Inhibition of HIV-LTR gene expression by oligonucleotides targeted to the TAR element

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ABSTRACT

All human immunodeficiency virus mRNAs contain a sequence known as TAR (trans-activating responsive sequence). The TAR element forms a stable RNA stem-loop structure which binds the HIV tat (trans-activator) protein and mediates increased viral gene expression. In principle, molecules which bind to the TAR RNA structure would inhibit trans-activation by perturbing the native RNA secondary structure. We have constructed a series of phosphodiester and phosphorothioate antisense oligonucleotides which specifically bind to the HIV TAR element. Specific binding to the TAR element was demonstrated *in vitro* with enzymatically synthesized TAR RNA. The TAR-directed phosphorothioates inhibited trans-activation in a sequence-dependent fashion in a cell culture model using an HIV LTR/human placental alkaline phosphatase gene fusion and tat protein supplied in trans. The molecules also inhibited HIV replication in both acute and chronically infected viral assays, but without sequence specificity. We have constructed a series of vectors consisting of the MMTV promoter and 5'-untranslated region of four different mRNAs, including the TAR region, to study the effect of TAR on gene expression in heterologous systems. The results suggest that, in the absence of the HIV LTR, the TAR element has a repressive effect on gene expression, which is relieved by tat.

INTRODUCTION

Traditionally, investigators attempting to inhibit gene expression with antisense oligonucleotides avoid targeting regions on the mRNA which have significant secondary structure. RNA folding programs are often used in an attempt to find single stranded regions, which presumably have the greatest accessibility for antisense binding (1). We have begun to investigate the feasibility of deliberately targeting antisense oligonucleotides to RNA secondary structures to delineate the rules for strand invasion and to measure the effects of disrupting RNA secondary structure on gene expression. The human immunodeficiency virus (HIV)

provides several well characterized examples of stable RNA secondary structures with important biological functions to test this strategy.

All HIV mRNAs contain a stable RNA stem-loop structure known as TAR on the extreme 5' end of the message. This region has been shown to specifically bind an HIV regulatory protein known as tat (trans-activator). The mechanisms mediating increased gene expression have been intensely studied and may involve increased transcription, translation and other aspects of mRNA metabolism (reviewed in 2-4). Although the molecular mechanisms of TAR/tat trans-activation are complex, it is clear that disruption of the natural TAR/tat interaction would inhibit the normal HIV life cycle and provide the opportunity for novel therapeutics in the treatment of AIDS (5).

There have been numerous recent reports of inhibition of HIV by antisense oligonucleotides (6-9) and oligonucleotide analogs including methyl phosphonates (10-13), phosphoramidates (14), phosphoroselenates (9), 2'-O-Methyl analogs (9), phosphorothioates (8,14-18) and a variety of pendant group conjugated oligonucleotides (12,19). Phosphorothioates have recently emerged as a well studied analog because they have several desired properties including solubility, specific hybridization (20), nuclease resistance (21,22), cell penetration (23), and have been shown to specifically inhibit gene expression in a number of experimental systems (24-26). In HIV infected cells phosphorothioates have been shown to have a strong sequence-independent antiviral activity which is not mediated by an antisense mechanism and may involve inhibition of viral reverse transcriptase (17), viral entry, or uncoating inside the cell.

To circumvent the difficulties in evaluating the activities of phosphorothioate antisense oligonucleotides due to non-antisense mechanisms and still test the hypothesis of binding antisense oligonucleotides to HIV RNA secondary structures, we constructed a highly quantitative and sensitive trans-activation assay in a non-viral cell culture system using the human placental alkaline phosphatase reporter gene fused to the HIV LTR. We designed and synthesized a series of phosphodiester and phosphorothioate antisense oligonucleotides targeted to the TAR element and tested their activities in binding to enzymatically synthesized target RNA *in vitro*, inhibition of trans-activation

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in the non-viral cell culture model and inhibition of HIV in both acute and chronic viral model systems. We also tested oligonucleotides targeted to the *tat* and *rev* mRNAs in each system.

The results show that phosphodiester and phosphorothioate oligonucleotides targeted to specific regions of the TAR element can bind TAR RNA in vitro and specifically inhibit trans-activation in the non-viral cell culture model. The sequence-specific effects were not sufficient to be observed above the non-specific antiviral effects of phosphorothioates in the viral assays. Finally, we exploited the trans-activation assay to study the mechanism of TAR/*tat* trans-activation by comparing the levels of gene expression of TAR-containing mRNA's with 5'-leader sequences from three other mRNAs.

MATERIALS AND METHODS

Plasmid construction

pHIV-PAP and pHIV-PAP-A31 were constructed by ligating the 5.2 kb HindIII/AatII fragment of pSV2Apap (27) which contains the human placental alkaline phosphatase gene (PAP) in its entirety plus RNA processing signals, to the 2.4 kb AatII/HindIII fragment of pHIVCAT0 or pHIVCAT1 (28) containing the HIV LTR or the HIV LTR with a U→A mutation in TAR position 31, respectively.

The pMMTV-X-pap series of plasmids (Fig. 1) were constructed as follows. The vector, pMAMneo (Clontech), was digested with NheI, then the ends partially filled with Klenow polymerase in the presence of dCTP and dTTP to create a 2 bp overhang. pSV2Apap was digested to completion with HindIII, then the ends partially Klenow filled with dATP and dGTP to create 2bp sticky ends complementary to the partially filled NheI ends of pMAMneo. Both plasmids were then digested with SacI, and the PAP fragment from pSV2Apap ligated directionally into the pMAMneo vector to give pMMTVpap.

