Efficient and Isoform-Selective Inhibition of Cellular Gene Expression by Peptide Nucleic Acids†

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ABSTRACT: Peptide nucleic acids (PNAs) are a potentially powerful approach for the recognition of cellular mRNA and the inhibition of gene expression. Despite their promise, the rules for using antisense PNAs have remained obscure, and antisense PNAs have been used sparingly in research. Here we investigate the ability of PNAs to be effective antisense agents inside mammalian cells, to inhibit expression of human caveolin-1 (hCav-1), and to discriminate between its α and β isoforms. Many human genes are expressed as isoforms. Isoforms may play different roles within a cell or within different tissues, and defining these roles is a challenge for functional genomics and drug discovery. PNAs targeted to the translation start codons for the α and β isoforms inhibit expression of hCav-1. Inhibition is dependent on PNA length. The potency and duration of inhibition by PNAs are similar to inhibition of gene expression by short interferring RNA (siRNA). Expression of the α isoform can be blocked selectively by a PNA. Cell proliferation is halted by inhibition of expression of both hCav-1 isoforms, but not by inhibition of the α hCav-1 isoform alone. Efficient antisense inhibition and selective modulation of isoform expression suggest that PNAs are versatile tools for controlling gene expression and dissecting the roles of closely related protein variants. Potent inhibition by PNAs may supply a "knock down" technology that can complement and "cross-check" siRNA and other approaches to antisense gene inhibition that rely on oligomers with phosphate or phosphorothioate backbone linkages.

Using oligonucleotides to inhibit gene expression should be straightforward. Once a target sequence is chosen, a complementary oligomer can be designed, introduced into cells, bind the mRNA, and reduce translation. Over the past two decades, however, antisense oligonucleotides have not proven to be a robust technology (*1*). This situation has begun to change dramatically because of the discovery that RNA interference (RNAi) by duplex RNA (siRNA)[1] can silence gene expression in mammalian cells (*2*) and because of continuing progress of traditional single-stranded antisense oligonucleotides in clinical trials (*3*). These advances are impressive, but there continues to be a need to improve antisense reagents for cell culture and in vivo applications.

Peptide nucleic acid (PNA) is a DNA/RNA mimic that presents a dramatically different chemical option for developing antisense gene inhibition (*4*). PNA oligomers (PNAs) bind by Watson−Crick pairing, and hybridization is characterized by elevated melting temperature (T_m) values (*5*). PNAs have a neutral amide backbone that resists hydrolysis by nucleases or proteases (*6*) and leads to less nonspecific association with proteins (*7*).

The dramatic differences in the chemical and biological properties of PNA relative to siRNA or classical antisense oligonucleotides lead to the hypothesis that PNAs may have advantages for the development of improved experimental tools or therapeutics. One advantage is that PNAs do not recruit RNase H or promote the degradation of target mRNAs (*8*), suggesting that PNAs can direct isoform expression by shifting usage of splice junctions (*9*−*11*) or alternate start codons. Another advantage is that PNAs are synthesized by protocols adapted from peptide synthesis, making it straightforward to modify PNAs with peptides to improve cellular uptake (*11*, *12*) or tissue targeting (*13*).

Previous studies have reported that PNAs can inhibit gene expression inside mammalian cells (*12*, *14*) but have not elucidated whether the inhibition of gene expression by PNAs confers advantages relative to classical antisense approaches or RNAi. More importantly, these studies have not presented sufficient data to develop rules for predicting the efficacy of antisense PNAs. As a result, over 10 years after the discovery of PNA the potential value of antisense PNAs for blocking gene expression within mammalian cells has remained obscure, and antisense PNAs have been largely ignored as tools for inhibiting mammalian gene expression.

