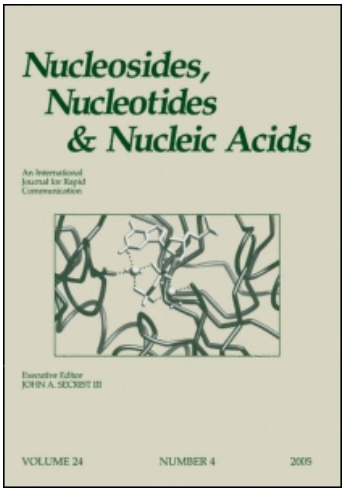

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Inhibition of Transcription by bisPNA-Peptide Conjugates

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Inhibition of Transcription by bisPNA-Peptide Conjugates

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ABSTRACT

Homopyrimidine bisPNAs have been reported to arrest transcription elongation by invading double-stranded DNA and forming a stable $(PNA)_2$/DNA complex. We previously reported that attachment of a designed cationic peptide to the bisPNA enhances the efficiency of strand invasion. Here we investigate whether conjugation to cationic peptides can also improve inhibition of transcription. We observe that a conjugate between a bisPNA and a peptide containing eight lysines is a superior agent for inhibition of transcription, but that inhibition of transcription is reduced as pH and the concentration of magnesium are increased. Our studies provide useful characterization of bisPNAs as agents for inhibiting transcription.

Key Words: Peptide nucleic acid; PNA; Peptide; Transcription inhibition.

INTRODUCTION

Peptide nucleic acid (PNA) is a DNA/RNA mimic that contains an amide backbone (Fig. 1).[1] In spite of this dramatic departure from the chemical composition of

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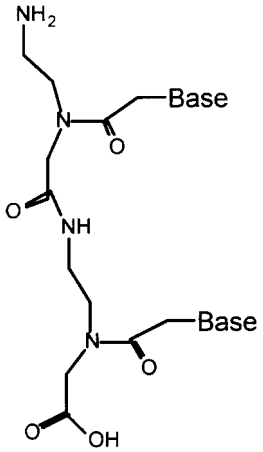

Figure 1. Structure of peptide nucleic acid (PNA) dimer.

DNA or RNA, PNAs are able to bind to complementary sequences by Watson-Crick base-pairing with high affinity and specificity.[2] The hybridization properties of PNA have been intensively investigated, and one of the most exciting findings has been that PNAs are superior agents for the recognition of DNA by strand invasion.[3]

To successfully invade duplex DNA, a PNA must bind to one strand of the duplex and displace the second strand. PNAs containing all four bases, A, C, G, and T, can readily invade supercoiled DNA,[4,5] but invasion of linear DNA is retarded by the absence of negative torsional stress. Polypyrimidine PNAs can, however, overcome the obstacles to strand invasion posed by the topology of linear DNA to form four-stranded complexes, in which one PNA strand binds by Watson-Crick base-pairing and the second strand binds by Hoogsteen base-pairing to the PNA-DNA duplex.[6–8] The rate of formation of this PNA-DNA complex can be greatly enhanced by reducing the entropic cost through linking PNA strands to form a bisPNA.[6]

This ability to invade duplex DNA sets PNA apart from other nucleic acid mimics or chemically optimized nucleic acids.[9] Strand invasion by PNAs might allow control of gene expression at the chromosome level, or permit mutations of chromosomal DNA to be efficiently directed to a specific location.[7] There have been several reports of PNAs binding duplex DNA and either inhibiting[10–13] or activating transcription.[14,15] However, to become a widely used strategy for recognizing chromosomal DNA, strand invasion by PNAs will have to be highly efficient and occur under physiological conditions.

We have previously reported that conjugates between bisPNAs and designed cationic peptides are superior agents for recognition of duplex DNA at neutral or basic pH and in solutions that contain concentrations of mono or di-valent cations similar to those found in cells.[16] We now investigate the influence of positive charge on the ability of PNAs to bind duplex DNA and block transcription.

