

Anti-TAR Polyamide Nucleotide Analog Conjugated with a Membrane-Permeating Peptide Inhibits Human Immunodeficiency Virus Type 1 Production

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The emergence of drug-resistant variants has posed a significant setback against effective antiviral treatment for human immunodeficiency virus (HIV) infections. The choice of a nonmutable region of the viral genome such as the conserved transactivation response element (TAR element) in the 5' long terminal repeat (LTR) may potentially be an effective target for drug development. We have earlier demonstrated that a polyamide nucleotide analog (PNA) targeted to the TAR hairpin element, when transfected into cells, can effectively inhibit Tat-mediated transactivation of HIV type 1 (HIV-1) LTR (T. Mayhood et al., *Biochemistry* 39:11532–11539, 2000). Here we show that this anti-TAR PNA (PNA_{TAR}), upon conjugation with a membrane-permeating peptide vector (transportan) retained its affinity for TAR *in vitro* similar to the unconjugated analog. The conjugate was efficiently internalized into the cells when added to the culture medium. Examination of the functional efficacy of the PNA_{TAR}-transportan conjugate in cell culture using luciferase reporter gene constructs resulted in a significant inhibition of Tat-mediated transactivation of HIV-1 LTR. Furthermore, PNA_{TAR}-transportan conjugate substantially inhibited HIV-1 production in chronically HIV-1-infected H9 cells. The mechanism of this inhibition appeared to be regulated at the level of transcription. These results demonstrate the efficacy of PNA_{TAR}-transportan as a potential anti-HIV agent.

In AIDS patients, rapid turnover and high mutation rates result in a diverse population of human immunodeficiency virus type 1 (HIV-1) quasispecies. Efforts to control the AIDS epidemic have relied on interactions between viral proteins or enzymes and new drug candidates. Current combination therapy that utilizes drug cocktails of inhibitors of viral protease and reverse transcriptase can initially reduce the viremia levels (1). However, the rapid emergence of variants due to mutations in the reverse transcriptase and/or protease gene eventually make chemotherapy ineffective.

To overcome the barrier of virus mutability and consequent drug resistance, the viral targets selected for drug intervention should include conserved regulatory regions resistant to mutational changes. The 5' nontranslated region of the viral genome (5' long terminal repeat [LTR]) comprises several important domains essential for viral replication and gene expression. These regions individually or collectively could be potential targets for drug design and development.

The HIV-1 LTR contains a unique region known as the transactivation response (TAR) region which is critical for transcriptional activation by the transactivator protein Tat (13). The TAR element extends between nucleotides +1 and +59 and forms a unique, stable stem-loop RNA structure (3, 46). Intensive research over the last decade on the transacti-

vation mechanism governing the Tat-TAR interaction has yielded significant biological and virological insights. It has now been established that the Tat protein of HIV-1 is a potent transactivator of viral gene expression and is essential for viral replication (25).

The primary role of Tat is in regulating productive and processive transcription after binding to its RNA target, the TAR element in the HIV-1 LTR (21). The structure of TAR has been well studied, and the salient features essential for its interaction with Tat, centering on a U-rich bulge near the apex of the loop, have been characterized (10). The structural integrity of TAR is important because natural or induced mutations that destabilize TAR by disrupting base pairing in the stem region abolish Tat-stimulated transcription, resulting in premature transcription termination at random locations downstream of the viral RNA start site (59). Thus, given the functional importance of the Tat-TAR interaction to the viral life cycle, the Tat protein and the TAR element both present attractive targets for drug design.

Agents affecting the interactions of Tat and TAR prevent transcriptional activation of the HIV-1 genome either by steric hindrance, a shear displacement mechanism, or deprivation of the functional molecules. For example, shielding the bulge-loop region of TAR with Tat peptides or analogs was shown to inhibit transactivation of HIV-1 LTR (6, 14, 26). Alteration of the stem-loop conformation of TAR by neomycin induces dissociation of Tat from TAR by an allosteric mechanism (65). In other strategies, either Tat protein or TAR RNA has been captured by a TAR RNA decoy (4, 37, 40, 60), TAR circle (3), anti-Tat monoclonal antibody and single-chain antibody (41, 54), ALX40-C (an oligocationic peptide) (49), or CGP64222 (a

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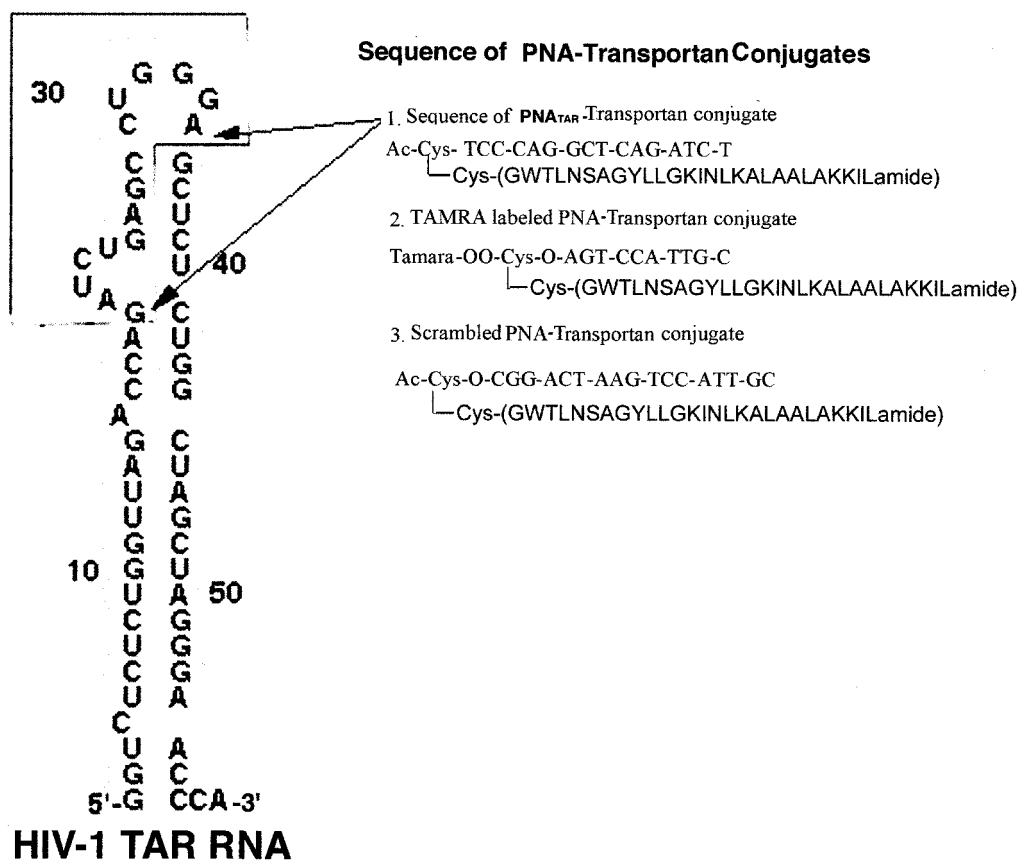


FIG. 1. Sequence of PNA-transportan conjugates. Secondary structure of the HIV-1 TAR RNA is as shown. The TAR sequences interacting with the PNA_{TAR}-transportan conjugate are indicated by arrows. The inset corresponds to the sequences of PNA_{TAR}, TAMRA-tagged PNA, and scrambled PNA conjugated with the 27-amino-acid transportan peptide.

9-residue basic oligomer of Tat) (19) to intervene in their functional interaction, thereby reducing transcription and ultimately the viral load.

The oligodeoxyribonucleotides complementary to the stem-loop and bulge regions of the TAR RNA have been employed successfully to block transcriptional activation of the HIV-1 viral genome. Several chemical modifications have been made in oligodeoxyribonucleotides in order to improve their stability and effectiveness in the cellular milieu. The therapeutic potential of phosphoramidates has recently been evaluated against the HIV-1 untranslated region as the target (11). In 1991, Nielsen described the synthesis of a polyamide nucleotide analog (PNA) in which the bases are linked with peptide bonds instead of a sugar phosphate backbone (48). The PNA molecules are resistant to nucleases and proteases (8) and have strong affinity for the cDNA or RNA sequences (31). Its ability to form an energetically favorable structure that can bind to its target in an orientation-independent manner has made it an excellent molecule for gene expression studies, but the low solubility of PNA in aqueous media and its inefficient cellular uptake are major drawbacks.