Here we investigate whether PNAs can inhibit a chromosomally expressed gene and whether inhibition is sufficiently selective to be specific to just one isoform. Many genes involved in normal physiology, lineage choice, and disease are expressed as multiple isoforms that arise from initiation of translation at alternate start codons (*15*). The total number of genes affected by alternate initiation is not known, but it is estimated that approximately 35% of human genes have multiple AUG codons prior to identified translation initiation

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[1] Abbreviations: peptide nucleic acid, PNA; short interfering RNA, siRNA; hCav-1, human caveolin 1.

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sites (*16*). Alternate initiation may arise from a second internal start codon within a single mRNA, from internal ribosomal entry sites, or from splicing to produce a second mRNA that does not contain the original upstream AUG codon (*17*). The functions of many translational isoforms are unknown but are likely to be essential for understanding normal biology and disease progression.

Investigating the roles of protein isoforms is complicated by their similarity. One strategy for understanding the cellular roles of proteins is to block their expression and study the resultant phenotype. Human caveolin 1 (hCav-1) is a 23-kDa protein that is critical for the formation of caveolae, flask-shaped invaginations of the plasma membrane (*18*, *19*). hCav-1 is expressed as two isoforms, α and *β* (*20*), that are differentially expressed in tissues (*21*). The function of hCav-1 in normal and diseased tissue and the significance of differential expression of the α and *β* isoforms have not been fully elucidated. It is known, however, that hCav-1 binds cholesterol (*22*) and that hCav-1-deficient mice are resistant to diet-induced obesity (*23*). We demonstrate that hCav-1 expression can be efficiently inhibited by PNAs with potencies comparable to siRNA and that inhibition can be isoform specific.

MATERIALS AND METHODS

Materials. PNAs were synthesized using an Applied Biosystems (Foster City, CA) Expedite 8909 synthesizer (*24*). DNA and RNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and The Center for Biomedical Inventions at The University of Texas Southwestern Medical Center at Dallas, respectively. Rabbit polyclonal antibody raised against hCav-1 was purchased from BD Transduction Laboratories (Lexington, KY) and goat polyclonal antibody against actin was from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated anti-rabbit and anti-goat IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Lipid-Mediated Transfection of PNA/DNA and siRNA Duplexes. HeLa cells (American Type Culture Collection, Manassas, VA) were plated at around 20 000 cells/well in a 24-well plate the day before transfection so that they would be 30−50% confluent at the time of transfection. The cells were incubated in Dulbecco's minimal essential medium (DMEM) supplemented with glutamine, 10% fetal bovine serum (FBS, Atlanta Biologicals Norcross, GA), 500 U/mL penicillin, and 0.1 mg/mL streptomycin and were incubated at 37 °C at 5% CO_2 overnight before transfection. Cells were transfected with PNA/DNA complexes or siRNA duplexes using OligofectAMINE (Invitrogen, Carlsbad CA) as described (*14*, *25*−*27*) in OptimMEM media (Invitrogen) without antibiotics. The identities of the DNA oligonucleotides used to assist transport of PNAs were 5′-GCAT-GTCTGGGGGCAGCCA-3′ (**PNA 1**, **PNA 7**, and **PNA 8**), 5′-CAAGGCCATGGCAGACGAGCAAGG-3′ (**PNA 2** and **PNA 6**), 5′-GCGCACACCAGTGT-3′ (**PNA 3**), 5′-CTCA-GACGAGCTGAGCGAG-3′ (**PNA 4**), and 5′-GTCTGGG-GGCACAT-3′ (**PNA 5**).

