Inhibition of Gene Expression Inside Cells by Peptide Nucleic Acids: Effect of mRNA Target Sequence, Mismatched Bases, and PNA Length

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ABSTRACT: Genome sequencing has revealed thousands of novel genes, placing renewed emphasis on chemical approaches for controlling gene expression. Antisense oligomers designed directly from the information generated by sequencing are one option for achieving this control. Here we explore the rules governing the inhibition of gene expression by peptide nucleic acids (PNAs) inside cells. PNAs are a DNA/RNA mimic in which the phosphate deoxyribose backbone has been replaced by uncharged linkages. Binding to complementary sequences is not hindered by electrostatic repulsion and is characterized by high rates of association and elevated affinities. Here we test the hypothesis that the favorable properties of PNAs offer advantages for recognition of mRNA and antisense inhibition of gene expression in vivo. We have targeted 27 PNAs to 18 different sites throughout the 5′-untranslated region (5′-UTR), start site, and coding regions of luciferase mRNA. PNAs were introduced into living cells in culture as PNA−DNA−lipid complexes, providing a convenient high throughput method for cellular delivery. We find that PNAs targeted to the terminus of the 5′-UTR are potent and sequence-specific antisense agents. PNAs fifteen to eighteen bases in length were optimal inhibitors. The introduction of one or two mismatches abolished inhibition, and complementary PNAs targeted to the sense strand were also inactive. In striking contrast to effective inhibition by PNAs directed to the terminal region, PNAs complementary to other sites within the 5′-UTR do not inhibit gene expression. We also observe no inhibition by PNAs complementary to the start site or rest of the coding region, nor do we detect inhibition by PNAs that are highly C/G rich and possess extremely high affinities for their target sequences. Our results suggest that PNAs can block binding of the translation machinery but are less able to block the progress of the ribosome along mRNA. The high specificity of antisense inhibition by PNAs emphasizes both the promise and the challenges for PNAs as antisense agents and provides general guidelines for using PNAs to probe the molecular recognition of biological targets inside cells.

Genome sequencing has revealed the identities of many genes that encode proteins whose functions are unknown. One strategy for understanding the cellular roles of these proteins is to inhibit gene expression using synthetic compounds. Oligonucleotides and oligonucleotide mimics offer important advantages as synthetic tools for selective gene inhibition because knowledge of the target gene sequence provides sufficient information for inhibitor design (*1*). Synthesis of oligomers can be performed by straightforward protocols, and many chemical options are available to tailor the oligomer properties for selected applications. Once synthesized, the effects of fully complementary and mismatch-containing oligomers can be compared, providing an important control to confirm that observed effects are due to inhibition of target gene expression rather than to unintended interactions with other cellular components. Oligonucleotides also represent an exquisitely specific tool for investigating nucleic acid structure and function inside cells under physiologically relevant conditions. Using them in this capacity, however, will require a basic understanding of the rules governing their recognition of targets inside cells and how these rules vary depending on the chemical characteristics of the type of oligomer being used.

Despite their inherent advantages, the control gene expression by oligonucleotides is often confounded by nonspecific interactions and by an inability to readily identify susceptible target sites (*2*). These problems have led to the development of sophisticated chemical modifications and screening methodologies. Modified DNA and RNA oligonucleotides have been shown to effectively inhibit gene expression in cell culture, and high throughput screening methods now allow the rapid identification of inhibitory oligonucleotides (*3*). These advances have led to successful treatment of disease, with one phosphorothioate-modified DNA oligomer approved to treat cytomegalovirus (CMV) retinitis (*4*), and other DNA and DNA-2′-O-alkyl RNA chimeric oligomers being tested in clinical trials (*5*). Thus it is clear that oligonucleotides can be used to sequence-selectively block gene expression within cells. However, oligomers that

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contain phosphodiester or phosphorothioate linkages have a high propensity to interact with cellular proteins, and therefore are not ideal for all applications. It has also been noted that only 1 in 8 to 1 in 15 oligonucleotides tested appear to be active (*2*). As a result, it is important to continue to develop novel oligomer chemistries and to critically evaluate whether they possess improved properties as antisense agents.

Peptide nucleic acids (PNAs)[1] (*6*) are a DNA/RNA mimic in which the phosphate deoxyribose backbone has been replaced by uncharged N-(2-aminoethyl)glycine linkages with the nucleobases attached through methylene carbonyl linkages to the glycine amino group (*7*−*9*). Because PNAs have a neutral backbone, their hybridization is not affected by the interstrand electrostatic repulsion that characterizes DNA and RNA duplexes. As a result, the hybridization of PNAs to complementary sequences is characterized by high affinities and by good mismatch discrimination (*10*, *11*). PNAs also possess high rates of association for duplex DNA (*12*) and a remarkable propensity for the invasion of double-stranded structure (*6*). PNAs are not hydrolyzed by nuclease or proteases (*13*) and are less likely than phosphorothioate oligomers to bind to proteins through nonsequence-specific interactions (*14*).

Their favorable properties have led to use of PNAs for many applications and it is not unreasonable to suggest that PNAs are one of the most successful designed macromolecules. Applications for PNAs include evaluating telomere length (*15*), inhibiting of human telomerase (*16*−*18*), capturing target nucleic acids (*19*, *20*), screening for genetic mutations (*21*, *22*), detecting specific sequences in unamplified DNA (*23*), and labeling plasmids with fluorophores (*24*). The structure of PNAs is dramatically different from DNA or RNA, but the array of successful applications supports the conclusion that PNAs have significant advantages for nucleic acid recognition outside of cells. The unresolved question for basic science is whether PNAs possess similar advantages for controlling cellular processes.

PNAs can be delivered into cells as conjugates with import peptides (*25*, *26*), by electroporation (*27*), or as complexes with DNA and cationic lipid (*18*) or polyethyleneamine (*28*). These delivery methods allow antisense inhibition by PNAs to be evaluated to determine whether PNAs possess advantages as antisense agents relative to oligonucleotides that contain 2′-*O*-alkyl RNA or DNA. Unlike RNA/DNA duplexes, RNA/PNA duplexes are not substrates for RNAse H (*29*), and the rules governing antisense inhibition of gene expression by PNAs are likely to be substantially different from those governing inhibition by oligomers that exploit RNase H. Several studies have described the ability of PNAs to block gene expression in cell extract (*29*−*34*), but only a handful of antisense PNAs have been tested for effects within mammalian cells (*8*, *26*, *35*). It has not been possible to determine whether antisense PNAs offer advantages relative to DNA or DNA-2′-*O*-alkyl chimera or what rules govern their efficacy.

