Inhibition of Tat-Mediated Transactivation of HIV-1 LTR Transcription by Polyamide Nucleic Acid Targeted to TAR Hairpin Element†

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ABSTRACT: Tat, an essential human immunodeficiency virus type 1 protein interacts with the transactivation response element (TAR) and stimulates transcription from the viral long-terminal repeat (LTR). Blockage of Tat−TAR interaction halts viral transcription and hence replication. We have found that polyamide nucleic acid (PNA), targeted to the TAR sequences of viral RNA genome is able to prevent Tat−TAR interaction by efficient sequestration of the TAR. Anti-TAR PNA competes for TAR and prevents Tat-mediated stimulation of HIV-1 LTR transcription in vitro but has no influence on the basal level of transcription in the absence of Tat. Using a reporter gene construct pHIV LTR−CAT and pCMV−Tat in cell culture, we have further shown that anti-TAR PNA is able to block Tat-mediated transactivation of HIV-1 LTR transcription in vivo as judged by the extent of LTR driven CAT gene expression in the absence and presence of anti-TAR PNA. Supplementation of 100 nM of anti-TAR PNA into the culture medium further enhances the suppression of transactivation. Nonspecific scrambled PNA had no influence on Tat−TAR interaction and LTR-driven CAT gene expression in cell culture. These results suggest that PNA targeted to the TAR sequence of the viral genome may be a potential inhibitor of HIV-1 gene expression.

HIV-1 is the etiological agent for acquired immune deficiency syndrome (AIDS). Its RNA genome encodes the common retroviral structural gene products *gag*, *pol*, *env*, and two essential regulatory proteins, Tat and Rev (*1*). The Tat protein is a potent transactivator that is essential for HIV-1 replication. Transcriptional activation of HIV-1 gene involves complex formation of Tat with an RNA target sequence called TAR (trans-activation-response-element), located downstream of the transcription start site in the viral long-terminal-repeat (LTR) (*2*). Tat stimulates transcription both at the level of initiation and elongation (*3*−*6*). The binding site for Tat is centered around a U-rich bulge, near the apex of the TAR RNA stem-loop structure (*7*). The functional importance of Tat−TAR interaction in the viral life cycle makes it an attractive target for potential inhibitors.

In an attempt to reduce viral transcription, a number of reports have suggested various chemicals and/or genetic inhibitors for inhibition of Tat-activated transcription. Here, they are arbitrarily classified as either general or specific inhibitors of Tat transactivation. The general inhibitors range from IL-6 (*8*), insulin-like growth factor I (*9*), R05−3335 and R024−7429, both bezadiazapin derivatives (*10*−*12*), DB60 and DB75, diphenylfuran derivatives which bind to TAR by shape recognition (*13*), Daunomycin (*14*), plant lignan, 3′-*O*-methyl nordihydroguaiaretic acid (*15*), thiamine disulfide, α-lipoic acid, and *N*-acetyl cysteine (*16*). Pearson et al. (*17*) have shown that a truncated form of Tat, containing the wild-type core and a truncated basic domain, inhibited HIV-1 Tat-induced gene expression. Similarly, Green et al. (*18*) reported that Tat peptides spanning amino acid residues 37−72 could inhibit Tat transactivation of HIV LTR. Tat peptide analogues have also been shown to down regulate transcription, as well as inhibit HIV-1 particles by approximately 85% (*19*).

Other Tat and TAR-related inhibitors include TAR RNA decoys (*20*, *21*), TAR circle (*22*), TAR ribozyme (*23*, *24*), Tat basic domain inhibitor of double-stranded (ds) RNA-dependent protein kinase (DAI) (*25*), ALX40−4C, an oligocationic peptide of basic nature (*26*), CGP64222, a 9 residue basic oligomer of Tat (*27*), a chimeric EIAV/HIV Tat protein (*28*), extra cellular anti-Tat monoclonal antibody (*29*), and single-chain anti-Tat antibodies (*30*). The ultimate goal of these studies has been to sequester Tat's function, thereby reducing transcription and viral load.

A somewhat new and important approach employed to block Tat−TAR binding is the use of synthetic oligodeoxyribonucleotides complementary to the HIV-1 TAR apical stem-loop and bulge region. Recently, in vitro results have shown that synthetic oligodeoxyribonucleotides can block the Tat−TAR interaction in gel mobility shift assays (*31*). In 1991, Nielsen described the synthesis of a new type of DNA analogue called polyamide ("peptide") nucleic acid (PNA) complex. PNAs are DNA homologues containing a peptide backbone of 2-aminoethylglycine units to which purine and pyrimidine bases are linked (Figure 1A). These polyamide oligomers have been shown to have interesting properties. For instance, they are very stable in human serum and cell extracts and show antisense activity (*32*). Other