Western Analysis. Cells were collected 3 days post-transfection, and cell pellets were washed with cold PBS and solubilized with lysis buffer (50 mM Tris-Cl, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA-Na, 1 mM dithiothreitol, 10 mM *β*-glycerophosphate, 0.1 mM NaF, 0.1 mM sodium orthovanadate, pH 7.4) containing Complete Protease Inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Cell suspension was incubated on ice for 10 min in the lysis buffer and then centrifuged at 8000 rpm for 5 m at 4 °C. Cell suspension containing whole cell lysate was analyzed for protein concentration using BCA Protein assay (Pierce, Rockford, IL). Ten micrograms of total protein was prepared in 5× SDS sample buffer (60 mM 1 M Tris-HCl, pH 6.8, 25% v/v glycerol, 2% w/v SDS, 0.1% w/v bromophenol blue, and 14.4 mM *β*-mercaptoethanol) and was separated using a 12% SDS−PAGE gel at constant current of 25 mA/gel. Close adherence to this protocol was necessary to resolve the α and *β* isoforms. Proteins were transferred to immobilon-P polyvinylidene fluoride (PVDF) microporous transfer membranes (Millipore Corporation, Billerica, MA) in buffer containing 25 mM Tris, pH 8.3, 150 mM glycine, and 20% methanol. Membranes were blocked with 1× TBS buffer (Amresco Corp., Solon, Ohio) containing 0.05% Tween-20 and 5% nonfat dried milk (blocking buffer) for 1 h at room temperature or overnight at 4 °C.

The membrane was washed in TBS-Tween buffer and was cut horizontally at the level of 30-kDa protein according to a prestained molecular weight standards (BioRad Laboratories, Hercules, CA) on the membrane. The upper part was incubated with anti-actin antibody (1:200 diluted in TBS-Tween), and the lower part was incubated with anti-hCav-1 antibody (1:5000) for 1 h at room temperature. After extensive washing with TBS-Tween buffer, the membranes were then incubated with peroxidase-conjugated secondary IgG antibodies at 1:5000 in blocking buffer for 1 h at room temperature and immunodetection was performed using the Supersignal Western Chemiluminescent Substrates (Pierce). Chemiluminescent signals were visualized by exposure to a Hyperfilm ECL film (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England).

Cell Proliferation Assay. HeLa cells were seeded in 12-well dishes with 40 000 cells per well. Cells were transfected with PNAs or siRNAs (200 nM) the next day as described above. Three days after transfection, cells were trypsinized and cell numbers of each well were determined using a Coulter Z Series Cell counter (Beckman Coulter, Fullerton, CA). Cells from each well were counted three times, and the average numbers were used for the comparison among different treatment groups and cell numbers were expressed as percentages of lipid controls. Cells were transferred to and reseeded at 35 000 per well in a new dish and were retransfected with the corresponding oligonucleotides the following day. Cell numbers were determined and transfected repeatedly at indicated time points after the initial seeding until the cells in any group could not be recovered for further transfection. All assays were performed in triplicate, and the reported values for inhibition are averages of these triplicate determinations.

RESULTS AND DISCUSSION

Experimental Design. The identities of the PNAs used in these studies were confirmed by mass spectrometry and are listed in Table 1. To enable efficient uptake of PNAs by cells, PNAs were annealed to DNA oligonucleotides and then complexed with cationic lipid (*14*, *25*−*27*). The lipid binds

Table 1: Sequence of PNAs Used in These Studies, the Rationale for Their Use, Calculated Molecular Weights and Observed Molecular Weights[a]

PNA	PNA sequence	comment/mRNA target site	mass	
			calc	obs
1	TGCCCCCAGACATGCTGGC	α hCav-1 start codon	5293.8	5294.6
2	CTCGTCTGCCATGGCCTTG	*β* hCav start codon	5290.7	5296.5
3	CTTCTCGCTCAGCTCGTCT	downstream of **PNA 2**	5225.6	5237.8
4	TGGTGTGCGCGTCGTACAC	downstream of **PNA 3**	5379.8	5395.5
5	TGCCCCCAGACGTGGTGGC	mismatch analogue of **PNA 1**	5349.8	5367.4
6	CTCGTCTGCCTTGACCTTG	mismatch analogue of **PNA 2**	5265.7	5266.6
7	TGCCCCCAGACATGC	15-base analogue of **PNA 1**	4193.6	4194.3
8	TGCCCCCAGACATGCTG	17-base analogue of **PNA 1**	4751.2	4758.2

[a] PNAs are listed N- to C-termini. Underlined letters indicate mismatched bases.

to the DNA, allowing it to pass through the cell membrane, while the hybridized PNA is carried along as cargo.