Recently, methods have been developed for the delivery of exogenous proteins into living cells with the help of membrane-permeating carrier peptides derived from HIV-1 Tat (residues 48 to 60) and antennapedia (residues 43 to 58) (9, 12, 57). The

basic nature of these peptides and the locations of aromatic groups on the peptides enable them to penetrate the cell membrane. Using a range of basic peptides, Futaki et al. have demonstrated that a peptide containing eight arginine residues can efficiently translocate across the membrane (16). Recently, a chimeric peptide derived from galparan and transportan has been used as an effective peptide vector for biodelivery of PNA molecules (52).

Previously, we have shown that transfection of an anti-TAR PNA complementary to the stem-loop and bulge regions of HIV-1 TAR can effectively inhibit the Tat-mediated transactivation of HIV-1 LTR in CEM cells (40). In this report, we conjugated the anti-TAR PNA with a membrane-permeating peptide vector, transportan, and examined the functional efficacy and antiviral activity of this chimeric molecule (PNA_{TAR}-transportan conjugate) in cell culture. Our results demonstrate that the PNA_{TAR}-transportan conjugate retained affinity for its target sequence and was able to effectively block Tat-mediated transactivation and HIV-1 production when added to the culture medium.

MATERIALS AND METHODS

PNA oligomers. The sequence of PNA_{TAR}-transportan conjugate, tetramethylrhodamine (TAMRA)-labeled PNA-transportan conjugate, and scrambled-PNA-transportan conjugate are shown in Fig. 1. PNA oligomers and their con-

jugated derivatives were obtained from Applied Biosystems. To avoid the problem of precipitation of PNA at high concentrations, the working stocks of PNA oligomers as well as the PNA-transportan conjugates were maintained at a 100 μ M concentration and stored at 4°C as recommended. The stocks were prepared by dissolving the PNA in water and heating to 50°C for 10 min, followed by incubation at 37°C for 30 min prior to quantification. Unused portions of the PNA stocks were aliquoted after 2 weeks, lyophilized, and stored at 4°C. The TAMRA-labeled PNA was protected from light to avoid photo-bleaching during its preparation and during the experiments.

Plasmid constructs. Plasmids for expression in mammalian cells were as follows: pHIV-1 LTR-Luc (a kind gift from M. B. Mathews), containing the firefly luciferase gene cloned downstream of the HIV-1 LTR; pDNA3-Tat (pCMV-Tat), encoding the 72-amino-acid Tat protein under the control of the cytomegalovirus (CMV) promoter (15); pCMV-R.Luc (Promega, Madison, Wis.), for expressing *Renilla* luciferase under the control of the CMV promoter; and pDNA3.1 (Invitrogen Corp., Carlsbad, Calif.), encoding the CMV promoter.

Plasmids used for preparing transcripts for gel shift assays. pEM7 and pTAR-BS were used for generating the wild-type TAR RNA and its mutant derivative carrying a deletion at the top of the stem (18), and pET-28a-RT was used for transcribing a 427-base-long HIV-1 reverse transcriptase coding fragment (27).

Plasmids used for generating probes for RNase protection assay. The plasmids used for generating probes for the RNase protection assay were as follows: plasmids pSP-luc+ and pSP-luc (Promega), encoding the firefly and *Renilla* luciferase gene cassettes, respectively, and having an opposing T7 promoter located downstream of the luc+ and rluc insert, whereas pGEM23 (34) has an opposing SP6 promoter located downstream of the TAR gene insert.

Cell culture and transfection. CEM and Jurkat T-cell lymphocytes were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, 100 U of penicillin, and 100 μ g of streptomycin per ml at 37°C in 5% CO₂-containing humidified air. The chronically HIV-1-infected H9 cells were maintained under identical conditions except that they were supplemented with 20% fetal calf serum. Jurkat T cells (5.0×10^6 cells in 300 μ l of RPMI 1640 supplemented with 20% fetal calf serum) were transfected with requisite amounts of the experimental plasmids pHIV-1 LTR-Luc and pCMV-Tat by electroporation using a Bio-Rad gene pulser II at 280 V and 975 μ F capacitance. In order to monitor the efficiency of transfection, the cells were cotransfected with the reporter plasmid pCMV-R.Luc. The cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 20 ng/ml) and the calcium ionophore A23187 (1 μ M) at 24 h posttransfection.

CEM cells were washed with equal volumes of phosphate-buffered saline (PBS) (without Ca²⁺ or Mg²⁺) before transfection. After washing, cells were resuspended in unsupplemented RPMI 1640 medium (5.0×10^6 cells in 250 μ l) and electroporated with experimental and reporter plasmids at 230 V and 800 μ F capacitance. Cells were then grown in 10 ml of complete RPMI 1640 medium.

Cellular uptake of PNA-transportan conjugate. The uptake of TAMRA-tagged PNA-transportan conjugate was examined in CEM cells and Jurkat cells. Briefly, the log-phase cells were washed with PBS and resuspended in a six-well plate in serum-free RPMI medium at a cell density of 3×10^5 cells/ml. The TAMRA-PNA-transportan conjugate (75 nM) was added to the culture medium, and the cells were incubated at 37°C. At various time intervals, aliquots (700 μ l) of the cells were removed, washed with PBS, and examined by fluorescence microscopy. Simultaneously, the effect of PNA-transportan conjugate on cell viability was examined using the calcein AM component from the live-dead viability kit (Molecular Probes) as per the manufacturer's protocol.

Electrophoretic mobility shift assay. The affinity and specificity of the PNA_{TAR}-transportan conjugate for the TAR RNA was examined by gel mobility shift analysis using the wild-type TAR RNA probe, a mutant TAR RNA probe, and the HIV-1 reverse transcriptase RNA probe. The ³²P-labeled TAR and TAR BS mutant RNA probes were prepared by *in vitro* transcription of the linearized plasmid templates as described previously (40). The 427-base HIV-1 reverse transcriptase (RT) RNA probe was generated by digesting pET-28a-RT with *EcoRV* and transcribing it using the T7 RNA polymerase as per the manufacturer's protocol (Roche Biochemicals). TAR RNA transcript (5×10^3 Cerenkov cpm) was incubated with PNA_{TAR}-transportan conjugate, unconjugated PNA_{TAR}, or scrambled PNA-transportan conjugate at various molar ratios. In another set, PNA_{TAR}-transportan conjugate was incubated with the mutant TAR BS or the HIV-1 RT radiolabeled probe at various molar ratios in order to evaluate its binding specificity.

Incubations were carried out for 3 h at 37°C in a binding buffer containing 50 mM Tris-HCl (pH 7.8), 60 mM KCl, 5 mM MgCl₂, 10 mM dithiothreitol (DTT), 10% glycerol, 0.01% bovine serum albumin, 0.01% NP-40, and 500 ng of poly(I-R-C), in a final volume of 15 μ l. Then 3 μ l of RNA gel loading dye (0.27%

bromophenol blue and 20% glycerol) was added to the samples and subjected to gel retardation analysis on native 6% polyacrylamide gel in Tris-borate-EDTA (TBE) buffer, pH 8.2. The gels were routinely run at 100 V for 30 min at 4°C in TBE buffer. The various complexes were resolved at a constant voltage of 150 V at 4°C. The gels were dried, visualized on a PhosphorImager (Molecular Dynamics), and quantified using Image-Quant software (Molecular Dynamics).

