Controlling Human Immunodeficiency Virus Type 1 Gene Expression by Unnatural Peptides†

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ABSTRACT: Small unnatural peptides that target specific RNA structures have the potential to control biological processes. RNA−protein interactions are important in many cellular functions, including transcription, RNA splicing, and translation. One example of such interactions is the mechanism of *trans*-activation of human immunodeficiency virus type 1 (HIV-1) gene expression that requires the interaction of Tat protein with the *trans*-activation responsive region (TAR) RNA, a 59-base stem−loop structure located at the 5′-end of all nascent HIV-1 transcripts. We report here a synthetic peptide derived from Tat sequence (37−72), containing all D-amino acids, that binds in the major groove of TAR RNA and interferes with transcriptional activation by Tat protein in vitro and in HeLa cells. Our results indicate that unnatural peptides can inhibit the transcription of specific genes regulated by RNA−protein interactions.

Three approaches for the development of synthetic molecules to control transcriptional regulation have been reported (*1*−*5*). The first two approaches rely upon oligodeoxynucleotides that form triplex structures in the major groove of double-helical DNA (*2, 6*) and carbohydrate-based ligands that interfere with transcription factor function (*3, 4*). Triple-helix oligodeoxynucleotides and oligosaccharides cannot recognize a broad range of DNA sequences and suffer from cellular uptake problems. Recently, pyrrole−imidazole polyamides have been shown as a new class of small molecules that can inhibit 5S RNA gene expression in *Xenopus* kidney cells (*5*). These synthetic transcriptional antagonists are designed to target DNA sequences and therefore cannot regulate gene expression that is controlled by RNA−protein interactions.

Trans-activation of HIV-1[1] gene expression is regulated at the level of RNA−protein interactions. The promoter of HIV-1, located in the U3 region of the viral long terminal repeat (LTR), is an inducible promoter that can be stimulated by the *trans*-activator protein Tat (*7*). As in other lentiviruses, Tat protein is essential for *trans*-activation of viral gene expression (*8*−*12*). HIV-1 Tat is an 86−102 amino acid protein with varying C-terminus depending upon the type of isolate. Only the first 72 residues are required for biological activity and these can be divided into five regions (Figure 1A). A basic domain (amino acids 48−59) in Tat forms the Tat nuclear/nucleolar localization signal (*13, 14*), and this domain is also essential for TAR RNA binding in vivo and sufficient for TAR recognition in vitro (*15*−*17*). The activation or cofactor binding domain of Tat contains the core and cysteine-rich elements combined with less well-defined sequences located toward the N-terminus of the protein (*7, 17, 18*). Tat protein interacts with the *trans*-activation responsive region (TAR) RNA, a 59-base stem−loop structure located at the 5′-end of all nascent HIV-1 transcripts (*7*). Subsequent deletion studies have established that the region from +19 to +42 incorporates the minimal domain that is both necessary and sufficient for Tat responsiveness in vivo (*19*−*21*). TAR RNA contains a six-nucleotide loop and a three-nucleotide pyrimidine bulge that separates two helical stem regions (Figure 1B). The trinucleotide bulge containing residues U23, C24, and U25 is essential for high affinity and specific binding of the Tat protein (*7*).

Syntheses of two D-enantiomeric proteins demonstrated that these proteins have optical properties, substrate specificity (*22*), and a structure (*23, 24*) that mirrors those of the naturally occurring L-proteins. A powerful method to identify D-peptide ligands through mirror-image phage display has been recently discovered and a cyclic D-peptide was identified that interacts with the Src homology 3 domain of c-Src (*25*). Due to the difference in chirality, D-peptides are resistant to proteolytic degradation and cannot be efficiently processed for major histocompatibility complex class II-restricted presentation to T helper cells (T_H cells). Consequently, D-peptides would not induce a vigorous humoral immune response that impairs the activity of L-peptide drugs (*26*). The D-peptide ligands may provide useful starting points for the design or selection of novel drugs.

Tat-derived peptides containing the arginine-rich RNA-binding domain of Tat are able to form in vitro complexes with TAR RNA (*16, 27*−*32*). Structural studies of Tat protein show that the RNA-binding domain is not a rigid helix (*33*). We reasoned that since the RNA-binding domain of Tat has a flexible structure, symmetry of small peptides containing arginine-rich sequences may not be crucial in TAR RNA

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[1] Abbreviations: HIV-1, human immunodeficiency virus type 1; TAR, *trans*-activation responsive region; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase.