DNA oligonucleotides are chosen to form PNA−DNA complexes with measured T_m values of 65−75 °C. T_m values below 65 °C risk having the complexes be too unstable to successfully transport the PNA across the cell membrane, while T_m values higher than 75 °C risk having the PNA be inefficiently released from the DNA once inside the cells. Because it is difficult to accurately estimate DNA−PNA T_m values with the required precision, we synthesize two or three carrier DNAs for each PNA and empirically determine the best combination.

Microscopy and fluorescent assisted cell sorting (FACS) analysis using a fluorophore-labeled PNA analogous in sequence to **PNA 1** confirmed uptake by over 95% of cultured HeLa cells (results not shown). We have previously used this approach to deliver PNAs to inhibit human telomerase (*25*, *26*), cause telomeres to shorten (*26*), and block expression of luciferase encoded by a plasmid that had been transfected into cells (*14*). Western analysis was used to detect expression of the α and *β* isoforms of hCav-1.

Inhibition of hCav-1 Expression by PNAs. The α and *β* isoforms of hCav-1 were originally thought to arise from the same transcript (*20*). More recent studies have reported that the *β* isoforms in cells from mice (*28*) and humans (*29*) are encoded by separate mRNA transcripts, a finding that we have confirmed (results not shown). We synthesized antisense PNAs complementary to the translation start sites for both the α and *β* isoforms (**PNA 1** and **PNA 2**), and to two sequences downstream of the *β* translation start site (**PNA 3** and **PNA 4**) (Figure 1A). The target site for **PNA 1** is within the mRNA encoding the α isoform. The exact sequence of the 5′-untranslated region of the *β* mRNA is unknown and the presence of the target site for **PNA 1** is uncertain.

Upon transfection into cultured HeLa cells, **PNAs 1**−**3** efficiently inhibited expression of hCav-1 (Figure 1B, lanes 1−3). **PNA 1** directed to the upstream translation start site specifically inhibited expression of the α isoform of hCav-1 but not the *β* isoform (Figure 1B, lane 1), consistent with the prediction that the target site for **PNA 1** is not present within the *β* mRNA. **PNA 2** and **PNA 3** inhibited both isoforms, consistent with presence of their target sequences within both the α and the *β* mRNAs. **PNA 4** did not significantly inhibit expression of either isoform. Failure of **PNA 4** may be due to inadequate delivery into the cell, an inability to efficiently bind to its target mRNA sequence, or an inability to stop the ribosome from continuing translation.

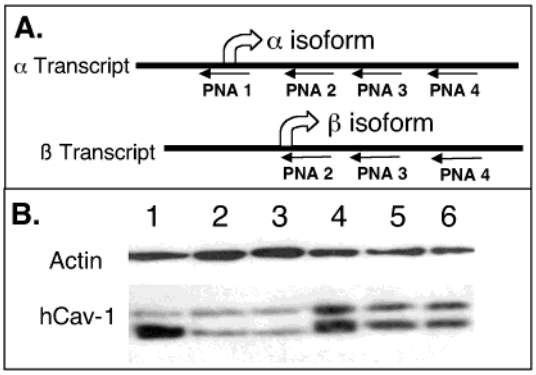

FIGURE 1: Target sites and inhibition of hCav-1 expression by PNAs. (A) Relative locations of the target sites for **PNAs 1**−**4** within the mRNAs encoding the α and *β* isoforms of hCav-1. The two different start sites for the α and *β* isoforms are indicated. The schematic is not drawn to scale. (B) Western analysis of inhibition of hCav-1 gene expression by **PNAs 1**−**4**. Lane 1, **PNA 1**; lane 2, **PNA 2**; lane 3, **PNA 3**; lane 4; **PNA 4**; lane 5, mismatch **PNA 5**; lane 6, mismatch **PNA 6**.

