Extending Recognition by Peptide Nucleic Acids (PNAs): Binding to Duplex DNA and Inhibition of Transcription by Tail-Clamp PNA−Peptide Conjugates†

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ABSTRACT: Peptide nucleic acids (PNAs) are a powerful tool for recognition of double-stranded DNA. Strand invasion is most efficient when pyrimidine PNAs are linked to form a bisPNA in which one strand binds by Watson−Crick base pairing while the other binds by Hoogsteen base pairing to the newly formed PNA−DNA duplex. Within many genes, however, polypyrimidine target sequences may not be located in optimal positions relative to transcription factor binding sites, and this deficiency may complicate attempts to identify potent antigene PNAs. To increase the versatility of strand invasion by PNAs, we have synthesized bisPNAs and bisPNA−peptide conjugates containing a mixed base extension of the Watson−Crick polypyrimidine strand. We find that these tail-clamp PNAs (TC-PNAs) bind duplex DNA and inhibit transcription. DNA recognition occurs with single-stranded or TC-bisPNAs and requires attachment of positively charged amino acids. Association rate constants, k_a, for binding to DNA by TC-PNAs are as high as 35000 M^{-1} s^{-1} and are usually only a fewfold lower than for analogous PNAs that lack mixed base extensions. The ability to bind duplex DNA is not always necessary for inhibition of transcription, possibly because PNAs can bind to accessible DNA within the transcription bubble created by RNA polymerase. These results, together with similar findings independently obtained by Nielsen and colleagues [Bentin, T., Larsen, H. J., and Nielsen, P. E. (2003) *Biochemistry 42*, 13987−13995], expand the range of sequences within duplex DNA that are accessible to PNAs and suggest that TC-PNA−peptide conjugates are good candidates for further testing as antigene agents.

Peptide nucleic acid (PNA)[1] is a DNA/RNA mimic in which the phosphate backbone has been replaced by a neutral amide backbone composed of *N*-(2-aminoethyl)glycine linkages (*1*). PNA offers important advantages for recognition of nucleic acids including high-affinity binding (*2*), resistance to degradation by nucleases or proteases (*3*), and low affinity for proteins (*4*). Perhaps the most unique strength of PNA is its high propensity for invading duplex DNA (*5*, *6*). This ability makes PNAs a leading candidate for developing strategies that aim to control transcription or introduce mutations into chromosomal DNA. To date, applications for strand invasion have included purification of genomic DNA (*7*, *8*), cleavage of DNA (*9*), creation of artificial primosomes (*10*, *11*), inhibition of transcription (*12*−*14*), activation of transcription (*15*, *16*), site-directed mutagenesis (*17*, *18*), noncovalent labeling of plasmids with fluorophores (*19*), recruitment of transcription factors to an artificial promoter (*20*), and recognition of duplex DNA by molecular beacons (*21*).

PNAs that selectively recognize DNA sequences inside cells would be valuable agents for controlling gene expression and probes for the structure and function of chromosomal DNA. However, before recognition of duplex DNA by PNAs can become a general approach to artificial gene regulation, it is necessary to (1) identify biologically significant sites that can be blocked by PNA binding and (2) design and synthesize PNAs capable of efficiently recognizing those sites.

There are several strategies for designing PNAs to recognize duplex DNA. PNAs containing mixtures of the A, C, G, and T bases can hybridize to supercoiled DNA (*11*, *22*, *23*). Hybridization promoted by the negative torsional stress of supercoiling is most efficient at inverted repeats capable of forming cruciforms and within AT-rich regions (*23*). Another approach to duplex recognition is use of pseudocomplementary PNAs (pcPNAs) (*24*, *25*). pcPNAs contain modified bases designed to minimize the potential for intramolecular base pairing and hairpin formation but to permit Watson−Crick recognition of A, C, G, and T. These pcPNAs are able to hybridize to both strands of a duplex target to form a double-displacement loop.

Perhaps the simplest approach depends on the remarkable ability of PNAs to spontaneously invade polypyrimidine sites within relaxed DNA (*1*, *9*, *10*, *12*, *26*, *27*). 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 Hoogsteen base pairing while the other binds by Watson−Crick base pairing (*26*). The efficiency of strand invasion can be improved by tethering the two PNA strands to form a bisPNA that pays

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[1] Abbreviations: PNA, peptide nucleic acid; TC, tail clamp; NE, nonextended; hCav-1, human caveolin 1.

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FIGURE 1: Stand invasion by a tail-clamp bisPNA (TC-bisPNA). PNA bases are shaded; tail-clamp bases are in bold.

a lower entropic penalty upon binding (*27*). Strand invasion efficiency has also been enhanced by attachment of cationic amino acids (*22*, *28*) and most recently by conjugation to a DNA intercalator (*29*).

Our laboratory is investigating the rules governing strand invasion by PNAs inside cells. While designing our experiments, we noted that although polypurine−polypyrimidine bases containing eight or more base pairs occur within promoter regions, they are not always near important transcription factor binding sites. We reasoned that it might be possible to increase the disruption caused by PNAs by extending the Watson−Crick strand of a bisPNA past a homopyrimidine core sequence to allow it to bind mixed base sequences (Figure 1). Extensions might also stabilize binding by short bisPNAs, increasing the number of sites that can be targeted. Here we test bisPNAs that have been extended with mixed base sequences (tail-clamp PNAs or TC-PNAs)[2] and show that TC-PNAs can bind to duplex DNA and inhibit transcription.