In order to replace the 5' untranslated region of pMMTVpap with 5' regions from heterologous genes precisely, the 1313 bp ClaI/KpnI fragment from pMMTVpap was subcloned into pBluescript SK- (Stratagene) using the same sites in the poly-linker, to give pBSpap. PCR was then employed to remove a large part of the 5' untranslated region and to create a restriction site midway between the MMTV TATA box and CAP site. The 5' PCR primer was designed with homology to the ClaI site of pBSpap and bases surrounding it (GCAGGAATCGATATCA). This site lies near the 5' end of the MMTV LTR. The 3' primer contains sequences homologous to the MMTV TATA/ CAP region and non-homologous sequences 5' which constitute an NheI site (GCGCGCTAGCACTCTTTTATATC). The completed PCR product consists of the MMTV LTR from ClaI to 10 bases 3' of the TATA, where there is an NheI site. pBSpap was then digested with ClaI and NheI and the PCR product with the same ends ligated into the vector to give pBSpapΔ5'UT.

For pMMTV-5LOpap, a 102 bp synthetic piece of dsDNA was made by annealing the synthetic oligonucleotide, 5'-CTAGTTT-TTGAGTAAACTT AGATGCGGAC ACCTGGACCG CC-GCGC-3', to an oligo with the sequence 5'-GGGAGC CTCGGCGCGG CGGTCCAGGT GTCCGCATCT AAGTT-TACTA AAAAAA-3'. This forms a dsDNA with a 4 bp overhang complementary to an NheI site at the 5' end, followed by sequence that builds back the CAP site and 5-LO 5' untranslated sequences, which end in a 10 bp overhang at the 3' end. A second pair of oligonucleotides with the sequences

5'-CGAG GCTCCCGGCG CTCGCTGCTC CCGCGGCCCCG CGCCATGCTG GGGCCCTGCA TG-3' and 5'-AGGGCCC-CAG CATGGCACGG GCCGCGGGAGC AGCGAGCGC-3', were also annealed creating a dsDNA with a 10 bp overhang complementary to that of the other synthetic dsDNA and completing the 5-LO 5' UT up to the AUG, followed by sequences which reconstruct the PAP gene from the AUG to the SphI site. The two DNAs were then ligated to give the full 5-LO 5' untranslated region, CAP site, and translational start site with the NheI site at the 5' end and an SphI site at the 3' end as well as sequences to reconstruct the PAP gene from the AUG to the SphI site. The synthetic DNA was then ligated directionally into pBSpapΔ5'UT prepared by digesting the plasmid with a combination of SphI and NheI to give pBS-5LO. Finally, the ClaI/KpnI fragment from pBS-5LO was ligated to the KpnI/XbaI fragment of pMMTVpap and XbaI/ClaI digested pBluescript SK- to complete the construction.

For pMMTV-HSVpap, PCR primers were designed with complementarity to either end of the HSV-1 UL-13 5' untranslated region contained in the plasmid pBEDJ. The 5' primer also contained non-homologous sequences to build back the CAP site and create an NheI site (ACTTGAGCTA GCTT TTTGAGT AACTTGTGTTA AAATTCGCG). Non-homologous sequences in the 3' primer build back the PAP AUG to the SphI site (ATCGTAGCAT GCAGGGCCCC AGCATC CGTC CGCCAGGTCG CC). The PCR product was then ligated into pBSpapΔ5'UT at the SphI/NheI sites. From this point construction was completed as for pMMTV-5LOpap. pMMTV-TARpap was created as pMMTV-HSVpap with PCR primers designed with complementarity to the TAR region of pHIV-PAP.

Cell lines, transfections, and PAP assays

HeLa and SK-MEL-2 cell lines were maintained in DMEM plus 10% FCS. For antisense experiments, cells were seeded in 6 well dishes at 50% confluency the day prior to the experiment. For each dish, 1 μg of pHIV-PAP and 12 μg of pcDEBtat were precipitated in 500 μl of 1×HBS (136mM NaCl, 5 mM KCl, 0.8 mM Na₂HPO₄, 0.1% dextrose, 20 mM HEPES pH 7.05) and 32 μl of 2.5 M CaCl₂. The CaPO₄ precipitate was then divided evenly between the six wells and allowed to sit for 6 hours prior to removal of the precipitate and addition of fresh media. 16 hours later, antisense oligonucleotides were transfected by the same method. The next day cells were washed 2× with TBS (154 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 mM MgCl₂) and harvested in 500 μl of TBS of which 100 μl were used in the protein assay. The remaining 400 μl of cell suspension was pelleted, then resuspended in 50 μl TBS buffer. Next endogenous phosphatases were inactivated by heating to 65°C for 30 min. The heat stable human placental alkaline phosphatase activity was then assayed by the addition of 500 μl of 5mM PNPP(Sigma) in TEA buffer(1M Tris-HCl, pH 9.85, 280 mM NaCl, 0.5 mM MgCl₂) to the cell suspension, followed by incubation at 37°C. Phosphatase activity was determined at 30 minute intervals using 150 μl aliquots of the reaction mixture and measuring the absorbance at 405 nm with a Titertek Multiscan MCC/340 ELISA plate reader. The PAP activity was normalized to total protein in each well as determined by Bio-Rad protein assay, in which 1/5 of the harvested cells in TBS (0.1 ml) were added to 30 μl of Bio-Rad Protein reagent, then incubated for 10 minutes at room temperature, followed by measurement of absorbance at 595 nm using the Titertek plate reader.