Reverse transcription of TAR RNA transcript. Reverse transcription catalyzed by HIV-1 RT on the TAR RNA transcript in the presence or absence of PNA_{TAR}-transportan conjugate, unconjugated PNA_{TAR}, or scrambled PNA-transportan conjugate was monitored by extension of 5'-³²P-labeled 17-mer DNA primer annealed with the TAR RNA template. The PNA_{TAR}-transportan conjugate or PNA_{TAR} at 1 μ M concentration was incubated with 10 nM annealed template-primer at 37°C for 1 h in a reaction buffer containing 50 mM Tris-HCl (pH 7.8), 10 mM DTT, 100 μ g of bovine serum albumin per ml, 60 mM KCl, and 5 mM MgCl₂ and used in the extension reaction. Reverse transcription was initiated by the addition of 50 nM HIV-1 RT and 100 μ M each of the four deoxynucleoside triphosphates. The reaction mixture was incubated at 25°C, and the reaction was terminated at the indicated time by the addition of an equal volume of Sanger's gel loading solution (56). The products were resolved on an 8% polyacrylamide-urea gel and visualized by PhosphorImager.

Assay for Tat-mediated transactivation of HIV-1 LTR. The amounts of Tat required for the transactivation of the HIV-1 LTR were optimized in Jurkat and CEM cells. In Jurkat cells, the plasmid pHIV-1 LTR-Luc (4 μ g) was cotransfected with pCMV-R.Luc (1 μ g) and various amounts of the Tat expression clone (pCMV-Tat) and electroporated under standard conditions as described above. The cells were plated in 10 ml of complete RPMI 1640 medium and stimulated with PMA and the Ca²⁺ ionophore A23187 at 24 h posttransfection. Following 8 h of stimulation, the cells were harvested and assayed for luciferase activity.

In another set of experiments, CEM cells were transfected with identical amounts of the plasmid cocktail comprising pHIV-1 LTR-Luc and pCMV-R.Luc and various amounts of plasmid pCMV-Tat under standard reaction conditions and assayed for luciferase activity at 16 h posttransfection.

Tat-mediated transactivation of HIV-1 LTR in cell culture in the presence of PNA_{TAR}-transportan conjugate. The effect of the PNA_{TAR}-transportan conjugate on Tat-mediated transactivation of HIV-1 LTR was assessed in both CEM and Jurkat cells as described below. CEM and Jurkat cells (5.0×10^6 cells) were electroporated with a plasmid cocktail comprising pHIV-1 LTR-Luc (4 μ g) and pCMV-R.Luc (1 μ g) in the presence or absence of optimal amounts of plasmid pCMV-Tat as determined from the above experiment. The Jurkat and CEM cells were allowed to recover from the effects of electroporation in complete RPMI medium containing 10% fetal bovine serum for 12 h and 3 h, respectively.

To facilitate the uptake of PNA-transportan conjugate, the cells were washed with PBS and resuspended in RPMI medium containing various concentrations of the PNA_{TAR}-transportan conjugate. The Jurkat cells were then stimulated with PMA and Ca²⁺ ionophore at 20 h posttransfection and harvested 8 h after stimulation. The CEM cells were harvested at 20 h posttransfection. The cells were analyzed for the luciferase reporter activities in order to evaluate the effect of the PNA_{TAR}-transportan on Tat-mediated transactivation of the HIV-1 LTR. Identical sets of experiments were carried out to determine the effect of the unconjugated PNA_{TAR}, scrambled PNA-transportan conjugate, and transportan on Tat-mediated transactivation of the HIV-1 LTR.

In a similar set of experiments, the CEM and Jurkat cells were electroporated with the plasmid pCMV-R.Luc and incubated in the presence or absence of PNA_{TAR}-transportan, unconjugated PNA_{TAR}, scrambled PNA-transportan conjugate, or transportan. The cells were harvested at 16 to 24 h posttransfection and analyzed for *Renilla* luciferase activity as described below.

Luciferase assays. Luciferase assays were performed by using the Promega dual luciferase assay kit. Briefly, the cells harvested at 1,500 rpm for 7 min were washed once with PBS and lysed by the addition of 50 μ l of the reporter lysis buffer (Promega). Following incubation at 25°C for 15 min on a rocking shaker, the lysates were centrifuged at 15,000 rpm for 10 min, and the supernatant was collected in fresh tubes. Luciferase assays were performed by mixing 30 μ l of the supernatant with 75 μ l of the firefly luciferase substrate in a 96-well Fluorotrac plate, and the light emission was measured using a Packard Top Count luminescence counter. The firefly luciferase activity was quenched by the addition of Stop and Glo reagent, which also served as a substrate for estimating the *Renilla* luciferase activity. Transfection efficiencies were normalized by the expression levels of the *Renilla* luciferase reporter gene construct cotransfected along with the experimental plasmid. The results of at least three separate transfections were analyzed for each experiment.

HIV-1 production in H9 cells in the presence of PNA_{TAR}-transportan conjugate. The chronically HIV-1-infected H9 cells were centrifuged and washed extensively in PBS to remove the previously produced virions. The cells were

suspended in 1.0 ml of serum-free RPMI 1640 medium at 5×10^6 cells/well and incubated in a six-well culture plate at 37°C for 6 h in the absence or presence of various concentrations of PNA_{TAR}-transportan conjugate, unconjugated PNA_{TAR}, scrambled PNA-transportan conjugate, or transportan alone. The cells were then reconstituted to a final cell density of 10^6 cells/ml in complete RPMI 1640 medium and incubated at 37°C. The cells were harvested on the third day at 1,200 rpm for 7 min, and the levels of p24 antigen were analyzed in the supernatants with an enzyme-linked immunosorbent assay (ELISA) p24 antigen kit (Abbott Laboratories). The cells were also analyzed for HIV-1 mRNA levels after total RNA isolation and RNase protection analysis as described below.

Total RNA isolation and RNase protection assay. The plasmids pSP-luc+, pSP-rLuc, and pGEM23 were linearized with the restriction enzymes *HincII*, *BsaI*, and *XbaI*, respectively. The former two linearized plasmids were used to generate radioactively labeled riboprobes for the firefly and *Renilla* luciferase corresponding to 390 nucleotides and 245 nucleotides, respectively. Briefly, the digested plasmids were transcribed using T7 RNA polymerase in the presence of [α -³²P]UTP (Perkin Elmer Life Sciences, Inc.) as per the MAXIScript in vitro transcription kit (Ambion Inc., Austin, Tex.). A 195-nucleotide-long riboprobe for the TAR RNA was synthesized from linearized pGEM23 with SP6 RNA polymerase (New England Biolabs) and [α -³²P]UTP under standard reaction conditions. The DNA templates were removed by DNase I digestion, and the RNA probes were purified by gel electrophoresis.

For the RNase protection assay, transfection and other experimental conditions as described above were maintained in the CEM, Jurkat, and H9 cells. The cells were harvested at the stated time and washed with PBS, and total RNA was isolated from the immortalized cell lines using the RNAqueous kit (Ambion Inc., Austin, Tex.). Seven micrograms of total RNA extracted from the experimental and control samples was hybridized to 7×10^4 to 10×10^4 cpm of the individual riboprobe and analyzed as per the RPA III kit (Ambion Inc., Austin, Tex.). Protected fragments were separated on a 6% polyacrylamide-urea gel and detected by PhosphorImager analysis.

Determination of [³H]thymidine incorporation into cellular DNA in chronically HIV-1-infected H9 cells. Cellular proliferation of chronically HIV-1-infected H9 cells in the presence of PNA_{TAR}-transportan conjugate was determined by estimating the levels of [³H]thymidine incorporated in their nuclei. Briefly, freshly split cells were grown in the presence or absence of PNA_{TAR}-transportan conjugate (5 μ M concentration) and supplemented with 10 μ Ci of [*methyl*-³H]thymidine/ml (83.7 Ci/mmol). The cells were withdrawn at 3, 12, 24, 36, and 48 h, and the cell number was determined using a Coulter counter. The cells were harvested, washed with PBS, and resuspended in 200 μ l of lysis buffer containing 1% NP-40 in PBS. The nucleic acids were precipitated by adding cold 10% trichloroacetic acid (TCA). Precipitates were collected on GF/C glass fiber filters (Whatman, Inc., Maidstone, Kent, England) and washed extensively with ice-cold 10% TCA and once in 70% ethanol. Filters were dried and placed in scintillation vials, and radioactivity was counted in the scintillation counter. Protein content in each lysate was estimated by the Bio-Rad protein assay. Results were expressed as counts per minute per milligram of protein.