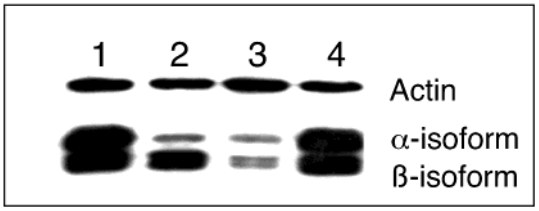

FIGURE 2: Comparison of inhibition of hCav expression by isoform-specific **PNA 1** and analogous **siRNA 1**. Lane 1, no PNA; lane 2, **PNA 1**; lane 3, **siRNA 1**; lane 4, mismatch-containing **PNA 5**.

Mismatch-containing **PNA 5** and **PNA 6** that were analogous in sequence to **PNA 1** and **PNA 2**, respectively, did not inhibit hCav-1 expression (Figure 1B).

We also tested inhibition of hCav expression by **siRNA 1**, which was directed to the same target sequence as **PNA 1**. Surprisingly, while **PNA 1** was able to inhibit hCav expression in an isoform-specific fashion, **siRNA 1** inhibited expression of both isoforms (Figure 2). One explanation for this result is that, because it exploits the protein machinery of the RISC complex, **siRNA 1** can bind to the *β* isoform mRNA whereas **PNA 1** cannot. This possibility raises the interesting speculation that siRNA might sometimes be too powerful for some subtle applications of inhibition of gene expression.

Inhibition of Gene Expression as a Function of PNA Length. In a previous study, we had targeted PNAs to 18 different sites within mRNA coding for luciferase and had observed inhibition of expression by PNAs directed to the

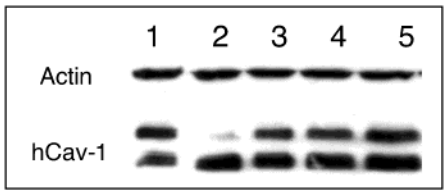

FIGURE 3: Inhibition by **PNA 1** and related PNAs as a function of length. Lane 1, no PNA; lane 2, 19 base **PNA 1**; lane 3, 17 base **PNA 8**; lane 4, 15 base **PNA 7**; lane 5, mismatch **PNA 5**. Cells were transfected by 200 nM PNA.

5′ terminus of the 5′-UTR but not by the 17 other PNAs targeted to downstream sites (*15*). This has led us to hypothesize that PNAs could block binding of the ribosome to the 5′-UTR but could not act as roadblocks to translation. We were surprised, therefore, by the efficiency of inhibition of hCav-1 expression by **PNAs 1**−**3**.

The previous work had employed PNAs that were 15 bases, while the PNAs targeting hCav-1 used in this study were 19 bases. To determine if PNA length contributed to the discrepancy in our observations, we synthesized 15-base **PNA 7** and 17-base **PNA 8** derived from isoform-specific **PNA 1**. **PNA 7** and **PNA 8** failed to efficiently inhibit expression of the α isoform of hCav-1 (Figure 3), suggesting that inhibition of gene expression is highly sensitive to PNA length. Successful inhibition using longer PNAs is consistent with findings that 21−25 base morpholino oligonucleotides that are directed to start codons are useful agents for controlling gene expression in Zebrafish (*30*).

The melting temperatures of **PNA 1**, **PNA 7**, and **PNA 8** were 85, 83, and 76.5 °C for hybridization to complementary sequences, respectively. These differences in melting temperature values are relatively small, but might be responsible for the enhanced potency of **PNA 1**. Alternatively, the additional length of **PNA 1** might be responsible for its improved ability to inhibit gene expression by increasing the size of the roadblock.