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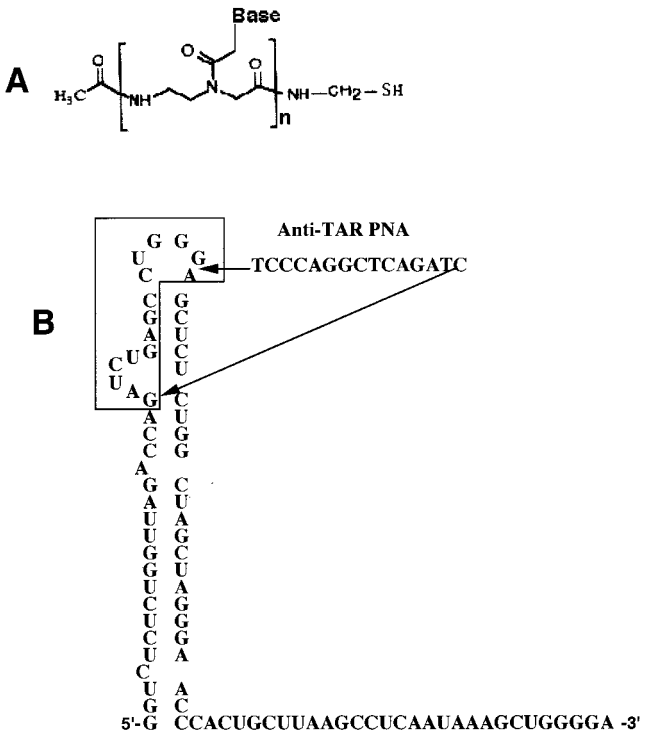

FIGURE 1: General structure and sequence of anti-TAR PNA targeted to HIV-1 TAR RNA stem-loop and bulge region. (A) In the structure of PNA, "b" represents purine and pyrimidine bases adenine, guanine, cytosine and thymine linked with polyamide backbone and "n" represents the number of bases in the molecule (*69*). The $(PNA)_{15}$ has a single Cys amino acid at the end of the sequence that corresponds to the 3′ terminus. (B) Secondary structure of HIV-1 TAR RNA stem-loop and bulge showing the region where the complementary 15 mer anti-TAR binds to TAR. The sequence of the anti-TAR PNA used for all experiment was [5′-(N) tcccaggctcagatc-Cys].

properties of PNA also include strand invasion of duplex DNA causing displacement of one of the DNA strands (*33*−*35*). Unlike oligodeoxyribonucleotides, whose binding polarity is determined by the deoxyribose sugar, the polyamide backbone of PNA can allow binding to target DNA in an orientation-independent manner (*34*). PNA binds with high affinity to both DNA and RNA and can be used as an anti-sense molecule for regulating gene expression. The double-stranded structures formed by the binding of polyamide oligomers with their targets are energetically more stable than DNA−DNA or DNA−RNA complexes, as evidenced by the comparatively higher melting temperatures (*33*, *36*). The high stability of PNA−nucleic acid complexes is reduced dramatically by single base pair mismatches, suggesting that PNA recognizes its target in a sequence specific manner (*32*, *33*, *36*).

In this communication, we present evidence to demonstrate that a 15 mer PNA sequence complementary to the minimal functional TAR sequence comprising the apical stem-loop and bulge region can specifically bind to TAR, thereby effectively inhibiting transactivation of HIV-1 LTR by Tat. This PNA sequence was chosen because the stem-loop and bulge of TAR RNA has been shown to be critical for Tat activated transcription (*6*). A scrambled PNA sequence was used in all experiments as a negative control to determine the specificity of anti-TAR PNA. Here we demonstrate that anti-TAR PNA physically blocks the Tat protein from forming a complex with the HIV-1 TAR RNA in vitro. PNA competes with Tat for binding to TAR as is evident in RNA band shift assays. The effect of anti-TAR PNA on Tat-mediated HIV-1 LTR transactivation was further examined in an in vitro transcription reaction, as well as transient transfection assays in cell culture. In all cases, anti-TAR PNA was able to effectively block HIV-1 LTR transactivation by Tat. The significance of these findings may lead to advances in HIV-1 therapeutic research.

MATERIALS AND METHODS

Gel Retardation Assay. The wild-type TAR RNA (pEM7) and its mutant derivatives were transcribed as described by Gunnery et al. (*37*). The TAR mutants used in this study carry deletion either in the apical loop (TAR S1) or at the top of the stem (TAR BS) or carry four-nucleotide insertions between the stem and loop region (TAR BF1). Briefly, the plasmid pEM-7 and its mutant constructs were linearized by digestion with *Hin*dIII and transcribed using T7 RNA polymerase as per the manufacturer's protocol (Boehringer Mannheim) and subjected to electrophoresis on a 10% polyacrylamide gel. The rNTP mixture contained 200 μM [α-^{32}P]UTP (specific activity 1 μCi/32 pmol; ICN). The major radioactive band was eluted and extracted with phenol/chloroform and precipitated with ethanol. In order to evaluate the affinity and specificity of the anti-TAR PNA for the TAR RNA, experiments were performed using varying molar ratios of PNA to wild-type TAR RNA or its mutant derivatives. To ascertain the ability of anti-TAR PNA to block the Tat−TAR interaction, gel retardation assays were performed as follows. Five nanograms of ^{32}P-labeled TAR RNA (2×10^4 cpm) was incubated with indicated concentrations of anti-TAR PNA or with scrambled PNA (synthesized by Research Genetics) for 30 min at room temperature in the absence or presence of Tat protein or 37 mer Tat peptide (spanning residues 36−72). The incubation buffer contained 30 mM Tris-HCl, pH 8.0, 75 mM KCl, 5.5 mM $MgCl_2$, 1.3 mM DTT, 12% glycerol, 0.01% NP-40, and 500 ng of r (I−C), in a final volume of 16 μL. Two microliters of RNA gel loading dye (0.27% bromophenol blue and 20% glycerol) was added to the samples and subjected to polyacrylamide DNA retardation analysis on a 4% polyacrylamide gel in Tris-glycine buffer. The gels were prerun at 100 V for 30 min at 4 °C in 10% Tris-glycine. The RNA−protein complexes were resolved from free RNA at a constant voltage of 175 V at 4 °C and subjected to phosphorImager analysis.

