RNase H* gene was constructed as described previously (Melekhovets and Joshi, 1996). The SV 40 promoter and the *tat-RNase H* gene were then subcloned in a retroviral vector using a two step PCR strategy. In the first PCR, the Tat-RNase H-5' primer (5'-CGG-AAG-ATC-TAA-TAC-GAC-TCA-CTA-T-3') and Tat-RNase H-3' primer (5'-GCG-CAT-CGA-TCT-ATA-GTA-CTT-TCC-TGA-TTC-C-3') were used to amplify the *tat-RNase H* gene from the pET-TH plasmid. This PCR product and the SV-5' primer (5'-CGG-AAG-ATC-TAA-TAC-GAC-TCA-CTA-T-3') were then used in a second PCR reaction with the pB1-SVR6842 plasmid DNA, to amplify the SV40 promoter and the *tat-RNase H* gene. The resulting PCR product contained a *Bgl* II restriction site, the SV40 promoter, the *tat-RNase H* gene and a *Cla* I restriction site. This cassette was digested with *Bgl* II and *Cla* I and cloned at the *Bam*H I and *Cla* I sites of the MoTN vector. Clones containing the SV40 promoter and the *tat-RNase H* gene in the correct orientation were confirmed using restriction enzyme and PCR analyses.

C. Transduction of MT4 cells and PBLs with MoTN and MoTN-TH

Amphotropic MoTN and MoTN-TH vector particles were produced as described previously (Joshi et al, 1990). Either MoTN or MoTN-TH vector particles were used to transduce the human $CD4^+$ T lymphoid (MT4) cell line (Pauwels et al, 1987; Larder et al, 1989) as described previously (Liem et al, 1993). Stable MT4 transductants containing either the MoTN or MoTN-TH vector were selected for resistance to the antibiotic G418 over a period of three weeks.

Human peripheral blood mononuclear cells were isolated using Ficoll-Hypaque gradient centrifugation. Following a phosphate buffered saline (PBS) wash, 1 x 10^6 cells/ml were cultured for a day at 37ºC in RPMI 1640 medium that contained 10% fetal bovine serum, 20 units/ml recombinant human interleukin (IL)-2 (Boehringer Mannheim) and 5 µg/ml phytohemagglutinin (Sigma). Cells in suspension were collected and cultured for two more days. T cell-specific monoclonal antibodies were used to determine T lymphocytes by flow cytometry. Approximately 1 x 10^6 PBLs were transduced with MoTN or MoTN-TH vector particles as described previously (Singwi et al, 1999) in the presence of 16 µg/ml polybrene and 20 units/ml IL-2. The cells were then collected by centrifugation at 200 x *g* for 1 hour at 32ºC and cultured for 16 hours at 32ºC. Prior to transduction, the cells were incubated at 37ºC for 6 hours in medium containing IL-2. Three rounds of transduction were carried out. Following the final round, transduced cells were selected for four days in medium containing G418 (500 µg/ml).

D. Detection of *tat-RNase H* DNA and RNA in transduced cells

Genomic DNA isolation from cells transduced with MoTN and MoTN-TH vector was carried out as described earlier (Sambrook et al, 1989). PCR analysis was performed as described earlier (Ramezani and Joshi, 1996) using the Tat-RNase H-5' and 3' primers. Total cellular RNA was extracted from MoTN and MoTN-TH vector transduced cells as described elsewhere (Chomezynski and Sacchi, 1987). Reverse transcription was performed using the Tat-RNase H-3' primer followed by PCR using the Tat-RNase H-5' and 3' primers as described previously (Ramezani and Joshi, 1996). PCR and RT-PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

E. HIV-1 susceptibility of MT4 and PBL transductants expressing Tat-RNase H

The pools of actively dividing stable MT4 transductants lacking or expressing Tat-RNase H (2×10^6 cells) were each infected with the HIV-1 strain NL4-3 (500 ng p24 equivalent) for 6 hours at 37ºC as described previously (Ramezani and Joshi, 1996). Transduced PBLs (5×10^6 cells) were infected with 1.5 ng (p24 equivalent) of a clinical isolate of HIV-1 for 16 hours at 37ºC as described previously (Singwi et al, 1999). Half of the culture supernatants was collected every third day and replaced with fresh medium. The amount of HIV-1 p24 antigen released in the cell culture supernatant was measured by enzyme linked immunosorbent assay (ELISA, Abbott) as described by the manufacturer.

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