RESULTS

Synthesis of PNA and PNA-transportan conjugates. PNA and PNA-transportan conjugates were synthesized at Applied Biosystems. The PNA molecules are linked to the transportan peptide through a disulfide linkage, as shown in Fig. 1. Secondary structure of the HIV-1 TAR RNA is also shown in this figure. The TAR sequences interacting with the PNA_{TAR}-transportan conjugate are indicated by arrows. The molecular mass of the PNA_{TAR}-transportan conjugate was analyzed by matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS). The observed mass of the conjugate (7,382.3 Da) corresponded to the calculated mass (7,377.3 Da) with a variance of -0.07% .

Affinity of PNA_{TAR}-transportan conjugate to TAR. In order to ascertain whether PNA_{TAR} conjugated with the transportan peptide vector retained its binding affinity for its target sequence on the TAR RNA, we performed gel mobility shift assays with the ³²P-labeled 82-base-long TAR RNA transcript and various concentrations of PNA_{TAR}-transportan conjugate,

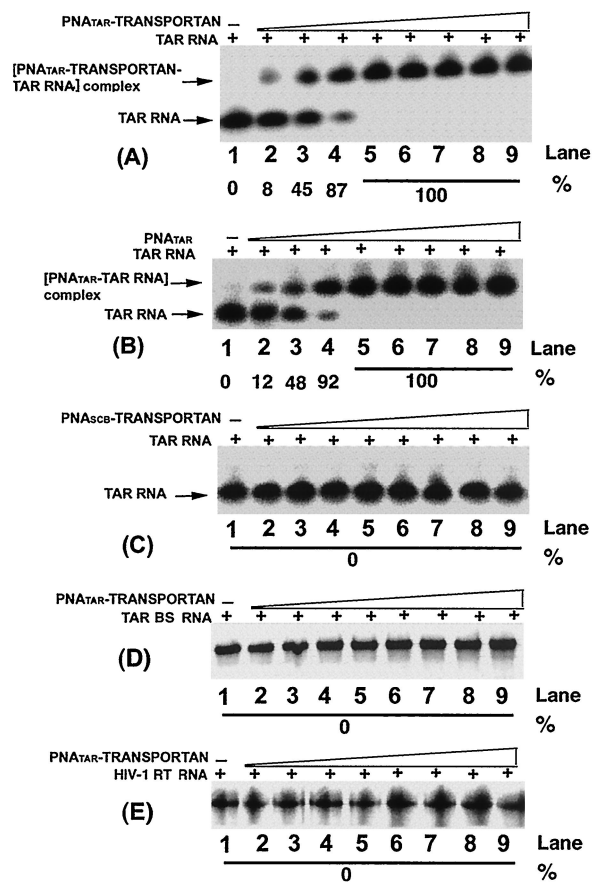


FIG. 2. Binding specificity of PNA_{TAR}-transportan conjugate to its target sequence. The affinity of the PNA_{TAR}-transportan conjugate (A) for its target sequence on the TAR RNA was assessed by gel mobility shift analysis as described in Materials and Methods. Unconjugated PNA_{TAR} (B) was included as a positive control, and scrambled PNA-transportan conjugate (PNA_{SCB}-transportan) (C) was included as a negative control. The specificity of this interaction was determined by analyzing the relative gel mobility shift upon interaction of PNA_{TAR}-transportan conjugate with a mutant TAR RNA (TAR BS; panel D) as well as with an unrelated RNA such as the HIV-1 RT RNA (panel E). In panel A, lanes 1 through 9 represent molar ratios of PNA_{TAR}-transportan conjugate to TAR RNA of 0, 0.1, 0.5, 0.8, 1.0, 2.5, 5, 7.5, and 10, respectively. Panel B and panel C show gel mobility shifts performed at similar ratios as indicated in panel A except that unconjugated PNA_{TAR} and scrambled PNA-transportan conjugate, respectively, were used. Similar ratios are also shown in panels D and E except that the mobility shift of the PNA_{TAR}-transportan conjugate was carried out with the TAR BS and HIV-1 RT RNA probes, respectively. The extent of gel shift was determined by quantifying the probe RNA band on the PhosphorImager using Image-Quant software (Molecular Dynamics). The percentage of labeled TAR RNA retarded due to PNA binding is indicated.

scrambled PNA-transportan conjugate, or unconjugated PNA_{TAR} (Fig. 2A to C). Titration of PNA_{TAR}-transportan conjugate with TAR RNA at molar ratios of the conjugate to TAR RNA of less than 1 resulted in a stoichiometric band shift of the labeled TAR RNA (Fig. 2A, lanes 2 to 4).

As shown in the figure, at molar ratios of 0.1, 0.5, and 0.8 of the conjugate to labeled TAR RNA, the extent of band shift corresponded to approximately 8, 45, and 87%, respectively. A complete shift in the mobility was achieved at an equimolar

ratio or a molar excess of the conjugate to TAR RNA (panel A, lanes 5 to 9). A similar titration was carried out in the presence of unconjugated PNA_{TAR} (panel B, lanes 2 to 9) and scrambled PNA-transportan conjugate (panel C, lanes 2 to 9). As shown in the figure, the binding of PNA_{TAR} to the TAR RNA was similar to that obtained with the PNA_{TAR}-transportan conjugate, suggesting that conjugation of PNA_{TAR} with the transportan peptide vector had no effect on its binding affinity. Scrambled PNA-transportan conjugate at similar molar ratios did not result in any gel shift, suggesting the specificity of the interaction. Further evidence of this specificity was demonstrated by our observation that interaction of PNA_{TAR}-transportan conjugate with TAR BS, a 63-base-long mutant TAR RNA carrying a deletion in the stem and bulge region (panel D), and with HIV-1 RT RNA, an unrelated RNA (panel E), also exhibited no shift in mobility.

Reverse transcription of TAR RNA region is blocked by PNA_{TAR}-transportan conjugate. Since the PNA_{TAR}-transportan conjugate displayed affinity for TAR RNA similar to unconjugated PNA_{TAR}, it was interesting to examine if the conjugate could effectively block reverse transcription of TAR RNA. Ability to block reverse transcription, in turn, would have multiple impacts on viral replication besides influencing Tat-mediated transactivation. For this purpose, the TAR RNA transcript primed with the labeled 17-mer DNA primer was incubated in the absence or presence of 100 nM PNA_{TAR}-transportan conjugate, PNA_{TAR}, or scrambled PNA at 37°C, followed by initiation of reverse transcription by HIV-1 RT. The results are presented in Fig. 3. PNA_{TAR} and PNA_{TAR}-transportan conjugate showed a similar pattern of reverse transcription, with a prominent pause at nucleotide position 42, prior to the loop site targeted by these two PNAs. These results suggest that conjugation of PNA_{TAR} with transportan did not alter the ability of PNA_{TAR} to block reverse transcription of TAR RNA.

Cellular uptake of PNA-transportan and its intracellular localization. Although PNAs display strong binding affinity to their target sequence and have great potential as antisense agents, their biodelivery in the cell is very poor (47). The biodelivery of PNA into cells has been enhanced by cationic detergents (39) and by linking them with membrane-translocating peptides (51). To evaluate the uptake of the PNA-transportan conjugate in our system, we used a transportan-conjugated 10-mer PNA tagged with a TAMRA fluorophore probe. The entry of the dye-linked conjugate into the cells was then monitored by fluorescence microscopy and analyzed by fluorescence-activated cell sorting (FACS) analysis. The results are shown in Fig. 4 and Table 1.

