Chemistry & Biology, Vol. 11, 749–758, June, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.chembiol.2003.09.014

Recognition of Chromosomal DNA by PNAs

Review

Kunihiro Kaihatsu, Bethany A. Janowski, and David R. Corey*
Departments of Pharmacology and Biochemistry
University of Texas Southwestern Medical Center at Dallas
5323 Harry Hines Boulevard
Dallas, Texas 75390

The recognition of cellular nucleic acids by synthetic oligonucleotides is a versatile strategy for regulating biological processes. The vast majority of published studies have focused on antisense oligonucleotides that target mRNA, but it is also possible to design antigene oligonucleotides that are complementary to chromosomal DNA. Antigene oligomers could be used to inhibit the expression of any gene or analyze promoter structure and the mechanisms governing gene regulation. Other potential applications of antigene oligomers include activation of expression of chosen genes or the introduction of mutations to correct genetic disease. Peptide nucleic acid (PNA) is a nonionic DNA/RNA mimic that possesses outstanding potential for recognition of duplex DNA. Here we describe properties of PNAs and the challenges for their development as robust antigene agents.

Introduction

DNA sequences can be viewed as an immense family of receptors and, like any receptors, are targets for the design of synthetic ligands for use as experimental tools and therapeutic leads. Potential applications that might benefit from recognition of duplex DNA include the inhibition or activation of gene expression, the investigation of DNA structure and promoter function, and the introduction or correction of mutations at specific sites. However, before these applications can become routine, it will be necessary to synthesize agents that efficiently and specifically recognize chromosomal DNA.

In this review we describe one synthetic approach toward meeting this challenge, the DNA/RNA mimic peptide nucleic acid (PNA) [1] (Figure 1). We focus on PNA because it has an unparalleled ability to recognize complementary sequences by strand invasion and may provide a general solution to the problem of gaining access to double-stranded DNA (dsDNA). We will briefly review relevant lessons from antisense oligonucleotides that target mRNA and then describe the development of PNAs as antigene agents.

Advantages of Oligonucleotides for Chemical Genetics

Nucleic acids possess significant advantages as chemical genetic tools for manipulating cellular processes [2, 3]. The most obvious advantage is that examination of a target sequence immediately suggests the design of a complementary oligonucleotide that is likely to be a high-affinity ligand. Once a sequence is chosen, efficient protocols allow rapid synthesis and testing of compounds for activity and make it possible to obtain hundreds or thousands of oligonucleotides for large-scale investigations of genome function. As potent inhibitory oligonucleotides are identified and tested, oligonucleotides that contain mismatched bases can serve as control molecules to establish the mechanism and specificity of the observed phenotype.

A practical advantage for therapeutic development is that one oligonucleotide, Fomivirsen, is an approved drug demonstrating that a member of this class of molecules can advance through the regulatory process, while several others are in clinical trials [2, 3]. Clinical experience demonstrates that oligonucleotides can be efficacious, exhibit low toxicity, and can be synthesized on a large scale for systemic administration. Oligonucleotides are, therefore, a realistic option for therapy, and their favorable properties will reduce the time needed to translate a lead compound into a drug that can be tested in the clinic.

What Have We Learned from Targeting mRNA?

The use of oligonucleotides to inhibit protein expression is not a new concept. Sequences within mRNA have been the primary intracellular targets for oligonucleotides, and the experience gained during 25 years of targeting mRNA is useful for planning experiments to target duplex DNA.

Many studies have demonstrated that oligonucleotides can act as potent and selective agents for inhibiting gene expression [2, 3]. Antisense oligomers, however, are notorious for forming unexpected interactions and producing phenotypes that are not related to binding to intended target sequences [4] (Figures 2A and 2B), and achieving useful results demands that experiments be well planned and well controlled. One cause for artifactual phenotypes is binding of oligonucleotides to RNA or DNA sequences that are similar to the intended target. Artifactual phenotypes may result from oligomers binding to proteins, and oligomers that form G quartet and other secondary structures are especially susceptible to such interactions [5]. Indeed, it is not clear that the molecules currently being tested in the clinic function through an antisense mechanism. There is every reason to believe that agents targeting duplex DNA will also produce misleading results if experiments are not well controlled. It is essential, therefore, that oligomers containing mismatched bases be used and that results be examined critically [6].

Another lesson from the study of antisense oligonucleotides is that successful use of oligonucleotides in cultured cells is governed by a window of efficacy bounded by a minimum dose needed to produce a phenotype and a maximum dose that marks the beginning of unacceptable toxicity or confounding nonspecific effects (Figure 2B). The size of the window will vary depending

*Correspondence: david.corey@utsouthwestern.edu

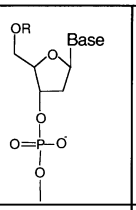
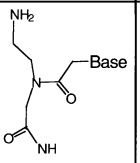
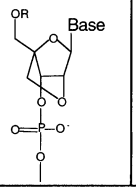
	**DNA**: Can recognize duplex DNA by triplex formation. Recognition by strand invasion is poor unless mediated by enzymes.
	**PNA**: Efficient synthesis methods. Spontaneous strand invasion of supercoiled and linear DNA. bisPNAs form a four stranded complex by strand invasion and triplex formation.
	**LNA**: Each LNA substitution confers a 5-10°C increase in T_m. Strand invasion properties not yet defined.

Figure 1. Structures of DNA, PNA, and LNA

The structures of DNA, PNA, and LNA are listed on the left. Characteristics relevant to strand invasion are noted on the right.

on cellular target, chemical properties of the oligomer, purity, method of delivery, and cell type. The challenge in all experiments is to work within this window and to increase its size through chemical innovation and wise choice of target. For example, the inhibition of gene expression by duplex RNA (siRNA) [7] has become a popular technique because the window for achieving useful results is large. We expect that the window for agents that target duplex DNA will need to be equally permissive before applications spread beyond a handful of specialist laboratories.

Chromosomal DNA as a Target for Oligonucleotides

Differences between Chromosomal DNA and mRNA

While it is important to appreciate lessons learned from work with antisense oligomers, there are fundamental differences between mRNA and duplex DNA that will influence plans for targeting chromosomes. One difference is that mRNA is partially single-stranded, while chromosomal DNA is almost entirely double-stranded and is packaged with protein into chromatin. Therefore, accessibility to target sequences within DNA will be limited, and initiating hybridization will be more challenging. Another difference is that most effective antisense agents induce the cleavage of mRNA (either by RNase H in the case of RNA-DNA hybrids [8] or by the RISC complex for duplex RNA [7]), while agents that target duplex DNA will usually be required to leave the DNA intact. Finally, the target sequence of an antisense oligonucleotide is carried on hundreds or thousands of mRNAs, while the target sequence for an antigene oligonucleotide is usually present only once or twice in the genome.

Recognition of dsDNA: Triplex Forming Oligonucleotides (TFOs)

Oligonucleotides can recognize duplex DNA by binding in the major groove through Hoogsteen base pairing and triple helix formation (Figure 3A) [9, 10]. Hoogsteen pairing involves bonding of protonated cytosine with AT Watson-Crick base pairs and thymine with GC pairs, and efficient recognition of duplex targets can be achieved by forming a parallel complex between polypyrimidine oligonucleotides and a polypyrimidine-polypurine sequence within dsDNA. Some progress has been made toward increasing the number of target sequences by developing strategies that allow recognition by oligonucleotides containing a few purine bases. The specificity of triplex recognition is stringent, with a single mismatched base reducing melting temperature (T_m) values by as much as 15°C [11, 12].