We have previously shown that PNAs complementary to the template region of the RNA domain of human telomerase (hTR) inhibit cellular telomerase and cause telomeres to shorten (*17*, *18*). These results demonstrated that PNAs delivered as PNA−DNA−lipid complexes can recognize a target inside cells and affect cellular function in a sequence-specific fashion. It was not clear, however, whether the ability to inhibit telomerase was relevant to understanding the potential for antisense inhibition of gene expression by PNAs. Telomerase is an unusually favorable target for inhibition by nucleic acids and their mimics because the template region of hTR binds to telomere ends and is therefore inherently accessible to hybridization by inhibitory oligomers. Furthermore, hybridization to hTR blocks a critical enzymatic activity, telomere elongation, providing an unambiguous confirmation that PNAs are successfully hybridizing to telomerase. Few other intracellular RNA target sequences combine this high level of accessibility with such functional importance.

mRNA, by contrast, is a more challenging target than hTR. mRNA possesses complex secondary structure that makes it difficult to accurately predict which target sites will be most accessible for hybridization. In addition, unlike antisense oligomers that contain DNA and modified DNA sequences, PNA-RNA hybrids do not support RNase H activity (*29*). Therefore, it will be necessary for PNAs to act as a roadblock against the action of the translation machinery because they cannot promote degradation of the target sequence. Because of the inability of PNAs to support RNase H activity, an individual PNA also cannot amplify its efficacy by helping to catalyze the inactivation of multiple mRNA molecules.

Here we describe experiments that establish guidelines for using antisense PNAs inside cells. We characterize the delivery of PNAs into cultured cells and correlate the ability of antisense PNAs to inhibit gene expression with the location of the mRNA.

MATERIALS AND METHODS

Materials. PNAs were synthesized using an Applied Biosystems (Foster City, CA) Expedite 8909 Synthesizer using monomer Fmoc reagents from Applied Biosystems (*36*). PNAs were purified by reversed-phase HPLC and analyzed by time-of-flight mass spectrometry (MALDI-TOF) as described (*36*). DNA oligonucleotides and LipofectAMINE were obtained from Life Technologies (Gaithersburg, MD) and solubilized according to the manufacturer protocol in sterile water. Both PNAs and oligonucleotides were quantitated based on spectrophotometric A_{260} values and the conversion factor of 30 μg/mL OD_{260}. 9-*cis*-Retinoic acid was obtained from Sigma (St. Louis, MO). Ligand stocks (10 mM) were dissolved in 80% ethanol/20% DMSO (v/v) and stored under nitrogen at −20 °C. All manipulations of 9-*cis*-retinoic acid (9-cisRA) were performed under yellow light to minimize the likelihood of isomerization. Plasmids pCMX-hRXRα, pCMX-β-GAL, and pTK−CRBPII-LUC were obtained from Dr. David Mangelsdorf (UT Southwestern Medical Center, Dallas, TX) (*37*).

Annealing of PNAs with DNA Oligonucleotides. PNA and DNA oligomer concentrations were determined as above and 100 μL volumes of a PNA:DNA heteroduplex mixtures (100 μM each) were prepared in thin-walled PCR tubes in 2.5 × PBS (phosphate buffered saline, pH 7.4, without calcium or

[1] Abbreviations: PNA, peptide nucleic acid; hTR, human telomerase; MALDI-TOF, matrix assisted laser desorption time-of-flight; FACS, fluorescence assisted cell sorting; RXR, retinoid X receptor; 9-cisRA, 9-*cis*-retinoic acid; 5′-UTR, 5′-untranslated region.

magnesium chloride, Life Technologies). Annealing of PNA and DNA oligomers was performed in a thermal cycler according to the following temperature profile. Reductions in temperatures occurred in one minute with hold times indicated (°C, min): 95, 5; 85, 1; 75, 1; 65, 5; 55, 1; 45, 1; 35, 5; 25, 1; and 15 °C, 1 min, hold at 15 °C. After annealing, the PNA:DNA complexes were maintained at 4 °C until use for transfections or evaluation of T_m.

Melting Temperature (T_m) Determination. Melting temperature studies were performed by measuring the change in absorbance at 274 nm with a 500 nm reference wavelength using an 8452A UV diode array spectrophotometer (Hewlett-Packard) and an HP 89090A Peltier temperature control accessory (TEMPCO Software). Determinations were performed in a 1.0 mL quartz cuvette (Spectrosil Far UV Quartz type 26.100 stoppered cell with a 10 mm path and a 15 mm Z-dimension (Uvonic Instruments, Plainview, NY). Sample was prepared by mixing 5 μL of 100 μM of annealed PNA−DNA heteroduplex stock solution with 145 μL of 0.1 M sodium phosphate (Na_2HPO_4, pH 7.4). Samples were overlaid with 145 μL of mineral oil to prevent evaporation at higher temperatures and to make the upper and lower baselines more consistent. Data were collected with the TEMPCO software from 9 to 96 °C and from 96 to 9 °C in 3 °C increments with an equilibration time of 0.1 min at each temperature after an initial 5 min equilibration prior to starting the temperature ramping. Data were collected in both directions (denaturation and annealing) to ensure that the observed curves were reversible. Data files were imported into Sigma Plot 4.0 for Windows (SPSS Science, Chicago, IL) for statistical and nonlinear curve fit analysis. Independent analyses were performed for the data corresponding to the denaturation and annealing profiles.

Lipid-Mediated Transfection of PNA-DNA Complexes. PNA−DNA heteroduplexes were prepared by equilibrating 15 μL of 100 μM PNA−DNA heteroduplex in 135 μL of Opti-MEM (Life Technologies). In a separate tube, 4.5 μL of (7 μg/mL) LipofectAMINE (Life Technologies) was activated in 145.5 μL of Opti-MEM by vigorously shaking for 5 s followed by equilibration for 5−10 min at room temperature. The PNA−DNA heteroduplex and LipofectAMINE aliquots (300 μL each) were mixed together and agitated vigorously for 15 s. Lipid complexes were allowed to form by incubating the mixture at room temperature for 15−20 min in the dark. The solution containing the PNA−DNA−lipid complex (600 μL) was diluted to 3 mL with Opti-MEM to afford a solution containing 1 μM PNA−DNA hybrid. This solution was then diluted to a final working concentration, which was 100 nM for most experiments.

COS-7 cells were plated at 11000−13000 cells/well in 48-well plates using Dulbecco's MEM (minimal essential media) with glutamine supplemented with 10% superstripped fetal calf serum, 20 mM HEPES buffer (final concentration, pH 7.4), 500 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.06 mg/mL anti-PPLO reagent (Life Technologies). Superstripped serum was used to ensure that competing ligands were removed from serum prior to addition of 9-cisRA. Ligand stripping was achieved by twice extracting serum with activated charcoal and cation exchange (CAG 1-X8 resin, Bio-Rad, Hercules, CA). Superstripped serum was doubly filtered through a 0.2 μm filter prior to addition to media. Cells were incubated at 37 °C at 5% CO_2 for a minimum of 6 h prior to initiating transfection. The cells were then washed once with 250 μL of Opti-MEM, followed by overnight transfection with the PNA−DNA−lipid complex lipid. A second transfection of reporter vectors was conducted subsequently as described below.