In Vitro Transcription. Transcription assays were performed using plasmids pHIV LTR−CAT (linearized by *Eco*R1) and pHTLV-1-LTR (linearized by *Hin*dIII) as the DNA template. The in vitro transcription reaction contained 20 mM HEPES (pH 7.9), 100 mM KCl, 1 mM DTT, 12.5 mM $MgCl_2$, 800 ng of linearized plasmid, 750 μM each of ATP, GTP, and CTP, and, 25 μCi of [α-^{32}P]UTP (0.4 μCi/pmol) and HeLa whole cell extracts equivalent to 60 μg of protein (7.5 μL) in a total volume of 20 μL. The transcription reactions were carried out in the absence or presence of purified Tat protein, anti-TAR PNA, or scrambled PNA and terminated by the addition of 350 μL of 150 mM NaCl and 0.2% SDS in TE buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA). The reaction products were extracted with equal volumes of phenol/chloroform, alcohol precipitated and

subjected to 4% polyacrylamide gel electrophoresis followed by phosphorimager analysis as described above.

Transfection Assays. Lymphocyte CEM CD4+ lymphocytes [12D7 (*38*)] were grown in a complete RPMI-1640 medium. Early to mid log phase, cells were harvested, centrifuged, and washed with an equal volume of phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+}. The cells (10^7 cells /mL) were resuspended in RPMI-1640 medium (without FCS, L-glutamine, or penicillin/streptomycin). A portion of cell suspension (250 μL; 2.5×10^6 cells) mixed with pHIV LTR−CAT (3 μg), pCMV−Tat (250 ng), anti-TAR PNA, or scrambled PNA (2.5, 5, and 10 μg) was electroporated at 230 V and then plated in 10 mL of complete RPMI-1640 media (*38*). In another set of experiments, anti-TAR PNA or scrambled PNA were supplemented in the media at a final concentration of 100 nM. Transfections of the CEM cells using 10 μg of pHIV LTR−CAT with and without 5 μg of anti-TAR or scrambled PNA were also performed to examine the basal level transcription. Cells were subsequently incubated at 37 °C for 18−24 h and harvested for the CAT assay, as described below. Control experiments were also performed using pU3R (HTLV-1 LTR−CAT, 3 μg), pC−Tax (l μg), and 5 μg of anti-TAR or scrambled PNA.

CAT Expression Assays. The harvested cells were washed with PBS without Ca^{2+} or Mg^{2+}. Cells were then resuspended in 100 μL of 250 mM Tris-HCl (pH 8.0) and lysed by freeze−thawing three times. Samples were then incubated at 65 °C for 5 min to inactivate deacetylases in the Lymphocyte extracts. Lysates were centrifuged at 12 000 rpm for 10 min at 4 °C, and the supernatants were transferred to fresh tubes. The protein concentration for each sample was determined using the Bio-Rad assay kit. The reaction mixture contained cell extract equal to 1.2 μg of protein, 0.4 mM of acetyl CoA, and 0.5 μCi of D-threo-[dichloroacetyl-1-^{14}C]-chloramphenicol (65 mCi/mmol; Amersham Life Sciences) in a total volume of 20 μL. Reactions were carried out at 37 °C for 7 min and terminated by the addition of 400 μL of ethyl acetate. The reaction mixtures were vortexed and centrifuged at room temperature for 10 min at 12 000 rpm. The organic top layer was transferred to fresh tubes, dried in a lyophilizer, and resuspended in 15 μL of ethyl acetate. Samples (10 μL) were spotted on TLC silica gel plates (J. T.Baker Inc) and acetylated reaction products were resolved for 2 h using chloroform/methanol (19:1 ratio) as the solvent system. The TLC plates were air-dried and subjected to phosphorImager analysis and quantitated using Imagequant (Molecular Dynamics). For CAT analysis of basal transcription of pHIV LTR−CAT cotransfected into CEM cells, with and without anti-TAR PNA or scrambled PNA, 2.5 μg of protein was used and the reactions were carried out at 37 °C for 30 min. The same procedure was also used with respect to the cells transfected with pU3R and pC−Tax in the presence and absence of anti-TAR PNA or scrambled PNA.

RESULTS

Binding Specificity of Anti-TAR PNA to HIV-1 TAR. The sequence of anti-TAR PNA and nonspecific scrambled PNA used in this study are shown in Figure 1A. Both anti-TAR PNA and scrambled PNA contain 15 bases linked with polyamide backbone in lieu of sugar phosphate backbone.