The great advantage of triple helix formation is that the polypyrimidine strand binds within the major groove of the target duplex. Therefore the target sequence does not need to become single stranded, removing an energetic barrier to hybridization. However, in spite of this strength, triplex formation has not yet achieved its full potential as a tool for recognition of chromosomal DNA inside cells. One reason is that target sequences are still largely restricted to polypurine-polypyrimidine duplexes, and these sequences may not always be near sites that are critical to expression of a target gene. Another deficiency is that binding by TFOs is relatively weak, especially at neutral or basic pH, and may not be adequate for routine recognition of cellular targets.

Recognition of dsDNA: Strand Invasion

In classic early studies, Radding and coworkers showed that single-stranded DNA can bind to supercoiled DNA by hybridizing to one strand of the duplex through Watson-Crick base pairing while displacing the other (Figure 3B) [13], a phenomenon known as strand invasion. This exciting result suggested that duplex DNA could be targeted regardless of sequence, but progress toward developing strand invasion as a strategy for the recognition of dsDNA by synthetic oligomers has been slow. The primary obstacle is that strand invasion must overcome preexisting base pairing of the duplex to initiate and maintain recognition. Compounding this difficulty, relatively low concentrations of cations stabilize duplex DNA and hinder strand invasion.

DNA oligonucleotides can invade supercoiled DNA at 37°C [14, 15]. However, this invasion is slow, inefficient, and largely restricted to sequences that are partially single stranded because they are AT-rich or form cruciforms. Rate constants for strand invasion of supercoiled plasmid DNA by DNA oligonucleotides can be enhanced up to 48,000-fold by conjugation to a cationic peptide or protein that acts to increase favorable electrostatic interactions with the duplex [14]. However, only a few sequences within supercoiled DNA could be recognized efficiently by the modified oligonucleotide, and relaxed DNA remained inaccessible [15].

PNAs: Powerful Tools for Recognition of dsDNA

The Need for a New Approach

Limitations on triplex formation or strand invasion are not major impediments for most work in cell-free sys-

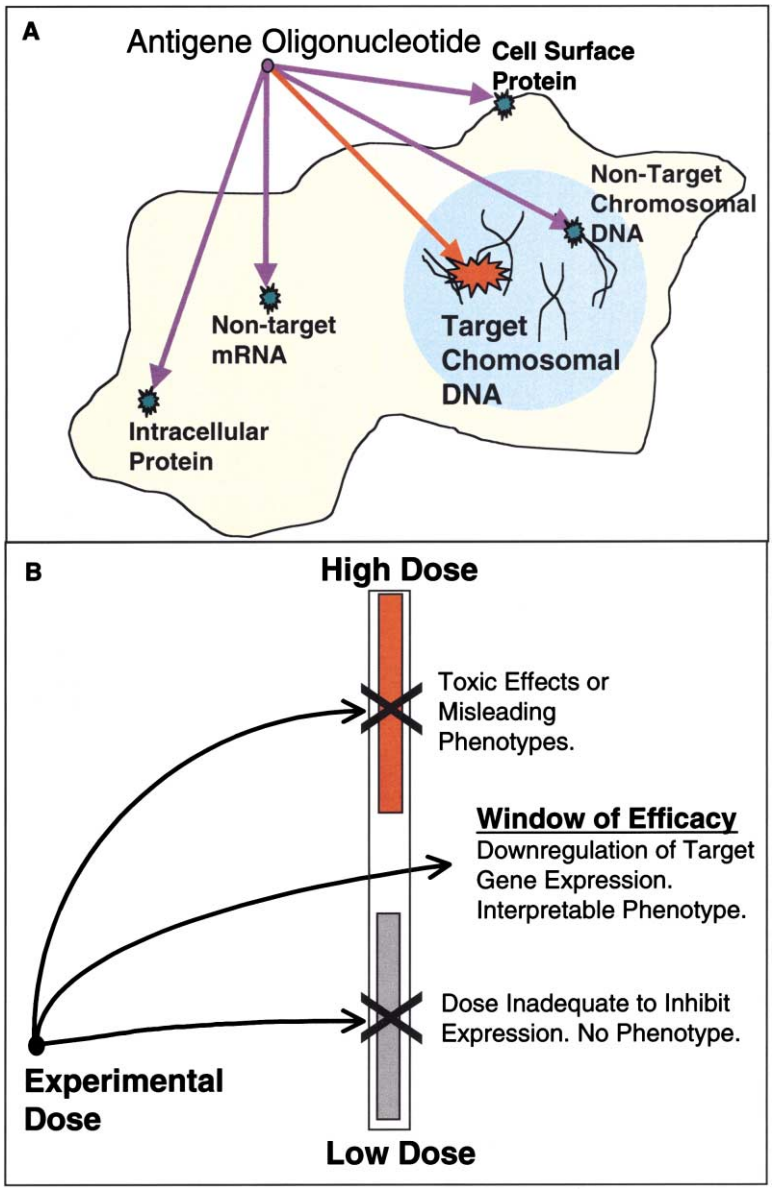


Figure 2. Challenges to the Observation of Accurate Phenotypes

(A) Observed phenotypes might result from specific or nonspecific interactions between antigene oligonucleotides and cellular components. Specific effects would result from binding to the intended target sequence on a chromosome. Misleading phenotypes might arise from binding to cellular proteins or to non-target mRNA and DNA sequences. Misleading phenotypes, especially reduced proliferation, can also result from contaminants introduced during cellular delivery of oligonucleotides.

(B) The window for successfully using oligonucleotides and PNAs to control cellular processes. Concentrations that are too high will produce toxicity and unintended phenotypes. Concentrations that are too low will not affect expression of the target gene. Chemical modifications and cellular delivery strategies that increase the range of effective doses will increase the likelihood that PNAs and other chemically modified oligomers will be widely useful for research and therapeutic development.

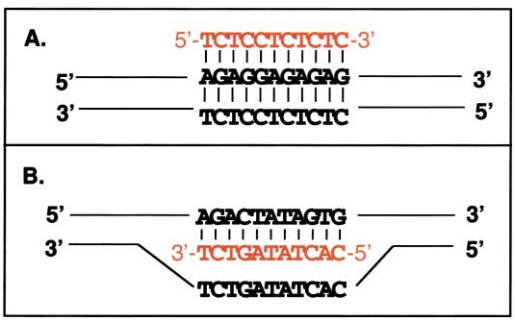


Figure 3. Recognition of Duplex DNA by Oligonucleotides

(A) Triplex helix formation at a polypurine-polypyrimidine sequence.
(B) Strand invasion at a sequence containing a mixture of A, C, G, and T.

tems, because experimental conditions can be optimized to promote recognition. For example, pH can be lowered to favor triplex formation, or DNA can be denatured by high pH or heat to facilitate strand invasion. Inside cells, however, it will be much more difficult to manipulate these variables to affect the environment of chromosomal target sequences. DNA oligonucleotides, therefore, appear to have limited potential for both triple helix formation and strand invasion inside cells. Chemical modifications to DNA or to compounds that mimic DNA, however, may provide the improvement in recognition necessary to make routine binding to cellular targets a practical goal.