It was observed that the PNA-transportan conjugate is able to enter the cells in a time-dependent manner, confirming that transportan linked with PNA retained its ability to cross the cell membrane (Fig. 4). At 12 h, approximately 90% of the CEM cells were found to display fluorescence, as against 52% of the Jurkat cells (Table 1). This suggests that the PNA-transportan conjugate can efficiently cross the membrane of both cell types, although the kinetics of cell entry varied. Furthermore, the PNA-transportan conjugate had no detrimental effect on cell viability up to the 10 μ M concentration tested in both CEM and Jurkat cells (results not shown). These results demonstrate that membrane-permeating peptide vectors such

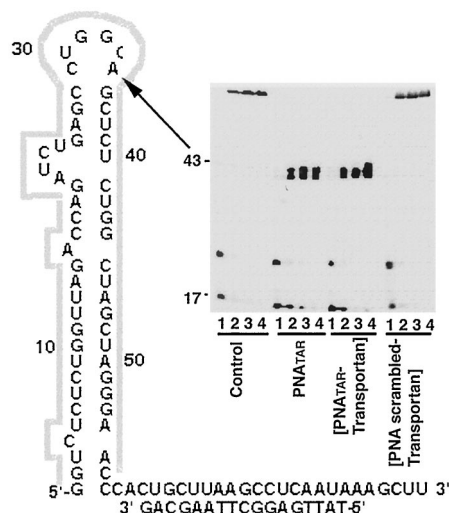


FIG. 3. Effect of PNA_{TAR}-transportan conjugate on reverse transcription on TAR RNA primed with the 17-mer DNA primer. TAR RNA template primed with the 5'-³²P-labeled 17-mer DNA primer was incubated in the absence or presence of PNA_{TAR}-transportan conjugate, unconjugated PNA_{TAR}, or scrambled PNA-transportan conjugate at 37°C for 1 h and used in the gel extension reaction as described under Materials and Methods. The reaction products were analyzed on a denaturing 8% polyacrylamide-urea gel and subjected to PhosphorImager analysis. Lanes 1 through 4 in each set represent the extension reactions at 25°C for 5, 10, 15, and 20 min, respectively. The control set represents reactions carried out in the absence of PNA_{TAR}-transportan conjugate, unconjugated PNA_{TAR}, or scrambled PNA-transportan conjugate. The arrow indicates the position on the gel corresponding to the sequence targeted by PNA_{TAR}-transportan conjugate as well as unconjugated PNA_{TAR} on TAR RNA.

as transportan may be effective carriers for intracellular delivery of PNA.

Inhibition of Tat-mediated transactivation of HIV-1 LTR by PNA_{TAR}-transportan conjugate. Since the transporter peptide was found to be an efficient vehicle for biodelivery of PNA, we examined the ability of PNA_{TAR}-transportan conjugate to block Tat-mediated transactivation of HIV-1 LTR in CEM and Jurkat cells. Using the reporter plasmid, we first established the optimum amounts of Tat required for the expression of the HIV-1 LTR in these cells. For this, we used the highly sensitive assay system involving expression of *Renilla* luciferase and firefly luciferase. Expression of the firefly luciferase (pHIV-1 LTR-Luc) and *Renilla* luciferase (pCMV-R.Luc) was under the control of the HIV-1 LTR and CMV promoter, respectively. The cells were transfected with these two plasmids in the presence of various concentrations of the Tat expression vector (pCMV-Tat).

In the Jurkat cells, the transfected cells were activated with PMA and Ca²⁺ ionophore for expression of the reporter plasmids. When Tat was expressed in *trans*, the firefly luciferase was transactivated severalfold, while the *Renilla* luciferase expression, used as an internal control to monitor transfection efficiency, remained constant. Expression of the firefly luciferase in CEM as well as Jurkat cells was found to be proportional to the concentration of pCMV-Tat used in the transfection. The amount of pCMV-Tat for optimum stimulation of HIV-1 LTR in CEM cells (100-fold stimulation) and Jurkat

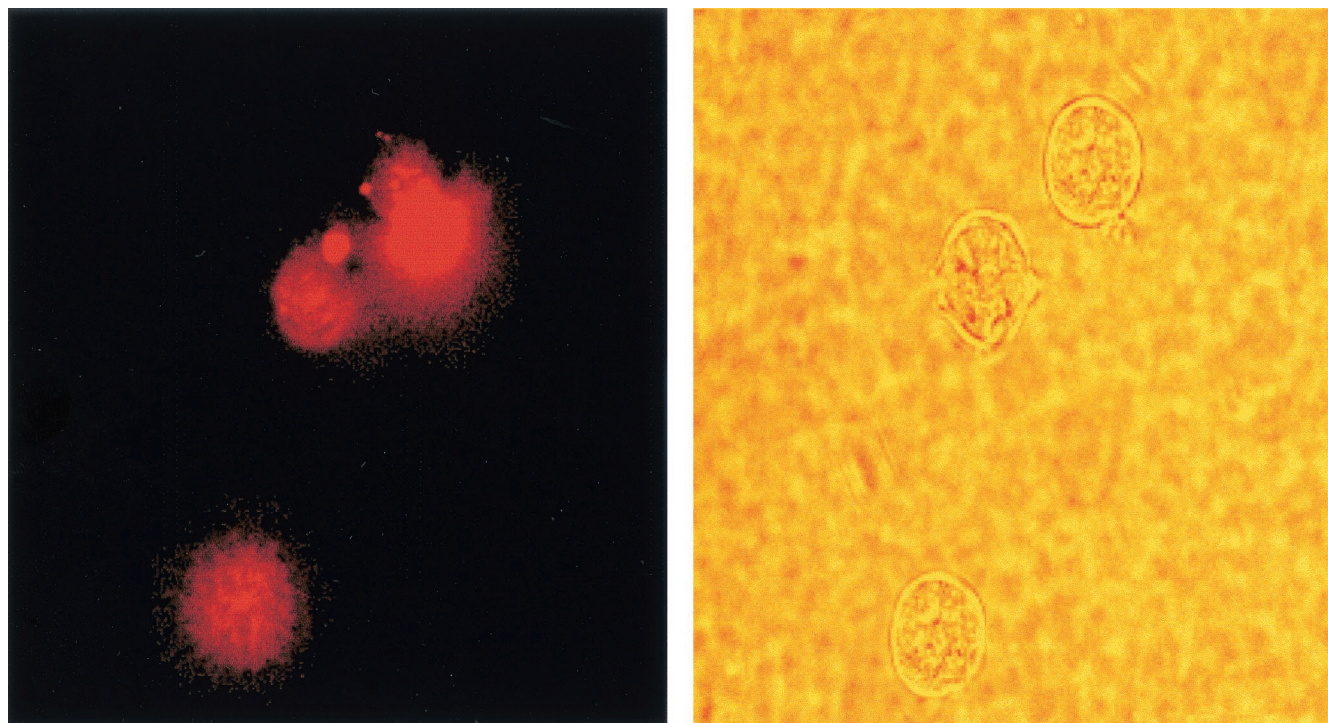


FIG. 4. Cellular uptake of PNA-transportan conjugate. CEM cells were incubated with 70 nM TAMRA-tagged PNA-transportan conjugate in a six-well plate in RPMI 1640 medium in the absence of serum. At various times, aliquots of the cells were washed, resuspended in PBS, and examined by fluorescence microscopy for monitoring cellular uptake. The panel on the left is the fluorescence image, and that on the right is the phase-contrast image of the same field.

cells (150-fold stimulation) was 1 μ g and 3 μ g, respectively (Fig. 5).

Using this standardized system, we determined the functional ability of the PNA_{TAR}-transportan conjugate to inhibit Tat-mediated transactivation of the HIV-1 LTR. The results are shown in Fig. 6. As shown in the figure, the PNA_{TAR}-transportan conjugate substantially inhibited Tat-mediated transactivation of the HIV-1 LTR, as judged from the expression levels of luciferase in both CEM and Jurkat cells. Approximately 55% inhibition was achieved at 500 nM PNA-peptide conjugate, which gradually leveled off to 70 to 80% inhibition at higher concentrations. Unconjugated PNA_{TAR}, scrambled PNA-transportan, and transportan itself had no effect on the luciferase activity under identical conditions. These results demonstrate that PNA_{TAR}-transportan present in the culture medium is able to enter the cells and prevent Tat-mediated transactivation by efficiently sequestering the TAR region of the HIV-1 LTR.