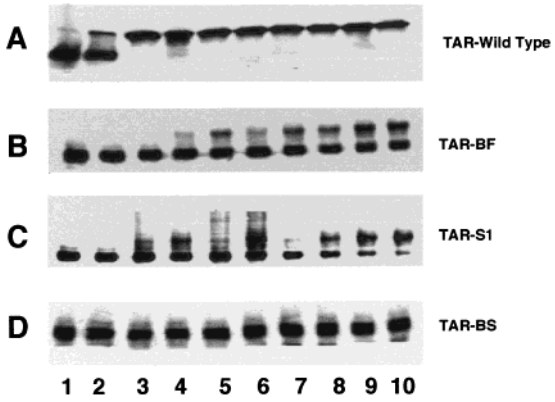

FIGURE 2: Binding of anti-TAR PNA to wild-type TAR RNA and its mutant derivatives. The binding affinity of anti-TAR PNA to TAR and its mutant derivatives (BF, S1, and BS) was assessed by gel retardation analysis as described in the Materials and Methods. Panels A, B, C, and D represent wild-type TAR, BF, S1, and BS, respectively. Lane 1−10 represent molar ratio of TAR to anti-TAR PNA of 1:0, 1:0.5, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, and 1:8, respectively.

The target sequence corresponding to the stem-loop and bulge of HIV-1 TAR RNA to which anti-TAR PNA binds is shown in Figure 1B. Initially, we determined the affinity of the anti-TAR PNA for the TAR RNA by gel retardation analysis. As seen in Figure 2A, anti-TAR PNA is able to bind stoichiometrically to TAR as judged by complete shift in the mobility of the TAR−PNA complex at a ratio of 1:1. To demonstrate the specificity of anti-TAR PNA, we have included three mutant derivatives of TAR carrying deletion either in the apical loop (TAR S1), or at the top of the stem (TAR BS) and a four-nucleotide insertion between the stem and loop region (TAR BF1). As expected, TAR mutants either do not form any complex with anti-TAR PNA (Figure 2D) or require high molar ratios of PNA to TAR to exhibit a significant shift in the mobility (Figure 2B and 2C).

Anti-TAR PNA Blocks Tat -TAR Interaction. To ascertain the ability of anti-TAR PNA to block the Tat−TAR interaction, gel retardation assays were performed with both full-length Tat protein (86 amino acids) and Tat peptide containing the basic domain (Figure 3). When the full-length Tat protein was examined for its ability to bind to TAR RNA, a specific Tat−TAR complex formation was discerned in a 4% nondenaturing PAGE (Figure 3A, lane 3). Anti-TAR PNA preincubated with TAR RNA for 30 min at room temperature displayed significant inhibitory effect on the formation of TAR−Tat complex (Figure 3A, lanes 4 and 5). As expected, scrambled PNA had no inhibitory effect on Tat−TAR interaction (Figure 3A, lanes 7 and 8). When anti-TAR PNA was preincubated with TAR RNA alone, a shift in TAR mobility was noted with the appearance of a band that ran slightly slower on the gel (Figure 3A, lane 2). This slower moving complex was not present when scrambled PNA was used in place of anti-TAR PNA (Figure 3A, lane 6).

Similar results were obtained when the Tat peptide was used for the gel shift retardation analysis (Figure 3B, lane 3). A similar Tat peptide without the basic domain did not show TAR binding (data not shown). The addition of anti-TAR PNA successfully blocked the formation of peptide−TAR interaction (Figure 3B, lane 4 and 5). Scrambled PNA showed no inhibitory effect on the formation of Tat peptide−

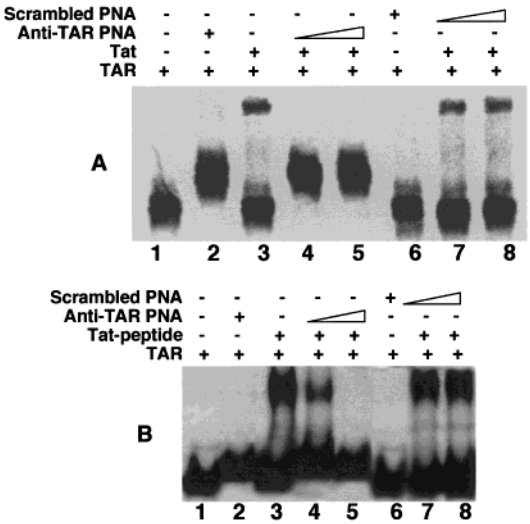

FIGURE 3: Anti-TAR PNA shields HIV-1 TAR RNA from forming a complex with Tat and a Tat peptide. (A) HIV-1 Tat protein was incubated with ^{32}P-labeled HIV-1 TAR RNA in the presence of anti-TAR PNA as described in Materials and Methods. The Tat−TAR complex formed in the absence or presence of anti-TAR PNA was resolved by nondenaturing gel retardation analysis using 4% polyacrylamide gel. Lane 1 (^{32}P-labeled TAR RNA alone); lane 2 (TAR + anti-TAR PNA); lane 3 (TAR+ Tat). Lanes 4 and 5 represent Tat incubated with TAR in the presence of 25 and 50 ng of anti-TAR PNA, respectively. TAR RNA was also incubated with Tat protein in the presence of nonspecific scrambled PNA. Lane 6, represents TAR RNA incubated with scrambled PNA alone. Lanes 7 and 8 represent TAR RNA incubated with Tat in the presence of 25 ng and 50 ng of scrambled PNA, respectively. (B) Ability of anti-TAR PNA to block the interaction of a 37 mer Tat peptide (spanning residues 36−72) with HIV-1 TAR RNA. Lane 1 (^{32}P-labeled TAR RNA alone); lane 2 (TAR + anti-TAR PNA); lane 3 (TAR+ Tat peptide). Lanes 4 and 5 represent Tat peptide incubated with TAR in the presence of 100 ng and 1 μg of anti-TAR PNA, respectively. Lane 6 represents TAR RNA incubated with scrambled PNA in the absence of Tat peptide. Lanes 6 and 7 represent Tat peptide incubated with TAR in the presence of 100 ng and 1 μg of scrambled PNA, respectively.