Lipid-Mediated Transfection of Reporter Vector Complexes into COS-7 Cells. COS-7 cells (ATCC CRL-1651) are an SV40 transformed African green monkey kidney cell line. Expression vectors (pCMX-βgal, 40 ng/well, pCMX-hRXRα, 20 ng/well and pTK-CRBPII-LUC, 40 ng/well) (*37*) were prepared for transfection by equilibrating plasmid DNA in 19.5 μL/well of Opti-MEM. Likewise, 0.2 μL of (7 μg/mL) LipofectAMINE was activated in 19.8 μL of Opti-MEM. The two components were mixed and complexed for 15−20 min as described above. The solution of lipid−plasmid complex was diluted with 10 μL of Opti-MEM/well and 50 μL of the vector mixture was dispensed into each well. The transfection was carried out for 6 h, after which the composite transfection mixture was removed by aspiration and replaced with the media. The media was supplemented with 1 μM 9-cisRA or a solvent control comprised of 80% ethanol/20% DMSO (v/v). Cells were harvested 40 h after addition of ligand and analyzed for luciferase and β-galactosidase activities.

Luciferase assays were conducted with 20 μL of cell lysate, 100 μL of assay buffer, and 100 μL of 1× luciferin (Biosynth, Naperville, IL) prepared in 0.1 M KH_2PO_4, pH 7.8, in an opaque, flat-bottom 96-well plate (Costar). Data were collected using enhance flash parameters on a model ML-3000 microplate luminescence system with BioLinx software v2.22 (Dynex Technologies, Inc., Chantilly, VA). β-Galactosidase assays were conducted using 40 μL of cell lysate and 125 μL of phosphate assay buffer containing 2 mg/mL *o*-nitrophenyl β-D-galactopyranoside (ONPG). Color development was conducted at 37 °C for 15 min or less depending on the rate of color development and the reaction was stopped with the addition of 50 μL of 1 M $NaCO_3$. Data were collected at 410 nm and a 630 nm reference wavelength on a model MR5000 microtiter plate reader with BioLinx software v2.22 (Dynex). All data points represent the mean of triplicate experiments normalized against β-galactosidase activity. Error bars represent the standard deviation of the mean.

Fluorescence Microscopy and FACS. PNA **I** was labeled with N-terminal tetramethyl rhodamine (Molecular Probes, Eugene, OR) (*38*) and cotransfected with DNA oligomer into DU145 cells as described above. Cells were rinsed four times with PBS and fresh media was added. Cells were then incubated for 2 h and rinsed an additional four times with PBS. This second incubation was added to increase the likelihood that cells would withstand the extended washing necessary to ensure removal of free rhodamine from cell surfaces. Cells for evaluation by microscopy were rinsed once more with distilled water, transferred onto slides, and mounted with GEL/MOUNT (Biomeda Corp., Foster City, CA). Cells were visualized using an Olympus (Melville, NY) fluorescent microscope at 100× magnification. Cells for FACS sorting were pelleted and resuspended in 1× PBS and were sorted using a FACStarPlus flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

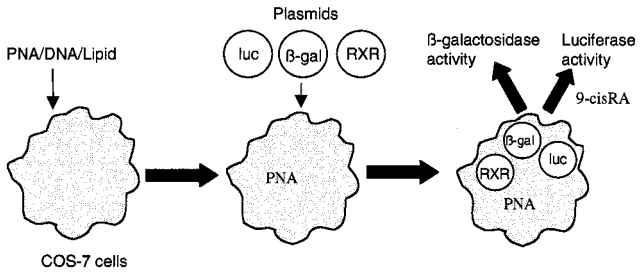

FIGURE 1: Use of luciferase to test antisense inhibition by PNAs. PNA is introduced into COS-7 cells in complex with DNA and lipid. Plasmids encoding luciferase (luc), β-galactosidase (β-gal), and the retinoid X receptor (RXRα) are cotransfected into cells. 9-*cis*-Retinoic acid is added to activate RXR, which then triggers expression of luciferase. β-galactosidase is constitutively expressed and serves as an internal control for normalization to allow comparison of experiments. Levels of β-galactosidase and luciferase are measured by colorometric or luminometric assays respectively (see Materials and Methods). The level of luciferase activity is a reporter for antisense inhibition of gene expression.

RESULTS AND DISCUSSION

Luciferase as a Target Gene for Characterizing Antisense Inhibition by PNAs. To characterize the ability of PNAs to recognize cellular mRNA and act as antisense agents, we developed a simple method for introducing PNAs into cells (*18*). In the first step, PNA and DNA are annealed to form a PNA−DNA heteroduplex. In the second step, cationic lipid is added to form a complex with the DNA oligomer. The lipid facilitates uptake of the DNA oligomer, and the PNA is transported into cells as passive cargo because of its association with DNA. This method takes advantage of lipid-based transfection protocols that are familiar to many laboratories and provides a robust method for the transfection of PNAs.

We chose luciferase as a model target for characterizing the rules governing antisense inhibition by PNAs in cells because luciferase activity can be detected by a rapid, sensitive, and quantitative assay. To test an antisense PNA, we first transfect the PNA−DNA duplex into cells as described above (Figure 1). We then introduce plasmid containing the gene encoding luciferase. This order of addition is advantageous because there is no luciferase present prior to addition of PNA. The absence of preexisting luciferase allows the effects caused by the PNA to be observed quickly without the need to wait for luciferase that was made prior to PNA addition to be degraded by the cell. This greatly increases the throughput and sensitivity of the assay. Adding PNA first also precludes the possibility that transfection of plasmid would interfere with subsequent introduction of the PNA, a variable that could easily confound comparison of different experiments.

To minimize background levels of luciferase, we exploit the fact that the luciferase gene promoter contains a response element for the retinoid X receptor (RXR). Luciferase expression can be induced by addition of 9-cisRA, an activator for RXR. There is very little expression of luciferase prior to induction with 9-cisRA, allowing the inhibition of luciferase activity upon addition of PNA to be cleanly evaluated. As an internal standard, we also transfected a constitutively expressed gene encoding β-galactosidase. This cotransfection and subsequent measurement of β-galactosidase activity provides an internal control for cell viability and transfection efficiency. All luciferase inhibition values are normalized to β-galactosidase values. The antisense effects of 53 different PNA−DNA combinations on cellular gene expression are examined in these studies. All assays for luciferase activity were performed in duplicate and usually in triplicate or greater. This comprehensive investigation would have been impractical had we not adopted the luciferase/RXR/β-galactosidase cotransfection as a high throughput model system.

Inhibition of Gene Expression by PNAs Targeted to Sequences within Luciferase mRNA. To establish rules correlating antisense inhibition by PNAs with target sequence we synthesized eighteen PNAs complementary to different sequences within luciferase mRNA (Table 1, Figure 2, PNAs **I**−**XVIII**). Values for three noncomplementary control PNAs are reported (**XIX**−**XXI**). PNAs **I**−**VI** target the 5′-untranslated region. PNAs **VI**−**VIII** are complementary to sites near the start codon. PNAs **VIII**−**XVIII** are directed to sequences throughout the coding region. It has been reported that antisense inhibition by PNAs in cell extract is enhanced by high affinity between PNA and the target sequences (*34*). PNAs **IX**, **XIII**, and **XVI** were chosen to be C/G rich and possessed melting temperatures for complementary sequences of greater than 95 °C (data not shown). The transport DNA oligonucleotides were designed to have between 8 and 15 base complementarity with their antisense PNA partner. We hypothesized that the affinity of the transport DNA for its PNA complement would be an important variable, so we measured melting temperature (T_m) values for all PNA−DNA duplexes. This hypothesis will be explored in detail below, and T_m values for PNA−DNA complexes ranged from 48 to 74 °C (Table 1). For some PNAs, more than one DNA was tested as a transport agent to identify a PNA−DNA complex that possessed a T_m near 52 °C, the lowest T_m value observed to promote uptake of a fluorescently labeled PNA (see below, Figure 6, Table 5). The values for inhibition are averages of duplicate or triplicate determinations.