For experiments in which pMMTV-X-pap plasmids were used,

SK-MEL-2 cells in 50% confluent 6-well dishes were CaPO₄ transfected as above with 2 μ g of pMMTV-X-pap and 1 μ g of pIP-RG7, a construct containing a full length glucocorticoid receptor under the transcriptional control of the RSV LTR. Cells were incubated for 40 hours at 37°C with or without 1×10^{-7} M dexamethasone. Alkaline phosphatase activity was measured for each condition and normalized to total protein as described above.

TAR RNA synthesis, characterization, and gel mobility shift assays

A template for the production of TAR RNA was produced by PCR. Primers were designed to pHIV-PAP such that the 5' primer consisted of non-homologous sequences making up the 17 bp T7 RNA polymerase promoter followed by 18 bases homologous to the first 18 nucleotides of the TAR stem/loop (5'-AATAGCACT CACTATAGG GTCTCTCTG GTTAGA-CCA-3'). The 3' primer consisted of 20 bases homologous to the last 20 bases of the stem/loop (5'-CCAGCATGT CTGG-AGGGC AG-3'). This PCR product was then used as a template for a T7 reaction. For a 1 ml reaction, template was added at 300 pM to a reaction mix containing 4 mM each of GMP, CTP, UTP and ATP, 2mM GTP, 40 mM Tris-HCl(pH 8.0), 1mM spermidine, 5 mM DTT, 0.01 % Triton X-100, 20% PEG 8000, 31 mM MgCl₂ and 10% T7 polymerase. The reaction was incubated for 4 hours at 37°C, the RNA product of the reaction de-salted on a Sephadex G-50 column, PAGE purified, dephosphorylated with calf intestine alkaline phosphatase, quantitated by U.V., and 32-P end labeled to high specific activity (6000 Ci/mmol) with polynucleotide kinase.

T7 synthesized TAR RNA was structurally characterized by base hydrolysis, RNase T1 (cleaves single stranded G's), RNase CL 3 (cleaves single stranded C's), RNase A (cleaves single stranded pyrimidines), and RNase V1 (cleaves double stranded regions). All reactions were carried out with end labeled TAR RNA at a concentration of 100 pM in a volume of 10 μ l. For the base hydrolysis, the RNA was added directly to a buffer consisting of 50 mM sodium bicarbonate, pH 9.2 and incubated for 10 minutes at 90°C. The reaction was stopped by the addition of 8 μ l of 7M urea then placed on dry ice until the reaction was loaded on the gel. All enzymatic reactions were carried out in a buffer consisting of 10 mM Tris pH 7.4, 50 mM NaCl, & 5mM MgCl₂. 3 μ g of tRNA (Sigma) was also added to each reaction along with the TAR RNA. 0.3 U RNase T1 (BRL), 5pg RNase A (Boehringer Mannheim), 0.1 U RNase CL 3 (Boehringer Mannheim) or 0.1 U RNase V1 (Pharmacia) were added to a given reaction, followed by a 5 minutes incubation at 22°C (T1, A, and V1) or for 15 minutes at 37°C (CL 3). Reactions were stopped by the addition of 8 μ l of 7M urea followed by heating to 90°C for 2 minutes, then were immediately loaded onto a 12% polyacrylamide gel with 50% w/v urea and separated by running at 2000V for 3 hours. The gel was then dried and exposed to film overnight.

Gel shift assays were performed by the addition of the TAR directed oligonucleotides at indicated concentrations to a 10 μ l reaction containing 1 nM of the labeled TAR RNA, 100 mM NaCl, 10mM Tris (pH 7.5), and 0.5 mM EDTA. Where indicated, a specific competitor DNA oligonucleotide analog of the TAR stem loop was added at 300 μ M (5'-GGGTCTCTCTGGTTAGACCA GATCTGAGCC TGGGAGCTCT CTGGC-TAACT AGGGAACC-3'). Sonicated E.coli RNA was added at indicated concentrations as a non-specific competitor. The

mixture was allowed to incubate 45 minutes at 37°C, then the TAR RNA/oligo complex separated from un-bound TAR RNA by electrophoresis on an 8% native acrylamide gel in 1 $\times$ TBE buffer at 200V for 2 hours. The gel was then dried and exposed to film.

Oligodeoxynucleotide synthesis and purification

Unmodified oligodeoxynucleotides were synthesized on an Applied Biosystems 380B DNA Synthesizer using standard phosphoramidite chemistry with oxidation by iodine. The reagents, both CPG-bound and β -cyanoethyl diisopropylphosphoramidites, were purchased from Applied Biosystems, Inc. (Foster City, CA). The standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide (29) in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation cycle wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG-column and deblocking in concentrated ammonium hydroxide at 55°C (18 hrs), the phosphorothioates were purified by trityl-on HPLC with a PRP-1 column using a gradient of acetonitrile in 50 mM of triethyl-ammonium acetate, pH 7 (4% to 32% in 30 minutes, flow rate of 1.5 ml/minute). Appropriate fractions were pooled, evaporated, and treated with 5% acetic acid at ambient temperature for 15 minutes. The solution was extracted with an equal volume of ethyl acetate, neutralized with ammonium hydroxide, frozen, and lyophilized. Analytical gel electrophoresis was accomplished in 20% acrylamide, 8 M urea, 45 mM tris-borate buffer, pH 7, 40 V/cm. Oligodeoxynucleotides and their phosphorothioate analogs were judged from HPLC analysis and by polyacrylamide gel electrophoresis to be greater than 95% full length material.