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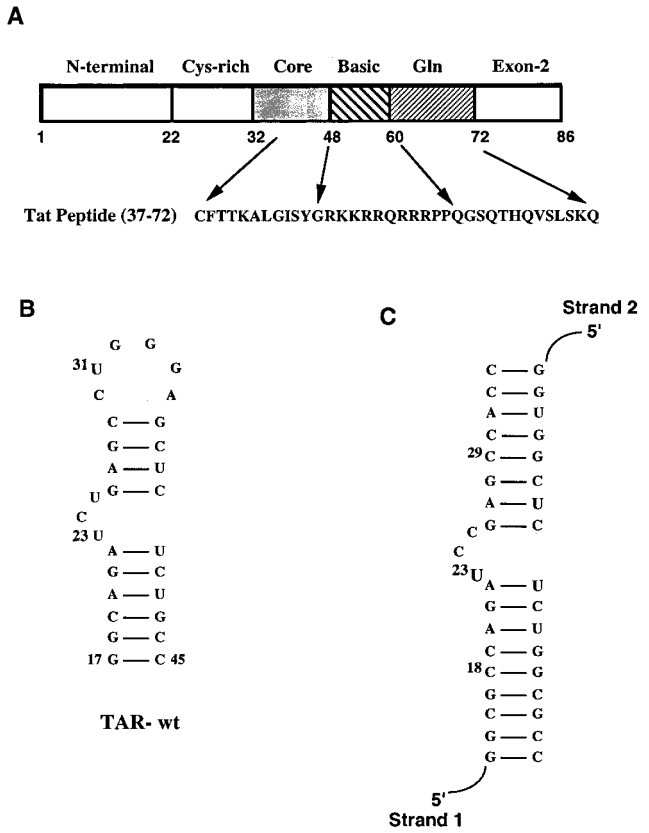

FIGURE 1: (A) Regions of the HIV-1 Tat protein and sequence of the Tat peptide (37−72). (B) Secondary structure of wild-type TAR RNA used in this study. Wild-type TAR RNA spans the minimal sequences that are required for Tat responsiveness in vivo (*19*) and for in vitro binding of Tat-derived peptides (*27*). Wild-type TAR contains two non-wild-type base pairs to increase transcription by T7 RNA polymerase (*45*). (C) Secondary structure of the designed duplex TAR RNA used in photo-cross-linking experiments (*41*). Duplex RNA contains the nucleotides C18−C29 and G36−G44 from the wild-type TAR RNA sequence and extra flanking base pairs to enhance hybridization of the two RNA strands. Uridine 23 was substituted with 4-thiouridine during T7 polymerase transcription reactions. Since substitution of UCU bulge sequence with UCC does not interfere with TAR RNA structure and function (*27*, *32*, *46*), we replaced U25 with C25 in strand 1 to incorporate 4-thioU at a single site by enzymatic methods. Numbering of nucleotides in the duplex TAR RNA corresponds to their positions in wild-type TAR RNA.

recognition. Two classes of unnatural peptides containing the RNA-binding domain of Tat have been shown to specifically recognize TAR RNA in vitro (*34*, *35*). An RNA-binding unnatural biopolymer consisting of chiral aminocarbonate monomers linked via a carbamate backbone was synthesized by solid-phase methods (*36*) and tested for TAR RNA binding. This Tat-derived unnatural biopolymer can specifically bind TAR RNA with high affinities (*34*). The second class of unnatural RNA-binding molecules consists of D-peptides. A Tat peptide, Tat(37−72), containing all D-amino acids was able to specifically bind TAR RNA and failed to bind mutant TAR RNAs (*35*). Measurements of equilibrium dissociation constants show that the D-Tat peptide binds TAR RNA with a K_D of 0.22 ± 0.05 μM, whereas under similar experimental conditions, L-Tat(37−72) binds TAR RNA with a K_D of 0.13 ± 0.08 μM (*35*). Since D-peptide ligands are resistant to degradation by naturally occurring enzymes, they can be applied to control protein−nucleic acid interactions in vivo. Here we show that a synthetic peptide derived from Tat sequence (37−72), containing all D-amino acids, that binds in the major groove of TAR RNA and interferes with transcriptional activation by Tat protein in vitro and in HeLa cells.

