Chapter 3

Aptamer RNA^{Tat} as an inhibitory molecule

Introduction

The product of *tat* gene plays many key roles besides *trans*-activating of HIV-1 genes. Tat influences the growth and metabolism of host cell (Ensoli et al., 1990, 1993) and is important for the efficient reverse transcription of HIV-1 (Harrich et al., 1997). Since HIV Tat proteins have diverse functions in the life cycle of HIV, it is an important and attractive target in efforts to develop weapons against HIV. Among various RNA-based strategies against HIV infection, the decoy strategy has a potential advantage over the use of other RNA inhibitors, such as short antisense RNAs and ribozymes, because the generation of escape mutants might be less frequent: alterations in Rev or Tat (HIV-1 protein) that prevent binding to a decoy would also prevent binding to native elements (such as RRE, Rev responsive element, and TAR sequences). Although decoys might act as much more efficient inhibitors, decoys might potentially be toxic to cells if they were to sequester cellular factors, in particular when the decoy RNA happens to include regions that can interact with cellular proteins. For example, cyclin T1 has been isolated that binds to Tat protein (Wei et al., 1998). The cyclin T1 binds to loop sequences of TAR RNA and Tat protein binds to the bulge residues of TAR RNA, in the cyclin T1-Tat-TAR ternary complex. Thus, cyclin T1 interaction with Tat promotes cooperative binding to the TAR RNA (Garber et al., 1998). I demonstrated, in Chapter 2, that authentic TAR-1 RNA inhibits transcription *in vitro* from CMV early promoter that is not related to the Tat/TAR interaction (Yamamoto et al., 1997). Since authentic TAR RNA of HIV-1 interacts with several cellular factors within the cell, in addition to inhibiting the transcription of unrelated genes, an authentic TAR RNA might not be the most suitable antagonist and specific inhibitor of Tat.

Thus, I used *in vitro* genetic selection method and isolated higher affinity aptamer, RNA^{Tat}, from the novel source of nucleic acids.

The selected aptamer was analyzed for its effect on inhibition of general transcription in cell free transcription assay. The selected aptamer did not effect the general transcription as oppose to the TAR-1 RNA (Yamamoto et al., 1998). In this study, I show that the selected aptamer recognizes Tat protein efficiently even in the presence of excess amount (100-fold) of authentic TAR RNA. Their interaction represents one of the highest affinities achieved, thus far reported for the RNA binding proteins. Moreover, the aptamer inhibited the Tat-dependent *trans*-activation of transcription both *in vitro* and *in vivo*.

Materials and Methods

Synthesis of Tat peptides and RNAs and 5'-end-labeling of RNA

Tat-1-derived peptide, CQ (37-72 aa), and Tat-2-derived peptide, CP (66-97 aa), were prepared as mentioned in Chapter 2. The amino acid composition of synthetic peptides after synthesis was confirmed by reverse phase HPLC analysis.

RNA^{Tat} is synthesized chemically on RNA/DNA synthesizer (Applied Biosystems Model 394) using Phosphoroamides from Glen research (Glen Corporation, USA). The synthesized RNA was deprotected and purified as mentioned before (Yamamoto, et al., 1998). The full length TAR-1 RNA as well as TAR-2 RNA (123 nts) were synthesized enzymatically by transcription *in vitro* using synthetic DNA template as mentioned before (Yamamoto et al., 1997). RNAs that are synthesized chemically are labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase and whereas for TAR-1 RNA synthesized by enzymatically first and dephosphorylated followed by the kinase reaction as mentioned above.

Competitive gel-shift binding assay

The purified TAR-1 RNA and RNA^{Tat} were denatured at 92 °C for 2 min and allowed to equilibrate at ambient temperature before the binding reactions. Both 5'-end labeled RNA^{Tat} and TAR-1 RNA were mixed at one to one ratio (each 15, 000 cpm) followed by the addition of 0.1 μM of unlabeled RNA^{Tat} and various amount of unlabeled TAR-1 RNA (0.1-10.0 μM). To this reaction mixture 0.1 μM of Tat-1 protein was added and equilibrated for 1 hour at 30 °C. Both free and complexed RNAs were separated on 15% non-denaturing polyacrylamide gel and analyzed. RNA^{Tat} was also analyzed in the presence of 0.1-4.0 μM of

TAR-2 RNA containing 100 nM of Tat-2 derived peptide, CP. This analysis was performed essentially under similar condition that used in Tat-1 except that labeled TAR-2 was not added initially.

The data points from the above two experiments were fit to the equation as mentioned below and calculated the EC_{50} values using Graphpad Prism program.

$Y = Bottom + (Top - Bottom)/(1+10^{X-logEC_{50}})$

Top and bottom, the Y values at the top and bottom plateau of the curve.

Transcription assay *in vitro* in the presence of RNA^{Tat} using CMV early promoter

In order to determine whether the isolated aptamer RNA^{Tat} could interfere or not with transcription of unrelated templates, I performed transcription assays *in vitro* with extracts of HeLa cell nuclei by using CMV DNA as the template as described above for studies of TAR-1 and its variants.