NMR analysis

The relative amounts of phosphorothioate and phosphodiester linkages obtained by our synthesis were determined by ³¹P NMR spectroscopy. The spectra were acquired on a Varian NMR spectrometer with a ³¹P frequency of 162 MHz. Typically, 1000 transients are co-added. A relaxation delay of 7.5 sec between transients is used to insure a fully relaxed spectrum. The ³¹P spectra are acquired at ambient temperature using deuterium oxide or dimethyl sulfoxide-d₆ as a solvent. Phosphorothioate samples typically contained less than one percent of phosphodiester linkages.

Acute infection assay

The human T-lymphoblastoid CEM cell line was maintained in an exponential growth phase in RPMI 1640 with 10% fetal calf serum, glutamine, and antibiotics. On the day of the assay, the cells were washed and counted by trypan blue exclusion. These cells (CEM-IIIb) were seeded in each well of a 96-well microtiter plate at 5×10^3 cells per well. Following the addition of cells to each well, the antisense oligonucleotide was added at the appropriate concentration. The concentrations utilized included a high test concentration of 25 μ M and five serial half log dilutions. Infectious HIV-1_{IIIb} was immediately added to each well at a multiplicity of infection determined to give complete cell killing at 6 days post-infection. Following 6 days of incubation at 37°C, an aliquot of supernatant was removed from each well prior to the addition of the tetrazolium dye XTT to each well. The XTT was metabolized to a formazan product by viable cells and the results calculated spectrophotometrically with a Molecular Devices Vmax Plate Reader. The XTT assay measures protection

Figure 1. General structure of the mammalian expression vectors. pHIV-PAP contains the HIV-1 LTR sequences (U3 in entirety and R through +78) controlling the expression of a reporter gene for human placental alkaline phosphatase (PAP), taken from pSV2Apap, (27) a gift of T. Kadesch. SV40 sequences were included to provide essential RNA processing signals. pMTV-X-pap represents a series of constructs with the human PAP gene under the control of the dexamethazone inducible MMTV LTR. The 5' untranslated region (5' UTR's) can be replaced exactly with heterologous 5' UTR's by cutting with NheI and SphI and replacing with 5' UTR fragments produced by PCR amplification of heterologous genes (see Methods). Substituted 5' UTR's are shown at the bottom of the figure.

from HIV-induced cell killing as a result of the addition of test compounds. The supernatant aliquot was utilized to confirm the activities determined in the XTT assay. Reverse transcriptase assays and p24 ELISA were performed to measure the amount of HIV released from the infected cells. Protection from killing results in an increased optical density in the XTT assay and reduced levels of viral reverse transcriptase and p24 core protein. The antisense oligonucleotides had no effect on the growth of uninfected CEM cells.

Chronic infection assay

Chronically infected, virus producing CEM cells were obtained from the outgrowth of viable cells following acute infection with

HIV-1_{IIIB}. These cells (CEM-IIIB) were seeded in each well of a 96-well microtiter plate at 5×10^3 cells per well. Antisense oligonucleotide was added to each well at the appropriate concentration, ranging from a high test concentration of $25 \mu\text{M}$. Following 6 days of incubation at 37°C the supernatants were removed from each well and the cells were washed two times with PBS. Following washing, the cells were disrupted in an ice-cold buffer of PBS plus 20mM Triton and 0.5% EDTA. The cell lysate was assayed for content of p24 by ELISA. The supernatants were analyzed for content of virus by reverse transcriptase assay and p24 ELISA. The antisense oligonucleotides had no effect on the growth of chronically infected CEM cells.

Figure 2. Trans-activation of the pHIV-PAP constructs by cotransfection with the tat-expression plasmid pcDEBtat. Cells (SK-MEL-2) were transfected by the calcium phosphate method in a 10cm dish with 2 μ g pHIV-PAP or pHIV-PAP-A³¹ and either 10 μ g pcDEBtat or bluescript. 48 hours post transfection cells were assayed for PAP activity as described in the methods. ($\square$) = pHIV-PAP + pcDEBtat ($\diamond$) = pHIV-PAP + bluescript, (*) = pHIV-PAP-A³¹ + pcDEBtat, ($\star$) = bluescript alone

p24 quantitation

The DuPont p24 ELISA Kit (NEK-046) was used according to the manufacturer's instructions to quantitate p24 capsid antigen. Absorbances were read at 492 nm using a plate reader. Viral protein concentration was determined from these absorbances using a standard curve and is expressed as nanograms per milliliter of culture supernatant.

Reverse transcriptase assay

Reverse transcriptase assays were performed by mixing 15 μ l of cell-free supernatant and 10 μ l of RT reaction buffer. The total 25 μ l reaction mixture consisted of 2.5 μ Ci of tritiated thymidine triphosphate (TTP) (ICN.80 Ci/mmol), poly rA (50 ng/ml):oligo dT (10 ng/ml) template:primer (Pharmacia), 1- mM Tris, pH 7.6, 8 mM magnesium chloride, 10 mM dithiothreitol (DTT), and, in the standard reaction, 0.5% Triton X-100. The reactions were incubated for 60 min. at 37°C. The reaction volume was then spotted onto DE81 chromatography paper (Whatman), washed extensively in 5% dibasic sodium phosphate, and counted for incorporated radioactivity in a toluene-based scintillation cocktail.