EXPERIMENTAL PROCEDURES

Buffers. All buffer pH values refer to measurements at room temperature. *TK buffer*: 50 mM Tris-HCI (pH 7.4), 20 mM KCI, and 0.1% Triton X-100. *Transcription buffer:* 40 mM Tris-HCI (pH 8.1), 1 mM spermidine, 0.01% Triton X-100, and 5 mM DTT. *TBE buffer*: 45 mM Tris-borate, pH, 8.0, and 1 mM EDTA. *Sample loading buffer:* 9 M urea, 1 mM EDTA, and 0.1% bromophenol blue in 1× TBE buffer. *Binding buffer:* 25 mM Tris-HCI (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, and 0.1% Triton X-100. *Hydrolysis buffer:* 50 mM Na_2CO_3/$NaHCO_3$, pH 9.2. *Elution buffer:* 1× TBE and 10% sodium acetate (3 M), pH 5.5. *Digestion buffer:* 100 mM Tris-HCl (pH 7.8) and 10 mM $CaCl_2$.

Oligonucleotide Synthesis: (A) DNAs. All DNAs were synthesized on an Applied Biosystems ABI 392 DNA/RNA synthesizer. The template strands encode the sequences for the duplex and wild-type TAR RNA (Figure 1). The top strand is a short piece of DNA complementary to the 3′ end of all template DNAs having the sequence 5′-TAATAC-GACTCACTATAG-3′. DNA was deprotected in NH_4OH at 55 °C for 8 h and then dried in a Savant speed-vac. The samples were resuspended in sample loading buffer and were purified on 20% acrylamide/8 M urea denaturing gels, 50 cm × 0.8 mm. Gels were run for 3 h at 30 W until xylene cyanol tracking dye was 5 cm from the bottom of the gel. DNAs were visualized by UV shadowing, excised from the gel, and eluted in 50 mM Tris, 16 mM boric acid, 1 mM EDTA, and 0.5 M sodium acetate. DNAs were ethanol-precipitated and resuspended in DEPC- (diethyl pyrocarbonate-) treated water. Concentration of DNAs was determined by measuring absorbance at 260 nm in a Shimadzu UV spectrophotometer.

(B) RNAs. All RNAs were prepared by in vitro transcription (*37*). The template strand of DNA was annealed to an equimolar amount of top-strand DNA and transcriptions were carried out in transcription buffer and 4.0 mM NTPs at 37 °C for 2−4 h. For reactions (20 μL) containing 8.0 pmol of template DNA, 40−60 units of T7 polymerase (Promega) was used. For the synthesis of 4-thioU-labeled RNA, UTP was replaced with 4-thio-UTP (4 mM final concentration) in the transcription buffer. 4-thio-UTP was synthesized according to the method of Stade et al. (*38*). Transcription reactions were stopped by adding an equal volume of sample loading buffer. RNA was purified on 20% acrylamide/8 M urea denaturing gels as described above. RNAs were stored in DEPC water at −20 °C.

RNAs were 5′-dephosphorylated by incubation with calf intestinal alkaline phosphatase (Promega) for 1 h at 37 °C in 50 mM Tris-HCl, pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1 mM spermidine. The RNAs were purified by multiple extractions with Tris-saturated phenol and one extraction with 24:1 chloroform−isoamyl alcohol followed by ethanol precipitation. The RNAs were 5′-end-labeled with 0.5 μM [γ-^{32}P]ATP (6000 Ci/mmol) (ICN)/100 pmol of RNA by incubating with 16 units of T4 polynucleotide kinase (New England Biolabs) in 70 mM Tris-HCl, pH 7.5, 10 mM

$MgCl_2$, and 5 mM DTT (*39*). RNAs were gel-purified on a denaturing gel, visualized by autoradiography, and recovered from gels as described above.

Peptide Synthesis. All D-Fmoc-amino acids were purchased from Bachem. Other peptide synthesis reagents including piperidine, 4-(dimethylamino)pyridine, dichloromethane, *N*,*N*-dimethylforamide, 1-hydroxybenzotriazole (HOBT), 2-(1*H*-benzotriazo-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), diisopropylethylamine, and 4-(hydroxymethyl)phenoxy- (HMP-) linked polystyrene resin were obtained from Applied Biosystems Division, Perkin-Elmer. Trifluoroacetic acid, 1,2-ethanedithiol, phenol, and thioanisole were from Sigma. Tat-derived peptides containing all D-amino acids (37−72 and 44−72 with Ala in place of Arg at positions 49, 52, 53, 55, 56, and 57) were synthesized on an Applied Biosystems 431A peptide synthesizer by standard FastMoc protocols. Cleavage and deprotection of the peptide was carried out in 2 mL of reagent K for 6 h at room temperature. Reagent K contained 1.75 mL of TFA, 100 μL of thioanisole, 100 μL of water, and 50 μL of ethanedithiol (*40*). After cleavage from the resin, peptide was purified by HPLC on a Zorbax 300 SB-C_8 column. The mass of fully deprotected and purified peptides was confirmed by fast atom bombardment (FAB) mass spectrometry.