Of the 18 antisense PNAs tested, **I** was the only PNA to significantly inhibit luciferase activity, blocking 80% of activity when delivered to cells as a PNA−DNA−lipid complex (Table 1, Figure 2) at a concentration of 100 nM. Other PNAs, including the three targeted to the start codon (**VI**−**VIII**), the three chosen to have exceptionally high affinities for the target (**IX**, **XIII**, and **XVI**), and the five targeted to other sites within the 5′-UTR did not significantly inhibit luciferase at concentrations as high as 500 nM. Once the combination of PNA **I** and DNA **1** was shown to inhibit luciferase activity, it was included as a routine positive control on all assays and the observed inhibition has been reproduced routinely. PNA **I** is targeted to the extreme 5′ end of the untranslated region, and its effectiveness as an inhibitor supports the hypothesis that PNAs can block binding of the translation apparatus to the 5′-UTR. The ineffectiveness of the other 17 PNAs tested is consistent with the suggestion that PNAs are less effective as agents for blocking movement of the translation apparatus along mRNA.

Once PNA **I** was identified as the most promising inhibitor, we investigated whether the observed inhibition was through an antisense mechanism by assaying PNAs **XIX** and **XX** that contain the sense sequence that is complementary to PNA **I** (Table 1, Figure 2). Testing sense PNAs is a

Table 1: Inhibition of Gene Expression by PNAs Targeted to Sites throughout the mRNA Encoding Luciferase and Its 5′-Untranslated Region[a]

PNA	Sequence DNA Complement	Tm °C	Inhibition %
I	**AGGGTCGCTCGGTGT**		
	AGCGAGCCACATCCC (DNA1)	67	80
II	**TAAGCGGGTCGCTGC**		
	GCCCAGCGACGATTC (DNA 2)	76	10
III	**CGCTGTTACGCTGC**		
	CAATGCGACGAGCGA (DNA 3)	54	34
IV	**AGATCTGCGGCATCG**		
	CTAGACGCCGTAGCT (DNA 4)	66	12
V	**GGAATGCCAAGCTCC**		
	ACGGTTCGAGGCCTT (DNA 5)	54	4
VI	**TTTACCAACAGTACC**		
	GGTTGTCATGGAAAT (DNA 6)	51	21
VII	**GTCTTCCATTTTACC**		
	GAGAAGGTAAAATGG (DNA 7)	52	26
	GAAGGTAAATGGATAC (DNA 8)	47	14
VIII	**TTTGGCGTCTTCCAT**		
	CCGCAGAAGGTTAAA (DNA 9)	64	24
IX	**AATGGCGCCGGGCCT**		
	GGCCCGGATTAC (DNA 10)	68	6
X	**CGGTTCCATCCTCTA**		
	CAAGGTAGGAGAGATATGC (DNA 11)	51	15
	CCAAGGTAGGAGATATGC (DNA 12)	58	4
XI	**TGTATTCAGCCCA**		
	CATAAGTCGGGTATAA (DNA 13)	60	15
XII	**AACGCGCCCAACA**		
	GCGCGGGTTGTATTT (DNA 14)	74	1
XIII	**GTCGTTCGCGGGCGC**		
	GCCCGCGCAGC (DNA 15)	63	10
XIV	**CATACTGTTGAGC**		
	TATGACAACTCGATAG (DNA 16)	63	0
XV	**ATAAATCGTATTT**		
	TATTTAGCATAAA (DNA 16)	48	7
XVI	**CCGACCGCGCCCGGT**		
	GCGGGCCAGGCT (DNA 17)	58	10
XVII	**GTAGCCATCCATC**		
	ATCGGTAGGTAGATAC (DNA 18)	62	9
XVIII	**TCGAAGATGTTGG**		
	GCTTCTACAACCATAA (DNA 19)	70	10
XIX	**ACACCGAGCGACCCT** (sense PNA)		
	GGGATGTGGCTCGCT (DNA 20)	77	23
XX	**ACCGAGCGACCCT** (sense PNA)		
	TGGGTCGCTGGGA (DNA 20)	62	12
XXI	**CAGTTAGGGTTAG** (control)		
	ATCCCAATCGTCA (DNA 21)	58	23

[a] PNAs are listed N- to C-termini and are in boldface. DNA oligomers are listed 3′ to 5′.

necessary control to support an antisense mechanism of action because PNAs have been demonstrated to have an unusually high ability to invade double-stranded DNA at polypurine-polypyrimidine sites (*6*) and at sites containing mixtures of all four bases (*12*, *39*). While we consider it unlikely that PNAs would exert an effect by strand invasion rather than binding to mRNA the ability of PNAs to invade duplex DNA forces consideration that inhibition of luciferase activity by **PNA I** might be due binding to duplex DNA rather than to mRNA. We did not observe any inhibition of luciferase activity by PNAs **XIX** or **XX**, consistent with the conclusion that the observed inhibition of luciferase activity is not due to binding to DNA. However, because the propensity for strand invasion will not necessarily be the