Potency and Time Dependence of Inhibition of hCav-1 Expression. siRNA provides a useful benchmark for evaluating the potential value of other strategies for inhibiting gene expression. To evaluate the efficiency of antisense PNAs, we compared the potencies of **PNA 1** and **PNA 2** with **siRNA 2**, an siRNA targeted to the same sequence as **PNA 2** (Figure 4). Dose response profiles revealed IC_{50} values of approximately 25 nM for inhibition of the α isoform by **PNA 1** and 25−30 nM for inhibition by **PNA 2** or **siRNA 2** (Figure 4). These IC_{50} values are similar to those we had previously determined for an siRNA and an siRNA-locked nucleic acid (LNA) chimera targeted against a nearby site in hCav-1 (*31*). Transfection of **siRNA 2** reduced mRNA levels, while transfection with **PNA 1** or **PNA 2** did not, consistent with the inability of PNA to recruit enzymatic cleavage of RNA (*8*−*10*).

We hypothesized that addition of **PNA 1** might facilitate inhibition of the β isoform by **PNA 2**. To test this hypothesis, we added **PNAs 1** and **2** together but observed the same ∼12−25 nM IC_{50} value observed upon individual addition of the PNAs (Figure 5), suggesting that their inhibitory effects were not additive.

We examined the duration of action of **siRNA 2** and **PNAs 1** and **2** (Figure 6). For both the siRNA and the PNAs, we observed efficient inhibition up to 6 days after transfection

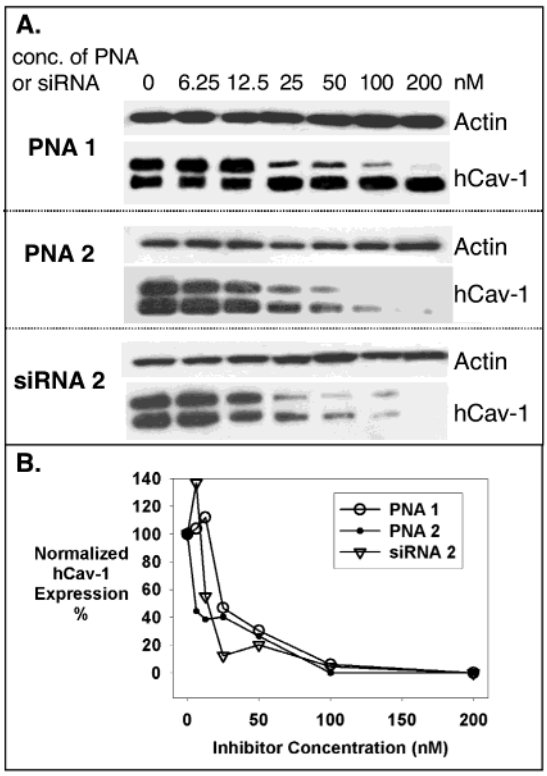

FIGURE 4: Inhibition of hCav-1 expression by varying concentrations of **PNA 1**, **PNA 2**, and **siRNA 2**. (A) Western analysis. (B) Graphical analysis of data shown in part A. Values were normalized to the actin internal control for each point and then normalized to the 0 nM inhibitor (lipid control) for each series. For **PNA 1**, inhibition of the α isoform is quantified.

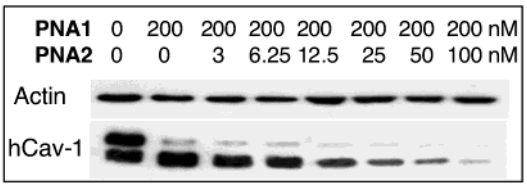

FIGURE 5: Inhibition by adding varying concentrations of **PNA 1** and **PNA 2** in combination.

with approximately 90% of expression blocked. During these 6 days, HeLa cells double 3−4 times, suggesting that inhibition persists despite substantial dilution of PNA. Much less inhibition was observed after 9 days, and no inhibition was observed at 12 days. Taken together, these results indicate that inhibition of gene expression by PNAs approximate the potency and longevity of inhibition by siRNA.