Site-Specific Photo-Cross-Linking Reactions. RNA−RNA and RNA−protein photo-cross-linking reactions were performed as described earlier by Wang and Rana (*41*). After irradiation, 2 mL of yeast tRNA (20 mg/mL) and 20 mL of sample loading buffer were added to each sample and the mixture was electrophoresed on 8 M urea−20% polyacrylamide gels.

Preparation of HeLa Cell Nuclear Extract. HeLa S3 cells were grown in a spinner flask [6 L, Dulbecco-modified minimal essential medium (DMEM) supplemented with 10% fetal calf serum] to a density of 5×10^5 cells/mL. The cells were washed twice in PBS by centrifugation at 2000 rpm for 5 min in a GSA rotor (Sorvall). HeLa cell nuclear extracts were prepared according to published procedures (*42, 43*) with the following modifications. All procedures were carried out at 4 °C, unless otherwise stated. HeLa cells were lysed in hypotonic buffer A (20 mM HEPES, 10 mM NaCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.35 M sucrose, and 0.5 mM PMSF, pH 7.9) containing 0.5% Triton X-100 on ice for 5 min. Nuclei were centrifuged at 1000*g* for 5 min, washed 3 times with Triton-free buffer B (same as buffer A, except 0.35 M sucrose) and extracted on ice for 30 min with buffer C (buffer A without sucrose, containing 0.42 M NaCl, and 20% glycerol). The crude extract in buffer C was centrifuged at 25000*g* for 20 min, and the supernatant was dialyzed twice for 5 h against buffer D (buffer A without sucrose, containing 80 mM KCl and 20% glycerol). The dialysate was centrifuged at 25000*g* for 15 min, frozen in liquid nitrogen, and stored at −80 °C.

Inhibition of Tat Trans-Activation in Vitro. The p10SLT plasmid that contains HIV-1 5′ long terminal repeat was used in the cell-free transcription experiments (*44*). Template DNAs were linearized with *Bam*HI to give 530-nucleotide runoff transcripts. Transcription reaction mixtures (25 μL) contained 0.5 μg of template DNA, 12 μL of HeLa cell nulear extract, 50 mM KCl, 6 mM $MgCl_2$, 6 mM sodium citrate, 10 mM Hepes (pH 7.9), 1 mM DTT, 10 mM phosphocreatine, 200 μM CTP, 200 μM ATP, 200 μM UTP, 20 μM GTP, and 10 μCi of [α-^{32}P]GTP (800 Ci/mmol, ICN; final concentration 0.5 μM). Increasing amounts of D-Tat were added to the transcription reactions as indicated in Figure 4. After incubation for 60 min at 30 °C, 175 μL of stop solution (0.3 M Tris-HCl, pH 7.4, 0.3 M sodium acetate, 0.5% SDS, and 2 mM EDTA) was added. The mixture was extracted with 200 μL of phenol−chloroform−isoamyl alcohol (50:48:2) and then with chloroform (200 μL). RNA transcripts were precipitated with ethanol and analyzed on 6% polyacrylamide−7 M urea gels. Efficiencies of *trans*-activation were determined by a PhosphorImage analysis (Molecular Dynamics).

Inhibition of Tat Trans-Activation in Vivo. We used HL3T1 cells, a HeLa cell line derivative containing an integrated HIV-1 LTR promoter and CAT reporter gene. Cells were grown in 2 mL of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum in 60 mm dishes at 37 °C in 5% CO_2 in a tissue culture incubator. Cells were refreshed by 2 mL of DMEM before transfection. Transfection was started by dropwise addition of 2×250 μL HeBS (Hepes-buffered saline) and then keeping at room temperature for 10 min. Approximately 15 μg of plasmids (pSV2Tat and pAL) and increasing amounts of D-Tat were transfected in the presence of $CaCl_2$ (final concentrated 125 mM) and the cells were incubated for 4 h at 37 °C in a tissue culture incubator. The medium was then discarded and the cells were subjected to glycerol (1.5 mL of 15%) shock for 45 s. Finally the cells were washed twice with PBS (5 mL) and were grown in 3 mL of DMEM. The cells were harvested 48 h posttransfection, with changing with fresh DMEM at 24 h, and lysed in reporter lysis buffer (Promega). Aliquots were used for CAT and luciferase assays. Both activities were normalized to protein concentration determined by using a modified Bradford assay (Bio-Rad).