MATERIALS AND METHODS

Synthesis of PNAs and PNA−Peptides. PNA monomers, Fmoc-T-OH, Fmoc-C(Bhoc)-OH, Fmoc-G(Bhoc)-OH, and Fmoc-A(Bhoc)-OH were obtained from Applied Biosystems (Foster City, CA). Linker molecule Fmoc-2-(aminoethoxy)-2-ethoxyacetic acid (AEEA)-OH and activators of base coupling *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and 1-hydroxy-7-azabenzotriazole (HOAt) were obtained from Applied Biosystems. Fmoc-XAL-PEG-PS resin was from Applied Biosystems. Amino acid monomers Fmoc-Ala-OH, Fmoc-Lys-(Boc)-OH, Fmoc-D-Ala-OH, and Fmoc-D-Lys(Boc)-OH were from Novabiochem. PNAs were synthesized on an Expedite 8909 synthesizer (Applied Biosystems, Foster City, CA), using standard fluorenylmethoxycarbonyl (Fmoc) chemistry and purified as described (*28*, *30*). PNA concentrations were quantified on the basis of spectrometric A_{260} values using the molar extinction coefficients 8800 (T), 6600 (C), 13700 (A), and 11700 M^{-1} cm^{-1}.

Preparation of Duplex DNA Targets for the Gel Shift Analysis. A pair of synthetic oligonucleotides encoding the polypurine PNA binding site (5′-gcgcggcagt cacgacgttg taaaacgacg gccagtgcca agcttga**aag gaggaga**gtc gacctcgagg catgtgctct gtatcgcgcc-3′ and 5′-ggcgcgatac agagcacatg cctcgaggtc gac**tctcctc ctt**tcaagct tggcactggc cgtcgtttta caacgtcgtg actgccgcgc-3′; target site underlined in boldface) was obtained from Dr. Bo Liu and Dr. Thomas Kodadek (University of Texas Southwestern Medical Center) (*20*). Another pair of synthetic oligonucleotides encoding the caveolin cDNA promoter site (*31*) (5′-cctccagcgc cgtgtgttcg cgccatacaa tacaagatc**t tccttcctc**a gttcccttaa agcacagccc agggaaacct cctcacagtt-3′ and 5′-aactgtgagg aggtttccct gggctgtgct ttaagggaac t**gaggaagga a**gatcttgta ttgtatggcg cgaacacacg gcgctggagg-3′; target site underlined in boldface) was obtained from Sigma Genosys. Each pair of complementary oligonucleotides was annealed, purified by 2% agarose gel electrophoresis, and extracted from agarose gel by the QIAquick gel extraction kit (QIAGEN Inc.). Purified double-stranded DNAs were amplified by polymerase chain reaction (PCR) and purified by the QIAquick PCR purification kit (QIAGEN, Valencia, CA) using standard procedure. Double-stranded DNA concentrations were quantified on the basis of spectrometric A_{260} values and the conversion factor of 50 μg/mL per OD. The dsDNAs were labeled by standard procedures using [γ-^{32}P]ATP and T4 polynucleotide kinase. The γ-^{32}P-labeled dsDNAs were desalted using Bio-Spin 6 chromatography columns (Bio-Rad, Hercules, CA).

Strand Invasion by PNAs. Hybridization of dsDNA and PNA or PNA−peptide conjugates was accomplished by mixing 12.5 nM γ-^{32}P-labeled dsDNA with 1−100 equiv of PNA in 10 mM sodium phosphate, pH 6.9, and 1 mM EDTA for 2 h at 37 °C. PNAs tend to aggregate upon storage. To ensure that PNAs were present in soluble active form, they were preheated at 75 °C for 5 min and then cooled to 37 °C gradually before being added to the DNA solution. For assays of strand invasion γ-^{32}P-labeled dsDNA was dissolved in 10 mM sodium phosphate (pH 6.9). After addition of PNAs, the reactions were incubated for 2 h at 37 °C. The strand invasion reactions were terminated by placement in an ethanol/dry ice bath. All experiments were performed in siliconized tubes.

Gel Shift Analysis of the Association of PNA and DNA. A gel shift assay was used to separate dsDNA duplexes complexed with PNA from unbound dsDNA. DNA and DNA/PNA mixtures were loaded onto the gel using a solution of bromophenol blue (FisherBiotech), xylene cyanol (Sigma), and Acid Orange G (Sigma) each 0.02% and 10% of glycerol (Sigma) dissolved in water. For a 5 μL DNA/PNA mixture, 3 μL of loading buffer was added, and DNA/PNA mixtures were electrophoresed at 300 V for 2 h on a 10% nondenaturing polyacrylamide gel using 1 × TBE as a running buffer (89 mM Tris base, 89 mM borate, 2 mM EDTA, pH 8.1) (Amresco, Solon, OH). Gel electrophoresis was performed at 4 °C. The products were visualized by autoradiography and quantified by using a Molecular Dynamics (Sunnyvale, CA) Model 425F phosphorimager. The absolute efficiency of the strand invasion was estimated by analyzing the ratio of the dsDNA band and shifted bands by 10% polyacrylamide gel electrophoresis.