Isoform-Specific Reduction in Cell Proliferation. To begin to dissect the relative functions of the α and β isoforms of hCav-1, we used PNAs and siRNAs to inhibit expression of one or both isoforms. We observed that inhibition of both isoforms by **siRNA 2** or **PNA 2** caused a dramatic reduction in cell proliferation (Figure 7). Addition of **PNA 1**, however, caused only a small decrease in proliferation, indicating that expression of the β isoform alone is sufficient to maintain cell proliferation. Addition of mismatch-containing PNAs or lipid alone did not cause proliferation to decrease significantly. These results suggest that the α isoform is at least partially redundant for growth in culture and demonstrate the usefulness of isoform-specific inhibition of gene expression for investigating the cellular function of proteins.

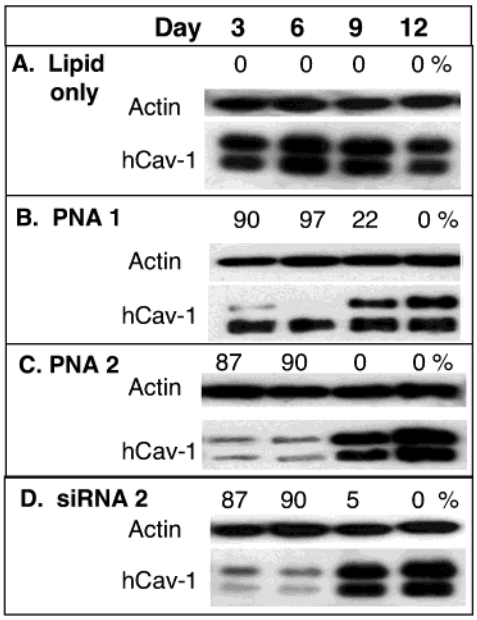

FIGURE 6: Timecourse of inhibition of hCav-1 expression by PNAs and siRNAs. Percent inhibition is noted above each lane. For cells treated with **PNA 1**, the percent inhibition refers to inhibition of the α isoform. Percentages are calculated by comparison with cells treated with lipid alone. (A) Lipid only; (B) **PNA 1** added; (C) **PNA 2** added; and (D) **siRNA 2** added.

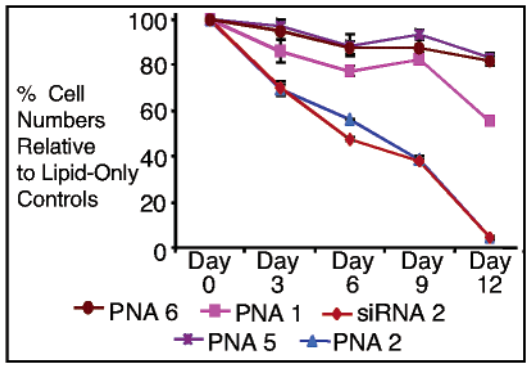

FIGURE 7: Effect on proliferation of cultured HeLa cells of inhibition of expression of hCav-1 α alone by **PNA 1** or hCav-1 α in combination with hCav-1 *β* by **PNA 2** or **siRNA 2**. **PNA 5** and **PNA 6** are mismatch-containing controls. Values are relative to the cell numbers of cultures that had been treated with cationic lipid only. Cells were transfected with **siRNA 2** (200 nM) or **PNAs 1**, **2**, **5**, or **6** (200 nM) at three-day intervals.

Mechanisms for Inhibition of Translation and Selective Targeting of Isoform Expression by Antisense PNAs. PNAs can inhibit gene expression by at least three different mechanisms. The first mechanism was revealed by our previous work with 15-base PNAs targeting plasmid-encoded luciferase mRNA (*14*). This study had demonstrated that PNAs directed to the terminus of the 5′-UTR could block gene expression, presumably by interfering with initial association between the mRNA and the ribosome.