Properties of PNAs

PNA is an RNA/DNA mimic in which the phosphate deoxyribose backbone is replaced by a neutral amide backbone composed of N-(2-aminoethyl) glycine linkage (Figure 1) [1]. Base pairing by PNAs is not affected

Table 1. Advice for Successful Use of PNAs

Realize that almost any PNA or PNA-peptide can be synthesized
Appreciate that the physical properties of PNA are more similar to peptides than to DNA or RNA
As with peptides, repeat the coupling of linkages with higher failure rates during synthesis
Make stock PNA solutions at low pH (~5) to increase solubility
Heat PNAs solutions prior to use to reverse aggregation that occurs during storage
Add charged residues if additional solubility is desired
Be prepared to obtain several PNAs for systematic and well-controlled experiments
Carefully desalt PNAs prior to adding them to cells
As with classical antisense oligonucleotides, demand robust and reproducible results

by intrastrand electrostatic repulsion and occurs with high affinity and enhanced rates of association [16] with strict sequence specificity [17–19]. Other properties of PNAs that encourage their use inside cells include resistance to degradation by nucleases or proteases [20] and low affinity for proteins [21]. Unlike DNA oligonucleotides that form RNA-DNA hybrids that can be cleaved by RNase H, RNA-PNA hybrids are not cleaved [22], reducing the likelihood of nonsequence-specific phenotypes.

PNAs are synthesized using methods adapted from peptide synthesis, and almost any PNA, PNA-peptide, or PNA-small molecule conjugate can be obtained, including sequences that are long (40–50 couplings), purine rich, or hairpins [23]. The ease with which PNAs can be derivatized facilitates combining the DNA binding properties of PNA with functional peptides and small molecules to enhance hybridization or uptake by cells, or to improve pharmacokinetic properties. PNAs are not as soluble as DNA or RNA, but their use is straightforward when simple precautions are taken [23, 24] (Table 1).

Strand Invasion by PNAs

PNAs have a remarkable ability to invade dsDNA that sets PNAs apart from other chemically modified nucleic acids and nucleic acid mimics. Strand invasion by PNAs is more efficient than strand invasion by modified DNA or RNA because the uncharged PNA backbone is not repelled by the negatively charged phosphodiester backbone of the duplex. This absence of repulsion increases the on rate for hybridization and decreases the off rate. Applications for strand invasion by PNAs have included purification of genomic DNA [25, 26], cleavage of DNA [27, 28], creation of artificial primosomes [16, 29], inhibition of transcription [30, 31], activation of transcription [32, 33], site-directed mutagenesis [34], noncovalent labeling of plasmids with fluorophores [35], recruitment of transcription factors to an artificial promoter [36], and recognition of duplex DNA by molecular beacons [37].

Strand invasion of both linear and supercoiled duplex DNA by PNAs is spontaneous, and there are several strategies for designing PNAs to recognize duplex DNA. PNAs containing mixtures of A, C, T, and G can hybridize to supercoiled DNA [16, 38, 39] (Figure 4A). Hybridization is promoted by the negative torsional stress of supercoiling and is most efficient at inverted repeats capable of forming cruciforms and within AT-rich regions that are relatively unstable.

A striking example of strand invasion by mixed sequence PNAs is the ability of a PNA that contains a fourteen base hairpin stem structure to bind to an inverted repeat within supercoiled DNA (Figure 4G) [16, 27]. One might have expected that this hairpin would be stable and that hybridization would be slow, but in fact the association rate constant (k_a) for hybridization by strand invasion to a cruciform site with supercoiled DNA is estimated at 500,000 M^{-1}, similar to the rate for hybridization of two unstructured single stranded DNA oligonucleotides. Strand invasion can also occur at sites lacking the potential to form non-B-type structure, albeit with lower efficiency, and can be promoted by attachment of cationic peptides [39]. Mixed sequence PNAs can also hybridize to linear duplex DNA, but nonphysiological hybridization temperatures, low pH, low ionic strength, or organic solvents will usually be necessary.

Another related approach to duplex recognition uses pseudocomplementary PNAs (pcPNAs) [40] (Figure 4B). pcPNAs contain modified bases such as diaminopurine (D) and 2-thiouracil (sU) designed to minimize the potential for base pairing between PNA strands but to permit Watson-Crick recognition of A, C, G, and T of both strands of the DNA target. These pcPNAs are able to hybridize to form a double-displacement loop and are able to invade relaxed DNA. PcPNAs have been demonstrated to block binding of proteins to DNA [41, 42], a property likely to be important for their eventual use as antigene agents.

Perhaps the simplest approach to strand invasion depends on the remarkable ability of PNAs to spontaneously invade polypyrimidine sites within relaxed DNA [43–46]. Strand invasion of relaxed DNA by PNAs can occur at polypurine-polypyrimidine sites through formation of a four-stranded complex in which one PNA strand binds by Hoogstein base pairing while the other binds by Watson-Crick base pairing (Figure 4C). Mechanistic studies suggest that association of the bisPNA with its DNA target is initiated by Hoogstein pairing between the DNA and one of the PNA strands, with the second PNA strand then invading the duplex [43]. The efficiency of strand invasion can be improved by tethering the two PNA strands to form a bisPNA, because attaching the strands reduces the entropic penalty paid upon binding [44] (Figure 4D). Stand invasion by bisPNAs can be further improved by attaching positively charged amino acids [44, 45] (Figures 4E and 4H) or DNA intercalators [46]. Polypyrimidine PNAs that contain extensions with mixtures of purines and pyrimidines (termed tail clamp PNAs) also hybridize to duplex DNA (Figure 4F) [72, 73], extending the range of accessible target sites.

Most of the reports of strand invasion noted above have used low ionic strength to promote unwinding of the target duplex and initiation of hybridization by PNAs. Since higher ionic strength conditions will be encountered inside cells, it is not clear that existing PNA designs will be able to accomplish strand invasion with the efficiencies observed in cell-free systems. One solution to this problem is to attach positively charged amino acids to the PNAs, a simple alteration that allows PNAs to invade duplex DNA at physiologic concentrations of magnesium, sodium, and potassium [38, 44, 45]