TABLE 1. Time course of TAMRA-tagged PNA-transportan conjugate uptake in cells^a

Cells	Uptake of PNA-transportan (%) at time posttransfection:				
	1 h	2 h	4 h	6 h	12 h
CEM	5	12	35	55	90
Jurkat	0	3	16	27	52

^a The extent of uptake of TAMRA-tagged PNA-transportan conjugate was monitored in CEM and Jurkat cells as described in Materials and Methods. The values represent averages of three independent experiments.

PNA_{TAR}-transportan inhibits at the level of transcription. A substantial reduction in the expression of the luciferase reporter in both CEM and Jurkat cells suggested that the PNA_{TAR}-transportan inhibits the Tat-mediated transactivation of the HIV-1 LTR. To determine if the inhibition was at the level of transcription, we performed RNase protection assays. CEM and Jurkat cells transfected with an HIV-1 LTR promoter-driven luciferase reporter plasmid in the absence or presence of a Tat expression plasmid were treated with various concentrations of PNA_{TAR}-transportan, PNA_{TAR}, scrambled PNA-transportan, or transportan and harvested at the specified times for total RNA isolation. In addition, the *Renilla* luciferase reporter driven by a CMV promoter was included as a control for monitoring transfection efficiency. The RNA samples were hybridized with the ³²P-labeled pSPLuc+ and pSPrluc probes, digested with the RNase A and T₁ mix, and examined by denaturing gel electrophoresis for protected fragments corresponding to the firefly and *Renilla* transcripts, respectively.

A representation of one such analysis is presented in Fig. 7. Our results indicated that the accumulation of firefly RNA was inversely proportional to the concentration of PNA_{TAR}-transportan in CEM and Jurkat cells (Fig. 7A). This provides direct evidence that the PNA_{TAR}-transportan interferes with the transcription process, presumably by binding to the TAR region of the HIV-1 LTR, thereby preventing Tat-mediated transactivation. Neither the unconjugated PNA_{TAR}, transportan itself, nor scrambled PNA-transportan affected the firefly RNA expression levels, indicating the specificity of the interaction of PNA_{TAR}-transport-

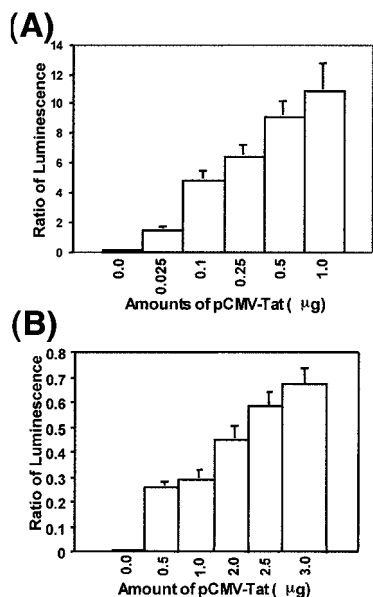


FIG. 5. Tat-mediated transactivation of HIV-1 LTR. The indicated amounts of pCMV-Tat were cotransfected along with a plasmid cocktail comprising pHIV-1 LTR-Luc (4 µg) and pCMV-R.Luc (1 µg) in Jurkat and CEM cells (5.0×10^6 cells) in order to establish the extent of Tat-mediated transactivation of the HIV-1 LTR. The highly sensitive luciferase reporter was used for this analysis. The extent of stimulation of the HIV-1 LTR was monitored as a function of the expression of the firefly luciferase gene, with the *Renilla* luciferase reporter serving as an internal control for normalizing the transfection efficiency. The results are expressed as the ratio of firefly to *Renilla* luciferase activity. Panels A and B represent the extent of transactivation in CEM and Jurkat cells, respectively, as a function of pCMV-Tat concentration. The results are presented as an average of three independent experiments. The bars represent the standard deviation.

tan with its target sequence. Furthermore, the effect of PNA_{TAR}-transportan on the HIV-1 LTR appeared to be promoter specific, as noted from our observation that PNA_{TAR}-transportan, PNA_{TAR}, transportan, and scrambled PNA-transportan did not affect the expression levels of the *Renilla* RNA driven by the CMV promoter (Fig. 7B).

Effect of PNA_{TAR}-transportan on HIV-1 production in chronically HIV-1-infected H9 cells. Since PNA_{TAR}-transportan was able to enter the cells and interfere with Tat-mediated transactivation, we examined its antiviral efficacy in H9 cells chronically infected with HIV-1. These cells were grown in the absence or presence of PNA_{TAR}-transportan (1.0 to 5.0 µM), unconjugated PNA_{TAR} (5.0 µM), transportan (5.0 µM), or scrambled PNA-transportan (5.0 µM) for 3 days, and their culture supernatants were analyzed for levels of p24 antigen by ELISA, since the levels of p24 antigen provided a measure of viral titers. The results are shown in Fig. 8.

The levels of p24 antigen decreased by 60% at 1 µM PNA_{TAR}-transportan conjugate. Further increases in the concentration of the PNA_{TAR}-transportan conjugate resulted in a substantial decrease (70 to 90%) in the p24 levels, suggesting its potential for blocking HIV-1 production (Fig. 8A). Our observation that unconjugated PNA_{TAR} at similar concentrations did not decrease viral production indicated the efficacy of PNA_{TAR}-transportan. Furthermore, the effect of PNA_{TAR}-transportan appeared to be quite specific, since neither scam-

bled PNA-transportan nor transportan by itself decreased HIV-1 production under identical conditions (Fig. 8B).

PNA_{TAR}-transportan inhibits transcription of HIV-1 mRNA in chronically HIV-1-infected H9 cells. We also examined the mechanism by which PNA_{TAR}-transportan inhibited HIV-1 production in chronically HIV-1-infected H9 cells. Total RNA isolated from the cells treated with PNA_{TAR}-transportan, unconjugated PNA_{TAR}, transportan, or scrambled transportan were hybridized to the radiolabeled probe synthesized from pGEM23 and subjected to an RNase protection assay. The results are shown in Fig. 9.

The mRNA initiating at +1 of the HIV-1 LTR protected an 83-nucleotide fragment of the riboprobe corresponding to the anti-TAR sequence. A decrease in levels of HIV-1 mRNA in cells treated with PNA_{TAR}-transportan suggested that the inhibition occurred at the transcription step. The specificity of the effect of PNA_{TAR}-transportan was seen from the observation that neither transportan itself nor scrambled PNA-transportan altered the HIV-1 mRNA levels. Furthermore, PNA_{TAR} alone also did not influence the levels of HIV-1 mRNA synthesis, indicating the efficiency of transportan as a vehicle for delivering PNA_{TAR} to its target site.

PNA_{TAR}-transportan conjugate has no effect on cellular pro-

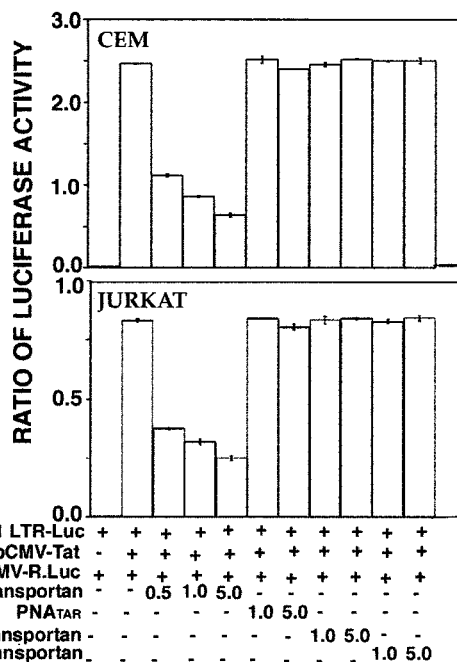


FIG. 6. Effect of PNA_{TAR}-transportan conjugate on Tat-mediated transactivation of HIV-1 LTR. CEM and Jurkat cells (5.0×10^6 cells), cotransfected with the plasmid cocktail comprising pHIV-1 LTR-Luc (4 µg) and pCMV-R.Luc (1 µg) in the presence or absence of the Tat expression clone pCMV-Tat, were incubated with the indicated concentrations of the PNA_{TAR}-transportan, unconjugated PNA_{TAR}, transportan, or scrambled PNA-transportan (PNA_{scb}-transportan) under the conditions described in Materials and Methods. At 20 h (CEM) or 28 h (Jurkat) posttransfection, cell lysates were assayed for firefly and *Renilla* luciferase activities. The results are presented as the relative ratio of firefly to *Renilla* luciferase activity. The amounts of the various plasmids, transportan, and PNA used in the experiment are indicated. The results are presented as an average of three independent experiments. The bars represent the standard deviation.