RESULTS AND DISCUSSION

D-Tat Interacts with TAR RNA in the Major Groove. The D-Tat peptide, Tat(37−72), contains the RNA-binding region and part of the core domain of Tat protein (Figure 1A). Since this D-peptide binds TAR RNA specifically and lacks the activation domain of Tat, it can be used to abolish Tat−TAR complex formation and thus inhibition of Tat *trans*-activation. To design unnatural ligands for nucleic acids recognition, it is important to determine whether D-Tat recognizes TAR RNA in a manner similar to Tat.

We examined the mode of RNA−protein interactions in the D-Tat−TAR complex and protease stability of D-Tat peptide by site-specific photo-cross-linking. Recently, we used a site-specific cross-linking method based on 4-thiouracil (4-thio-U) photochemistry to determine the conformation of TAR RNA and its interaction with Tat protein under physiological conditions (*41*). We synthesized a TAR RNA duplex containing 4-thio-U at position 23 and performed photo-cross-linking experiments (Figure 2). Irradiation of the D-Tat−RNA complex yields a new band with electrophoretic mobility less than that of the RNA (Figure 2A, lane 4). Both D-Tat and UV (360 nm) irradiation are required for the formation of this cross-linked RNA-peptide complex (see lanes 3 and 4). Since the cross-linked D-Tat−RNA complex is stable to alkaline pH (9.5), high temperature (85 °C) and

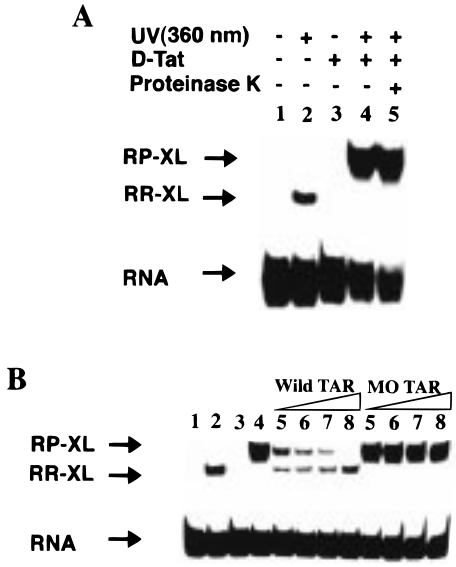

FIGURE 2: (A) Site-specific photo-cross-linking reaction of TAR RNA duplex labeled with 4-thiouracil at position 23 with the D-Tat peptide. Photo-cross-linking experiments were performed as described earlier by Wang and Rana (*41*). The RNA−RNA and RNA−peptide cross-link are indicated by RR-XL and RP-XL, respectively. (B) Specificity of the D-Tat−TAR cross-linking reaction determined by competition assays. RNA−protein complexes were formed between 40 nM ^{32}P-5′-end-labeled TAR RNA duplex modified with 4-thioU at position 23 and 0.4 μM D-Tat in the presence of unlabeled wild-type TAR RNA or mutant TAR RNA (M0) lacking the trinucleotide bulge sequence. Lane 1, duplex RNA without irradiation; lane 2, duplex RNA with irradiation; lane 3, control lane containing RNA−peptide complex without UV irradiation; lane 4, RNA−peptide complex with irradiation; lanes 5−8, RNA−peptide complex irradiated in the presence of increasing concentrations (0.02−1 μM) of unlabeled wild-type TAR or mutant M0 TAR RNA. The RNA−RNA and RNA−peptide cross-link are labeled as RR-XL and RP-XL, respectively.

denaturing conditions (8 M urea, 2% SDS), we conclude that a covalent bond is formed between TAR RNA and the peptide during the cross-linking reaction. To test the protease stability of the D-Tat−RNA complexes, we subjected the D-Tat−RNA cross-link products to vigorous proteinase K digestion (10−20 μg of proteinase K at 55 °C for 15 min), which showed that the complexes were completely stable and there were no signs of D-Tat peptide degradation (lane 5). Under similar proteinase K treatment, L-Tat−TAR photo-cross-link products resulted in a complete loss of RNA−protein cross-link and a gain in free RNA as observed by band intensities on the gel (*41, 45*).