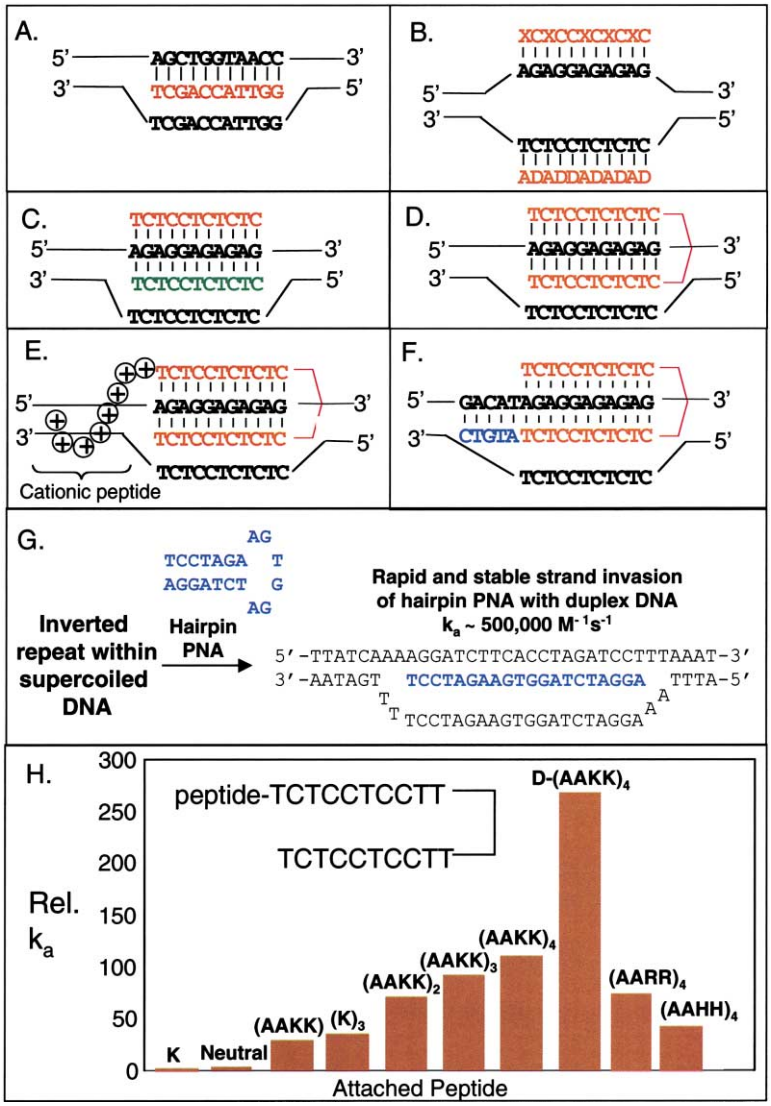


Figure 4. Strategies for Strand Invasion by PNAs and Examples of the Efficient Strand Invasion by PNAs and PNA-Peptide Conjugates

(A) Strand invasion by a mixed sequence PNA.

(B) Strand invasion by two psuedocomplementary PNAs to form a double D loop. X, 2-thiouracil; D, diaminopurine.

(C) Strand invasion of a polypyrimidine-polypurine site by two polypyrimidine PNAs to form a four-stranded complex in which one strand binds by Hoogstein base-pairing and one strand binds by Watson-Crick base pairing.

(D) Strand invasion by a bis-polypyrimidine bisPNA in which the two PNA strands are attached by a flexible linker.

(E) Strand invasion by a bisPNA-peptide conjugate containing cationic amino acids.

(F) Strand invasion by a tail clamp PNA containing a mixed base extension from a polypyrimidine core sequence.

(G) PNA hybridization to structured DNA sequences. A hairpin PNA complementary to an inverted repeat (target sequence shaded) within supercoiled duplex DNA overcomes intramolecular base pairing and hybridizes with a rate association constant of 500,000 M^{-1} s^{-1} [16, 27].

(H) Positive charge attached to the N terminus of PNAs dramatically increases the rate of strand invasion of linear nonsupercoiled DNA [43]. Indicated peptides were attached to the N terminus of the bisPNA shown. k_a values are relative to the bisPNA with one lysine on the N terminus (k_a = 340 M^{-1} s^{-1}). The highest k_a value was achieved with attached peptide D-$(AAKK)_4$, 90,000 M^{-1} s^{-1}. D denotes D amino acids.

While this review focuses on PNAs, other modified bases and nucleotides can also be incorporated into oligomers with dramatically improved recognition properties. For example, introduction of a single locked nucleic acid (LNA) base (Figure 1) can raise the melting temperature of an oligonucleotide by up to 10°C, allowing extremely avid binding to be achieved [47]. Comparison of strand invasion by PNAs with that of other chemically modified oligomers will be an ongoing priority.

Cellular Uptake of PNAs

Cellular uptake is a major barrier to successful use of oligonucleotides inside cells. Fortunately, several methods have been developed to promote entry of PNAs into cells.

One strategy for achieving cellular uptake involves annealing PNAs to negatively charged DNA oligonucleotides and then complexing the heteroduplex with cationic lipid [48]. In this variation of standard protocols for delivering RNA or DNA into cells, the lipid binds to the DNA, allowing it to pass through the cell membrane. The hybridized PNA is carried along as cargo and then released once inside the cell by melting of the complex.

PNAs delivered into cells by this method have been shown to inhibit telomere synthesis by human telomerase [49] and to bind mRNA and inhibit expression of luciferase [50], human caveolin [75], and human progesterone receptor (B.A.J., unpublished data). The inhibition of mRNA by PNAs delivery by this strategy is as efficient and as prolonged as inhibition by analogous siRNAs [75]. Advantages of this method are that the PNA does not require chemical modification and standard transfection protocols can be used. A disadvantage is that use of lipid adds an extra experimental step. It is likely that this simple method has not become more widely used because its successful use requires that a laboratory be familiar with the chemical properties of PNAs and possess expertise performing transfections of mammalian cell culture.

PNAs have also been introduced into cells by electroporation to inhibit telomerase activity [51] and alter mRNA splicing [52]. This approach does not require

modification of the PNA, but does require specialized equipment and expertise. Microinjection of oocytes has become a widely used method for delivery of morpholino oligonucleotides in studies of inhibition of specific genes on early development [53], and it is likely that PNAs can be delivered using a similar method. Use of oocytes for chemical genetic analysis by antigene PNAs would be an important breakthrough. Cell volume does not increase during early development, allowing PNAs to be active throughout many cell divisions and facilitating the study of embryonic phenotypes that often also have relevance to adult animals.

Spontaneous uptake of PNAs by cells can be achieved by attachment of peptide sequences that promote translocation across cell membranes [54, 55]. One advantage of this approach is that lipid is not necessary, making the procedure more straightforward. Another advantage of this approach is that the peptide might also be used to promote nuclear uptake. Disadvantages are that additional synthetic steps are needed to add the peptide to the PNA and relatively high concentrations of PNA are required. In animals, uptake of PNAs tagged with four lysine residues has been shown to occur in a variety of tissues in mice and alter splicing [56]. Development of conjugates between PNAs and peptides or peptoids may ultimately be the method needed to achieve breakthrough results in vivo. PNAs with minimal modification can enter cultured cells and alter splicing, but only when high concentrations are present in the growth medium [57].

The success of antigene PNAs requires that they enter the nucleus. To date, it is not clear whether nuclear uptake will present a significant obstacle for PNAs intended to bind chromosomal DNA. Fluorophore-labeled PNAs do not appear to accumulate in the nucleus (K.K. and C. Nulf, unpublished data), but failure to observe uptake by microscopy does not necessarily indicate that insufficient amounts are entering for regulation of gene expression. Indeed, the experiments that report alteration of splicing provide a clear indication that some PNA can enter the nucleus [53, 56, 57]. If nuclear uptake is problematic for some applications, PNAs can be modified with peptides containing nuclear localization sequences (NLS), and PNA-NLS conjugates have shown improved localization [58].

Recognition of Chromosomal DNA

Choosing Targets for Chromosomal Recognition

Strand invasion by PNAs is remarkably efficient in cell-free systems, but success is less certain in complex cellular environments. To improve the likelihood that experiments inside cells will produce useful data, it is necessary to carefully consider the choice of target genes. There should be a rapid and sensitive assay for its expression so that experiments capable of detecting modest levels of inhibition can be done quickly. It is also essential that the gene sequence and promoter sequence be well characterized to allow the identification of unambiguous sequences near sites important for transcription.

It is possible to envision several different types of target sequence for PNAs (Figures 5A–5C). Transcription factor binding sites represent one class of target, because hybridization should disrupt assembly or regulation of the transcriptional apparatus. To date, no data is available regarding how near PNAs must be to a target site to be able to disrupt protein binding, and this will be an important variable to investigate. The transcriptional start site is another target, because the open complex formed by RNA polymerase is likely to offer a single-stranded region that Sigman and coworkers have demonstrated to be susceptible to binding by synthetic oligomers [59]. It may also be possible for oligomers to bind downstream of the start site and act as a transcriptional roadblocks. It is not known, however, whether binding is strong enough to stop the forward progress of the transcription machinery in mammalian cells.