RESULTS

HIV-LTR trans-activation model using the human placental alkaline phosphatase reporter

A number of groups have used a 'reporter gene' system to study the control of the HIV LTR, which generally involves cotransfection of a plasmid which constitutively expresses the HIV tat protein along with a plasmid which contains the HIV LTR fused to a gene encoding an easily assayable enzyme. In most mammalian cells cotransfection of the plasmids results in

length	28	18	26
P=S	1972	1308	1307
P=O	1971	1116	1118

Figure 3. Antisense oligonucleotides directed against the HIV TAR element. The oligonucleotide sequences are complimentary to the tar sequences where indicated.

expression of the tat protein which trans-activates the HIV LTR. This, in turn, results in the expression of the reporter protein which can be assayed to quantitate tat dependent expression. The reporter gene used most frequently is the *cat* (chloramphenicol acetyltransferase) gene(30)(28)(31).

Recently several alkaline phosphatase genes or cDNAs have been used as an alternative to the *cat* gene. Berger et al (31) have used a secreted alkaline phosphatase cDNA as a reporter fused to the HIV LTR and measured trans-activation by assaying the media for alkaline phosphatase activity. We have constructed a vector, pHIV-PAP (Fig 1) using the human placental alkaline phosphatase gene (PAP) which expresses a membrane-bound form of the enzyme which is assayed by harvesting the cells and measuring PAP activity in the cell membrane (27). The basis for use of the membrane-bound PAP gene as a reporter is that this enzyme is stable to heat treatment which inactivates all normal cellular alkaline phosphatases (27). We found that some cell lines (such as A431 cells) contained an alkaline phosphatase activity that is not heat labile, and therefore gave an unacceptably high background PAP activity. SK-MEL-2 cells had essentially no heat stable alkaline phosphatase activity while HeLa cells had a small but measurable heat stable alkaline phosphatase activity which was measured and subtracted from each experiment. Both (cell lines) gave essentially identical trans-activation results. When the pHIV-PAP plasmid was cotransfected with the tat expressing plasmid pcDEBtat (28) into HeLa or SK-MEL-2 cells trans-activation of the HIV LTR was apparent from measurement of the membrane-bound heat stable alkaline phosphatase (Fig. 2). Replacement of pcDEBtat plasmid with a control plasmid (bluescript) resulted in a slight amount of PAP activity above background, which we attributed to the basal level of gene expression from the HIV LTR. Transfection of a constant amount of (2 μ g) of pHIV-PAP plasmid with different amounts of pcDEBtat ranging from 1–20 μ g (keeping the total DNA constant with bluescript) showed that trans-activation could be increased

Figure 4. Structural characterization of TAR RNA. T7 synthesized TAR RNA was mapped by base hydrolysis, RNase T1, RNase V1, RNase A, and RNase CL 3. RNase T1 is specific for single stranded G's; the three major cleavage sites in this lane corresponding with the position of the loop GGG. RNase CL 3 and RNase A are also single strand specific, with RNase CL 3 cleaving preferentially at C's and U's and RNase A at pyrimidines. The major hits in these lanes correspond to the CU of the loop and the UCU bulge. RNase V1 is specific for double stranded regions but has no nucleotide specificity. Major hits in this lane correspond to the predicted stem areas of the sequence.

by adding more tat plasmid until about 10 μ g, followed by no additional increase with more plasmid (data not shown). For most antisense experiments we transfected 12 μ g of pcDEBtat in order to be in slight tat excess. The PAP assay provided the desired highly quantitative measure of LTR gene expression, where production of chromophore (p-nitrophenol) in the PAP assay could be continuously measured in 96 well plates over time to obtain accurate enzyme quantitation (Fig 2)

Using HIV LTR-CAT plasmids it has been reported that any mutations in the conserved TAR loop sequence CUGGG results in a complete loss of tat-mediated trans-activation (28). We tested

Figure 5. Gel mobility shift assay of TAR RNA. (A) 1 nM of end-labeled TAR RNA was incubated as described in Materials and Methods with TAR-specific oligonucleotides 1116 (phosphodiester), 1308 (phosphorothioate with the same sequence), and the randomized sequences 1485, or 1497 at 200 μ M for 45 minutes, with no competitor (0), non-specific RNA competitor (sonicated *E. coli* RNA) at 1000 $\times$ over oligo (-), or specific competitor (a DNA oligonucleotide version of TAR) at 300 μ M (+). Oligonucleotide/RNA complex was separated from unbound TAR RNA by electrophoresis on an 8% acrylamide gel. (B) Oligonucleotides 1118 and 1971 were incubated as above at the indicated concentrations in the absence of competitor.

this result in the PAP reporter system by constructing a plasmid with a U $\rightarrow$ A mutation in loop position 31 (pHIV-PAP-A31). The results (Fig. 2) show that there was an 80% decrease in trans-activation in the mutant TAR compared with wild type. This result is in general agreement with the results obtained from TAR HIV LTR-CAT experiments and confirms the TAR sequence-dependence of the pHIV-PAP construct.