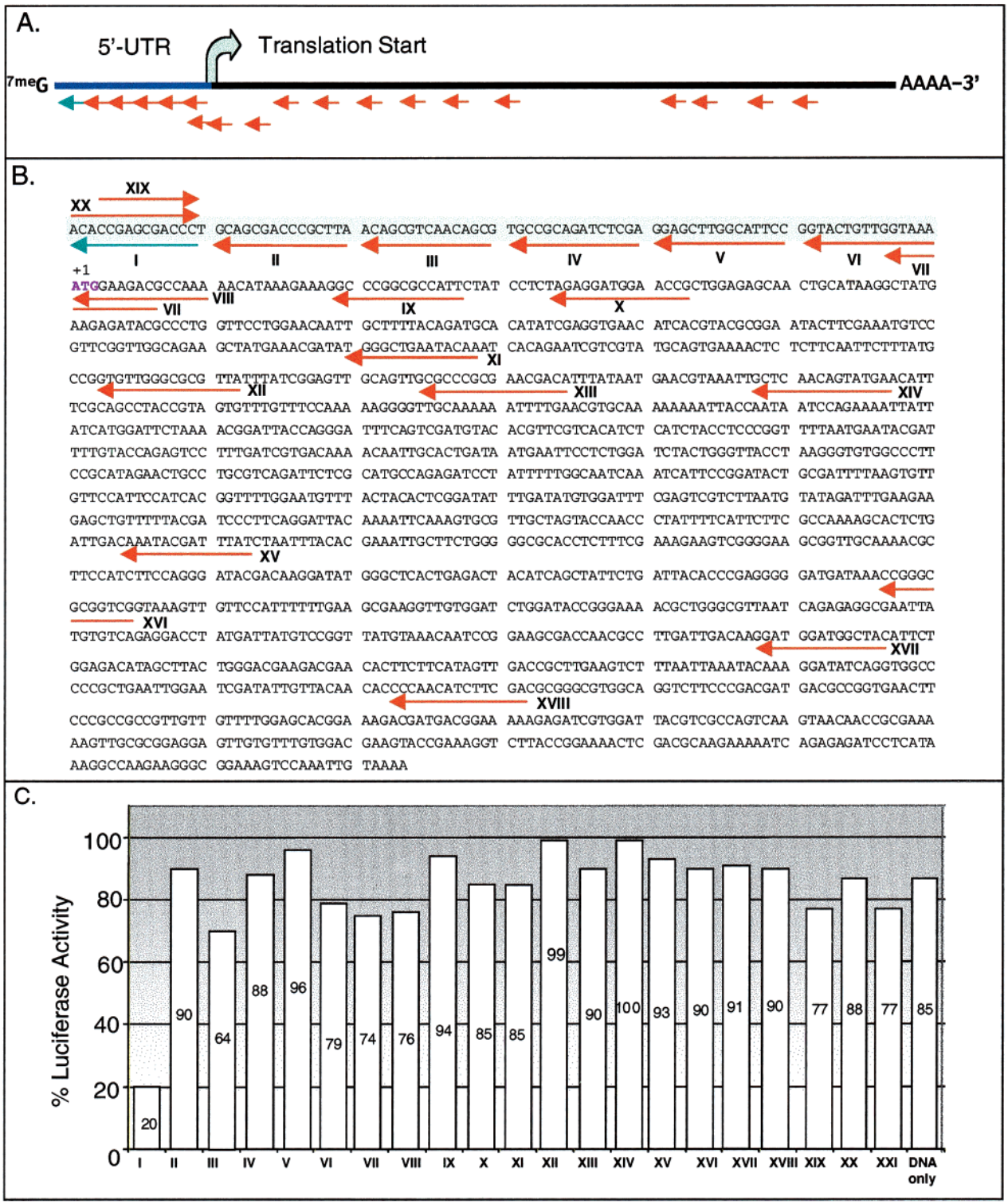

FIGURE 2: Effect of target site on inhibition of luciferase activity by PNAs (100 nM final concentration). (A) Schematic of the location of PNAs **I**−**XVIII** targeted to the 5′-UTR, the start codon, and the coding region of luciferase. (B) Location of PNAs **I**−**XXI** relative to the sequence of luciferase mRNA. (C) Bar graph showing the inhibition of luciferase expression by PNAs **I**−**XXI** and DNA **1** added alone.

same for PNAs targeted to both strands of the duplex, this possibility cannot be automatically ruled out.

As a further control, we measured the effects of PNA **XXI** which lacks complementarity to luciferase. PNA **XXI** is a particularly useful control because it is complementary to the RNA template of human telomerase and has already been demonstrated to enter cells, bind a target RNA, and alter cellular function in a sequence-specific fashion (*17*). Like PNA **I**, PNA **XXI** also contains three consecutive guanines, a motif in PNAs previously shown to lead to high affinities for complementary sequences (*40*). We observed that PNA **XXI** did not significantly block luciferase activity. Because **XXI** can be successfully introduced into cells we can eliminate lack of uptake as an explanation its inability to inhibit luciferase activity. We also transfected transporter DNA **1**, the DNA partner for PNA **I**, by itself, and found that it did not block luciferase activity. Similarly, the transfected duplex of DNA **1** and DNA **20** also did not block

Table 2: Effect of PNA Length and Placement on Inhibition of Luciferase by PNAs Targeted to the Terminal Region of the 5′-UTR[a]

PNA	Sequence DNA Complement	Tm °C	Inhibition %
I	**AGGGTCGCTCGGTGT** (15 bases)		
	AGCGAGCCACATCCC (DNA 1)	67	80
XXII	**AGGGTCGCTC** (10 bases)		
	AGCGAGCCACATCCC (DNA 1)	47	13
	CCAGCGAGTC (DNA 22)	53	0
	TCCCAGCGAG (DNA 23)	71	9
XXIII	**CGCTCGGTGTC** (11 bases)		
	TCCCAGCGAGCCACA (DNA 24)	43	18
	GAGCCACAGGC (DNA 25)	52	7
	GCGAGCCACAG (DNA 26)	67	3
XXIV	**GGTCGCTCGGT** (11 bases)		
	GCCACCAGCGA (DNA 27)	50	27
	AGCGAGCCACATCCC (DNA 1)	63	43
XXV	**AGGGTCGCTCGGT** (13 bases)		
	TCCCAGCGAGCCACATCCC (DNA 28)	84	31
	GCGAGCCACATCCC (DNA 29)	56	60
XXVI	**AGGGTCGCTCGGTGTTCG** (18 bases)		
	AAGCTCCCAGCGAGCCAC (DNA 30)	>90	28
	AGCGAGCCACATCCC (DNA 1)	66	88

[a] PNAs are listed N- to C-termini and are in boldface. DNA oligomers are listed 3′ to 5′.

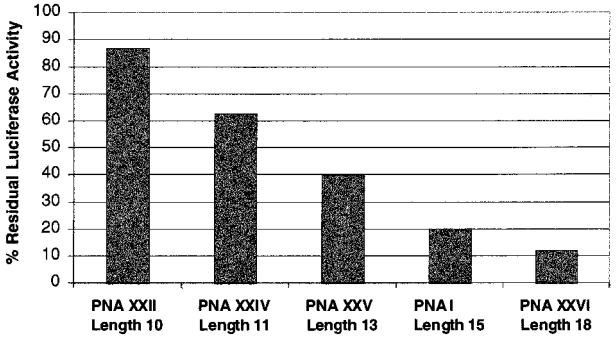

FIGURE 3: Inhibition of luciferase activity by PNAs directed to the terminal region of the 5′-UTR as a function of PNA length.

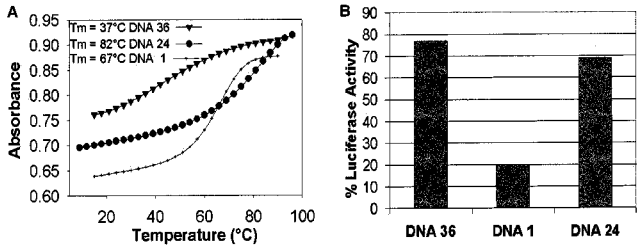

FIGURE 4: Correlation of melting temperatures of hybrids between PNA **I** and varied transporter DNAs with inhibition of luciferase activity. (A) Melting temperature determinations of PNA/DNA hybrids between PNA **I** and DNAs **1**, **24**, and **36**. (B) Bar graph showing the inhibition of luciferase activity by PNA **I** complexed with DNAs **1**, **24**, and **36**.

activity. Taken together, the results of these control experiments support the hypothesis that PNA **I** inhibits luciferase activity by binding to its intended target site at the 5′-termini of the 5′-UTR of luciferase mRNA.