TAR complex (Figure 3B, lanes 7 and 8). The high specificity of anti-TAR PNA to TAR region was evident from the fact that TAR incubated with anti-TAR PNA, but not with scrambled PNA, formed a slow migrating complex (Figure 3B, lanes 2 and 6).

In Vitro Tat-Mediated Transactivation of HIV-1 LTR Is Inhibited by Anti-TAR PNA. Since HIV-1 Tat enhances transcription elongation via binding to the viral TAR RNA stem-loop structure, it was of interest to probe if anti-TAR PNA targeted to the stem-loop region of TAR could block the function of Tat in vitro. To examine this possibility, the purified Tat protein was added to the in vitro transcription reactions using HeLa whole cell extract (as a source of cellular RNA polymerase II and other cellular factors) and *Eco*RI-linearized HIV LTR−CAT DNA template in the presence and absence of anti-TAR PNA and nonspecific scrambled PNA. Tat was found to stimulate the synthesis of the full-length 375 base long HIV-1 LTR transcript as compared to the basal levels of transcription of HIV-1 LTR (Figure 4A, lanes 2 and 3). To demonstrate whether Tat transactivation could be inhibited by anti-TAR PNA, 50 nM (lane 4) and 100 nM (lane 5) of the PNA were added to the in vitro transcription reactions. At 100 nM concentration of anti-TAR PNA, transactivation of the HIV-1 LTR by Tat was effectively blocked (lane 5). On the other hand, scrambled PNA did not affect LTR transactivation by Tat either at lower or higher concentration (Figure 4A, lane 6 and 7). To determine whether PNA affected basal levels of transcription, reactions were carried out in the absence of Tat and in the presence of anti-TAR PNA and scrambled PNA (Figure 4A, lane 8 and 9). Both, anti-TAR PNA (lane 8) and scrambled PNA (lane 9) did not exhibit any inhibitory effect on the basal transcription level (lane 2). To demonstrate the specificity of anti-TAR PNA-mediated inhibition on transcriptional activation, we examined the in vitro transcription levels of the HTLV promoter in the presence or absence of anti-TAR PNA and scrambled PNA. As seen in Figure 4B, both anti-TAR PNA or scrambled PNA did not exhibit any significant influence on the transcription levels of the HTLV promoter.

Anti-TAR PNA Blocks Transient Transactivation of pHIV LTR−CAT in the Presence of pHIV-1 Tat In Vivo. To test the ability of anti-TAR PNA to block in vivo trans-activation of HIV-1 LTR by Tat, the reporter plasmid pHIV LTR−CAT was transfected into CEM (12D7) cells, with or without the Tat expression vector along with anti-TAR PNA or nonspecific scrambled PNA. The expression of LTR was then determined by CAT analysis. Tat was found to transactivate HIV-1 LTR at higher than the basal levels as judged by the CAT expression (Figure 5A, lane 2). However, more than 80% CAT expression was found to be inhibited when cotranfection of anti-TAR PNA was carried out at 5 μg or higher concentrations (Figure 5A; lanes 4 and 5). As a control, nonspecific scrambled PNA was cotransfected into CEM cells at 2.5, 5, and 10 μg with HIV LTR−CAT and CMV−Tat. The ability of Tat to transactivate the HIV-1 LTR−CAT gene was not hindered by the scrambled PNA (Figure 5A; lanes 6−8) as compared to the anti-TAR PNA.

Experiments were also carried out to ascertain if the anti-TAR PNA could passively enter CEM cells to further inhibit Tat-mediated transactivation. After CEM cells were cotransfected, as described above, anti-TAR PNA was added to the complete RPMI-1640 medium at a final concentration of 100 nM for 18−24 h period. As shown in Figure 5A (lane 3), a 1.4-fold decrease in LTR−CAT activation was observed when 2.5 μg of anti-TAR PNA was transfected. This inhibition was further increased by 4.2-fold when 100-nM anti-TAR PNA was supplemented to the culture medium (Figure 5B; lane 2). When the concentration of anti-TAR PNA was increased to 5 μg in the initial transfection along with the presence of 100 nM anti-TAR PNA in the media, a 22-fold inhibition of CAT activity was observed (Figure 5B; lane 3). A similar control experiment was carried out with scrambled PNA, having a base composition similar to anti-TAR PNA, but with scrambled sequences. Under identical conditions, scrambled PNA had no effect on HIV LTR−CAT expression even when added to the media at a final concentration of 100 nM (Figure 5B, lanes 5 and 6). These results suggest that PNA can passively enter the CEM cells albeit at a low efficiency.