Preparation of Duplex DNA Targets for in Vitro Transcription Assay. A 168 bp DNA duplex fragment that contains the PNA target sequence of 5′-aaggaggaga-3′ was PCR amplified from the modified pUC18 vector. The primers used were 5′-taatacgactcactatagggtgctgcaaggcgattaag-3′ and 5′-aattaaccctcactaaagggttttatatac-3′ (Sigma Genosys). The underlined part within the primer encodes for a T7 promoter that provides a recognition sequence for T7 RNA polymerase. The PCR product was purified by 1% agarose gel electrophoresis and extracted from agarose gel by the QIAquick gel extraction kit (QIAGEN, Inc.).

In Vitro Transcription Assay. Hybridization of duplex DNA and PNA was accomplished by the same protocol used for the gel shift assay except that unlabeled duplex DNA

[2] The term tail-clamp PNA was coined by Nielsen and Bentin (*39*).

Table 1: Observed Kinetic Association Constants for Selected PNA and PNA−Peptide Conjugates Used in These Studies[a]

PNA	sequence	k_a (M^{-1} s^{-1})	k_{rel}
1	TCTCCTCCTT-(AEEA)$_3$-TTCCTCCTCT	340	1
2	TCTCCTCCTT-(AEEA)$_3$-TTCCTCCTCT-D-(AAKK)$_4$	94000	268
3	**TCGAC**TCTCCTCCTT-(AEEA)$_3$-TTCCTCCTCT	nd	
4	**TCGAC**TCTCCTCCTT-(AEEA)$_3$-TTCCTCCTCT-D-(AAKK)$_4$	35000	102
5	**CGAGGTCGAC**TCTCCTCCTT-(AEEA)$_3$-TTCCTCCTCT-D-(AAKK)$_4$	860	2.5
6	TCCTCCTT-(AEEA)$_3$-TTCCTCCT	nd	
7	TCCTCCTT-(AEEA)$_3$-TTCCTCCT-D-(AAKK)$_4$	1400	4.1
8	**GACTC**TCCTCCTT-(AEEA)$_3$-TTCCTCCT	nd	
9	**GACTC**TCCTCCTT-(AEEA)$_3$-TTCCTCCT-D-(AAKK)$_4$	2400	7.2
10	TTCCTCCTCT	nd	
11	TTCCTCCTCT-D-(AAKK)$_4$	1600	4.7
12	TTCCTCCTCT**CAG**-D-(AAKK)$_4$	nd	
13	TTCCTCCTCT**CAGCT**-D-(AAKK)$_4$	620	1.8
14	TTCCTTCCTC-(AEEA)$_3$-CTCCTTCCTT	nd	
15	TTCCTTCCTC-(AEEA)$_3$-CTCCTTCCTT-D-(AAKK)$_4$	48000	138
16	**AGATC**TTCCTTCCTC-(AEEA)$_3$-CTCCTTCCTT	nd	
17	**AGATC**TTCCTTCCTC-(AEEA) $_3$-CTCCTTCCTT-D-(AAKK)$_4$	5900	17
18	TCTCATCCTT-(AEEA)$_3$-TTCCTACTCT	nd	
19	TCTCĀTCCTT-(AEEA)$_3$-TTCCTĀCTCT-D-(AAKK)$_4$	nd	
20	**TCGAC**TCTCATCCTT-(AEEA)$_3$-TTCCTACTCT	nd	
21	**TCGAC**TCTCĀTCCTT-(AEEA)$_3$-TTCCTĀCTCT-D-(AAKK)$_4$	nd	
22	TCTCCTCCTT-(AEEA)$_3$-TTCCTACTCT	nd	
23	TCTCCTCCTT-(AEEA)$_3$-TTCCTĀCTCT-D-(AAKK)$_4$	12000	35
24	**TCGAC**TCTCCTCCTT-(AEEA)$_3$-TTCCTACTCT	nd	
25	**TCGAC**TCTCCTCCTT-(AEEA)$_3$-TTCCTĀCTCT-D-(AAKK)$_4$	26000	77
26	**TCAAC**TCTCCTCCTT-(AEEA)$_3$-TTCCTC̄CTCT	nd	
27	**TCĀAC**TCTCCTCCTT-(AEEA)$_3$-TTCCTCCTCT-D-(AAKK)$_4$	34000	101

[a] PNAs and PNA−peptides are listed from C to N termini. All PNAs have a single C-terminal lysine. Boldface bases are extensions; underlined bases are mismatched relative to target duplex DNA. k_{rel} values are relative to unmodified bisPNA **1**. nd: strand invasion not detected.

was used. After hybridization at 37 °C for 2 h, 3 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM UTP, 0.5 mM GTP, 0.0125 mM CTP (Ambion, Austin, TX), and 2 μCi of [α^{32}-P]CTP were added, and the in vitro transcription was initiated by adding 40 units of T7 polymerase (Ambion). After incubation at 37 °C for 1 h, the reaction was stopped by adding 1 μL of 0.5 M EDTA. The samples were mixed with an equal volume of formamide, heated at 95 °C for 3 min, and loaded on 5.5% polyacrylamide gels containing 8 M urea (Ameresco). The relative amount of transcribed RNA was quantified using a Molecular Dynamics Model 425F phosphorimager.