Effect of PNA Length, Placement, and Mismatches on Inhibition of Luciferase Activity. To further examine the terminal sequence of the 5′-UTR as a target for PNAs, we synthesized a series of PNAs **XXII**−**XXVI** (Table 2, Figure 3) analogous to **PNA I** but that vary in length from 10 to 18 bases. PNAs **XXII** and **XXIII** contained 10 or 11 bases and did not inhibit luciferase activity. PNA **XXIV** contained 11 bases, and inhibited activity slightly. Related PNAs that were longer, 13 base PNA **XXV** and 18 base PNA **XXVI**, blocked 60 and 88% of luciferase activity, respectively. We also tested PNAs **XXV** and **XXVI** in combination with DNA partners **28** and **30** that formed hybrids with T_m values of 84 and >90 °C, respectively. These high-affinity complexes did not yield significant inhibition, suggesting that the duplex is too stable and that the PNAs are not being released within the cell. This issue will be further explored below. The correlation between PNA length and the ability to inhibit translation supports the conclusion that high affinity for target site is an important consideration for antisense activity.

We also examined the effect of mismatched bases on inhibition of luciferase activity by PNAs related to PNA **I** (Table 3). PNAs containing either one (PNAs **XXVIII** and **XXIX**) or two mismatches (**XXVII** and **XXX**) do not substantially inhibit luciferase activity. This result provides further support for the conclusion that PNAs **I**, **XXV**, and, **XXVI** inhibit luciferase expression by hybridizing to luciferase mRNA. This observation is important because it has been noted that antisense oligonucleotides with phosphorothioate backbones can inhibit gene expression by a sequence-specific mechanism, such recognition of G-quartets by proteins, that does not involve binding to the intended mRNA target (*41*).

Effect of DNA/PNA Complementarity on Inhibition of Luciferase Expression by a PNA Directed to the Terminus of the 5′-UTR. Our transfection protocol requires that PNAs

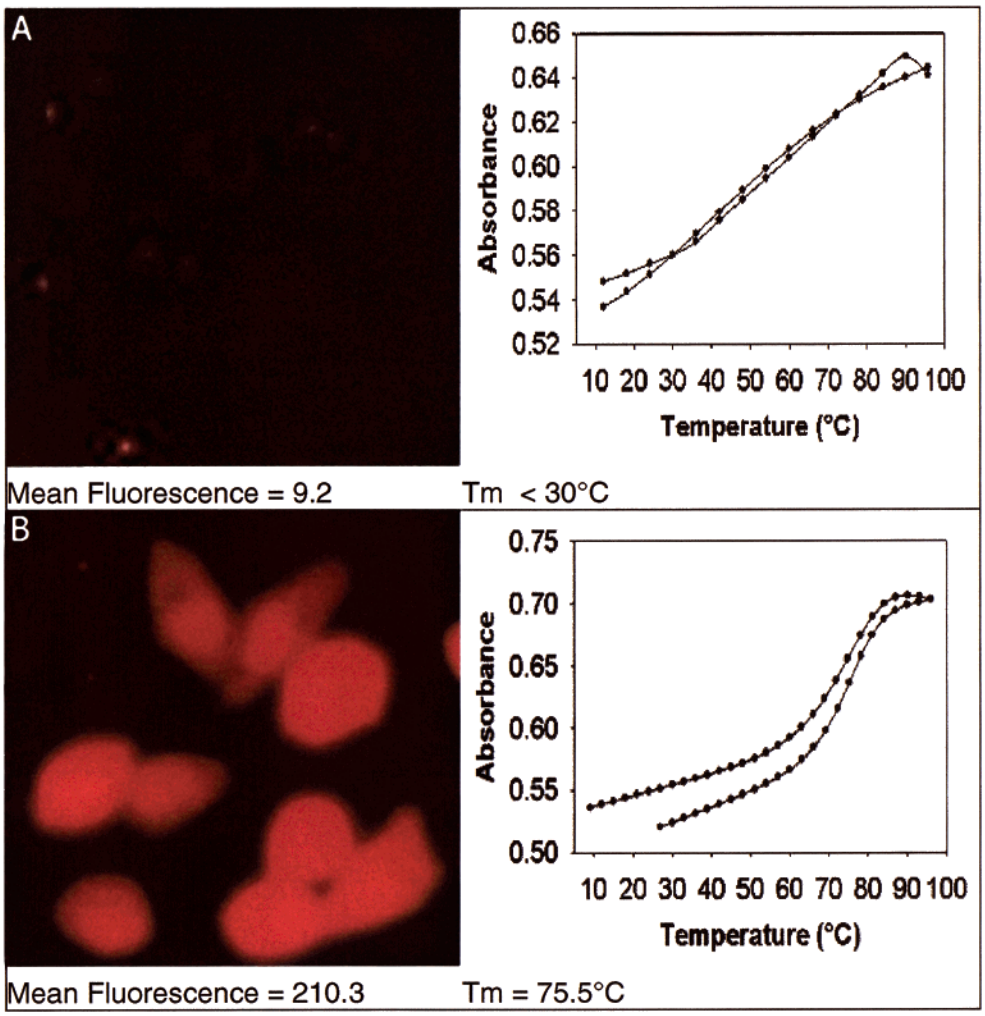

FIGURE 5: Cellular uptake of DNA-PNA complexes. Melting temperature evaluations were performed by first monitoring hypochromicity over increasing temperature and then monitoring rehybridization as temperatures were decreased. Both evaluations are shown in this figure. (A) *Right*: uptake of rhodamine labeled PNA **XXXI** mixed with a DNA oligomer (DNA **46**) containing minimal complementarity measured by fluoresence microscopy and FACS analysis. Left: Measurement of the association of PNA **XXXI** and a DNA oligomer with minimal complementarity, as a function of temperature. No melting temperature could be measured. (B) Right: uptake of rhodamine labeled PNA **XXXI** mixed with a complementary DNA measured by fluoresence microscopy and FACS analysis. Left: Measurement of the association of PNA **XXXI** and DNA **42** as a function of temperature. The measured melting temperature was 75 °C. Fluorescence microscopy showing the effects of DNA-PNA overlap on cellular uptake of a rhodamine-labeled PNA.

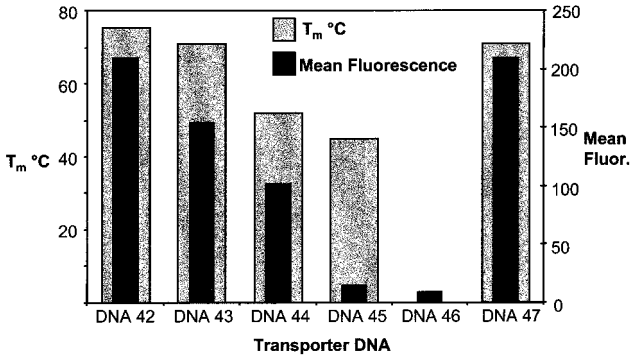

FIGURE 6: Correlation of melting temperature with cellular uptake of complexes between rhodamine-labeled PNA **XXXI** and different DNA oligomers. Cellular uptake was monitored by FACS.

associate with DNA prior to entry into cells. As a result, it is likely that the affinity of the PNA−DNA duplex is an important variable and that accurate prediction of optimal PNA−DNA overlap will be essential for using transfected PNAs to affect cellular processes. To establish guidelines for PNA delivery, we examined the inhibition of luciferase expression by PNA **I** upon formation of complexes with DNA oligonucleotides **24** and **32**−**40** (Table 4) and compared them to inhibition by PNA **I** in complex with DNA **1** (Figure 4). T_m values for the duplexes between DNAs **24** and **32**−**40** and PNA **I** varied from 37 to 82 °C reflecting the differing complementarity of the complexes.