Transcription assay *in vitro* in the presence of RNA^{Tat} using LTR promoter

To analyze whether the selected RNA^{Tat} inhibit the Tat-1 mediated *trans*-activation, cell free transcription assay was used in the nuclear extracts of HeLa cells (Promega). For this, HIV-1 LTR was used as a template instead of CMV early promoter as mentioned previously. HIV-1 LTR DNA template (851 nts) was generated by PCR amplification from the plasmid containing full length HIV-1 LTR and generates RNA product (692 nts) when transcribed *in vitro*. Initially, the RNA^{Tat} and TAR-1 RNA were denatured in H_2O, independently, at 94 °C for 5 min and equilibrated at ambient temperature. To allow complex formation between the Tat-1 protein and RNA^{Tat} or TAR-1 RNA, RNA samples

were equilibrated either in the presence or absence of Tat-1 protein (0.2 μg) on ice in reaction buffer (as used before). To this, another mixture containing reaction buffer, $MgCl_2$, template DNA, RNase inhibitor, [α-^{32}P]CTP and nuclear extract was added as mentioned before (Yamamoto et al., 1997). Transcription reaction was carried out for 40 min at 37 °C and terminated by the addition of stop solution and the newly transcribed RNA was isolated from the reaction mixture using the procedure reported (Yamamoto et al., 1997).

Analysis of aptamer RNA^{Tat} and TAR-1 RNA *in vivo* using a reporter system in HeLa cells

Construction of expression vectors: For the expression of both TAR-1 RNA and RNA^{Tat} transcripts, U6 promoter-containing plasmids were constructed. A set of primers containing an *Eco* T14 I site and either the TAR/*Xba* I or RNA^{Tat}/*Xba* I fragment was inserted into vector, pU6-V. The details of construction of pU6-V were described previously (Koseki et al., 1998). The integrity of secondary structures of TAR-1 RNA and RNA^{Tat} transcribed from the expression vectors were initially evaluated by Zuker method (1989). Transcription from the vectors produced either TAR-1 RNA or RNA^{Tat} flanked by a small U6 RNA sequence (23 nts) and UUUUU (terminating signal extra sequences) at the 5'- and the 3'-end, respectively. Construction of the Tat-expression vector pADβ-tat was reported by Koseki et al. (1998). In the vector pADβ-tat, expression of Tat is under the late promoter of adenovirus 2.

Inhibition assay: In these studies, previously established HeLa cells that were stable transfected with a chimeric HIV-1 LTR-Luc gene (Koseki et al., 1998). Initially, about 3 x 10^5 cells were distributed into six-well plates and incubated for 24 h at 37 °C in an atmosphere of 5% CO_2 in air. Cells were transfected with 2.0 μg of decoy plasmid (either pU6-TAR-1

or pU6-RNA^{Tat}), 0.5 μg of Tat-expressing plasmid (pADβ-tat) and 0.5 μg of β-galactosidase-expressing plasmid using 6 μl of TransIT-LT1 reagent (Pan Vera, USA) and incubated further 4 h at 37 °C. Luciferase activity of each sample was then estimated as described previously (Koseki et al., 1998). For controls, a plasmid with the U6 promoter (pU6-V) was used for co-transfection with 0.5 μg of Tat-expressing plasmid (pADβ-tat).

Results

Comparison of the relative affinities of TAR-1 and RNA^{Tat} for Tat-1 protein

To block interactions between two molecules by an effector molecule as an effective inhibitor, that competes with the native molecule (here TAR RNA) for binding to the receptor (Tat), one needs to evaluate the binding ability of an effector (in our case the RNA^{Tat}) molecule in the presence of authentic molecule (TAR RNA). A good inhibitory molecule (effector) should interact with the receptor even in the presence of an excess amount of native molecule for example, TAR RNA. To compare directly interaction between the RNA^{Tat} and the Tat-1 protein in the presence of native TAR-1 RNA, I performed a competitive binding assay. For this study, the RNA^{Tat} and Tat-1 protein were initially allowed to bind followed by equilibrating the complex with authentic TAR-1 RNA at various levels (1:1 to 1:100 molar ratio). Initially, the amount of complex formed by the RNA^{Tat} and Tat-1 protein at 1:1 molar ratio was established as 100% complex in the absence of TAR-1 RNA. Upon formation of this complex, various level (ranging from 0.1-10.0 μM) of unlabeled TAR-1 RNA was added to the mixture and incubated further. The free and complexed RNAs^{Tat} were separated on non-denaturing polyacrylamide gel. As the TAR-1 RNA concentration increases the level of complex formed by the RNA^{Tat} was reduced (Fig. 20A). Data collected at various concentrations of TAR-1 RNA were fit to the equation as mentioned in materials and methods and calculated the value of EC_{50} (effector concentration). The EC_{50} calculated for the TAR-1 RNA was about 10 μM, where the amount of the RNA^{Tat}-Tat-1 complex was reduced to half (Fig. 20B). Thus, in agreement with the previously mentioned kinetic binding studies, the effector RNA (RNA^{Tat}) interaction