Antisense oligonucleotide design and TAR binding in vitro

Phosphodiester and phosphorothioate oligonucleotides targeted to the HIV TAR element were designed (Fig. 3 and Table 1) to complement the loop and bulge region of TAR, which includes the site where the Tat protein binds (32–37). To determine if antisense oligonucleotides were capable of invading and disrupting the TAR secondary structure, we synthesized the complete stem loop (bases 1–59) of TAR using T7 RNA polymerase. The template for the T7 reaction was generated by PCR, utilizing a plasmid containing the HIV-1_{III} LTR and PCR primers complementary to the plasmid in the TAR region with non-complementary T7 promoter sequences on the 5' end of the 5'-primer. The PCR transcription template was subcloned and sequenced to assure the sequence fidelity of the template. The TAR RNA was gel purified, and 5' end-labeled for RNA structure probing and measurement of the binding of antisense oligonucleotides in vitro. A battery of enzymes which cut RNA with sequence and/or structural specificity was used to determine the secondary structure of the TAR RNA. Results obtained were consistent with previously described TAR RNA secondary structure (Fig. 4.) (38); the loop and bulge regions were most susceptible to single strand-specific enzyme cleavage while the double stranded regions were most susceptible to the enzyme V1,

Table 1. Description of oligonucleotides

Compound #	Length	Analog	Sequence	Target
1308	18	P=S	GCT CCC AGG CTC AGA TCT	TAR
1116	18	P=O	" "	TAR
1497	18	P=S	GCA CCG CTA GCT CCA TGT	None (Randomized 1308)
1485	18	P=O	" "	None (Randomized 1116)
1307	26	P=S	GCC AGA GAG CTC CCA GGC TCA GAT CT	TAR
1118	26	P=O	" "	TAR
1720	26	P=S	TGA CGC AGC AAT TCC AAC TCC GGG GC	None (Randomized 1307)
1716	26	P=O	" "	None (Randomized 1307)
1972	28	P=S	CCA GAG AGC TCC CAG GCT CAG ATC TGG T	TAR
1971	28	P=O	" "	TAR
1334	20	P=S	GGC TCC ATT TCT TGC TCT CC	tat-AUG
1337	20	P=S	CCGT CCC CTC GCC TCT TGC CG	tat 5'-uT
1968	27	P=S	TCG TCG CTG TCT CCG CTT CTT CCT GCC	rev

which has specificity for duplex RNA or single stranded regions with stacked bases.

Gel shift experiments (Fig. 5A lanes 4,5,7,8) showed that both phosphodiester and phosphorothioate oligonucleotides complementary to the TAR loop and bulge region could bind with sequence specificity. Binding of each of the TAR-specific oligonucleotides to TAR RNA could be reversed by an excess of a competing DNA oligonucleotide with the same sequence as TAR RNA. (Fig. 5A lanes, 6 and 9). Randomized versions of the TAR-specific phosphodiester and phosphorothioate oligonucleotides did not bind to TAR (Fig. 5A, lanes 10–15). The binding of oligonucleotides 1118 and 1971 was measured more precisely in a concentration dependent gel-shift experiment (Fig. 5B). Densitometry measurements of the exposed film showed that each oligonucleotide shifted 50% of the TAR RNA at approximately 0.3 μ M oligonucleotide concentration. The phosphorothioate oligonucleotides generally bound 50% of the TAR at about 5 fold higher concentration than the phosphodiesters (not shown). These results demonstrate that antisense oligonucleotides targeted to the bulge and loop regions of TAR are capable of binding and disrupting the native TAR structure at pharmacologically reasonable concentrations. Additional phosphodiester and phosphorothioate oligonucleotides with shorter lengths (12–14) or sequences complementary to the stem region of TAR did not bind to TAR RNA in vitro (data not shown).

Antisense inhibition of trans-activation

The TAR-specific oligonucleotides and randomized controls with the same base composition were tested for inhibition of trans-activation in a cell culture model using the HIV LTR fused to the human placental alkaline phosphatase gene as described above. We have shown that expression of the reporter gene is strictly dependent upon the presence of the tat (Fig. 2), and would therefore expect that specific disruption of the TAR/tat interaction by oligonucleotides would inhibit gene expression. Specific inhibition of HIV LTR gene expression was demonstrated with TAR-specific phosphorothioates (Fig. 6, 1307, 1308, 1972), relative to sequence composition matched controls (1497, 1720). The randomized control phosphorothioates had a dose-dependent effect on gene expression in this model system, which was less

Figure 6. Antisense oligonucleotide inhibition of pHIVpap. (a) SK-MEL-2 cells seeded at 50% in 6-well dishes were CaPO4 co-transfected with pHIVpap and pcDEBtat. 16 hours later oligonucleotides were transfected at the indicated doses. The following day the cells were harvested and PAP activity was determined and normalized to total cellular protein as described in Materials and Methods. (b) Oligonucleotides 1308 and 1497 were transfected into HeLa cells in triplicate for each indicated dose, 16 hours after the initial transfection of the reporter plasmids, pHIVpap and pcDEBtat, into the cells. PAP activity was assayed the following day as above. Assays were performed in triplicate, standard deviations were approximately 15%.