If a polypyrimidine-polypurine sequence is present within a promoter, it may be possible to disrupt transcription with a bisPNA through strand invasion and formation of a four-stranded complex. Alternatively, PNAs can be targeted to sequences that form non-B-type structures. The advantage of targeting non-B-type structures is that they often contain some single-stranded bases, providing a convenient place for the initiation of strand invasion. PNAs bind within the hairpin stems of DNA cruciforms [16, 60] and within guanine-rich DNA that forms G quadruplex structure [61, 62].

As an example of the type of non-B-type structure that PNAs might target, Hurley and colleagues have recently raised the exciting hypothesis that G quadruplexes form within chromosomal DNA and act as important regulatory sites for gene expression (Figure 5B) [63]. Binding of a PNA to the G-rich strand might prevent the quadruplex from forming and disrupt its ability to regulate expression. Conversely, binding of a PNA to the C-rich strand might promote quadruplex structure and increase regulation.

The gene for the human progesterone receptor [64] offers an example of a gene with several promising target sites (Figure 5C). These include several relatively long polypyrimidine sequences that might be ideal sights for polypyrimidine bisPNAs and a long inverted repeat that might form a cruciform capable of readily binding to mixed-base PNAs. The human progesterone receptor has two distinct promoters that regulate the transcription of two different isoforms. Targeting these promoters separately would offer an approach for isoform-specific inhibition of gene expression. Thus, there are numerous plausible target sites within the gene that are both functionally important and have sequences that are susceptible to strand invasion.

Inhibition of Transcription

Published data indicate that PNAs can efficiently block transcription in cell-free systems [30, 31, 65]. There have also been reports of antigene PNAs being used to inhibit gene expression in cultured cells and in animals [66, 67]. While these results are promising, the data for antigene effects inside cells are not as clear or well controlled as the work in cell-free systems, and more work will be necessary to establish the potential of antigene PNAs for the regulation of cellular processes. Future studies will need to be systematic and include ample controls to demonstrate that observed effects are due to PNAs

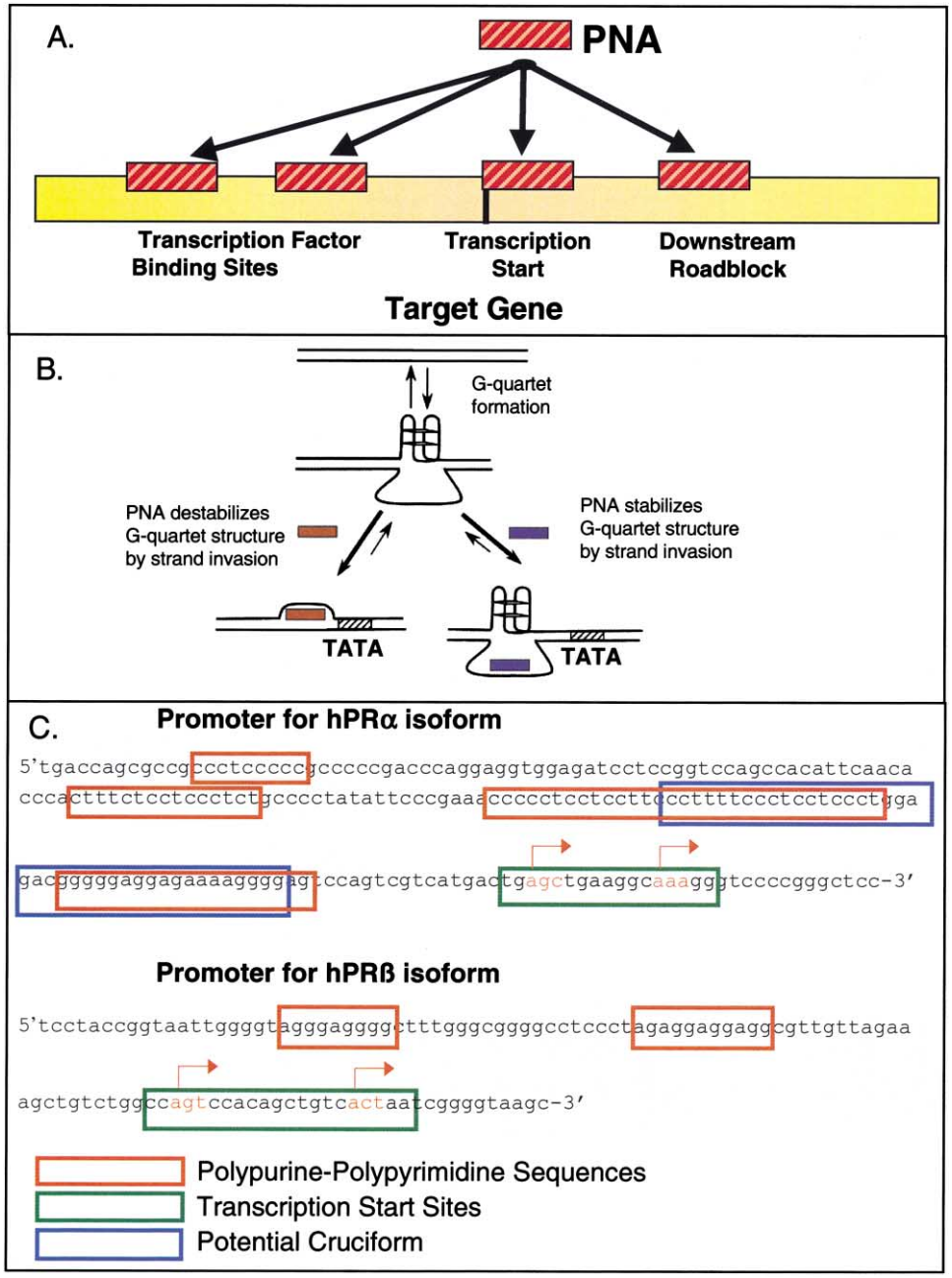


Figure 5. Antigene PNAs, General Scheme, and Specific Promising Targets

(A) Types of potential targets for antigene PNAs.

(B) Targeting PNAs to G quartet secondary structure. PNAs can disrupt G quartet structure by binding to G-rich sequences. PNAs may be able to stabilize G quartet structure by binding to the complementary strand. We depict G quartet formation activating transcription, but G quartet formation may also repress transcription [63].

(C) Targets for antigene PNAs in the human progesterone receptor [64]. Targets include multiple polypurine-polypyrimidine sequences, multiple transcription start sites, two isoforms, and a partial inverted repeat/cruciform.

binding to their intended targets. Once an unambiguous antigene effect is established, an important goal for these studies will be to establish predictive rules governing selection of target sequences (i.e., polypurine-polypyrimidine, non-B-type, open complex, etc.).

Activation of Transcription

Activation of gene transcription is another potential application for duplex recognition by PNAs (Figure 6). Studies have reported that simply hybridizing PNAs within the promoter region can cause a modest increase in gene expression [32, 33]. The mechanism of this upregulation is not clear but may involve recognition of the displaced strand by proteins involved in transcription.