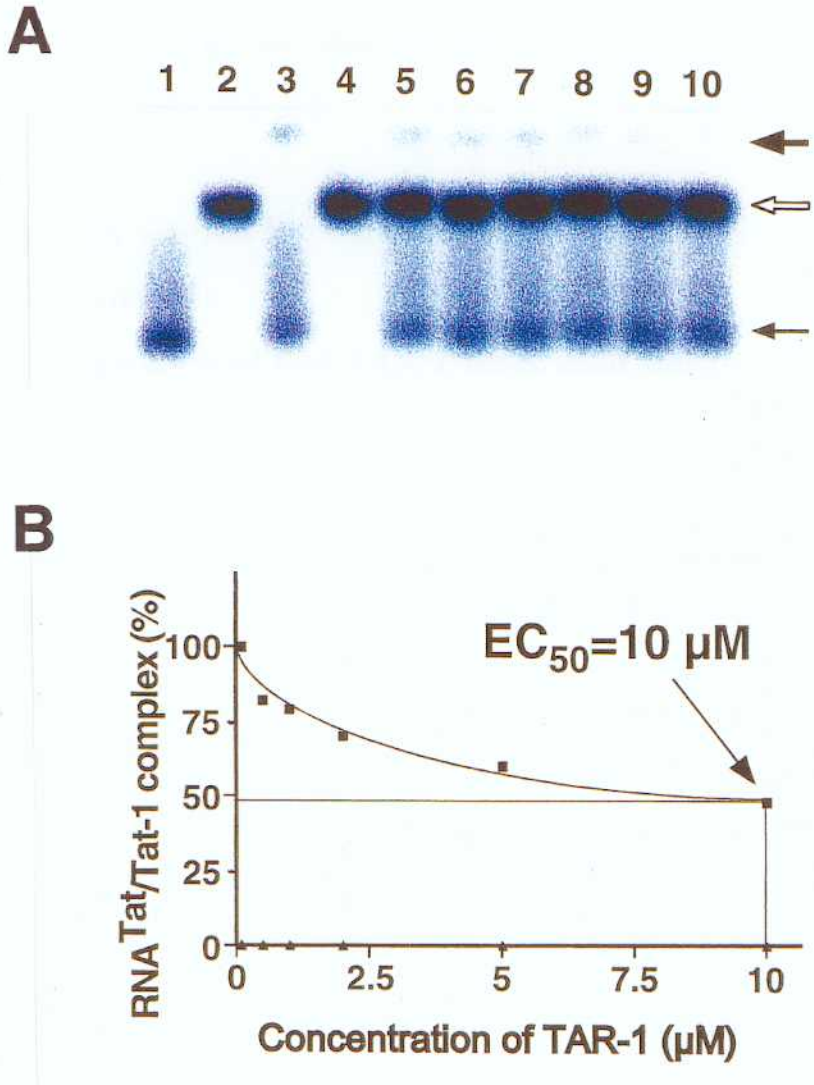

Figure 20. Competitive gel-shift binding assay with TAR-1 RNA for binding to Tat protein of HIV-1. A, Representative autoradiogram of competitive gel-shift binding assay with TAR-1 for binding to 0.1 μM Tat-1. Lane 1, labeled RNA^{Tat} and unlabeled RNA^{Tat} (0.1 μM); lane 2, labeled TAR-1 and unlabeled TAR-1 (0.1 μM); lane 3, labeled RNA^{Tat}, 0.1 μM unlabeled RNA^{Tat}, and Tat-1; lane 4, labeled TAR-1, 0.1 μM unlabeled TAR-1 and Tat-1; lanes 5-10, labeled RNA^{Tat}, 0.1 μM unlabeled RNA^{Tat}, increasing amount of unlabeled TAR-1 (0.1, 0.5, 1, 2, 5, and 10 μM) and Tat-1. Thin and out-lined arrows indicate the position of free RNA^{Tat} and TAR-1, respectively. RNA^{Tat}-Tat-1 complex is indicated by bold arrow. B, Competitive binding curve is showing the effector concentration (EC_{50}, 10 μM).

efficiently takes place even in the presence of large excess of TAR-1 RNA (about 100-fold). Similar study was performed with TAR-1 RNA and RNA^{Tat} in the presence of RE peptide (Yamamoto et al., 1998). The EC_{50} calculated for the TAR-1 RNA was about 3.2 μM against 40 nM of RNA^{Tat}, suggesting that obtained data from this study with Tat-1 protein is close to the data with RE.