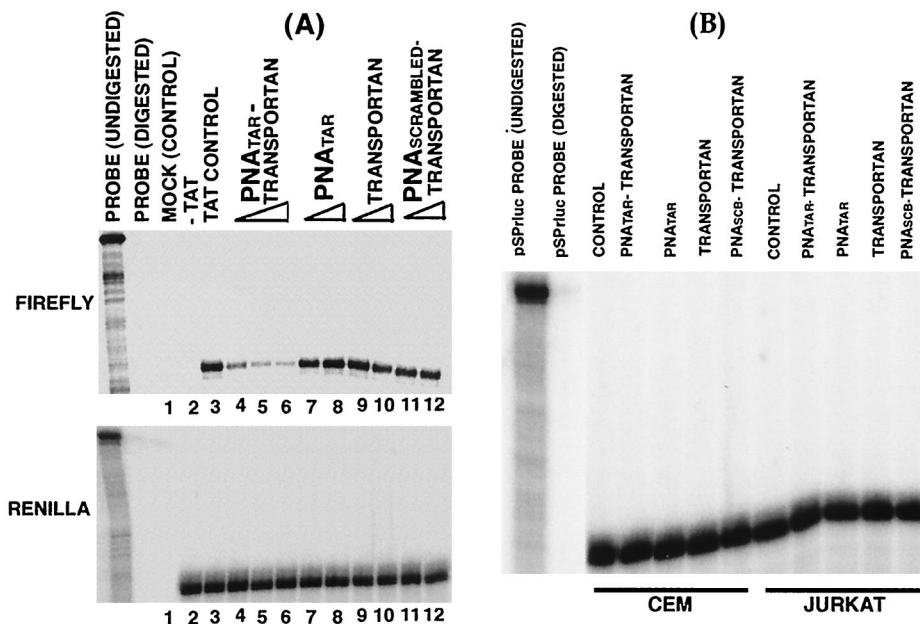


FIG. 7. RNase protection assay. (A) Jurkat cells were transfected with 4 μg of pHIV-1 LTR-Luc and 1 μg of pCMV-R.Luc in the absence or presence of pCMV-Tat (2.5 μg). The cells were grown in the presence of PNA_{TAR}-transportan (0.5 to 5.0 μM), PNA_{TAR} (1.0 and 5.0 μM), transportan (1.0 and 5.0 μM), or scrambled PNA-transportan (1.0 and 5.0 μM). At 28 h posttransfection, total RNA was isolated from the cells and hybridized with RNA probes for the different reporter transcripts (firefly and *Renilla* luciferase). The pSPrluc probe coding for the *Renilla* RNA served as a transfection control. The firefly RNA levels were normalized to the *Renilla* RNA signal. The lanes are as follows: mock-transfected cells (lane 1); luciferase expression in the absence of Tat (lane 2); and luciferase expression in the presence of Tat and the indicated inhibitors (lanes 3 to 12). (B) CEM and Jurkat cells were transfected with 1 μg of pCMV-R.Luc and incubated in the absence or presence of PNA_{TAR}-transportan, unconjugated PNA_{TAR}, transportan, or scrambled PNA-transportan (PNA_{SCB}-transportan) at 5 μM in order to investigate the promoter specificity of PNA_{TAR}-transportan. Total RNA was isolated from cells and subjected to the RNase protection assay with the pSPrluc probe. The protected fragments were analyzed on a denaturing polyacrylamide-urea gel and visualized on a PhosphorImager. *Renilla* RNA was normalized to total RNA in the cell. The lane marked control represents *Renilla* RNA signal in transfected cells in the absence of the inhibitor.

liferation of chronically HIV-1-infected H9 cells. We examined the incorporation of [³H]thymidine into cellular DNA in order to determine if the PNA_{TAR}-transportan conjugate impacted cellular proliferation of chronically HIV-1-infected H9 cells. The cells were grown in the presence or absence of PNA_{TAR}-

transportan and supplemented with [³H]thymidine. At the indicated time points, aliquots were withdrawn, and the DNA was precipitated with TCA to determine thymidine incorporation. The results of this analysis are shown in Fig. 10. As shown in the figure, similar amounts of [³H]thymidine were incorporated into cellular DNA in both the treated and untreated cells at all time points analyzed, indicating that cellular proliferation was not altered in the presence of PNA_{TAR}-transportan.

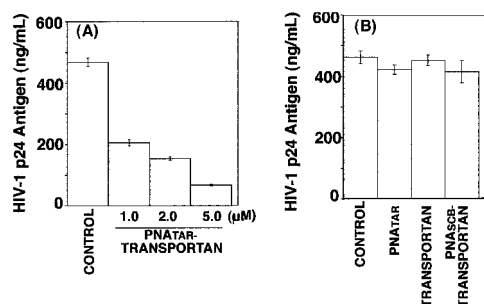


FIG. 8. Effect of PNA_{TAR}-transportan conjugate on HIV-1 production in chronically HIV-1-infected H9 cells. The indicated concentrations of PNA_{TAR}-transportan (A), unconjugated PNA_{TAR}, transportan, or scrambled PNA-transportan (PNA_{SCB}-transportan) (B) were added to the culture medium of chronically HIV-1-infected H9 cells, and their ability to inhibit HIV-1 production was examined by monitoring the levels of p24 antigen in the culture supernatant. The results are presented as relative amounts of HIV-1 p24 antigen present in the supernatants of H9 cells cultured in the absence (control) or presence of PNA_{TAR}-transportan (1.0 to 5.0 μM), unconjugated PNA_{TAR} (5.0 μM), transportan (5.0 μM), or scrambled PNA-transportan (5.0 μM). The results are expressed as mean values along with standard deviation for three independent experiments.

DISCUSSION

In spite of massive efforts for the prevention of AIDS, the AIDS epidemic has reached alarming proportions worldwide. The current antiviral treatment for HIV-1 infection is restricted to drugs that mainly target two key viral enzymes in its life cycle, reverse transcriptase and protease (7, 22, 23, 28, 32, 42–44, 55, 67). Successful HIV-1 therapy, such as the highly active antiretroviral therapy, is hampered by the appearance of drug-resistant strains of HIV-1, which have been directly correlated with mutations in the genes encoding the viral reverse transcriptase and protease (1, 5).

In the present study we have used specific PNA-transportan conjugate to target the TAR RNA, an important regulatory element in the nontranslated 5' end of the retroviral genome. The rationale for targeting TAR was that it forms a stable nuclease-resistant stem-loop structure which is averse to mutational changes (25, 59). HIV-1 Tat, a nuclear transcriptional activator, interacts with TAR to enhance the processivity of

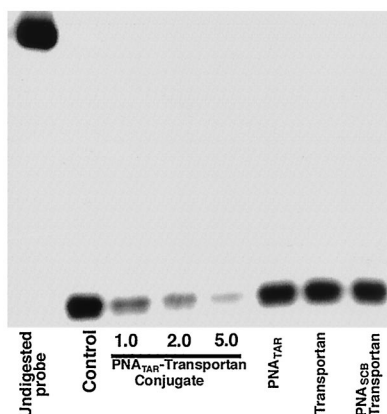


FIG. 9. PNA_{TAR}-transportan inhibits transcription of the HIV-1 mRNA in chronically HIV-1-infected H9 cells. Total RNA was isolated from chronically HIV-1-infected H9 cells treated with the indicated concentrations of PNA_{TAR}-transportan or 5.0 μ M PNA_{TAR}, transportan alone, or scrambled PNA-transportan (PNA_{SCB}-transportan). The RNA was hybridized with the pGEM23 probe and digested with the RNase A and T₁ mix, and the protected fragments were separated on a polyacrylamide-urea gel and detected by PhosphorImager analysis. The undigested probe corresponds to a 195-base fragment, whereas HIV-1 mRNA initiating at +1 of the HIV-1 LTR protected an 83-nucleotide fragment of the riboprobe corresponding to the anti-TAR sequence.