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RESULTS AND DISCUSSION

Experimental Design

In previous work using a 90 base pair (bp) duplex DNA target, we discovered that attachment of peptide to the bisPNA molecule greatly increased the efficiency of strand invasion.[16] Improved strand invasion, however, may not necessarily correlate with improved inhibition of transcription, because the action of RNA polymerase may affect hybridization of the PNA. Therefore, to directly test inhibition of transcription by bisPNA peptide conjugates we introduced the target sequence from the 90 bp duplex into a duplex DNA fragment of 168 bp that contains a promoter for transcription by T7 RNA polymerase.

Strand Invasion by bisPNA-Peptide Conjugates

Before investigating the inhibition of transcription, we tested the ability of the three related PNAs to bind the longer 168 bp duplex (Table 1). One of these PNAs was conjugate bisPNA-D$(AAKK)_4$, which we had shown to be a superior agent for strand invasion in previous studies.[16] As a control, we also tested an analog of conjugate bisPNA-D$(AAKK)_4$ that contained mismatched bases. The third PNA tested was PNA-3K, a conjugate that contained three lysines. We chose to add three lysines because that design has been used by other workers in the field and has been demonstrated to yield efficient strand invasion.[7,17]

We assayed strand invasion at both pH 6.9 and 7.4. We chose pH 6.9 because it is optimal for strand invasion, while pH 7.4 is more favorable for transcription and provides a better test for hybridization under physiologic pH. As we had observed with the 90 bp duplex,[16] strand invasion by conjugate bisPNA-D$(AAKK)_4$ was superior to strand invasion by conjugate bisPNA-3K at pH 6.9 and pH 7.4 (Fig. 2). The bisPNA-D$(AAKK)_4$ conjugate containing mismatched bases did not

Table 1. Composition of the bisPNA and bisPNA-peptide conjugates used in these studies with their predicted and observed molecular weights.

	Molec. Wt.	
PNA	Calc.	Found
LysTCTCCTCCTT-$(AEEA)_3$-TTCCTCCTCT	5755	5755
LysTCTCCTCCTT-$(AEEA)_3$-TTCCTCCTCT-D$(AAKK)_4$	7349	7347
LysTCTCCTCCTT-$(AEEA)_3$-TTCCTCCTCT- 3K	6140	6142
LysTCTCATCCTT-$(AEEA)_3$-TTCCTACTCT	5804	5805
LysTCTCATCCTT-$(AEEA)_3$-TTCCTACTCT-D$(AAKK)_4$	7398	7397
LysTCCTCCTT-$(AEEA)_3$-TTCCTCCT	4721	4726
LysTCCTCCTT-$(AEEA)_3$-TTCCTCCT-D$(AAKK)_4$	6314	6322
LysTCCTCCTT-$(AEEA)_3$-TTCCTCCT-3K	5101	5113

D:D amino acids; K:Lysine; A:Alanine. Molecular weights were determined by MALDI-TOF mass spectroscopy. PNAs are listed from C to N termini.

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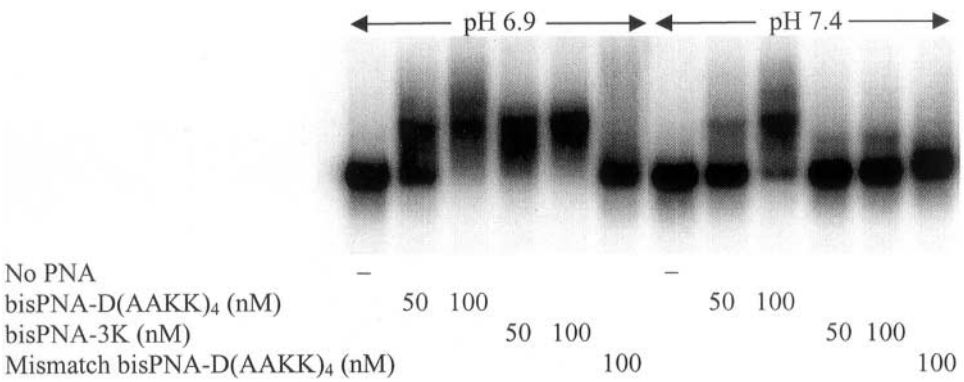