Comparison of the relative affinities of TAR-2 and RNA^{Tat} for Tat-2 peptide

Earlier studies showed that TAR-1 RNA itself function as an effector (or decoy) when expressed in HIV-1 infected cell-lines, either in single or multiple RNA (Lisziewicz et al., 1991, 1993). It showed previously that the TAR-2 can bind to the Tat-1 protein and vice versa (Guyader et al., 1987; Elangovan et al., 1992). Moreover, *in vitro* and *in vivo* studies on TAR-2 RNA and Tat-2 suggest that their interactions are efficient compared to the TAR-1 RNA binding to the Tat-2 protein (Emerman et al., 1987; Rhim and Rice, 1993). It is therefore, possible that the TAR-1 RNA could not be function as an effector molecule for the HIV-2 infection or co-infection cases. The TAR-2 has two TAR-1-like motifs (Fig. 2A) and these two TAR-1 like regions are found to be important for the *trans*-activation as well as for efficient binding to the Tat-2 (Garcia-Martinez et al., 1995). Although, the RNA^{Tat} was originally selected against the Tat-1 protein, I found that RNA^{Tat} binds to Tat-2-derived peptide (CP) efficiently, even though the efficiency is not as much as to Tat-1-derived peptides. As performed in the case of TAR-1 studies, I performed competitive binding assay using Tat-2-derived peptide (Fig. 21A). The EC_{50} value for the RNA^{Tat} (100 nM)-CP complex in the presence of TAR-2 RNA was found to be 4 μM, suggesting about 40-fold higher affinity to the CP by the RNA^{Tat} (Fig. 21B).

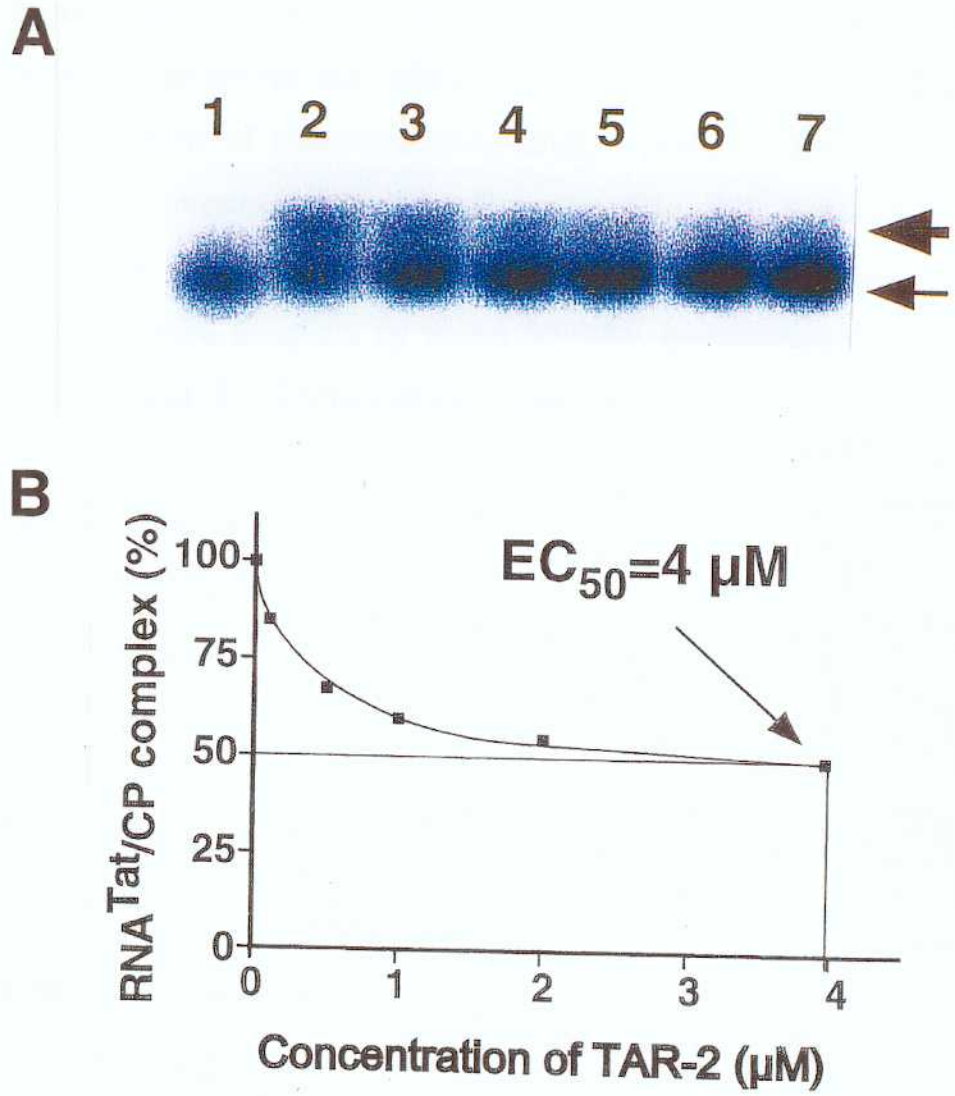

Figure 21. Competitive gel-shift assay with TAR-2 RNA for binding to the Tat-2 peptide, CP. A, Representative autoradiogram of competitive gel-shift assay with TAR-2 for binding to CP. Unlabeled RNA^{Tat} (0.1 μM) and labeled RNA^{Tat} were mixed and denatured (lane 1) and allowed to form complex in the presence of 0.1 μM CP (lane 2). To this, increasing amount of unlabeled TAR-2 was added (lane 3, 0.1 μM; lane 4, 0.5 μM; lane 5, 1.0 μM; lane 6, 2 μM; lane 7, 4 μM) as a competitor. Thin and bold arrows indicate the position of free and complexed $RNAs^{Tat}$ with CP, respectively. B, Competitive binding curve is showing the EC_{50} (4 μM).