RESULTS AND DISCUSSION

Design of TC-BisPNAs and TC-BisPNA−Peptide Conjugates. We showed previously that attachment of PNAs to a cationic peptide, (D-AAKK)$_4$, containing D-amino acids dramatically increases strand invasion (*28*) and inhibition of transcription (*32*). Several laboratories have reported that attachment of peptides to PNAs can promote cellular uptake (*14*, *33*−*35*). More recently, we have found that peptide (D-AAKK)$_4$ also introduces PNAs into cultured cells and that antisense PNA−(D-AAKK)$_4$ conjugates targeted to mRNA selectively inhibit gene expression (Kaihatsu, unpublished results). These data from experiments using cells demonstrate that biologically significant quantities of the PNA−(D-AAKK)$_4$ conjugate enter cells, that attachment of the peptide does not prevent hybridization to a nucleic acid target inside cells, and that attachment of the peptide does not lead to toxicity or obvious non-sequence-selective phenotypes.

The favorable properties of peptide (D-AAKK)$_4$ suggested that it might also improve strand invasion by TC-PNAs. To facilitate comparisons, we synthesized PNAs with and without the (D-AAKK)$_4$ peptide and with and without the tail-clamp extension. PNAs and PNA−peptide conjugates were obtained by automated synthesis, routinely purified as a single product by HPLC, and characterized by mass spectral analysis. The two strands of the bisPNAs were connected by three 2-(aminoethoxy)-2-ethoxyacetic acid linker molecules. Gel mobility shift assays were used to visualize the binding of PNA to duplex DNA and calculate association rate constants (Table 1). In all experiments pH was neutral, and PNAs were present at a concentration of 125 nM, 10-fold above the concentration of duplex DNA.

Recognition of Duplex DNA by TC-BisPNAs. To evaluate the effect of adding tail-clamp extensions to bisPNAs, we compared recognition of duplex DNA by TC-PNAs and nonextended bisPNAs (NE-bisPNAs) that do not contain tail-clamp sequences. NE-bisPNA **1** invaded duplex DNA in a gel shift assay with a k_{obs} value of 340 M^{-1} s^{-1}, but analogous TC-bisPNA **3** that contained a tail clamp consisting of two purine and three pyrimidine bases did not detectably bind to DNA (Figure 2A, Table 1).

The failure of TC-bisPNA **3** to recognize duplex DNA indicated that additional chemical modifications would be necessary; so to facilitate binding, we attached cationic peptide (D-AAKK)$_4$ to afford TC-bisPNA peptide conjugate **4**. This modification allowed **4** to bind with a k_{obs} value of 35000 M^{-1} s^{-1}, only 2.7-fold less than the k_{obs} of strand invasion of the analogous NE-bisPNA−peptide conjugate **2**.

We then examined DNA recognition in the presence of $MgCl_2$ and KCl because the ability to invade duplex DNA in the presence of mono- and divalent cations is likely to be critical for recognition of chromosomal DNA. We observed that TC-bisPNA−peptide conjugate **4** was able to invade duplex DNA in the presence of up to 7 mM $MgCl_2$ or 75 mM KCl (Figure 3), concentrations of cations similar to those found inside cells. The tolerance of the high ionic strength

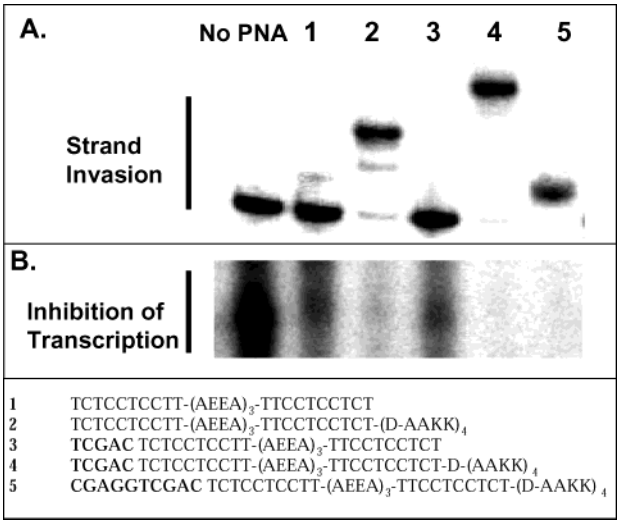

FIGURE 2: (A) Recognition of duplex DNA by bisPNAs and bisPNA−peptide conjugates **1**−**5** monitored by a gel shift assay. (B) Inhibition of transcription by bisPNAs and bisPNA−peptide conjugates **1**−**5**. PNAs were present at 125 nM, a 10-fold excess over the concentration of target DNA (12.5 nM). Boldface bases in PNAs **3**−**5** constitute the tail clamp.