Figure 2. Strand invasion of bisPNA-peptide conjugates monitored by gel shift assay. The duplex DNA fragment (5 nM) was labeled with ^{32}P and incubations with PNA were carried out at pH 6.9 or pH 7.4.

bind to duplex DNA under any of the conditions tested, demonstrating that attachment of a cationic peptide does not cause indiscriminate nonspecific binding.

Inhibition of Transcription by bisPNAs

After establishing that strand invasion of the target 168 bp duplex was efficient, we examined the inhibition of transcription by bisPNA-D(AAKK)$_4$, bisPNA-3K, and a bisPNA containing only one lysine at its C-terminus (Fig. 3). In our first experiments, duplex DNA was incubated with bisPNA under conditions that are ideal for strand invasion, 37°C for 2 h, 10 mM Tris-Cl, pH 6.9. Nucleotide triphosphates (NTPs), [α-^{32}P]CTP and 3 mM $MgCl_2$ were added subsequent to incubation with PNA. Finally, transcription was initiated by adding T7 RNA polymerase to the reaction. This order of addition was chosen so that strand invasion could occur under optimal conditions of low pH and the absence of divalent cation, prior to raising the concentration of magnesium so that transcription by T7 RNA polymerase could proceed.

We observed that bisPNA-(AAKK)$_4$ and bisPNA-3K inhibited transcription more efficiently than the bisPNA with one lysine at its C-terminus (Fig. 3, lanes 2, 4, and 5). The analogous bisPNAs that contained mismatched bases did not inhibit transcription (Fig. 3, lanes 3 and 6). To further characterize improved inhibition of transcription by PNAs containing multiple cationic amino acids, we examined inhibition as a function of PNA concentration (Fig. 4). These dose response data indicated that bisPNA-D(AAKK)$_4$ or bisPNA-3K were efficient inhibitors at concentrations under 25 nM, while the bisPNA with just one lysine at the C terminus required concentrations greater than 50 nM. These data are consistent with the strand invasion data (Fig. 2) suggesting that attachment of positive charge improves DNA recognition. In these and subsequent experiments we did not observe a substantial accumulation of truncated products. The lack of truncated products is probably observed because only a small amount of radiolabel was incorporated into

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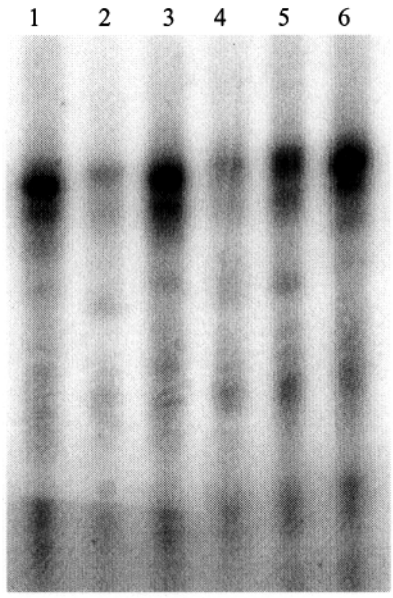

Figure 3. Effect of addition of bisPNAs and bisPNA-peptide conjugates on transcription. Complexes were formed at 37°C for 2 h in 5 mM NaCl, 10 mM Tris-Cl, pH 6.9, with 5 nM DNA duplex and 100 nM bisPNA. Mg^{2+} (final concentration of 3 mM) and NTPs were added after binding and in vitro transcription was allowed to proceed at 37°C for 1 h.

them prior to termination and because their small size made them more susceptible to degradation by nucleases.