Table 2. Inhibition of HIV by Antisense Oligonucleotides in Acute Infection Model

Compound #	Length	Target	IC ₅₀ μ M Reverse transcriptase	IC ₅₀ μ M Cytopathic Effects
1308	18	TAR Loop	2.0	1.8
1497	18	Randomized 1308	1.3	1.7
1334	20	Tat AUG	6.0	4.1
1337	20	Tat 5'-UT	1.5	0.6
1307	26	TAR Loop	0.3	0.6
1720	26	Randomized 1307	0.15	0.3
1968	27	Rev AUG	0.2	0.2
1972	28	TAR Loop	2.0	1.8

Inhibition of HIV in the acute model by phosphorothioate antisense oligonucleotides was determined both by the inhibition of cytopathic effects on the CEM cells or by directly measuring the virus levels by reverse transcriptase assays. The IC₅₀ values shown above were calculated from a dose-response experiment where oligonucleotides were added to microtiter wells at 10 μ M and 6 serial half log dilutions. Each concentration was assayed in triplicate. Cytopathic effects and reverse transcriptase were determined after 6 days.

that what was observed with the sequence-specific oligonucleotides (Fig. 6). Two tat-mRNA directed antisense oligonucleotide phosphorothioates (Table 1) complementary to the initiation of translation (1334) and in the 5'-untranslated region (1337) also had significant effects on gene expression.

In each case, the phosphodiester analogs of the active sequences were ineffective in inhibiting gene expression (not shown). To obtain inhibition with the sequence-specific phosphorothioate oligonucleotides with this system, it was necessary to increase the penetration of the oligonucleotides into the cell. Simple addition of the phosphorothioates after transfection of the plasmids did not result in activity. The data shown in Fig. 6 were obtained by calcium phosphate transfecting the oligonucleotides into the cells. We have obtained similar data when the oligonucleotides were delivered to the cells via lipofection, or by electroporation (data not shown). However, in the case of both the TAR-directed or the tat mRNA directed phosphorothioates, sequence-specific and dose-dependent inhibition of gene expression could be measured.

Acute infection model

Antiviral activity was measured in CEM cells infected with HIV-1_{IIIB}. Antisense oligonucleotides were added at a high test concentration of 10 μ M. HIV-1_{IIIB} was added to CEM cells at a multiplicity of infection previously determined to give total cell killing at 6 days post-infection. Following 6 days of culture a sample of supernatant from each well was removed for confirmatory testing and the tetrazolium dye, XTT was added to each well.

The results of the XTT assay were confirmed by performing a reverse transcriptase assay on the sample supernatants. The activity of each compound was determined by calculation of an IC₅₀ value based upon the data from the XTT and reverse transcription assays. This data are presented in Table 2. The results show that the compounds tested generally had significant antiviral activity with IC₅₀s as low as 150–300 nM. The results from the XTT and reverse transcriptase assays were in good agreement. However, in contrast to the trans-activation assays, the randomized sequence controls showed activity equal to the sequence specific compounds, suggesting that phosphorothioates have other non-antisense antiviral activities in the acute model as previously described (16,39,40).

Figure 7. Time course for inhibition of HIV by antisense oligonucleotides in chronically infected CEM cells. Three compounds 1307-(tar) 1720-(randomized 1307) and 1968 (rev) were incubated with HIV-1_{IIIB} chronically infected CEM cells at 10 μ M. Samples were removed on days 3, 4, and 5 and 6. On each of these days the cells were removed from triplicates of each well, washed three times in tissue culture medium without FBS, and lysed in PBS containing 0.5% Triton X-100 and 0.01% sodium azide. A p24 ELISA was performed on each of the cell lysates to quantitate intracellular p24. The data are reported directly on O.D.'s from the ELISA.

Chronic infection model

It has previously been shown that a 27-mer phosphorothioate directed to the initiation of translation of the *rev* gene mRNA has a sequence-dependent activity in a chronic infection model (16). Therefore, we tested the TAR-directed antisense oligonucleotides in a similar chronic infection model using CEM cells and included the anti-*rev* phosphorothioate 27 mer. After six days the reverse transcriptase activity was measured in the cell supernatant and the cells were lysed and tested for p24 gag. AZT was run as an additional control since it is known to have no effect on chronically infected cells. The results of a dose response experiment with a 25 μ M high dose and six serial half log dilutions demonstrate that the TAR-directed compound 1307 and the *rev* directed compound (1968) had the greatest antiviral activities as measured by reduction of p24 and reverse transcriptase activity (data not shown). However, randomized compounds also had activity. We also subsequently tested the TAR-directed 26-mer (1307), its randomized control (1720) and the anti-*rev* compound for activity as a function of the number of days of treatment at 10 μ M. The results (Fig. 7) showed that the TAR-directed phosphorothioate and its randomized control had identical activities, while the anti-*rev* compound had slightly more activity with a similar dose-response.

Mechanism of TAR trans-activation

A wealth of recent studies on the mechanism of TAR/tat trans-activation indicate that there is a direct interaction between the tat protein and the TAR stem loop structure. Recent reports have

Figure 8. Regulation of gene expression in HIV LTR-PAP and MMTV-pap fusion constructs. The plasmids shown in Figure 1 were cotransfected into SK-MEL cells along with a plasmid supplying the glucocorticoid receptor and, where indicated, a plasmid supplying tat (pcDEBtat). Cells were incubated for 48 h in the presence or absence of 10^{-7} M dexamethasone. Cells were harvested and PAP activity was assayed as described in the Materials and Methods.

clearly shown that at least one function of TAR is to bring the activating portion of tat protein in close proximity to the HIV LTR. This results in stimulation of the HIV LTR through interactions between the activation portion of the tat protein and requires non-TAR HIV LTR sequences (41–43). However, translational effects of TAR have been observed and the TAR/tat interaction may be important in other areas of RNA metabolism (2–4,44–46). It has also previously been shown that, in the presence of tat, TAR is at least partially stimulatory when attached to leader mRNAs in the 5'-untranslated regions downstream of constitutive heterologous promoters such as SV40, RSV or CMV. (47,48). We have used the human placental alkaline phosphatase reporter system to investigate the role of the TAR element by placing it downstream to the steroid-inducible MMTV promoter. To determine the impact of the TAR element on gene expression levels, we constructed three other plasmids which contain the MMTV promoter driving reporter gene expression, but with the 5'-untranslated sequences from other mRNAs as the leader replacing the TAR-HIV (Fig.1) Each of the constructs were transfected into HeLa cells and gene expression was measured in the presence or absence of dexamethasone (dex) inducer.