RNA polymerase II complexes (25). The importance of the Tat-TAR interaction in regulation of gene expression has been well established (21, 25). We had earlier demonstrated that a novel inhibitor, PNA, targeted to the TAR sequences of the viral RNA genome blocks the Tat-TAR interaction, resulting in the inhibition of transactivation of the HIV-1 LTR (40). In another study, PNA has been employed to inhibit *in vitro* protein synthesis at both the transcriptional and translational levels (20, 31).

Effective antisense inhibition in cells requires efficient cell entry. Though PNAs have been shown to inhibit gene expression and have several advantages over conventional DNA or RNA antisense oligomers, their use as an alternative therapy has been limited chiefly by their inability to cross the cell membrane efficiently (47). More recent findings demonstrating that PNAs conjugated to transporter peptides exhibit efficient biodelivery into cells have redefined its potential as a therapeutic agent (52).

For the present studies, we used the transportan peptide vector for biodelivery of PNA_{TAR}. Transportan is a 27-amino-acid chimeric peptide consisting of 13 amino acids from the amino terminus of the neuropeptide galanin and 14 amino acids from the amino terminus of the wasp venom toxic peptide mastoparan (33). Transportan penetrates every cell type in a rapid and efficient way and is localized mostly in membranous structure; upon prolonged incubation, it concentrates in the nucleus (50). In moderate concentrations (10 μ M), it does not affect the growth of cells in culture (50). These unique properties of the chimeric transportan peptide make it potentially useful as a vector for biodelivery of different proteins or nonprotein drugs into cells. We used this approach to address the issues of (i) uptake of the PNA-transportan conjugate by cells and its effect on cell viability and (ii) functional efficacy of the PNA_{TAR}-transportan conjugate in

blocking the Tat-TAR interaction, thereby inhibiting viral transcription and replication.

To ascertain the ability of the PNA-transportan conjugate to enter the cells, we tagged it with TAMRA, a fluorescent probe, and monitored its uptake in the cell. A substantial accumulation of the fluorophore in the cell in 12 h (Table 1) indicated efficient uptake, though the overall kinetics of its internalization was slower than with transportan alone (50, 51). In marked contrast to an earlier report demonstrating that cell type did not influence the cell-penetrating ability of transportan (51), the uptake of the PNA-transportan conjugate by CEM and Jurkat cells varied significantly (Table 1). This may be attributed to a difference in the morphology and membrane composition of these two cells, or it is likely that the presence of the PNA moiety influences uptake. Nonetheless, the accumulation of the PNA-transportan conjugate in the cells is encouraging and suggests that the transportan vector may be used effectively for biodelivery of this class of compounds.

PNA_{TAR}-transportan substantially inhibited Tat-mediated transactivation of the HIV-1 LTR in both CEM and Jurkat cells (Fig. 5). The PNA-peptide conjugate may have dual functions; it may prevent the TAR-Tat interaction by sequestering the TAR sequence of the nascent RNA, or it may prevent transcription directly by interacting with the TAR sequence on the proviral DNA. One may visualize that the large transportan moiety may interfere with the PNA, targeting its site inside the cell. However, this did not appear to be true in this study, since the PNA_{TAR}-transportan conjugate displayed similar binding affinity for its target sequence on the TAR RNA (Fig. 2) and was also able to block reverse transcription to a similar extent as the unconjugated PNA_{TAR} (Fig. 3). Moreover, a substantial inhibition of Tat-mediated transactivation was also achieved by PNA_{TAR}-transportan conjugate in cell culture (Fig. 6). The mechanism of this inhibition appeared to be at the level of transcription (Fig. 7).

Our results demonstrating that PNA_{TAR}-transportan can enter chronically HIV-1-infected H9 cells and inhibit virus production are very promising (Fig. 8). This is the first report

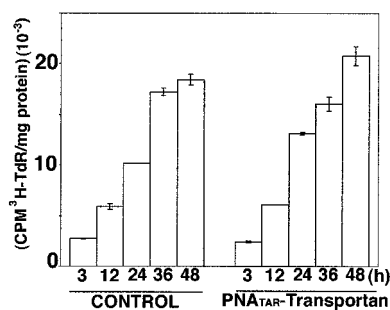


FIG. 10. [³H]thymidine incorporation into cellular DNA in chronically HIV-1-infected H9 cells. Chronically HIV-1-infected H9 cells grown in the presence or absence of 5.0 μ M PNA_{TAR}-transportan were supplemented with 10 μ Ci of [*methyl*-³H]thymidine/ml. At the indicated time points, the cells were harvested into DNA and assayed for the amounts of [³H]thymidine (TdR) incorporated into DNA by TCA precipitation of the acid-insoluble material onto glass fiber filters. The total amount of radioactivity was determined by scintillation counting, and the results are represented as counts per minute incorporated per milligram of protein. The results are expressed as average values along with the standard deviation for three independent experiments.

on the use of a PNA-transporter peptide conjugate to inhibit the replication of HIV-1. Interestingly, recent studies by Sei et al. (58) have demonstrated that PNA targeted to the Gag-Pol transframe domain can arrest virus production. However, very high concentrations of PNA (100 μ M) were required to achieve this effect. In contrast, the PNA-transportan conjugates used in this study were able to inhibit virus replication at significantly lower concentrations. It may also be noted that the effect of the PNA_{TAR}-transportan conjugate on transactivation of the HIV-1 LTR appeared to be more prominent in the transfected cells than in H9 cells. This is not surprising, as the events associated with the replication of HIV-1 in H9 cells are far more complex than those associated with the expression of the luciferase gene in cells.

The choice of antisense targets appears quite challenging, since targeting the various regulatory genes of the HIV-1 genome has not always been effective against diverse HIV-1 strains (2, 29, 30, 36–38, 66). The unique 5' untranslated region (U5) (nucleotides 1 to 333) of the HIV-1 genome, containing several critical domains essential for viral replication, may be an ideal target for drug intervention. These critical domains include (i) the primer-binding site (nucleotides 183 to 201), essential for tRNA₃^{Lys}-primed initiation of reverse transcription (45, 53, 62); (ii) the A-loop region located upstream of the primer-binding site (nucleotides 168 to 173), essential for the selection and interaction of tRNA₃^{Lys} primer (24, 35, 63); and (iii) the LTR sequences at the 5' and 3' ends, essential for viral transcription and integration (61).

Studies in our laboratory are under way to analyze the effect of PNA-transportan conjugates targeted against these sites as effective antiviral agents. A PNA-transportan conjugate directed against galanin receptor mRNA has been shown to inhibit the expression levels of galanin receptor in Bowes melanoma cells in cell culture and also alleviate pain in the rat model (52). Recently, Good et al. have shown that PNA conjugated with a 10-mer peptide vector targeted against rRNA and against mRNA of Acp protein of bacteria can effectively cure HeLa cells infected with *Escherichia coli* K-12 strains, raising the possibility of anti-infective drug development (17).

In summary, our studies have demonstrated that cellular uptake of anti-TAR PNA is significantly enhanced upon conjugation with a transportan peptide, resulting in effective blockage of the Tat-TAR interaction at relatively low inhibitor concentrations and inhibiting transcription. These studies with transportan peptides as the vector for biodelivery of PNA have created a therapeutic niche for the application of PNA against HIV-1. Thus, our approach using PNA-transportan to target the critical regions of the HIV-1 genome in the quest for novel inhibitors of HIV-1 appears quite promising. Optimal chemical modification of the existing transporter peptides by treatment with polyethylene glycol (64) in order to increase the circulating half-life of PNA-transporter conjugates will further advance the efforts to develop potent antiviral therapeutics targeting viral genes.

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The first two authors made an equal contribution.

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