Influence of pH and Ionic Strength on Transcription Inhibition

To be useful in vivo, strand invasion will need to occur under conditions of physiologic ionic strength and pH. Therefore, we examined transcription under more stringent conditions, with PNA added after addition of divalent Mg^{2+} and in solutions at pH 7.4. Addition of divalent cations has the potential to reduce strand invasion by stabilizing the target duplex and raising the energetic cost of strand separation. Raising the pH decreases protonation of cytosine, reduces the efficiency of triplex formation, and lessens formation of the three stranded PNA-DNA-PNA complex.

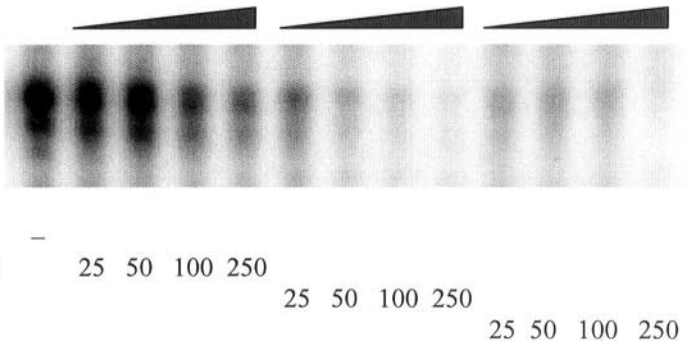

Figure 4. Effect of increasing the concentration of bisPNA and bisPNA-peptide on the inhibition of transcription. Complexes were formed at 37°C for 2 h in 5 mM NaCl, 10 mM Tris-Cl, pH 6.9, with 5 nM DNA duplex and 100 nM bisPNA. Mg^{2+} (final concentration of 3 mM) and NTPs were added after binding and in vitro transcription was allowed to proceed at 37°C for 1 h.

We observed that both bisPNA-D(AAKK)$_4$ and bisPNA-3K were able to inhibit transcription at pH 6.9 in the presence of 3 mM $MgCl_2$ (Fig. 5 A). Differences in the efficiency of inhibition between bisPNA-3K and bisPNA-D(AAKK)$_4$ were not apparent unless 5 mM $MgCl_2$ was included (Fig. 5 B). Addition of NaCl in addition to $MgCl_2$ did not significantly decrease the inhibition of transcription, suggesting that monovalent cations have less impact on recognition than do divalent cations (Fig. 6). At pH 7.4 transcription was not inhibited when $MgCl_2$ was present during annealing regardless of whether bisPNA, bisPNA-(AAKK)$_4$ or bisPNA-3K were used (Fig. 5 A). These data show that, at pH 6.9, inhibition of transcription tolerates high concentrations of $MgCl_2$ and NaCl, but that at pH 7.4 inhibition is much more sensitive to the presence of divalent cations. Reduced inhibition of transcription in the presence of divalent cations is consistent with previous reports.[12]

Inhibition of Transcription by Shorter bisPNAs

To further explore the relationship between strand invasion and inhibition of transcription we synthesized shorter PNAs that contained only eight bases in each arm (Table 1). We first examined strand invasion and demonstrated that the bisPNAs could bind to the target duplex, and that binding was more efficient at pH 6.9 than at pH 7.4, and that attachment of peptide D(AAKK)$_4$ promoted binding more than attachment of three lysines. We then examined transcription in the presence of $MgCl_2$ at pH 6.9 and 7.4, but did not observe significant levels of inhibition upon addition of the shorter bisPNAs. These data suggest that the shorter bisPNAs can bind duplex DNA, but this binding is not stable enough to allow inhibition of transcription even when transcription is allowed to proceed at a pH, pH 6.9, that is optimal for strand invasion.