The results (Fig. 8) show that for the three MMTV-X-pap constructs not containing the TAR element, there was efficient dex regulation of the MMTV promoter as measured by an alkaline phosphatase reporter gene assay. With the construct containing the HIV TAR and 5'-leader sequences there was only slight induction by dex (Fig. 8, 3-rd set of bars), suggesting that this particular leader sequence repressed gene expression when induced by a heterologous promoter. However, when the tat-expressing plasmid was cotransfected, dex induction of the MMTV promoter was restored to normal levels (4-th set). This result suggests that the TAR-containing HIV leader sequence behaved as a general repressor of gene expression which was reversed by the presence of tat protein. It is noteworthy that the tat protein did not induce gene expression in the absence of dex (4-th set, 1-st bar), suggesting that the HIV LTR is required in addition to tat in order for tat to mediate gene expression.

DISCUSSION

One of the objectives of this work was to determine if antisense oligonucleotides targeted to regions of RNA secondary structure would be effective in binding to the target and disrupting its natural structure. To test this possibility, we chose the HIV TAR element as a target because of its well characterized structure and the importance of the structure to the biological function of the RNA in the viral life cycle. Although the TAR element forms a stable hairpin in solution, as determined by the enzymatic structure probing experiments, there are two GU pairs, several single base and one three-base bulge in the hairpin which weaken the hairpin structure. We have found that both phosphodiester and phosphorothioate oligonucleotides ranging from 18 to 28 bases in length will bind to TAR RNA, with binding constants for the phosphorothioates in the micromolar range as determined by gel shift assays. Phosphorothioates bind with about a 4–5 fold lower affinity than the diesters. It is noteworthy that shorter phosphodiester and phosphorothioate oligonucleotides in the 12 to 14-mer range did not bind to the TAR RNA in gel shift experiments (not shown), suggesting that longer oligonucleotides are needed in order to gain sufficient thermodynamic stability to disrupt the existing structure with oligodeoxynucleotides.

It is significant that the phosphorothioate oligonucleotides bind to the TAR element *in vitro*. In order to be able to inhibit TAR activity in the cell culture trans-activation assay, nuclease resistant oligonucleotides such as the phosphorothioates are required. We have not observed inhibition of trans-activation in the cell culture model with the phosphodiester oligonucleotides. The most likely explanation is that the phosphodiester compounds are rapidly degraded in the cell and the culture medium. In contrast, the phosphorothioates inhibited trans-activation in a sequence-dependent fashion. This result suggests that phosphorothioates are capable of inhibiting gene expression, even when targeted to areas of significant RNA secondary structure. It should be noted that when the compounds were simply added to the culture media no activity was observed. This may be due to low levels cellular uptake since oligonucleotides introduced into the cells by transfection, were active. Although oligonucleotide uptake was not directly has measured in this study, uptake has been shown to vary with cell types; and transfection of oligonucleotides is not always necessary to get activity. (Cowsert et al, manuscript in preparation and 23–26).

In viral infectious assays, activity was observed without transfecting the oligonucleotides. However, antisense activity was most likely masked by phosphorothioate non-antisense antiviral activity such as inhibition of viral reverse transcriptase, viral entry, or uncoating inside of the cell. This work demonstrates that phosphorothioates targeted to the TAR element can disrupt trans-activation if sufficient quantities could be delivered to cells. Moreover, these molecules can be used as delivery agents to target chemical functionality to all HIV mRNAs. Methods are being undertaken to enhance cellular uptake, which might possibly allow for a significant decrease in the dosage at which activity is observed, thereby eliminating or reducing non-antisense effects.

It was also our intention to study the mechanism of TAR/tat transactivation. Other investigators have clearly shown that tat functions at the level of transcription by binding TAR resulting in stimulation of the HIV LTR through interactions between the activation portion of the tat protein and non-TAR HIV LTR sequences. However, TAR-containing RNA is not expressed when injected into *Xenopus* oocytes, while the same RNA without

the TAR element is readily translated (37). Moreover, coinjected tat protein with the TAR-containing RNA results in translation. One possibility is that the TAR element is a translational inhibitor of the RNA and that the binding of the tat protein alleviates the inhibition. Although it is clear that TAR is not a repressor of transcription in the HIV LTR, it is possible that it is a translational repressor. In order to study this effect, we have taken a slightly different approach and compared the HIV TAR element, out of the context of the HIV LTR, with the 5' leaders of other genes in a steroid inducible reporter system. A series of plasmids were constructed containing the MMTV LTR followed by the 5'-untranslated regions of four different mRNA's, including the HIV TAR element followed by the coding region of the human placental alkaline phosphatase reporter gene. Constructs not containing the TAR element were found to be steroid inducible, however, the construct containing the TAR element showed little dexamethasone inducible expression as compared to other constructs. Addition of a plasmid expressing the tat protein to the system resulted in normal levels of steroid induction of the PAP gene. This data is consistent with the hypothesis that TAR acts a translational repressor, with repression being alleviated by tat.

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