Effect of aptamer RNA^{Tat} on the CMV-template transcription in a cell-free transcription assay

As demonstrated in Chapter 2, authentic TAR-1 RNA inhibited the transcription of the CMV template in transcription assay *in vitro*. In order to examine the effect of the isolated aptamer (RNA^{Tat}) on transcription of unrelated templates, I performed further transcription assays in extracts of HeLa cell nuclei (Fig. 22). Addition of exogenous authentic TAR-1 RNA (100 pmol) inhibited the transcription of the CMV-derived template by about 50-60%, as mentioned above (Fig. 22A, lanes 3 and 4). Transcription from the CMV promoter in the absence (Fig. 22A, lane 1) or in the presence (Fig. 22A, lane 2) of 100 pmol of tRNA (total tRNA from yeast), and in the presence of 100 pmol of RNA^{Tat} (Fig. 22A, lane 5) was unaffected or only marginally affected. Quantification of the results of three independent transcription experiments revealed that only TAR RNA inhibited transcription to a significant extent (Fig. 22B).

Effect of aptamer RNA^{Tat} on the Tat-dependent transcription in cell free transcription assay

TAR-1 RNA appears to have some inhibitory effect on the general transcription (Yamamoto et al., 1997). To test directly whether the isolated RNA^{Tat} inhibit the Tat-dependent *trans*-activation in nuclear extracts of HeLa cells, I carried out an assay similar to that described previously (Yamamoto et al., 1997). In the present assay, I used luciferase gene under the control of the HIV-1 promoter (nt -159 to +692) as the reporter gene. *In vitro* transcription assay was carried out in the presence of 20-200 pmol of TAR-1 RNA or RNA^{Tat} and all samples contained similar amount of Tat-1 protein (20 pmol) and DNA template. Representative results are shown in Figure 23A. In the control assay,