A second mechanism is revealed by the observation in our current study of inhibition by **PNAs 1**−**3** that are directed at or near the start codons for the α and *β* hCav. These data suggest that PNAs can prevent the ribosome from initiating protein translation by obstructing recognition of the start codon. A third mechanism is revealed by inhibition of expression of the α mRNA by **PNA 2** and **PNA 3** that are targeted approximately 100 bases downstream from the α start site. Their ability to inhibit expression suggests that PNAs can also act as downstream roadblocks to obstruct the ribosome after recognition of the start codon. Alternatively, it is also possible that binding of **PNA 2** and **PNA 3** may affect RNA structure at a distance and alter recognition of the α mRNA start codon.

Since concluding these studies, we have observed that antisense PNAs complementary to the either the A or B isoforms of the human progesterone receptor are also effective antisense agents (Janowski, unpublished results). These recent data reinforce the suggestion that antisense PNAs will prove to be general tools for controlling gene expression.

Antisense PNAs and siRNA. siRNA has been widely recognized as an effective tool for inhibition of cellular gene expression. Given this success, it is important to carefully justify the goals for continued development of other antisense strategies.

It is now apparent that siRNA can yield nonselective phenotypes inside cells. Expression array analysis has revealed that the introduction of siRNAs into cells can have phenotypic consequences unrelated to recognition of their intended targets (*32*, *33*). Two other reports have demonstrated that siRNAs can activate the interferon response (*34*, *35*), suggesting a further complication for some experiments and a potential obstacle to development of siRNA therapeutics. As applications become more demanding it is likely that siRNA will encounter problems that are similar to those encountered by other antisense strategies and that the search for ideal antisense strategies will continue.

We have shown here that PNAs inhibit hCav expression with potencies similar to an analogous siRNA. More work will be needed to fully characterize the genome-wide specificity of PNA-mediated inhibition and to develop rules to guide optimal selection of target sites for PNAs and to establish whether PNA can offer a challenge to siRNA. However, the favorable properties of PNAs, such as their inability to act as substrates for RNAse H (*8*), their tight and selective binding to complementary sequences (*4*, *5*), and their low binding to proteins (*7*) suggest that there will be some circumstances in which the use of PNAs will be preferred.

It is also possible that antisense PNAs will be valuable as a complementary approach that can be used in tandem with siRNA. Confirming that an observed phenotype is due to inhibition of target mRNA has been an obstacle for antisense research (*1*). PNAs and siRNAs have different chemical properties and different mechanisms of inhibition of gene expression and are unlikely to cause the same nonselective phenotypes. Therefore, if treatment with an antisense PNA yields the same phenotype as treatment with an analogous siRNA, it is likely that the phenotype is valid. This PNA/siRNA complementation would provide a stringent test for the phenotypic consequences of gene silencing.

One focus of research on the inhibition of gene expression is the development of therapeutic agents. siRNAs have been shown to be active in vivo (*36*−*38*), but we have observed that their biodistribution is similar to traditional phosphorothioate DNA oligonucleotides (*39*). It is likely, therefore, that restricted tissue distribution will complicate the develop-

ment of therapeutic siRNAs, just as it has slowed development of traditional antisense oligomers. PNAs possess physical properties different from those possessed by siRNA or traditional antisense oligonucleotides, and PNAs can easily be derivatized with peptides or other molecules designed to modify pharmacokinetics and biodistribution. The full potential of PNA for in vivo applications is unknown, but it is clear that (i) PNAs can be effective antisense sense agents inside cells, and (ii) PNAs offer an important chemical and pharmacological alternative for therapeutic development.

Conclusion. Antisense PNAs can inhibit gene expression inside cells with potencies that are comparable to siRNA. Inhibition can be isoform specific and is a useful tool for exploring isoform-specific knock-down phenotypes. When used in combination with siRNA, PNAs can be used to "cross-check" phenotypes to help build a case that observed physiologic effects are due to inhibition of the intended target gene. The potency, isoform specificity, and advantageous chemical properties of antisense PNAs suggest the potential for wide application in basic research, target validation, and medicine.

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