The specificity of the cross-linking reaction was established by competition experiments. RNA−protein cross-link formation was inhibited by the addition of unlabeled wild-type TAR RNA and not by a mutant TAR RNA lacking the trinucleotide bulge (Figure 2B). Thus, formation of a specific RNA−protein complex between TAR RNA and D-Tat is necessary for photo-cross-linking. These results show that D-peptide interacts in the major groove of TAR RNA in a similar manner to Tat protein (*28, 41, 46*).

D-Tat Competes with L-Tat for TAR RNA Binding. To determine whether D-Tat can inhibit L-Tat−TAR complex formation in vitro, we carried out photo-cross-linking experiments of the L-Tat−TAR complex in the presence of increasing amounts of D-Tat followed by proteinase K digestion (Figure 3). An RNA−protein cross-link was

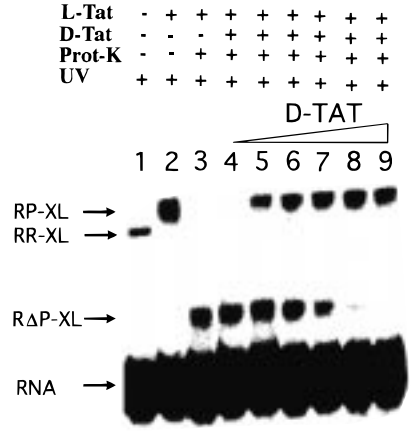

FIGURE 3: Inhibition of Tat−TAR complex formation by the D-Tat peptide. Reaction mixtures contained 0.04 μM 5′-end-labeled 4-thioU containing TAR RNA duplex and 0.3 μM L-Tat peptide. Photo-cross-linking of 4-thioU-containing duplex TAR RNA was performed with L-Tat peptide (lane 2) and the RNA−protein cross-link was digested with proteinase K (lane 3). Increasing amounts of D-Tat were added during RNA−protein complex formation followed by photo-cross-linking and protease digestion (lanes 4−9). The concentrations of D-Tat in lanes 4−9 were 0.1, 0.2, 0.3, 0.5, 1.0, and 1.5 μM, respectively. Lane 1 is a control lane without any peptide. The RNA−RNA and RNA-peptide cross-links and protease digest products containing small peptides are indicated by RR-XL, RP-XL, and RΔP-XL, respectively.

observed in L-Tat−TAR complex (lane 2) that was degraded to smaller RNA−peptide complexes by the addition of proteinase K enzyme (lane 3). Since D-Tat−TAR cross-link is resistant to proteolysis, increasing the amount of D-Tat prior to the cross-linking reactions resulted in an increase in RNA−protein cross-link after proteinase K digestion (lanes 4−9). We used 0.04 μM thio-U-labeled TAR RNA duplex, 0.3 μM L-Tat, and increasing concentration of D-Tat from 0.1 to 1.5 μM (lanes 4−9) to form RNA−protein cross-link products. A decrease in smaller RNA−peptide cross-link products was also observed as the concentration of D-Tat was increased. When D-Tat concentration became 1 μM (lane 8), a negligible amount of L-Tat−TAR cross-link products (RΔP-XL) was observed. These results demonstrate that D-Tat can compete with L-Tat to form a complex with TAR RNA in vitro.

D-Tat Inhibits Tat Trans-Activation in Vitro. We next tested the effects of D-Tat peptide on HIV-1 transcription in a cell-free system. In vitro transcription reactions were performed with HeLa cell nuclear extract and linearized HIV-1 DNA template (*47*). Tat produced a large increase in the synthesis of correctly initiated 530-nucleotide runoff RNA transcripts (Figure 4A). Transcription was stimulated by Tat at concentrations ranging from 50 to 300 ng/10 μL reaction. Quantitation revealed that 100 ng of Tat/reaction produced a 10−12-fold stimulation of HIV-1 transcription (Figure 4A, see lanes 1 and 2). In our Tat *trans*-activation experiments, we used 100 ng of Tat in each assay. Control experiments showed that Tat did not significantly increase transcription from an HIV-1 promoter with a mutated TAR element either in the stem region (G26−C26) or in the loop sequence (U31−G31) (data not shown). Increasing amounts of D-Tat resulted in a significant decrease in Tat-mediated transcriptional activation (Figure 4B). In the presence of 1 μg of D-Tat (∼3× to the wild-type Tat), more than 80% Tat transactivation was inhibited (see lanes 1 and 10 in Figure