Another strategy for accomplishing this end is to attach a PNA to a peptide or small molecule capable of binding to a transcription factor. The resulting conjugate

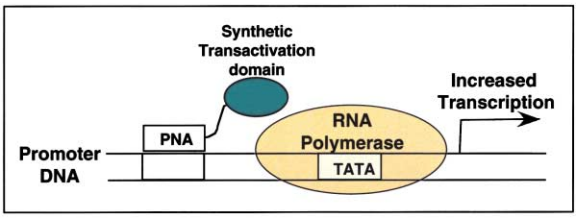


Figure 6. PNA Conjugates as Artificial Activators of Transcription

A PNA that binds within a promoter region and an attached transcription activation domain triggers transcription by recruiting RNA polymerase [36].

would be a synthetic transcription activator capable of recruiting the transcription machinery. Initial work from Kodadek and coworkers has shown that a conjugate consisting of a PNA attached to a peptide selected by phage display to bind to the transcription factor Gal 80 can successfully recruit Gal 80 to a target site on DNA [36]. More recently, PNA-peptide conjugates have also been shown to be able to activate gene expression in mammalian cell extract [74]. Such artificial transcription factors would be an elegant strategy for developing entirely synthetic agents for activating gene expression. Similar strategies have also been reported using polyamides [68] and TFOs [69].

Challenges for future research will include the design or selection of efficient transcriptional activators that will also be stable inside cells. When combined with improved strategies for efficient strand invasion and efficient methods for cellular delivery, such synthetic PNA activator conjugates may prove to be powerful tools for increasing transcription. Since there are no general methods for increasing gene expression in mammalian cells, synthetic activators would be widely useful.

Introduction of Mutations

Correction of the mutations that cause genetic disease is a major unmet therapeutic need. Glazer and colleagues reported that upon introduction into cultured mammalian cells, a PNA directed to the supFG1 gene was able to induce mutation frequencies at a frequency of 0.1% up to 10-fold higher than background [70]. Analysis of the mutations produced by the PNA suggested that the bound PNA acts as a mutagenic lesion and may cause replication slippage errors. More recently, Glazer and coworkers have synthesized a bifunctional PNA-DNA conjugate consisting of a PNA domain designed to anchor the conjugate at a target site and a DNA domain with a one-base mismatch relative to a duplex target [34]. They also tested the PNA and DNA added together but not attached. In cell-free extracts, this strategy yielded a recombination frequency at least 60-fold above background, with a rate of 0.05 to 0.1%.

Obviously, much work remains to increase the efficiency of mutagenesis, and significant improvement will require strategies for optimizing PNA binding to chromosomal DNA. It is also likely that greater knowledge of the rules for invasion of chromosomal DNA will contribute to the design of PNAs that yield increased mutation frequencies. This database of rules will likely arise from the combination of results from projects involving inhibition of transcription, activation of transcription, and introduction of mutations. PNAs have been shown to facilitate the action of the recombination protein RecA [71], and this ability to act in concert with recombination enzymes may permit useful mutagenesis frequencies to be achieved.

Where Do We Go from Here?

Ten years of work with PNAs has demonstrated that they can readily invade linear and supercoiled DNA and can function to control gene expression or introduce mutations. However, reports of effects inside cells are relatively few, and it is clear that the field has a long way to go before antigene PNAs become widely accepted tools. The challenge confronting workers in the field is to build upon previous work and develop reproducible protocols that can be successfully employed by laboratories that have never worked with PNAs before.

Specific experimental goals for future work include (1) continued development of new PNA designs and new strategies for strand invasion; (2) continued development of improved methods for cellular delivery, with a focus on optimal nuclear delivery; and (3) unambiguous and thoroughly controlled demonstrations that PNAs can recognize chromosomal DNA inside cells. Demonstrations may involve inhibition of gene expression, activation of gene expression, or introduction of mutations; (4) determination of the rules that govern successful strand invasion inside cells so that the wider community is convinced that PNAs can be useful tools; (5) optimization of pharmacokinetics and biodistribution, and (6) development of antigene PNAs for cell culture, animal studies, and clinical development.

To achieve these goals it will be necessary to demonstrate rules for the recognition of chromosomal DNA by PNAs. Such comprehensive studies will require a significant number of oligomers, but we emphasize that it is not difficult to synthesize, solubilize, or work with PNAs. Groups working with PNA should set high standards for themselves and be held to high standards by their colleagues. With systematically designed groups of PNAs in hand, it should be possible to set up a design cycle to test hypotheses about susceptible sequences, obtain data, and then redesign the experiment strategy to achieve improved results and facilitate in vivo application using these powerful molecular tools.

Acknowledgments

This work was supported by grants from the National Institutes of Health (GM-60642) and the Robert A. Welch Foundation (I-1244).

References

1. Nielsen, P.G., Egholm, M., Berg, R.H., and Buchardt, O. (1991). Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science *254*, 1497–1500.
2. Opalinska, J.B., and Gewirtz, A.M. (2002). Nucleic-acid therapeutics: basic principles and recent applications. Nat. Rev. Drug Discov. *1*, 503–514.
3. Braasch, D.A., and Corey, D.R. (2002). Novel antisense and peptide nucleic acid strategies for controlling gene expression. Biochemistry *41*, 4503–4510.
4. Stein, C.A. (1999). Keeping the biotechnology of antisense in context. Nat. Biotechnol. *17*, 209.
5. Stein, C.A. (1999). Two problems in antisense biotechnology: in