We find that the stability of the DNA−PNA duplex is a critical variable for inhibition of luciferase activity. DNAs **24** and **33** form the most stable PNA−DNA duplexes, with T_m values of 82 °C but cause the lowest levels of inhibition (Table 4, Figure 4), scarcely above the background levels produced by noncomplementary oligomer **XXI** (Table 1, Figure 2). The failure of these highly stable DNA−PNA hybrids to inhibit luciferase was also observed with combinations of PNA **XXV** with DNA **28** and PNA **XXVI** with DNA **30** (see above) (Table 2). We hypothesize that very stable PNA−DNA duplexes do not inhibit gene expression because they cannot release transfected PNAs at concentrations that are sufficient to cause antisense inhibition.

Table 3: Effect of Mismatches on Inhibition by PNAs Targeted to the Terminus of the 5′-UTR[a]

PNA	Sequence DNA Complement	Tm °C	Inhibition %
I	**AGGGTCGCTCGGTGT**	67	80
	AGCGAGCCACATCCC (DNA1)		
XXVII	**AGGGCTGCTCGGTGT**		
	GACGAGCCACATCCC (DNA36)	66	38
	AGCGAGCCACATCCC (DNA1)	67	37
XXVIII	**AGGGTCGCCCGGTGT**		
	AGCGGGCCACATCCC (DNA48)	69	30
XXIX	**AGGGTCGCTCGATGT**		
	AGCGAGCCACATCCC (DNA1)	48	1
XXX	**AGGGTCGCTCAATGT**		
	AGCGAGCCACATCCC (DNA1)	49	27

[a] PNAs are listed N- to C-termini and are in boldface. DNA oligomers are listed 3′ to 5′. Underlined bases denote mismatches relative to the target site of PNA II.

Table 4: Effect of DNA Complementarity on the Ability of a PNA/DNA Hybrid to Inhibit Gene Expression[a]

PNA	Sequence DNA Complement	Tm °C	Inhibition %
I	**AGGGTCGCTCGGTGT**		
	CGAGCCACAACCCT (DNA32)	60	38
	AGCGAGCCACATCCC (DNA 1)	67	80
	TCCCAGCGAGCCACA (DNA 24)	82	23
	ACATCCCAGCGAGCCACA (DNA 33)	82	25
	AGCCACATCCCAGCG (DNA 34)	67	86
	GACGAGCCACATCCC (DNA 35)	63	78
	GGCGAACCATATTCC (DNA 36)	37	31
	AGCGAGCCACAGAAA (DNA 37)	68	45
	AGCGAGCCACAAGGG (DNA 38)	67	55
	AGCGAGCCACACTTT (DNA 39)	69	50
	AGCGAGCCACACTCT (DNA 40)	71	62

[a] PNA I is listed from its N- to C-terminus and is in boldface. DNA oligomers are listed 3′ to 5′.

Table 5: FACS Analysis of Cellular Uptake and Melting Temperatures of Hybrids of Rhodamine Labeled PNA **XXXI** and Different DNA Transporter Oligonucleotides[a]

PNA	Sequence DNA Complement	Tm °C	Mean Fluoresence
XXXI	Rhodamine-**TAGGGTTAGCAA**		
	ATCCCAATCGTT (DNA42)	75	210
	TCAATCCCAATCT (DNA 43)	71	154
	GAGTCAATCCCAA (DNA 44)	52	101
	GAAGAGTCAATCC (DNA 45)	45	15
	CGGGAAGAGTCAA(DNA 46)	nd	9
	CGTTATCCCAATC (DNA 47)	68	210

[a] nd: not detected. PNA XXXI is listed from its N- to C-terminus and is in boldface. DNA oligomers are listed 3′ to 5′.

Consistent with this hypothesis, moderately stable PNA−DNA duplexes containing DNAs **32**, **34**, and **35** that possessed melting temperatures of 60, 67, and 63 °C inhibited 38, 86, and 78% of luciferase activity, respectively. DNA **36**, formed only a weak hybrid with PNA **I** (T_m = 37 °C) and did not significantly block luciferase activity (Table 4, Figure 4). Thus, it appears that the DNA−PNA duplex must be strong enough to successfully convey the PNA during the transfection but weak enough to release the PNA once cellular delivery is achieved.

We hypothesized that the use of DNAs to bridge two PNAs and possibly form PNA−DNA chains would lead to formation of higher order complexes that might improve inhibition. This design feature is built into the DNA **1**/PNA **I** hybrid and most of the other duplexes used in these studies. To test our hypothesis, we synthesized DNAs **37**−**40** to be

incapable of bridging PNAs. Each contain a different 5′-terminal sequence and we compared their effectiveness to that of DNA **I** which can bind two PNAs. DNAs **37**−**40** possess identical complementarity to PNA **I** and also possess very similar T_m values (Table 4). Despite this similarity in T_m values, complexes of PNA **I** with DNAs **37**−**40** inhibited luciferase activity at levels ranging from 45 to 62%, significantly below that afforded by use of DNA **1**. This observation suggests that the ability to bridge two or more PNAs modestly enhances their ability to act as antisense agents and may be a useful design feature to include.

Effect of PNA−DNA Complementarity on Uptake Monitored by FACS Analysis. Our luciferase assays measure activity but do not directly evaluate cellular uptake. As a direct test for the importance of basepairing on cellular entry of PNA−DNA complexes, we characterized PNA uptake by FACS and microscopy. We examined the uptake of rhodamine-labeled PNA **XXXI** in complex with a DNA oligonucleotides (DNA **42**−**47**) which varied from being fully complementarity (DNA **42**, T_m, 75) to noncomplementary (DNA **46**, no association detected) (Figure 5, Table 5). We found that melting temperatures closely correlated with uptake, with cellular fluorescence increasing 15-fold as the T_m for DNA−PNA binding increased from 45 to 75 °C (Figure 6, Table 5). Substantial uptake was observed for the hybrid between PNA **XXXI** and DNA, T_m of 52 °C, suggesting this as a lower limit of effective T_m values.

To further test whether DNA transporters that are capable of forming higher order PNA−DNA complexes are also able to promote PNA uptake, we designed DNA **47** to bridge two PNA molecules and possess the potential to form a PNA−DNA chain. Nine bases of DNA **47** were intended to pair to one PNA molecule, with the remaining four bases available to pair with a second. The PNA **XXXI**−DNA **47** complex possessed a melting temperature of 71 °C. Microscopy and FACS analysis revealed that cellular uptake was as efficient as that of the highly effective PNA **XXXI**−DNA **47** complex, which was capable of forming 13 base pairs with a single PNA molecule. Taken together with the results from our inhibition of luciferase activity, these data indicate that complexes with T_m values above 52 °C can enter cells at levels significantly above background levels, and the ability of DNA transporters to bridge PNAs does not interfere with uptake.