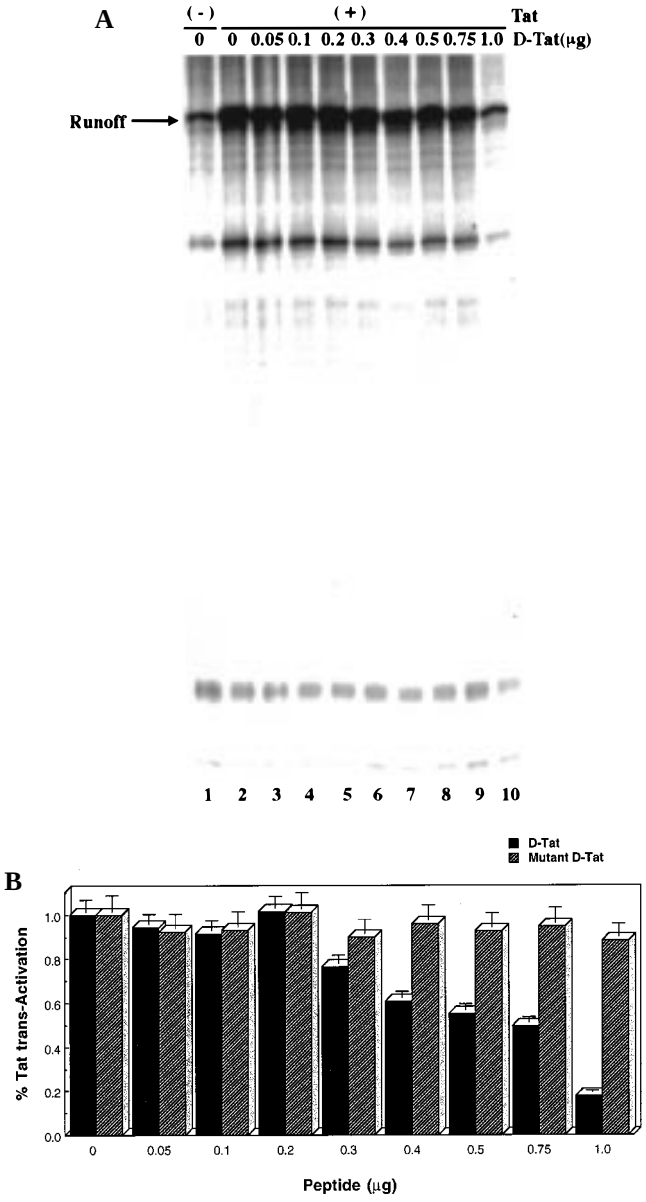

FIGURE 4: Inhibition of Tat transactivation by the D-Tat peptide in vitro. (A) The plasmid (p10SLT) that contains HIV-1 5′-LTR (*44*) was linearized by cleavage with *Bam*HI (+530) and used to perform in vitro transcription assays as described earlier by Wang and Rana (*47*). Lane 1, production of runoff transcripts without Tat; lane 2, transcription activation in the presence of 100 ng of Tat; lanes 3−10, transcription in the presence of 100 ng of Tat and increasing amounts of D-Tat as indicated. Full-length transcript is labeled as runoff. Tat transactivation experiments and preparation of HeLa cell extracts were carried out as described by Parada and Roeder (*52*). (B) Graphical representation of inhibition results. Transcriptional activities were measured from three sets of experiments and normalized to Tat activation without the D-Tat peptide inhibitor (100%). To address the specificity of D-Tat inhibitory activities, Tat *trans*-activation was carried out in the presence of a mutant D-Tat peptide, Gly44−Gln72, where all Arg residues in the RNA-binding region were replaced with Ala.

4A). The amount of recovered transcripts and the efficiency of transcription were normalized by including a labeled RNA not originating from HIV-1 LTR. To determine the specificity of transactivation inhibition by D-Tat, we synthesized a mutant D-Tat peptide, Gly44−Gln72, where all Arg residues in the RNA-binding region were substituted with Ala. The mutant D-Tat was unable to bind TAR RNA in electrophoretic mobility-shift experiments (data not shown) and did not inhibit Tat *trans*-activation in vitro (Figure 4B). These results indicate that D-Tat is able to specifically inhibit Tat transactivation in vitro.