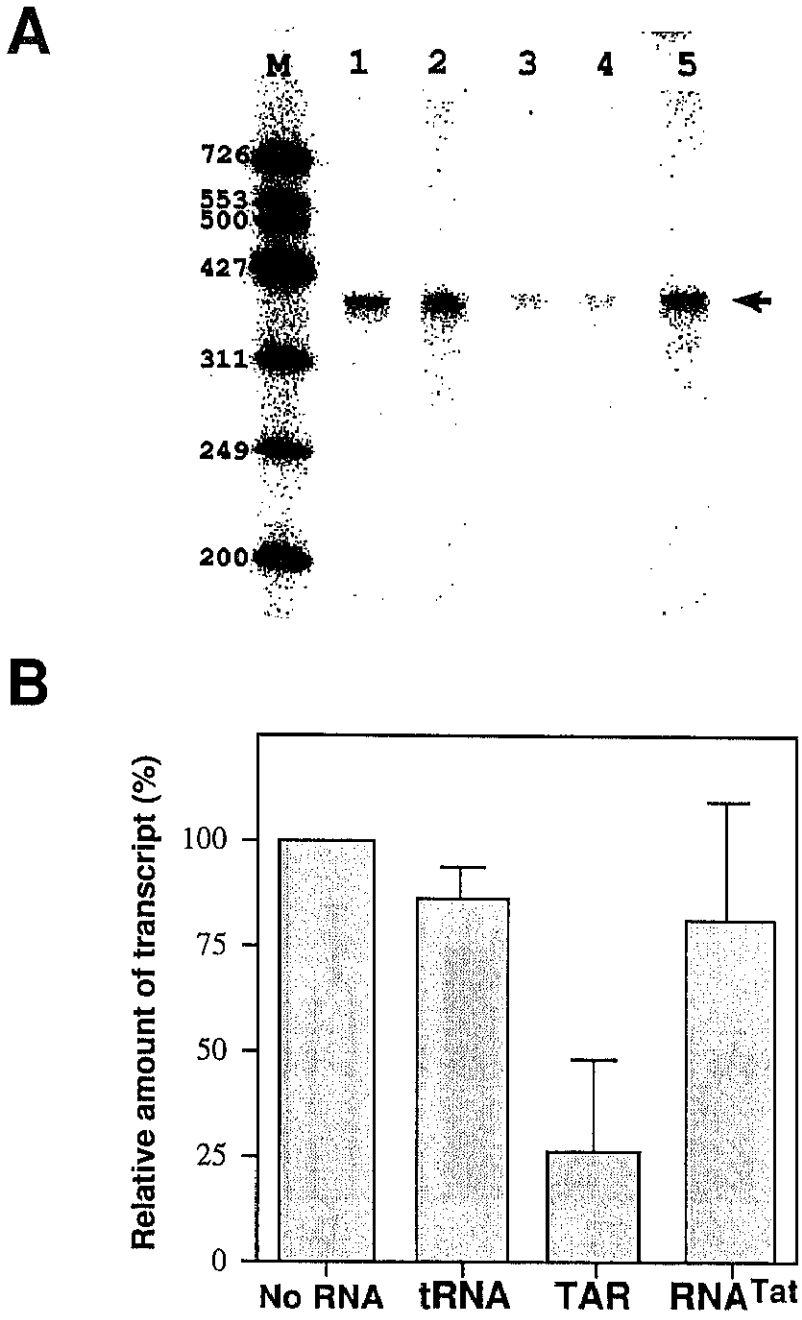

Figure 22. Inhibition of transcription from a CMV early promoter-driven template by RNA^{Tat} and TAR-1 RNA in an extract of HeLa nuclei. A, The template containing the early promoter of CMV was transcribed in the absence (lane 1) and in the presence (lanes 3 and 4) of 100 pmol of TAR-1 RNA, in the presence of 100 pmol of tRNA (total tRNA from yeast; lane 2), or in the presence of 100 pmol of RNA^{Tat} (lane 5). Single-stranded DNA markers were loaded in lane M. The newly synthesized transcript is indicated by an arrow. B, The relative level of transcript (364 nts) synthesized *in vitro* was quantitated in three independent experiments (experimental variations are indicated by error bars).

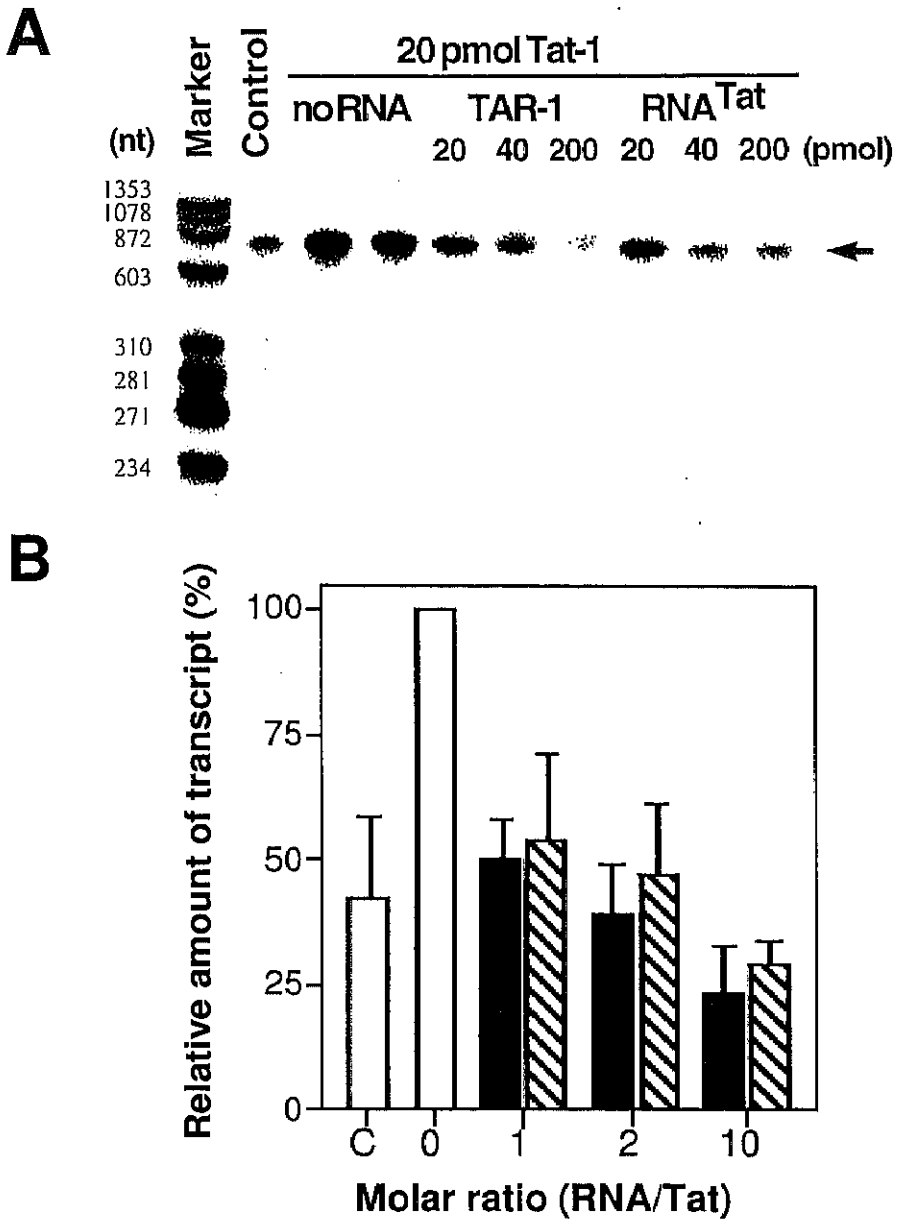

Figure 23. Inhibition of Tat-dependent *trans*-activation of transcription by the RNA^{Tat} *in vitro*. A, Inhibition of transcription from a HIV-1 LTR promoter-driven template by TAR-1 RNA and RNA^{Tat} in a HeLa nuclear extract. The template containing LTR that generates 692 nts upon transcription was transcribed in HeLa nuclear extracts in the absence (Control) or in the presence (no RNA) of Tat-1 (20 pmol). Similar reactions that contains Tat-1 protein were also performed in the presence of increasing amounts of exogenous TAR-1 (20-200 pmol) and exogenous RNA^{Tat} (20-200 pmol). The arrow indicates the newly synthesized transcript. B, The relative level of transcripts synthesized was quantified in four independent experiments (experimental variations are indicated by the error bars). Filled and hatched bars are for TAR-1 and the RNA^{Tat}, respectively.