Specificity of Anti-TAR PNA-Mediated Inhibition of Trans-Activation. Two sets of control experiments were performed to evaluate the specificity of anti-TAR PNA mediated inhibition of trans-activation. First, the basal expression level of HIV-1 LTR−CAT was examined in the absence of Tat, with and without anti-TAR PNA and scrambled PNA (Figure

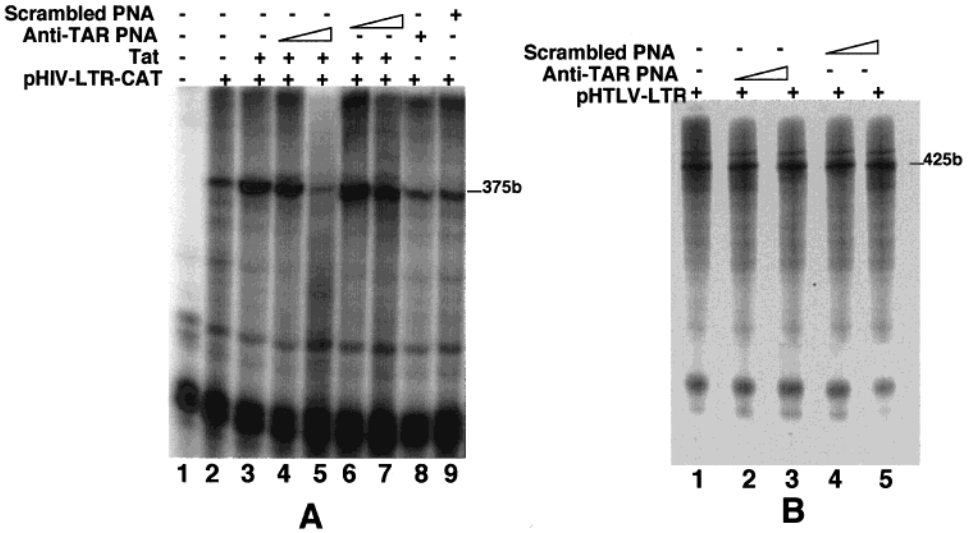

FIGURE 4: Tat-specific transactivation of HIV-1 transcription is blocked by anti-TAR PNA in vitro. (A) Linearized pHIV LTR−CAT DNA was incubated at 30 °C for 1 h in the absence (lane 2) or presence of 300 ng of purified Tat (lanes 3−7**)** to produce a 375 base nucleotide RNA transcript. Two hundred and fifty nanograms and 500 ng of anti-TAR PNA (lanes 4 and 5) and 250 and 500 ng of nonspecific scrambled PNA (lanes 6 and 7) were added to in vitro transcription reactions containing 800 ng of HIV LTR template in the presence of 300 ng of Tat. Control experiments were also carried out with 500 ng of anti-TAR PNA (lane 8) and scrambled PNA (lane 9) in the absence of Tat. (B) Reactions with the linearized pHTLV−LTR DNA in the absence of Tax were also carried out in the presence of 250 ng and 500 ng of anti-TAR PNA (lanes 2 and 3) or scrambled PNA (lanes 4 and 5) as described in panel A.

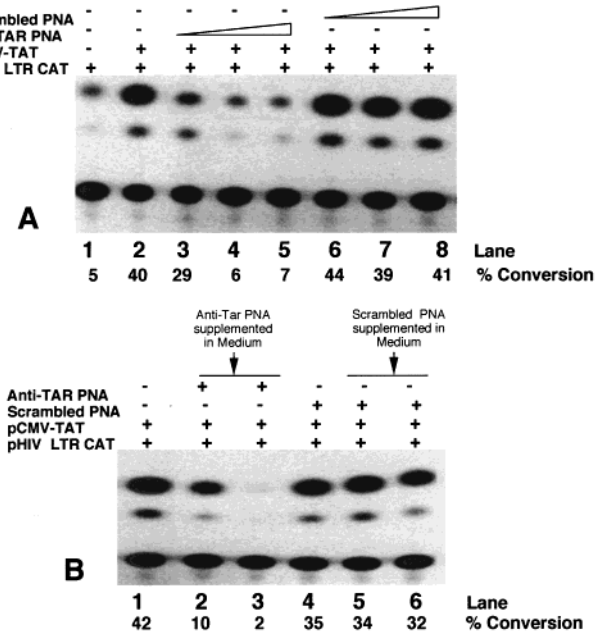

FIGURE 5: Effect of Anti-TAR PNA on Tat-mediated transactivation in CEM cells. (A) pHIV LTR−CAT (3 μg) construct was electroporated in 2.5 × 10^6 CEM cells (12D7; *38*, *68*) at 230 V in the absence (lane 1) or presence of 250 ng of Tat expression vector (lanes 2−8). Anti-TAR PNA was cotransfected at 2.5, 5, and 10 μg (lanes 3−5), as well as scrambled PNA at the same concentrations (lanes 6−8). Transfected cells were plated in 10 mL of complete RPMI-1640 for 18−24 h incubation at 37 °C (*37*). Panel B represents another set of experiments where culture medium was further supplemented with 100 nM of anti-TAR PNA (lanes 2 and 3) or scrambled PNA (lanes 5 and 6). Cells were harvested and cell extract equivalent to 1.2 μg of cellular proteins were used for CAT analysis (*70*). Values represent the percentage of conversion of the [^{14}C]chloramphenicol substrate in the CAT assay. The experiment is a representative from three independent assays.