vitro delivery and the design of antisense experiments. Biochim. Biophys. Acta *1489*, 45–52.
6. Crooke, S.T. (1996). Proof of mechanism of antisense drugs. Antisense Res. Dev. *6*, 145–147.
7. Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature *411*, 494–498.
8. Crooke, S.T. (1999). Molecular mechanisms of antisense drugs: RNase H. Antisense Nuc. Acid. Res. Dev. *9*, 377–379.
9. Gowers, D.M., and Fox, K.R. (1999). Towards mixed sequence recognition by triple helix formation. Nucleic Acids Res. *27*, 1569–1577.
10. Knauert, M.P., and Glazer, P.M. (2001). Triplex forming oligonucleotides: sequence-specific tools for gene targeting. Hum. Mol. Genet. *10*, 2243–2251.
11. Mergny, J.-L., Sun, J.-S., Rougee, M., Montenay-Garestier, T., Barcelo, F., Chomilier, J., and Helene, C. (1991). Sequence specificity in triple-helix formation: Experimental and theoretical studies of the effect of mismatches on triplex stability. Biochemistry *30*, 9791–9798.
12. Roberts, R.W., and Crothers, D.M. (1991). Specificity and stringency in DNA triplex formation. Proc. Natl. Acad. Sci. USA *88*, 9397–9401.
13. Holloman, W.K., Wiegand, R., Hoesli, C., and Radding, C.M. (1975). Uptake of homologous single-stranded fragments by superhelical DNA: A possible mechanism for genetic recombination. Proc. Natl. Acad. Sci. USA *72*, 2394–2398.
14. Corey, D.R. (1995). 48,000-fold Acceleration of hybridization of chemically modified oligomers to duplex DNA. J. Am. Chem. Soc. *117*, 9373–9374.
15. Ishihara, T., and Corey, D.R. (1999). Rules for strand invasion by chemically modified oligonucleotides. J. Am. Chem. Soc. *121*, 2012–2020.
16. Smulevitch, S.V., Simmons, C.G., Norton, J.C., Wise, T.W., and Corey, D.R. (1996). Enhanced strand invasion by oligonucleotides through manipulation of backbone charge. Nat. Biotechnol. *14*, 1700–1705.
17. Egholm, M., Buchardt, O., Christensen, L.R., Behrens, C., Freier, S.M., Driver, D.A., Berg, R.H., Kim, S.K., Norden, B., and Nielsen, P.E. (1993). PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. Nature *365*, 566–568.
18. Hamilton, S.E., Pitts, A.E., Katipally, R.R., Jia, X., Davies, B.A., Rutter, J.P., Wright, W.R., Shay, J.W., and Corey, D.R. (1997). Identification of Determinants for Inhibitor Binding within the RNA Active Site of Human Telomerase Using PNA Scanning. Biochemistry *36*, 11873–11880.
19. Ratilainen, T., Holmen, A., Tuite, E., Nielsen, P.E., and Norden, B. (2000). Thermodynamics of sequence-specific binding of PNA to DNA. Biochemistry *39*, 7781–7791.
20. Demidov, V.V., Potaman, V.N., Frank-Kamenetskii, M.D., Egholm, M., Buchardt, O., Sonnichsen, S.H., and Nielsen, P.E. (1994). Stability of peptide nucleic acids in human serum and cellular extracts. Biochem. Pharmacol. *48*, 1310–1313.
21. Hamilton, S.E., Iyer, M., Norton, J.C., and Corey, D.R. (1996). Specific and nonspecific inhibition of transcription by DNA, PNA, and phosphorothioate promoter analog duplexes. Bioorg. Med. Chem. Lett. *6*, 2897–2900.
22. Knudsen, H., and Nielsen, P.E. (1996). Antisense properties of duplex and triplex forming PNAs. Nucleic Acids Res. *24*, 494–500.
23. Mayfield, L.D., and Corey, D.R. (1999). Automated synthesis of peptide nucleic acids (PNAs) and peptide nucleic acid-peptide conjugates. Anal. Biochem. *268*, 401–404.
24. Braasch, D.A., and Corey, D.R. (2001). Synthesis, analysis, purification, and intracellular delivery of peptide nucleic acids. Methods *23*, 97–107.
25. Boffa, L.C., Carpaneto, E.M., and Allfrey, V.G. (1995). Isolation of active genes containing CAG repeats by DNA strand invasion by a peptide nucleic acid. Proc. Natl. Acad. Sci. USA *92*, 1901–1905.
26. Seeger, C., Batz, H.-G., and Orum, H. (1997). PNA-mediated purification of PCR amplifiable human genomic DNA from whole blood. Biotechniques *23*, 512–516.
27. Norton, J.C., Waggenspack, J.H., Varnum, E., and Corey, D.R. (1995). Targeting peptide nucleic acid protein conjugates to structural features within duplex DNA. Bioorg. Med. Chem. *3*, 437–445.
28. Footer, M., Egholm, M., Kron, S., Coull, J.M., and Matsudaira, P. (1996). Biochemical evidence that a D-loop is part of a four-stranded PNA-DNA bundle. Nickel-mediated cleavage of duplex DNA by a Gly-Gly-His bis-PNA. Biochemistry *35*, 10673–10679.
29. Demidov, V.V., Broude, N.E., Lavrentieva-Smolina, I.V., Kuhn, H., and Frank-Kamenetskii, M.D. (2001). An artificial primosome: design, function, and applications. Chembiochem *2*, 133–139.
30. Vickers, T.A., Griffith, M.C., Ramasmay, K., Risen, L.M., and Freier, S.M. (1995). Inhibition of NF-kappa B specific transcriptional activation by PNA strand invasion. Nucleic Acids Res. *23*, 3003–3008.
31. Larsen, H.J., and Nielsen, P.E. (1996). Transcription-mediated binding of peptide nucleic acid (PNA) to double-stranded DNA: sequence-specific suicide transcription. Nucleic Acids Res. *24*, 458–463.
32. Mollegaard, N.E., Buchardt, O., Egholm, M., and Nielsen, P.E. (1994). Peptide nucleic acid-DNA strand displacement loops as artificial transcription promoters. Proc. Natl. Acad. Sci. USA *91*, 3892–3895.
33. Wang, G., Jing, K., Balczon, R., and Xu, X. (2001). Defining the peptide nucleic acids (PNA) length requirement for PNA binding induced transcription and gene expression. J. Mol. Biol. *313*, 933–940.
34. Rogers, F.A., Vasquez, K.M., Egholm, M., and Glazer, P.M. (2002). Site-directed recombination via bifunctional PNA-DNA conjugates. Proc. Natl. Acad. Sci. USA *99*, 16695–16700.
35. Zelphati, O., Liang, X., Nguyen, C., Barlow, S., Sheng, S., Shao, Z., and Felgner, P.L. (2000). PNA-dependent gene chemistry: stable coupling of peptides and oligonucleotides to plasmid DNA. Biotechniques *28*, 304–315.
36. Liu, B., Han, Y., Corey, D.R., and Kodadek, T.K. (2002). Towards synthetic transcriptional activators: recruitment of transcription factors to DNA by a PNA-peptide chimera. J. Am. Chem. Soc. *124*, 1838–1839.
37. Kuhn, H., Demidov, V.V., Coull, J.M., Fiandaca, M.J., Gildea, B.D., and Frank-Kamenetskii, M.D. (2002). Hybridization of DNA and PNA molecular beacons to single-stranded and double-stranded DNA targets. J. Am. Chem. Soc. *124*, 1097–1103.
38. Bentin, T., and Nielsen, P.E. (1996). Enhanced peptide nucleic acid binding to supercoiled DNA: possible implications for DNA "breathing" dynamics. Biochemistry *35*, 8863–8869.
39. Zhang, X., Ishihara, T., and Corey, D.R. (2000). Strand Invasion by mixed base PNAs and PNA-peptide chimera. Nucleic Acids Res. *28*, 3332–3338.
40. Lohse, J., Dahl, O., and Nielsen, P.E. (1999). Double duplex invasion by peptide nucleic acid: a general principle for sequence-specific targeting of double-stranded DNA. Proc. Natl. Acad. Sci. USA *96*, 11804–11808.
41. Protozanova, E., Demidov, V.V., Soldatenkov, V., Chasovskikh, S., and Frank-Kamenetskii, M.D. (2002). Tailoring the activity of a restriction endonuclease PleI by PNA-induced looping. EMBO J. *3*, 956–961.
42. Protozanova, E., Demidovl, V.V., Nielsen, P.E., and Frank-Kamenetskii, M.D. (2003). Pseudocomplementary PNAs as selective modifiers of protein activity on duplex DNA: the case of type IIs restriction enzymes. Nucleic Acids Res. *31*, 3929–3935.
43. Kuhn, H., Demidov, V.V., Nielsen, P.E., and Frank-Kamenetskii, M.D. (1999). An experimental study of mechanism and specificity of peptide nucleic acid (PNA) binding to duplex DNA. J. Mol. Biol. *285*, 1337–1345.
44. Griffith, M.C., Risen, L.M., Greig, M.J., Lesnik, E.A., Sprankle, K.G., Griffey, R.H., Kiely, J.S., and Freier, S.M. (1995). Single and bispeptide nucleic acids as triplexing agents: Binding and stoichiometry. J. Am. Chem. Soc. *117*, 831–832.
45. Kaihatsu, K., Braasch, D.A., Canisoglu, A., and Corey, D.R. (2002). Enhanced strand invasion by peptide nucleic acid-peptide conjugates. Biochemistry *41*, 11118–11125.
46. Bentin, T., and Nielsen, P.E. (2003). Superior duplex DNA strand