transcription was allowed to proceed in the absence of Tat protein. The amount of transcript synthesized was about 2-3 times lower than that in the presence of Tat-1 protein (Fig. 23A). In the presence of 20 pmol TAR-1 RNA or RNA^{Tat}, the level of the transcript decreased to the level observed in the absence of Tat-dependent *trans*-activation (Figs. 23A and 23B). The amount of transcript decreased with increases in the concentration of RNA, irrespective of whether it was TAR-1 RNA or RNA^{Tat} (Figs. 23A and 23B). The extent of inhibition by TAR-1 RNA and RNA^{Tat} was nearly identical. These results indicated that both RNA^{Tat} and TAR-1 RNA (inhibitor) efficiently blocked the synthesis of RNA transcript (692 nts).

Evaluation of TAR RNA and RNA^{Tat} as decoy to sequester the Tat-1 in mammalian cells

In order to test directly the ability of RNA^{Tat} to act as a decoy, I used a model system that was developed in our laboratory to examine potential inhibitory effect (Koseki et al., 1998). To obtain more reproducible data, I used only LTR-Luc-transduced cells in the present studies. The transduced HeLa cells genome encodes additionally LTR and Luc genes (LTR derived from HIV-1 and a gene for luciferase). Since two genes were fused, it is therefore, the production of luciferase in the transduced cells depends upon the LTR promoter. The authentic TAR-1 RNA sequence is located in the LTR and binds to Tat-1 protein. Tat protein of HIV-1 stimulates transcription significantly upon binding to TAR-1 RNA and the transcriptional *trans*-activation by the Tat can be monitored directly in terms of the activity of the luciferase produced by the cultured cells (Fig. 24A). *Trans*-activation by Tat protein occurs in the nucleus and, thus, it is important that inhibitory molecules must be expressed in the nucleus. Therefore, I used the U6 promoter in this analysis (Koseki et