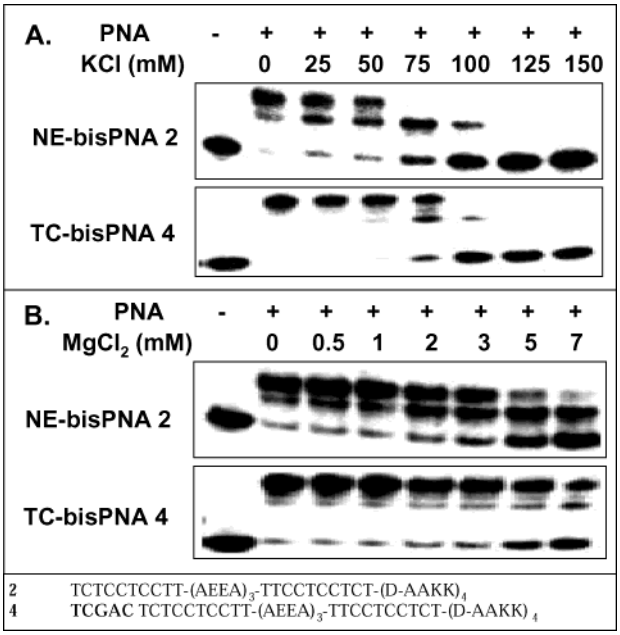

FIGURE 3: Recognition of duplex DNA by NE-bisPNA−peptide conjugate **2** and TC-bisPNA−peptide conjugate **4** in the presence of increasing concentrations of (A) KCl and (B) $MgCl_2$. Recognition was monitored by a gel shift assay. PNAs were present at 125 nM, a 10-fold excess over the concentration of target DNA (12.5 nM). Boldface bases in **4** constitute the tail-clamp region. Complexes were formed during a 2 h incubation at 37 °C in 10 mM sodium phosphate and 1 mM EDTA, pH 6.9, in the presence of the listed concentration of the mono- or divalent cation.

of TC-bisPNA **4** was similar to that of NE-bisPNA−peptide conjugate **2**. In some of these data it is apparent that more than one species is present. The ability of bisPNAs to produce multiple bands upon gel shift analysis has been analyzed by Nielsen and co-workers and attributed to the binding of bisPNAs in multiple geometries (*36*).

Recognition of Duplex DNA by a TC-PNA with a Ten-Base Tail Clamp. To further test the potential of tail clamps, we synthesized TC-bisPNA−peptide conjugate **5** that contained a ten-base tail clamp consisting of six purine and four pyrimidine bases (Table 1, Figure 2A). TC-bisPNA−peptide conjugate **5** invaded duplex DNA, but the k_{obs} value for **5** was 860 M^{-1} s^{-1}, approximately 40-fold lower than TC-bisPNA−peptide conjugate **4** that contained a five-base extension. These results demonstrate that PNAs containing tail clamps as long as ten bases can bind to duplex DNA but also suggest that increasing the length of extensions may lower the on-rate for strand invasion.

Inhibition of Transcription by TC-BisPNAs. After establishing the relative strand invasion properties of PNAs **1**−**5**, we evaluated their ability to inhibit transcription by phage T7 polymerase. We found that PNAs **1**−**5** each inhibited transcription to varying degrees. The most potent inhibition was achieved by PNAs **2**, **4**, and **5** (Figure 2B), results consistent with the efficient association with duplex DNA by these PNAs during the gel shift assay (Figure 2A).

We were more surprised to note inhibition of transcription by NE-bisPNA **1** and TC-bisPNA **3** because the gel shift assay had revealed little or no binding of DNA by these PNAs. These data, however, are consistent with findings by Larsen and Nielsen that had demonstrated that bisPNAs with four or five positive charges could inhibit transcription under conditions where strand invasion does not occur (*37*). They attributed this observation to the PNA binding to the single-stranded transcription bubble formed during RNA synthesis, a recognition mode that would make strand invasion unnecessary. Binding of PNAs to the transcription bubble would also be consistent with results from Sigman and colleagues that modified DNA oligonucleotides can block transcription by targeting the open complex (*38*).

DNA Binding and Inhibition of Transcription by Short TC-BisPNAs. If the length of the PNA strand that binds is increased using Watson−Crick base pairs, tail clamps may stabilize hybridization and enhance the ability of bisPNAs to bind to polypurine−polypyrimidine tracts that are relatively short. Increasing the ability to bind to relatively short polypurine−pyrimidine sequences is important because it would add to the number of sites within a promoter that can be targeted, making it more likely that a promising sequence will exist near a transcription factor binding site.

To test whether tail clamps can assist the binding of short bisPNAs, we synthesized PNAs **6**−**9** that were analogous to PNAs **1**−**4** but contain eight rather than ten bases in each pyrimidine arm of the bisPNA (Figure 4). NE-bisPNA **6** and TC-bisPNA **8** did not invade duplex DNA (Figure 4A). This result was not surprising because analogous bisPNAs **1** and **3** that contained ten pyrimidine bases in each arm (Figure 2A) had also recognized DNA poorly, and we would not have expected shorter PNAs to do better.

To achieve significant recognition of duplex DNA, we followed the same strategy described above and synthesized PNA conjugates with cationic peptide (D-AAKK)$_4$ to afford NE-bisPNA−peptide conjugate **7** and TC-bisPNA−peptide conjugate **9**. We found that association of **7** and **9** with DNA could now be readily observed. However, the k_{obs} values for **7** and **9** were 1400 and 2400 M^{-1} s^{-1}, respectively (Table 1), significantly below the k_{obs} values for analogous bisPNAs **2** and **4** (94000 and 35000 M^{-1} s^{-1}, respectively) that contained ten bases in each arm. Apparently, the length of the polypyrimidine core affects the rate at which DNA

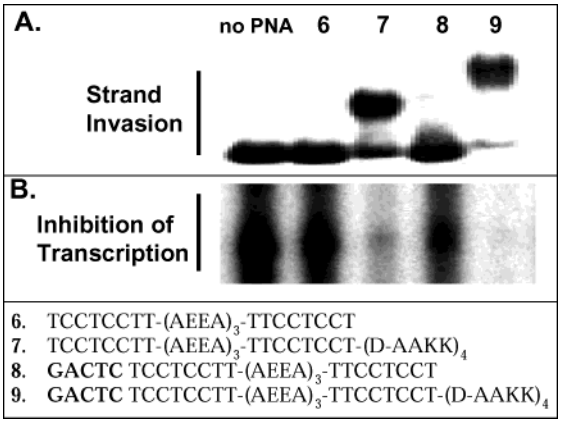

FIGURE 4: (A) Recognition of duplex DNA by bisPNAs **6** and **8** and bisPNA−peptide conjugates **7** and **9** monitored by a gel shift assay. (B) Inhibition of transcription by bisPNAs **6** and **8** and bisPNA−peptide conjugates **7** and **9**. PNAs were present at 125 nM, a 10-fold excess over the concentration of target DNA (12.5 nM). Boldface bases in **8** and **9** constitute the tail clamp.