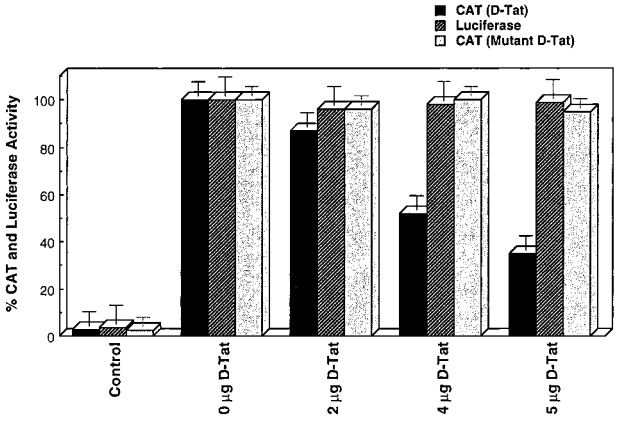

FIGURE 5: Inhibition of Tat transactivation by the D-Tat peptide in vivo. CAT activity expressed from the integrated HIV-1 LTR of HL3T1 cells with increasing amounts of D-Tat or mutant D-Tat peptide is shown. Mutant D-Tat peptide contains Ala in place of Arg at positions 49, 52, 53, 55, 56, and 57 in the RNA-binding region of the peptide. Luciferase activity was a control experiment to monitor the transfection inhibition of pSV2Tat by the addition of D-Tat. Transfection and enzymatic activity (CAT and luciferase) assyas were performed as described previously (*49*, *50*). CAT and luciferase activities were measured from five experiments and normalized to 100%. Control lane does not contain pSV2Tat or pAL and shows basal level of transcription.

D-Tat Inhibits Tat Trans-Activation in Vivo. It has been previously established that Tat peptides containing the basic domain are taken up by cells within less than 5 min and accumulate in the cell nucleus (*48*). Since our D-Tat peptide also contains the basic domain of Tat, we reasoned that this peptide would be rapidly taken up by HeLa cells and accumulate in the nucleus. Once D-Tat peptide reaches the nucleus, it would compete with Tat for TAR binding and lead to inhibition of Tat function. To test this hypothesis, we added D-Tat during transfection of pSV2-Tat (*49*) and pAL (*50*) plasmids into HeLa cells containing an integrated LTR−CAT reporter (*51*). Plasmids pSV2Tat and pAL express the first exon of Tat protein and luciferase enzyme, respectively. Transfection of pSV2Tat enhanced transcription as determined by CAT activity. As shown in Figure 5, increasing amounts of D-Tat resulted in a decrease of CAT activity while luciferase activity was not affected. Tat *trans*-activation was inhibited more than 60% by 5 μg (~0.5 μM) of D-Tat peptide. Further addition of D-Tat did not further inhibit Tat transactivation, probably because maximum peptide uptake efficiency is reached at 5 μg of D-Tat. Modifications of peptides to increase their cellular uptake are in progress. To rule out the possibility that the observed inhibition of transactivation is due to some nonspecific toxicity of the D-peptide or reduction of the pSV2Tat plasmid uptake, transcription of luciferase gene was monitored (Figure 5). Transcription of luciferase gene was not affected by D-Tat peptide as measured by luciferase enzymatic activity assays. Cell viability assays showed that cells were not killed by D-Tat treatment. Specificity of the inhibition was tested by adding a mutant D-Tat peptide, Gly44−Gln72, where all Arg residues in the RNA-binding region were substituted with Ala during transfection of plasmids and analyzing the

CAT and luciferase activities as described above for D-Tat. This mutant D-Tat peptide did not inhibit Tat *trans*-activation (Figure 5). Thus, these results indicate that the D-Tat peptide specifically inhibits *trans*-activation by Tat protein in vivo.

These findings show that a small Tat-derived D-peptide binds TAR RNA by interacting in the major groove of the RNA and selectively inhibits Tat *trans*-activation. Similarly, D-peptides can be designed to inhibit protein binding in the minor groove of nucleic acids. It remains to be determined whether a broad range of RNA−protein interactions can be selectively targeted. These results present the first example of the application of D-peptides as artificial regulators of cellular processes involving RNA−protein interactions in vivo.

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