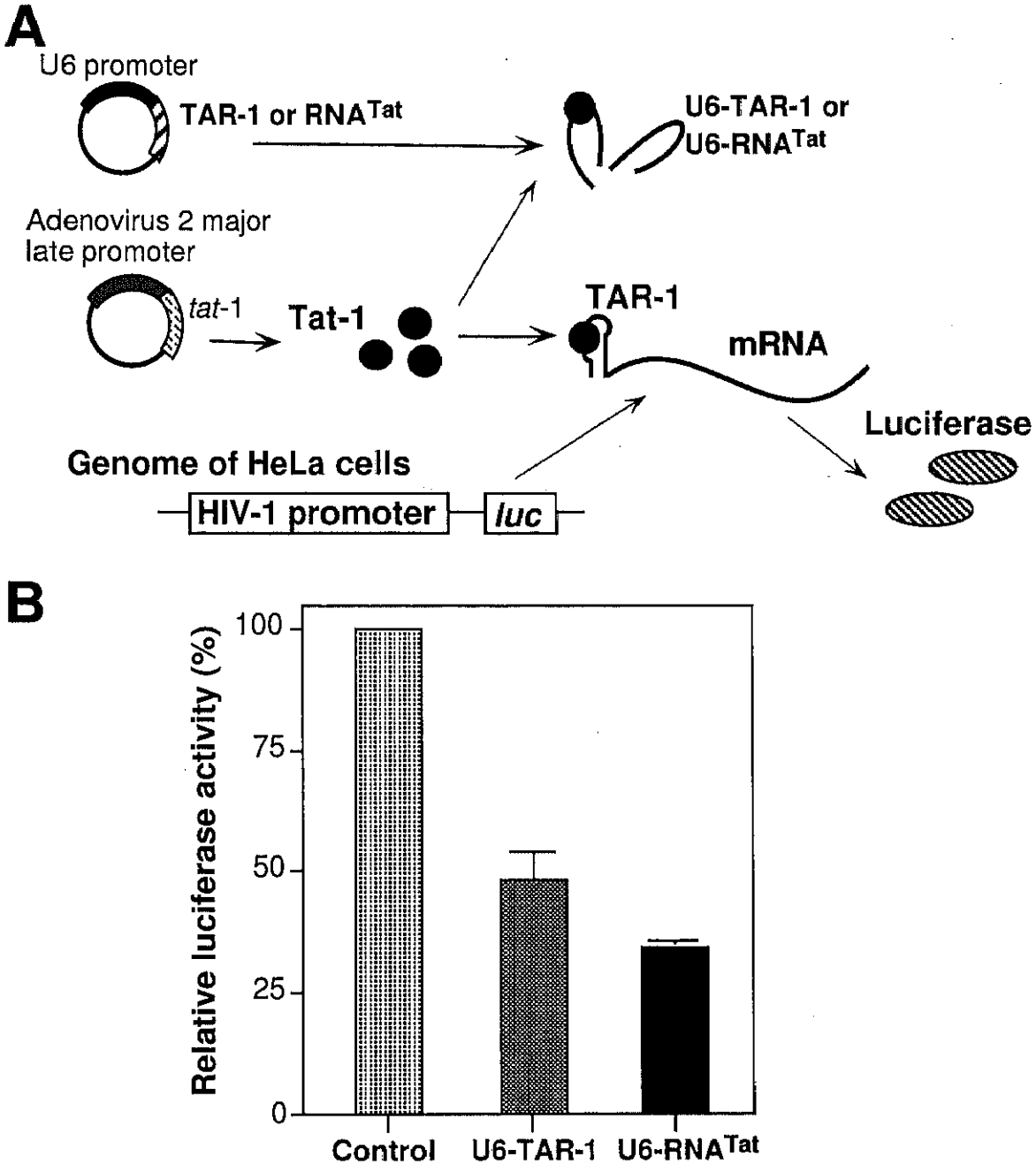

Figure 24. Inhibition of Tat-dependent *trans*-activation of transcription by the RNA^{Tat} *in vivo*. A, Schematic representation of *in vivo* assay system. Upon transfection of Tat-1 plasmid (pADβ) alone to LTR-Luc transduced cells, elongation is stimulated by the interaction of Tat-1 and TAR-1, thus resulting enhanced production of luciferase. When pU6-TAR-1 or pU6-RNA^{Tat} was co-transfected with pADβ, Tat produced should be trapped by the TAR-1 or RNA^{Tat}, resulting lower level of luciferase. B, Effect of endogenous expression of TAR-1 and RNA^{Tat} on the luciferase activity in HeLa cells expressing chimeric LTR-Luc gene. The relative level of luciferase was quantified from three independent experiments (experimental variations are indicated by error bars).

al., 1998). The Tat-expressing vector (pADβ-tat) was used either with pU6-TAR-1 or with pU6-RNA^{Tat} plasmids that generate, respectively, U6-driven TAR-1 and RNA^{Tat}, to co-transfect LTR-Luc-transduced cells, and luciferase activity was monitored. The amount of luciferase produced was inhibited by 50-70% of the control level after transfection with decoy vectors (Fig. 24B). The data clearly suggest that both TAR-1 RNA and RNA^{Tat} inhibited the Tat-mediated *trans*-activation of transcription. Similar inhibition was observed previously with TAR-1 RNA as the decoy (Lisziewicz et al., 1993).

Discussion

To block Tat/TAR interaction by the isolated aptamer RNA^{Tat} as an effective inhibitor, that competes with TAR RNA for binding to Tat, the binding ability of RNA^{Tat} needs to evaluate in the presence of authentic TAR RNA. Competitive binding assays suggested that RNA^{Tat} has strong affinity to the Tat even in the presence of a large excess of TAR RNAs of HIV-1 and HIV-2. Similar study was performed with TAR-1 RNA and RNA^{Tat} in the presence of Tat-1-derived peptide RE (Yamamoto et al., 1998). The EC_{50} calculated for the RE was close to the value of RNA^{Tat} with Tat-1 protein, suggesting that the binding ability of Tat-derived peptides containing the basic region is similar to that of full-length Tat protein.

To examine whether RNA^{Tat} affects transcription of unrelated templates, I performed transcription assays in extracts of HeLa cell nuclei. Although addition of exogenous authentic TAR-1 RNA inhibited transcription of the CMV-derived template, addition of exogenous RNA^{Tat} did not affect the transcription. The absence of inhibitory effects on general transcription by RNA^{Tat}, as compared to the inhibition by authentic TAR RNA, makes the aptamer RNA^{Tat} an attractive molecule for further analysis as a Tat decoy in infections by HIV-1 and/or HIV-2. Thus, I tested directly whether RNA^{Tat} inhibits the Tat-dependent *trans*-activation in nuclear extracts of HeLa cells with the LTR-Luc template. The results suggested that both RNA^{Tat} and TAR-1 RNA (as an inhibitor) efficiently blocked the synthesis of RNA transcript.

Since above data clearly suggest that RNA^{Tat} inhibits the Tat-mediated *trans*-activation of transcription without interaction to cellular factors (for example, cyclin T1) *in vitro*, I tested the ability of RNA^{Tat} to act as a decoy *in vivo*. I used a model system that was developed in our laboratory to examine potential inhibitory effect of decoy RNA and U6

promoter to express RNA^{Tat} in the nucleus because *trans*-activation by Tat protein occurs in the nucleus. The amount of luciferase that produced from LTR promoter was reduced to 30-50% of the control level after transfection with decoy vectors. Since RNA^{Tat} did not affect general transcription but specifically inhibited Tat-dependent *trans*-activation although the levels of the inhibition by either TAR RNA or RNA^{Tat} were similar, RNA^{Tat} has potential as a tool for gene therapy against HIV infection. The next challenge is to studies with mammalian cells transfected by live virus.