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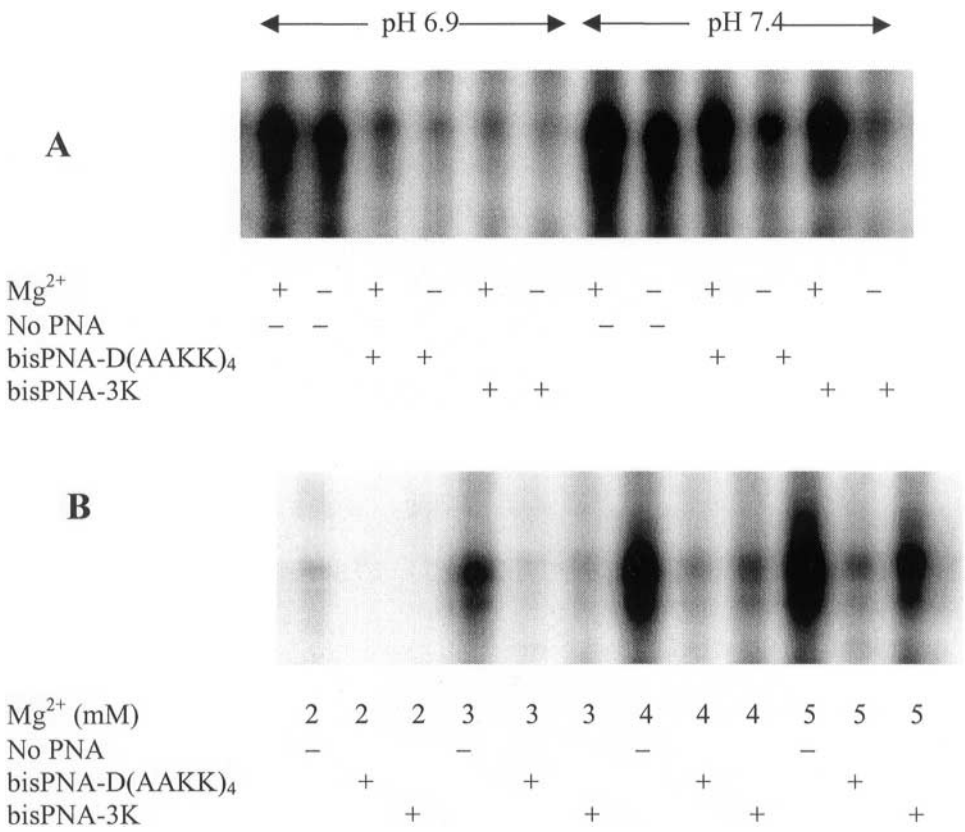

Figure 5. Influence of pH and the concentration of Mg^{2+} on the efficiency of transcription inhibition by bisPNA or bisPNA-peptide conjugates. Part A). Complexes were formed with 5 nM duplex DNA and 100 nM bisPNA or bisPNA-peptide in 10 mM Tris-Cl buffer at pH 6.9 or pH 7.4. The solution also contained 5 mM NaCl and was incubated at 37°C for 2 h with or without 3 mM Mg^{2+} as indicated. After binding, Mg^{2+} concentration was adjusted to 3 mM final and NTPs and T7 enzyme were added. Transcription was allowed to proceed at 37°C for 1 h. Part B). Complexes were formed with 5 nM duplex DNA and 100 nM bisPNAs in 10 mM Tris-Cl, pH 6.9, with 5 mM NaCl and indicated concentrations of Mg^{2+} at 37°C for 2 h. NTPs and T7 enzyme were added after binding. Transcription was allowed to proceed at 37°C for 1 h.

Conclusions

The ability of PNAs to inhibit transcription has been well established,[10–13] our goal in these experiments was to characterize inhibition, correlate it with strand invasion, and test whether its efficiency can be improved by simple chemical modifications. We observed that attachment of positive charge to a bisPNA improves the inhibition of transcription, and that bisPNA-D(AAKK)$_4$ is a slightly more effective agent than bisPNA-3K. Inhibition of transcription tolerates increased pH or ionic strength, but falls off dramatically when both pH and ionic strength is