invasion by acridine conjugated peptide nucleic acids. J. Am. Chem. Soc. *125*, 6378–6379.
47. Braasch, D.A., and Corey, D.R. (2001). Locked nucleic acids: Fine-tuning nucleic acid hybridization. Chem. Biol. *8*, 1–7.
48. Hamilton, S.E., Simmons, C.G., Kathriya, I., and Corey, D.R. (1999). Cellular delivery of peptide nucleic acids and inhibition of human telomerase. Chem. Biol. *6*, 343–351.
49. Herbert, B.-S., Pitts, A.E., Baker, S.I., Hamilton, S.E., Wright, W.E., Shay, J.W., and Corey, D.R. (1999). Inhibition of telomerase leads to eroded telomeres, reduced proliferation, and cell death. Proc. Natl. Acad. Sci. USA *96*, 14726–14781.
50. Doyle, D.F., Braasch, D.A., Simmons, C.G., Janowski, B.A., and Corey, D.R. (2001). Intracellular delivery and inhibition of gene expression by peptide nucleic acids. Biochemistry *40*, 53–64.
51. Shammas, M.A., Simmons, C.G., Corey, D.R., and Shmookler-Reis, R.J. (1999). Telomerase inhibition by peptide nucleic acids reverses "immortality" of transformed cells. (1999). Oncogene *18*, 6191–6200.
52. Karras, J.G., Maier, M.A., Lu, T., Watt, A., and Manoharan, M. (2001). Peptide nucleic acids are potent modulators of endogenous pre-mRNA splicing of the murine interleukin-5 receptor-chain. Biochemistry *40*, 7853–7859.
53. Nasevicius, A., and Ekker, S.C. (2000). Effective targeted gene 'knockdown' in zebrafish. Nat. Genet. *26*, 216–220.
54. Simmons, C.G., Pitts, A.E., Mayfield, L.D., Shay, J.W., and Corey, D.R. (1997). Synthesis and membrane permeability of PNA-peptide conjugates. Bioorg. Med. Chem. Lett. *7*, 3001–3006.
55. Pooga, M., Ursel, S., Hallbrink, M., Valkna, A., Saar, K., Rezaei, K., Kahl, U., Hao, J.-K., Xu, X.-J., Wiesenfeld-Hallin, Z., et al. (1998). Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo. Nat. Biotechnol. *16*, 857–861.
56. Sazani, P., Gemignani, F., Kang, S.H., Maier, M.A., Manoharan, M., Persmark, M., Bortner, D., and Kole, R. (2002). Systemically delivered antisense oligomers upregulate gene expression in mouse tissues. Nat. Biotechnol. *20*, 1228–1233.
57. Salzani, P., Kang, S.H., Maier, M.A., Wei, C., Dillman, J., Summerton, J., Manoharan, M., and Kole, R. (2001). Nuclear antisense effects of neutral, anionic, and cationic oligonucleotide analogs. Nucleic Acids Res. *29*, 3965–3974.
58. Braun, K., Peschke, P., Pipkorn, R., Lampel, S., Wachsmuth, M., Waldeck, W., Friedrich, E., and Debus, J. (2002). A biological transporter for the delivery of peptide nucleic acids (PNAs) to the nuclear compartment of living cells. J. Mol. Biol. *318*, 237–243.
59. Milne, L., Xu, Y., Perrin, D.M., and Sigman, D.S. (2000). An approach to gene-specific transcription inhibition using oligonucleotides complementary to the template strand of the open complex. Proc. Natl. Acad. Sci. USA *97*, 3136–3141.
60. Kushon, S.A., Jordan, J.P., Seifert, J.L., Nielsen, H., Nielsen, P.E., and Armitage, B.A. (2001). Effect of secondary structure on the thermodynamics and kinetics of PNA hybridization to DNA hairpins. J. Am. Chem. Soc. *123*, 10805–10813.
61. Datta, B., and Armitage, B.A. (2001). Hybridization of PNA to structured DNA targets: quadruplex invasion and the overhang effect. J. Am. Chem. Soc. *123*, 9612–9619.
62. Armitage, B.A. (2003). The impact of nucleic acid secondary structure on PNA hybridization. Drug Discov. Today *8*, 222–228.
63. Siddiqui-Jain, A., Grand, C.L., Bearss, D.J., and Hurley, L.H. (2002). Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress c-MYC transcription. Proc. Natl. Acad. Sci. USA *99*, 11593–11598.
64. Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H., and Chambon, P. (1990). Two distinct estrogen regulated promoters generate transcripts encoding two functionally different human progesterone receptor forms A and B. EMBO J. *9*, 1603–1614.
65. Zhao, X., Kaihatsu, K., and Corey, D.R. (2003). Inhibition of transcription by PNA-peptide conjugates. Nucleosides Nucleotides Nucleic Acids, in press.
66. Tyler, B.M., Jansen, K., McCormick, D.J., Douglas, C.L., Boules, M., Stewart, J.A., Zhao, L., Lacy, B., Cusack, B., Fauq, A., et al. (1999). Peptide nucleic acids targeted to the neurotensin receptor and administered i.p. cross the blood-brain barrier and specifically reduce gene expression. Proc. Natl. Acad. Sci. USA *96*, 7053–7058.
67. Boffa, L.C., Scarfi, S., Mariani, M.R., Damonte, G., Allfrey, V.G., Benatti, U., and Morris, P.L. (2000). Dihydrotestosterone as a selective cellular/nuclear localization vector for anti-gene peptide nucleic acid in prostatic carcinoma cells. Cancer Res. *60*, 2258–2262.
68. Ansari, A.Z., Mapp, A.K., Nguyen, D.H., Dervan, P.B., and Ptashne, M. (2001). Towards a minimal motif for artificial transcriptional activators. Chem. Biol. *8*, 583–592.
69. Stanojevic, D., and Young, R.A. (2002). A highly potent artificial transcription factor. Biochemistry *41*, 7209–7216.
70. Faruqi, A.F., Egholm, M., and Glazer, P.M. (1998). Peptide nucleic acid-targeted mutagenesis of a chromosomal gene in mouse cells. Proc. Natl. Acad. Sci. USA *95*, 1398–1403.
71. Belotserkovskii, B.P., and Zarling, D.A. (2002). Peptide nucleic acid (PNA) facilitates multistranded hybrid formation between linear double-stranded DNA targets and RecA protein-coated complementary single-stranded probes. Biochemistry *41*, 3686–3692.
72. Kaihatsu, K., Shah, R.H., Zhao, X., and Corey, D.R. (2003). Extending Duplex Recognition by Peptide Nucleic Acids (PNAs): Strand Invasion and Inhibition of Transcription by Tail Clamp PNAs and PNA-Peptide Conjugates. Biochemistry, in press.
73. Bentin, T., Larsen, H.J., and Neilsen, P.E. (2003). Combined triplex/duplex invasion of double-stranded DNA by "Tail Clamp" peptide nucleic acids (PNA). Biochemistry, in press.
74. Liu, B., Han, Y., Ferdous, A., Corey, D.R., and Kodadek, T. (2003). Transcription activation by a PNA-peptide chimera in a mammalian cell extract. Chem. Biol., *10*, 909–916.
75. Liu, Y., Braasch, D.A., Nulf, C.J., and Corey, D.R. (2004). Isoform-specific inhibition of cellular gene expression by peptide nucleic acid. Biochemistry *43*, 1921–1927.