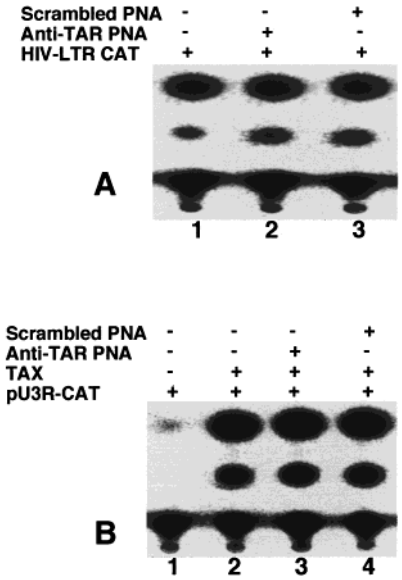

FIGURE 6: Anti-TAR PNA does not block transactivation of HIV-1 LTR in the absence of Tat or Tax mediated transactivation of HTLV-1 LTR. Panel A represents control experiments where CEM cells were transfected with 10 μg of pHIV LTR−CAT alone (lane 1) and in the presence of 5 μg of anti-TAR PNA (lane 2) or scrambled PNA (lane 3). Cell lysates equivalent to 2.5 μg of protein were used for CAT reactions. Panel B represent control experiments where CEM cells were transfected with 3 μg of pU3R (HTLV-1 LTR−CAT) in the absence (lane 1) or presence of 1 μg of pC−Tax (lanes 2−4). Five micrograms of anti-TAR PNA (lane 3) as well as scrambled PNA (lane 4) was cotransfected with pU3R and pC−Tax. Cell lysates equivalent to 2.5 μg of protein were used for CAT assays. Values represent the percentage of conversion of the [^{14}C]chloramphenicol substrate in the CAT assay. The experiment is a representative from three independent assays.

6A). When pHIV LTR−CAT was cotransfected with anti-TAR PNA (lane 2) and scrambled PNA (lane 3) into CEM cells**,** the CAT activity did not change significantly as compared to HIV-1 LTR−CAT alone (lane 1), providing further evidence that the inhibition of Tat−TAR interaction by anti-TAR PNA is a site specific event. Also, to determine the specificity of anti-TAR PNA for HIV-1 TAR RNA, the reporter plasmid pU3R−CAT (HTLV-1 LTR) was trans-

fected into CEM cells with or without Tax expression vector, along with anti-TAR PNA and scrambled PNA. Results shown in Figure 6B indicate that Tax protein considerably increased pU3R−CAT expression (lane 2) which remained unaffected in the presence of anti-TAR PNA (lane 3) or scrambled PNA (lane 4).

DISCUSSION

Genetic variation of the HIV-1 genome in AIDS patients, poses as one of the most difficult challenge in the quest for effective inhibitors of HIV-specific enzymes. The awesome daily production rate of as many as 10 billion virions per body can rapidly accumulate a significant number of drug resistant variants that make chemotherapy ineffective (*39*, *40*). This potent barrier can be overcome if the viral targets selected for drug intervention are resistant to mutational changes. The unique 5′ (U5) nontranstlated region (1−333 nucleotides) of the HIV-1 genome containing several critical domains essential for viral replication may be an ideal target for drug intervention. These critical domains comprise of (i) the primer-binding site (PBS; nucleotides 183−201) essential for $tRNA^{Lys3}$ primed initiation of reverse transcription (*41*−*45*); (ii) the A-loop region located upstream of the PBS (nucleotides 168−173) essential for the selection and interaction of $tRNA^{Lys3}$ primer (*46*−*48*); (iii) the LTR sequences at the 5′ and 3′ ends essential for viral transcription and integration (*49*); and (iv) the trans-activation response element (TAR) essential for viral gene expression via transcriptional activation (*50*−*52*) and probably having an additional role in the initiation of reverse transcription (*53*, *54*). These regulatory sequences in the 5′ nontranslated region are averse to mutational changes and, therefore, can be potential targets for arresting viral replication.

We have earlier shown that PNA targeted to PBS site of the viral genome blocks the initiation of reverse transcription (*33*). The anti-PBS PNA oligomer having a terminal DNA nucleotide annealed with the complementary RNA template is recognized as a bona fide primer by HIV-1 RT, resulting in abortive reverse transcription products (*33*, *36*, *55*). In the present study, we have used a specific PNA targeted to the TAR RNA stem-bulge region of the nascent RNA transcript. TAR is located downstream of the transcription initiation site at nucleotides +1 and +59 and forms a highly stable nuclease resistant, stem-loop structure (Figure 1B). HIV-1 Tat, a nuclear transcriptional activator, interacts with TAR to enhance the processivity of RNA polymerase II complexes (*52*, *56*). Tat contains an arginine rich motif (ARM), typical of sequence specific RNA binding proteins (*57*). The idea that TAR may be a novel target for drug intervention is supported by the fact that it is resistant to mutational changes and any natural or induced mutation that destabilizes the TAR by disrupting base pairing in the stem region also abolishes Tat-stimulated transcription (*58*) resulting in premature transcription termination at random locations downstream of the viral RNA start site (*39*).