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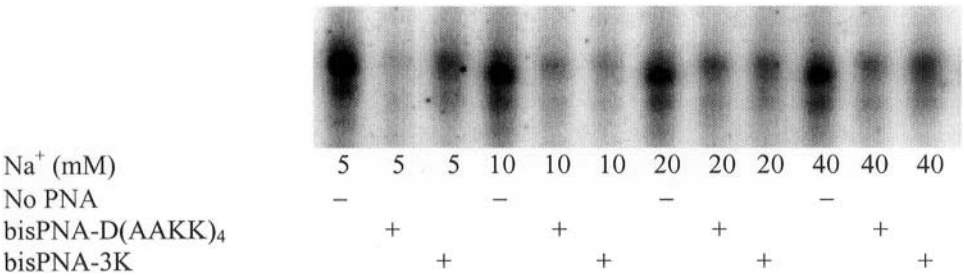

Figure 6. Influence of Na^+ on efficiency of transcription inhibition by bisPNA and bisPNA-peptide conjugates. Complexes were formed in 10 mM Tris-Cl, pH 6.9, with 3 mM $MgCl_2$ present and the indicated concentrations of Na^+ at 37°C. DNA and PNA were incubated for 2 h, after which NTPs and T7 enzyme were added. Transcription was allowed to proceed at 37°C for 1 h.

increased, or when PNA length is decreased. Our results demonstrate that inhibition of transcription can be efficient, but that ionic strength, pH, and bisPNA length are critical variables.

We note that addition of positive charges increases strand invasion substantially but only marginally increases the ability of PNAs to inhibit transcription. Why is this correlation poor? One possible explanation is that the open complex created during transcription is much more susceptible to PNA binding than is linear DNA itself, allowing easier access to PNAs that lack extensive chemical modifications. This is not a new hypothesis, having been put forth in 1996 by Larsen and Nielsen.[13] Further studies to explore the implications of this phenomenon are underway.

EXPERIMENTAL SECTION

Synthesis of PNAs and PNA-Peptide Conjugates

PNA monomers Fmoc-T-OH, Fmoc-C(Bhoc)-OH and Foc-A(Bhoc)-OH, linker molecule Fmoc-AEEA-OH, activators of base coupling O-(7-azabenzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), and 1-hydroxy-7-aza-benzotriazole (HoAt) were obtained from Applied Biosystems (Foster City, CA). Fmoc-XAL-PEG-PS resin, amino acid monomers Fmoc-Lys(Boc)-OH, Fmoc-D-Ala-OH, and Fmoc-D-Lys(Boc)-OH were from Novabiochem (Leufelfingen, Switzerland)

PNAs were synthesized on an Expedite 8909 synthesizer (Applied Biosystems), using standard fluorenylmethoxycarbonyl (Fmoc) chemistry.[18] After completion of synthesis, PNA-peptide conjugates were cleaved from the resin by trifluoroacetic acid (TFA), purified by HPLC on a C18 reverse phase column and analyzed by time-of flight mass spectrometry (MALDI-TOF).[18] PNA concentrations were quantified based on spectrometric A_{260} values using the conversion factor of molar extinction coefficients: 8800 (T), 6600 (C), 13700 (A) $M^{-1}cm^{-1}$.