Antisense Inhibition by PNAs. The inhibition of gene expression by oligonucleotides or PNAs presents a series of technical challenges. The first challenge is delivery of the oligomer across the cell membrane. Our transfection strategy for intracellular PNA delivery is advantageous because it does not require modification of the PNAs and because it is derived from standard lipid-mediated transfection protocols that are familiar to many laboratories. One disadvantage for our approach is that lipid-mediated transfections, while simple, require repeated manipulation of cells. An alternate strategy for delivery of PNAs is to attach PNAs to peptides known to promote cellular uptake (*25*, *26*). The disadvantage of this approach is that obtaining PNA−peptide conjugates requires more elaborate synthetic procedures. The compensation, however, is that peptide conjugates enter cells spontaneously upon addition to media, removing the need for repeated transfection and facilitating long-term studies.

A second challenge to successful antisense inhibition is the targeting of oligomers to susceptible mRNA target sequences. PNAs have shown outstanding potential for strand invasion of duplex DNA (*6*, *12*, *39*, *42*, *43*). PNAs also possess high affinities for complementary RNA sequences, so it is reasonable to assume that they may also be able to efficiently bind to a wider range of mRNA sequences than can oligonucleotides that contain negatively charged backbone linkages. Indeed, we have shown PNAs targeted to the RNA component of telomerase act as effective inhibitors of telomerase activity (*36*). Inhibition by PNAs targeted to the template region was not surprising given the necessity for the template to be accessible for hybridization to telomeric ends, but we also observed that 8 of 11 PNAs targeted to nontemplate sequences also blocked activity. This potential for superior binding is important because predicting the sequence of antisense oligomers has proven an elusive goal (*44*).

The third challenge is that, once bound, the oligomer must prevent translation of the target protein. For oligomers that contain DNA sequences, inhibition of expression is facilitated by the formation of a DNA−RNA hybrid that can act as a substrate for RNAse H. RNase H cleavage has two consequences. The bound RNA is degraded, preventing translation, and the efficacy of the oligomer is amplified because it can then bind additional mRNAs and cause their destruction as well. As we note above, PNAs cannot activate RNAse H (*29*). The inability of PNAs to activate RNase H likely contributes to our failure to observe inhibition of luciferase activity upon addition of PNAs **II**−**XVIII** (Table 1).

By contrast to our inability to detect inhibition of luciferase activity by PNAs **II**−**XVIII**, PNAs targeted to the terminus of the 5′-UTR of luciferase are potent antisense agents. Presumably, these PNAs act to block binding of the translation apparatus, and similar results have been obtained using 2′-modified RNA directed to the terminal portion of the 5′ UTR of intercellular adhesion molecule 1 (ICAM1) (*45*). The 5-UTR may be a predictable target sequence for antisense gene inhibition. 5′-UTR sequences, however, are frequently not well-known, although this situation will change as sequencing of model mammalian genomes is completed. We chose not to employ RNA structure prediction programs when choosing target sequences for PNAs **I** to **XVIII** because of the uncertainty of prediction of the entire mRNA. However, subsequent prediction of the structure of the 5′-UTR using the program RNA Structure 3.5 (*46*) revealed that the target site for **I** was highly accessible and this availability may also have contributed to its inhibitory potency. We note that there is one sequence within the coding region of RXR that shares 12−15 bases with the terminal 5′-UTR sequence of luciferase. We cannot rule out that PNA binding to this RXR sequence also contributes to the observed inhibition of luciferase expression, and we are currently investigating this possibility.

A fourth challenge to the development of oligomers as effective antisense agents is the possibility that their introduction into cells will cause phenotypes that are unrelated to binding to their intended targets. In the past, this has been a major problem for the interpretation of results, especially for oligonucleotides that contain phosphorothioate linkages. It is possible that PNAs may have inherent advantages as specific reagents because they lack a negatively charged

backbone and therefore will not bind to the many proteins inside and outside of cells that normally act to bind negatively charged macromolecules. In addition, the inability of PNAs to activate RNase H also eliminates the likelihood of unintended degradation of nontarget mRNAs, a potential source of the unintended effects caused by antisense phosphorothioate oligomers (*47*). None of the PNAs tested in this study produced toxic effects or unusual phenotypes when added to cells at 100 nM, the concentration needed to produce maximal inhibition by PNA **I**. Toxicity and nonspecific effects were observed at concentrations greater than 500 nM, suggesting that the window of effective concentrations for PNAs delivered in complex with DNA and LipofectAmine is relatively narrow. Use of other transfection reagents or cell types might allow application of higher concentrations of PNA.

We certainly do not exclude the possibility that it may be possible to successfully target PNAs to mRNA sites other than the 5′-UTR terminus. Our 17 inactive PNAs (**II**−**XVIII**) may have been targeted to inaccessible sites or may have been present at concentrations too low to be effective. We strongly believe, however, that our results affirm that antisense experiments that use PNAs targeting sequences other than the terminus of the 5′-UTR and that claim to show that these PNAs are effective in vivo should be accompanied by the well considered use of multiple control PNAs. In addition, our experiments used only standard unmodified PNAs. It is possible that chemical modifications may expand the potential for antisense PNAs. One promising option is the use of bis-PNAs that may form three stranded complexes with very high affinity, possibly acting as roadblocks capable of blocking translation within the coding regions.

Another strategy is to synthesize longer or C/G rich PNAs capable of tightly binding to mRNA (*34*). As described above, we found that these tight binding PNAs could not block expression in our experiments, but such PNAs may succeed when tested against other sequences. Alternatively, a compelling option is to use covalently linked PNA−DNA hybrids (*48*). Such options would combine the enhanced ability of PNAs to hybridize to nucleic acid targets with the ability of DNA to recruit RNase H. Such hybrids would combine enhanced hybridization by PNAs with two advantages conferred by the DNA portion. The first is that the strategy allows delivery of conjugates by cationic lipids, eliminating the need for complementary hybridization and simplifying transfection protocols. The second advantage is that once in cells, the DNA portion could recruit RNAse H to degrade mRNA targets. One final option to improve antisense PNAs is to attach functional groups capable of promoting RNA cleavage. This has already been achieved, and while the targeted RNA cleavage was reported to be slow, improved cleavage chemistries may make in vivo applications more practical (*49*).

Summary. Strategies for the convenient and effective antisense inhibition of gene expression promise to provide important tools for interpreting the physiologic significance of the seemingly overwhelming amount of genomic information now being generated. We have used a convenient transfection protocol to test the efficacy of PNAs as antisense agents in cultured cells and have obtained results that clearly define their strengths and weaknesses. Activity is effectively blocked by PNAs directed to the terminal region of the 5′-UTR, and occurs with high mismatch discrimination. Activity is not blocked by PNAs targeted to 17 other sites within the 5′-UTR, around the start codon, or the throughout the coding region. To be active inside cells, the complex between PNA and DNA transporter must have a T_m value of between 52 and 71 °C. We conclude PNAs are a useful addition to the chemical diversity available for antisense research and that exciting options exist for improvement of their properties through chemical modification and technical innovation.

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