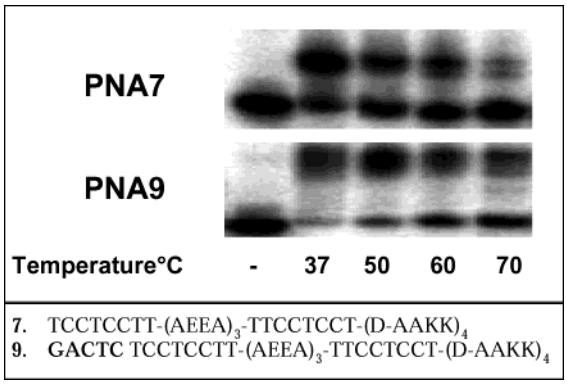

FIGURE 5: Stability of recognition of duplex DNA monitored by gel shift assays of eight-base bisPNAs with (PNA **9**) and without (PNA **7**) a tail clamp as a function of temperature.

recognition successfully initiates. Inhibition of transcription by PNAs **6**−**9** reflected their ability to invade duplex DNA, with **7** and **9** being more efficient inhibitors than **6** or **8** (Figure 4B).

To further address whether the tail clamp improves the stability of hybridization of relatively short bisPNAs, we examined the ability of NE-bisPNA **7** and TC-bisPNA **9** to recognize duplex DNA during incubations at varied temperatures (Figure 5). We observed that TC-bisPNA−peptide conjugate **9** was able to maintain binding at temperatures as high as 70 °C, whereas NE-bisPNA−peptide conjugate **7** was less efficient both at 70 °C and at lower temperatures. This observation suggests that the tail-clamp modification can confer a significant degree of additional stability when PNAs are relatively short and is consistent with findings by Nielsen and co-workers (*39*).

Recognition of Duplex DNA and Inhibition of Transcription by TC-PNAs Containing Just One Pyrimidine Stand. Single-stranded polypyrimidine PNAs can also invade duplex DNA, albeit at a slower rate than bisPNAs (*26*). To test whether single-stranded TC-PNAs can also invade duplex DNA, we compared DNA recognition and inhibition of transcription by PNAs **10**−**13** which contain from zero to five base extensions to a ten-base polypyrimidine core.

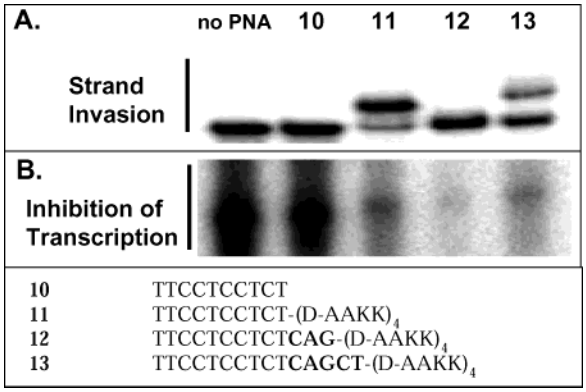

FIGURE 6: (A) Recognition of duplex DNA by single chain PNA **10** and single chain PNA−peptide conjugates **11**−**13**, monitored by a gel shift assay. (B) Inhibition of transcription by single chain PNA **10** and single chain PNA−peptide conjugates **11**−**13**. PNAs were present at 125 nM, a 10-fold excess over the concentration of target DNA (12.5 nM). Boldface bases in **12** and **13** constitute the tail clamp.

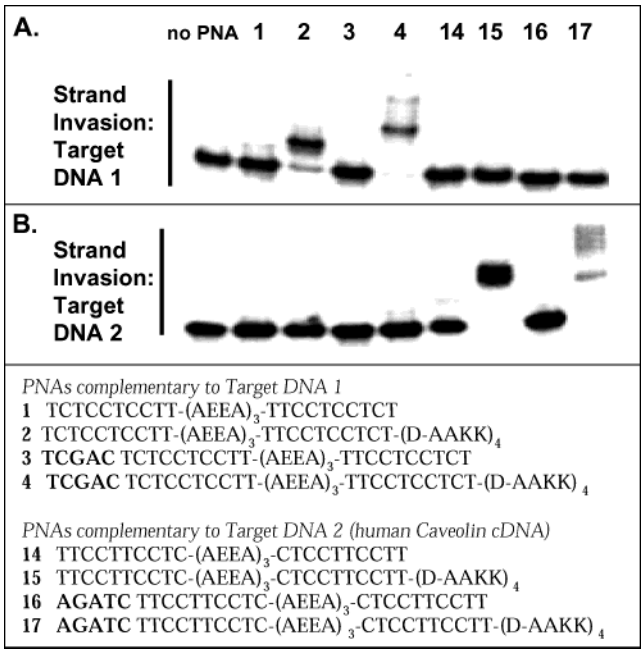

FIGURE 7: Specificity of binding to two different target DNAs by bisPNAs and bisPNA−peptide conjugates **1**−**4** and **14**−**17**. Target DNA **1** in (A) is a designed target sequence employed in previous studies of transcription factor recruitment (*20*), while target DNA **2** in (B) is derived from the genomic sequence of human caveolin 1 (*31*). PNAs were present at 125 nM, a 10-fold excess over the concentration of the target DNAs (12.5 nM). Boldface bases in **3**, **4**, **16**, and **17** constitute the tail clamp.