The anti-TAR PNA used in this study formed a tight complex with TAR RNA at molar ratios of 1:1, resulting in retarded mobility of the complex in gel mobility shift assays, thus exhibiting a great affinity for TAR RNA (Figure 2A). Scrambled PNA did not influence the mobility of TAR RNA, suggesting the specificity of interaction of anti-TAR PNA to its target sequence. The binding affinity of anti-TAR PNA is abolished or drastically reduced when TAR RNA contained mutational changes in its stem-loop region (Figure 2, panels B, C, and D). The anti-TAR PNA effectively blocked Tat−TAR interaction. Addition of anti-TAR PNA following the Tat−TAR binding also disrupted Tat−TAR interaction suggesting that TAR sequences covered by Tat are also prone to invasion by chargeless anti-TAR PNA. Similar results were obtained with the truncated form of Tat. It has been shown that the truncated form of Tat containing the wild type core and arginine rich sequence from the C-terminal domain, can also bind directly to TAR RNA (*19*, *59*, *60*) and inhibits HIV-1 Tat induced trans-activation of transcription. (*17*, *18*, *61*). We found that the binding affinity of this 37 mer peptide to TAR RNA is greater than the full-length Tat protein (Figure 3B). Anti-TAR PNA effectively competes with Tat peptide for binding to TAR RNA, whereas scrambled PNA has no influence on Tat peptide-TAR interaction. These results suggest that anti-TAR PNA may effectively be used to sequester TAR RNA to block Tat function via Tat−TAR interaction and thereby inhibit Tat-mediated transactivation of HIV-1 transcription. This premise is further supported by the results obtained from the in vitro transcription of the HIV LTR−CAT plasmid using HeLa cell nuclear extract in the presence and absence of Tat and anti-TAR PNA (Figure 4A). It was observed that accumulation of the full-length HIV-1 transcript (375 bases) is significantly increased in the presence of Tat but dramatically inhibited in the presence of anti-TAR PNA. As expected, anti TAR−PNA did not inhibit in vitro transcription of the pHTLV-LTR (Figure 4B). These observations suggest that anti-TAR PNA specifically prevents Tat−TAR interaction resulting in loss of Tat-mediated trans-activation of transcription.

Activation of transcription by HIV-1 Tat in the cell-free system and its inhibition by anti-TAR PNA would suggest that similar inhibition mechanism may be mimicked in vivo. It has been shown that Tat alters RNA polymerase II processivity and stimulates the synthesis of full-length transcript (for review, see ref *34*). The activation of RNA polymerase II occurs transiently during transcription through TAR and is independent of initiation (*63*). It has been demonstrated that both an intact loop sequence and an intact Tat-binding site are critical for active TAR element, and there is no complementation in cis between TAR element carrying mutations in loop or in the Tat-binding site (*63*). Keen et al. (*64*, *65*) have shown that Tat together with cellular factors gets attached to transcription complexes that are formed on templates carrying a functional TAR element, but does not associate with transcription complexes that are transcribed through TAR elements carrying inactivating mutations. TAR RNA is therefore simply used to recruit Tat and cellular cofactors and does not play a direct role in the activation of elongation.

We envisaged that PNA targeted to conserved sequences of TAR stem-loop and stem-bulge regions may provide a potential barrier in recruitment of Tat and associated cellular factors resulting in down regulation of transcription. To examine this proposition, we used pHIV-1 LTR−CAT and pCMV−Tat reporter gene constructs to transfect CEM cells in the presence and absence of anti-TAR PNA and nonspecific scrambled PNA. The basal level of transcription of

HIV-1 LTR as judged by CAT expression was significantly stimulated when pCMV−Tat was also cotransfected suggesting an in vivo like Tat−TAR interaction in the cell. Cotransfection of the anti-TAR PNA along with the reporter plasmids was further able to sequester HIV-1 LTR-TAR effectively as evidenced by significantly low levels of CAT expression (Figure 5A; lanes 3−5). Most significantly, supplementation of 100 nM of anti-TAR PNA in the culture medium efficiently blocked the CAT expression (Figure 5B; lane 3) suggesting that the chargeless anti-TAR PNA is able to enter into the cells. On the other hand, cotransfection and supplementation of nonspecific scrambled PNA had no influence on Tat−TAR mediated transactivation of CAT expression (Figure 5B; lanes 5−6).

The daunting task of arresting the mammoth HIV epidemic is highly challenging. The rapid emergence of drug resistant strains has considerably overshadowed the benefits of the 14 clinically available anti-HIV-1 drugs. Selection of the dominant, preexisting drug-resistant variants and the abundance of latently infected cells (which possess integrated proviral DNA) are the potential barriers encountered to effective drug therapy (*40*, *39*, *66*). Blocking the transactivation mechanism by targeting the conserved TAR element appears to be an attractive viral target since it is essential not only for viral gene expression but also for Tat-mediated activation of the proviral genome. TAR RNA decoy sequences have been used in vivo as a bait to trap Tat, resulting in the inhibition of viral replication (*23*, *67*). Recently, Hamy et al. (*27*) have identified a peptide compound (CGP64222) that binds directly to TAR RNA at the Tat-binding site and inhibits HIV replication. Mestre et al. (*31*) have shown that synthetic oligodeoxyribonucleotides can block the Tat−TAR interaction in gel mobility shift assays. In the present study, the use of an antisense PNA that binds TAR RNA at the Tat-binding site and blocks transactivation of HIV-1 LTR is unique in that it is sequence specific and is expected to exert no interference with the host cell genome and its metabolism. Therefore, this class of sequence specific inhibitors would have a better chance of blocking viral replication, and thereby preventing new cells from being infected. Currently efforts are underway in our laboratory to develop an appropriate vehicle to enhance bio-delivery of PNA into cells.

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