PNA **10** that did not contain an mixed base extension or peptide $(D\text{-AAKK})_4$ did not recognize DNA under the conditions used in our assays, but analogous PNA−peptide conjugate **11** could associate with DNA with a k_{obs} of 1600 M^{-1} s^{-1} (Figure 6A, Table 1). PNA−peptide **12** with a three-base tail clamp was not observed to associate with duplex DNA, but PNA−peptide **13** with a five-base tail clamp did bind with a k_{obs} value of 620 M^{-1} s^{-1}. These data demonstrate that even relatively simple single chain TC-PNA−peptide conjugates can bind duplex DNA. This result is significant because the ability to use easily synthesized single chain

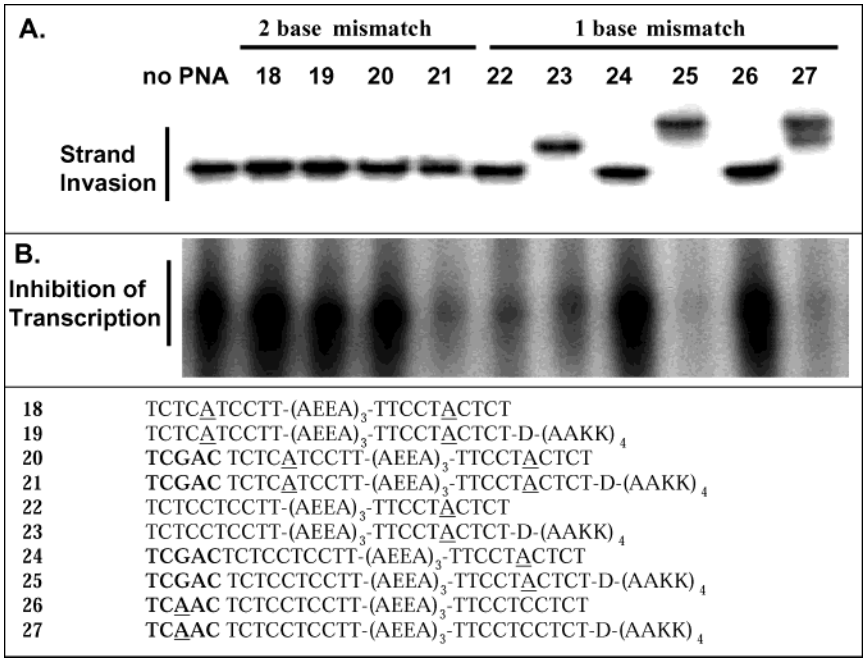

FIGURE 8: Effect on one- and two-base mismatches on (A) strand invasion monitored by a gel shift assay and (B) inhibition of transcription by PNAs and PNA−peptide conjugates. The sequences of PNA and PNA−peptide conjugates **18**−**27** are shown at the bottom of the figure. All PNA and PNA−peptide conjugates have a C-terminal lysine. Mismatched bases are underlined. PNAs were present at 125 nM, a 10-fold excess over the concentration of target DNA (12.5 nM). Boldface bases in **20**, **21**, and **25**−**27** constitute the tail-clamp region.

PNAs further expands the range of options for recognition of duplex DNA.

We also measured the ability of PNAs **10**−**13** to inhibit transcription (Figure 6B). PNA **10** which failed to associate with DNA also did not block transcription. Transcription was blocked by PNAs **11** and **13**, a result consistent with their ability to bind duplex DNA. As described above for bisPNAs **1** and **3**, we observed that lack of strand invasion was not equivalent to an inability to inhibit transcription. TC-PNA **12** effectively blocked RNA polymerization (Figure 6B) even though it did not appear to associate with duplex DNA in gel shift assays (Figure 6A).

Specificity of Target Recognition by TC-BisPNAs. To support the belief that TC-bisPNAs could be a general strategy for expanding recognition by PNAs, we also examined strand invasion by PNAs **14**−**17** that were targeted to the genomic sequence of human caveolin 1 (hCav-1) (*31*). We observed that bisPNAs **14**−**17** that were targeted to hCav-1 were unable to bind to the target DNA complementary to bisPNAs **1**−**4** (Figure 7A).

When we tested anti-hCav PNAs **14**−**17** for binding to duplex DNA containing the hCav-1 sequence, we observed that NE-bisPNA−peptide conjugate **15** and TC-PNA−peptide conjugate **17** readily hybridized to hCav-1 DNA with k_{obs} values (48000 and 5900 M^{-1} s^{-1}, respectively; Table 1) similar to those observed for bisPNAs **2** and **4** to their complementary target. BisPNA **14** and TC-bisPNA **16** that lacked an attached peptide did not appear to invade hCav-1 DNA. BisPNAs **1**−**4** were not able to bind to hCav-1 DNA (Figure 7B). Our data from two different duplex DNA targets, together with similar data from Nielsen and coworkers (*39*), are consistent with the conclusion that the recognition of duplex DNA by TC-bisPNA−peptide conjugates will be a general phenomenon.

Specificity of DNA Recognition and Inhibition of Transcription by TC-PNAs. To characterize the effect of mismatched bases on recognition by TC-PNAs, we investigated the effect of the introduced two mismatches into the pyrimidine core (PNAs **18**−**21**), one mismatch into the pyrimidine core (PNAs **22**−**25**) of the pyrimidine core or one mismatch into the tail clamp (PNAs **26** and **27**) (Figure 8A). When one purine base was introduced into each pyrimidine arm of NE-bisPNAs or TC-bisPNAs **18**−**21**, no strand invasion was observed regardless of whether the PNA contained an attached cationic peptide or a tail-clamp extension. This is consistent with our previous finding that a one-base mismatch in the DNA target prevents binding by bisPNAs and bisPNA−(D-AAKK)$_4$ conjugates (*28*).

These results suggest that the presence of mismatched PNA bases interferes with recognition of DNA, even when the cationic peptide is available to enhance binding and reinforces the conclusion that simply attaching a cationic peptide to a PNA is not sufficient for strand invasion. Only one of these mismatch-containing PNAs, TC-bisPNA−peptide conjugate PNA **21**, inhibited transcription (Figure 8B). As mentioned above and previously reported by Nielsen and colleagues (*13*), inhibition by PNA **21** may be due to binding to the open complex formed by T7 RNA polymerase, a task likely to be easier than binding to a closed duplex by strand invasion.

PNAs **22**−**27** that contained one mismatch exhibited varying abilities to invade DNA. PNAs **22**, **24**, and **26** that lacked an attached cationic peptide did not invade duplex DNA. Poor strand invasion by these PNAs was expected because their fully complementary analogues, NE-bisPNA **1** and TC-bisPNA **3**, also showed little or no association (Figure 2). By contrast, all of the PNAs with attached positively charged peptides, **23**, **25**, and **27**, associated with target DNA. k_{obs} values for **23**, **25**, and **27** were 12000, 26000, and 34000 s^{-1}, respectively, similar to k_{obs} for the analogous fully complementary NE-bisPNA−peptide **2** and TC-bisPNA−peptide **4** (Figure 8A, Table 1), suggesting that

the presence of a mismatched base within the Hoogsteen strand or within the tail clamp does not affect the kinetics of the association of bisPNA−peptide conjugates with DNA. Inhibition of transcription paralleled the results from the gel shift assay (Figure 8B).

These results with mismatch-containing PNAs suggest that hybridization of TC-PNAs can occur in the presence of one mismatch within the PNA but that specificity is much more stringent when two mismatches are present. Because attachment of the cationic peptide can increase the possibility of nonspecific binding, when using TC-PNA−peptide, it will be necessary to design PNAs to possess minimal complementarity to nontarget sequences. We note, however, that for TC-PNAs to yield a nonspecific phenotype inside cells they not only would have to bind nonspecifically but would need to do so at a site that would cause gene expression to be disrupted. The full impact of nonspecific binding on the production of phenotypes by antigene PNAs and PNA−peptide conjugates will await careful experimentation inside cells.

Implications for Targeting Chromosomal DNA within Cells and Applications of TC-PNAs. The ability of PNAs to bind to duplex DNA suggests that they are an exciting option for developing agents that can bind duplex DNA and inhibit gene expression. However, more than 10 years after the first demonstration of strand invasion by PNAs they have yet to become a routinely used tool for targeting chromosomal DNA. Among the challenges blocking more rapid progress is the need to optimize the efficiency of strand invasion to critical sequences within promoters. Efficient recognition is essential because lesser levels of hybridization are unlikely to yield levels of gene inhibition that would be discernible above background.

The results reported here suggest that TC-bisPNAs offer a strategy for increasing the size of a target sequence that can be disrupted by the binding of PNAs. Increased lesion size leads to a greater likelihood that strand invasion by PNAs will infringe on protein binding and that important protein functions can be disrupted. Nielsen and colleagues have independently investigated use of TC-PNAs (Nielsen, personal communication). Their studies focus on stand invasion by TC-PNAs containing polypyrimidine sequences that are shorter than the ones we have characterized. Using footprinting experiments they confirm that hexamer TC-bisPNAs can bind to duplex DNA and that strand invasion is enhanced by addition of positive charge. Unlike the hexameric or decameric bisPNAs we examine, the shorter PNAs display equilibrium binding allowing direct determination of K_d values, which are as low as 0.9 μM for a TC-bisPNA with five positive charges. This work is complementary to ours and reinforces the suggestion that TC-PNAs are a robust and general approach to recognition of duplex DNA. In particular, the studies suggest that short six-base pyrimidine core regions are adequate, increasing the number of genomic targets that might be accessible to TC-bisPNAs.

Taken together, our studies and those of Nielsen demonstrate that strand invasion by TC-PNAs can expand the versatility of strand invasion by PNAs. Developing robust protocols for targeting chromosomes inside cells is likely to be challenging, and the increased versatility demonstrated here should aid in the targeting of key sequences within promoters. Future goals for research include (i) intracellular delivery of antigene PNAs with efficiencies now achieved with antisense PNAs, (ii) optimization of nuclear uptake, and (iii) development of predictive rules for targeting chromosomal sequences inside cells.

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