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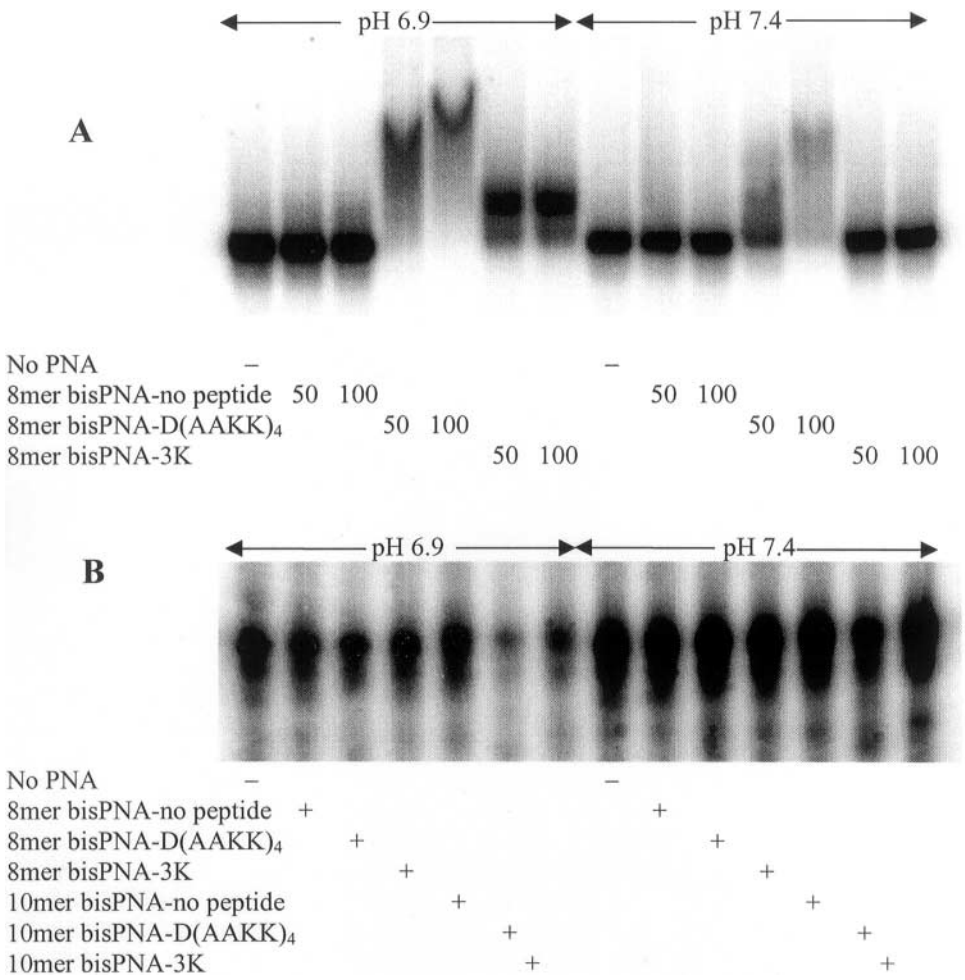

Figure 7. Strand invasion by a shorter bisPNA and bisPNA peptide conjugates, and their effect on transcription. PNAs contained either eight or ten bases in each arm. Part A) Strand invasion of duplex DNA (5 nM) by 50 or 100 nM eight base bisPNA, bisPNA-D(AAKK)$_4$ or bisPNA-3K. PNAs were incubated with duplex DNA for 2 h at 37°C in 10 mM Tris-Cl buffer at pH 6.9 or pH 7.4. with 5 mM NaCl, 3 mM $MgCl_2$ and NTPs. Part B) Inhibition of transcription by eight base (per arm) or ten base (per arm) bisPNA and bisPNA/peptide conjugate. Complexes were formed using 5 nM duplex DNA and 100 nM PNAs at 37°C for 2 h in 10 mM Tris-Cl, pH 6.9 buffer with 5 mM NaCl, 3 mM Mg^{2+} and NTPs present. T7 RNA polymerase was added after binding. Transcription was allowed to proceed at 37°C for 1 h.

Preparation of Duplex DNA Target

A 168 bp DNA duplex fragment which contains the PNA target sequence of 5′-aaggaggaga-3′ was PCR amplified from a modified pUC 18 vector.[19] The primers

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used were 5′-taatacgactcactatagggtgctgcaaggcgattaag-3′ and 5′-aattaaccctcactaaagggttttatatacatacagagc-3′ (Sigma Genosys). The underlined part within the primer encodes for a T7 promoter which did not anneal to the template vector but provide enough recognition sequence for T7 RNA polymerase. The PCR product was purified by 1% agarose gel electrophoresis and extracted from agarose gel by QIAquick Gel Extraction Kit (Qiagen, Valencia CA). Concentrations of double stranded DNA were quantified based on spectrometric A260 values and the conversion factor of 50 μg/mL/OD.

End Labeling of Duplex DNA

DNA fragments that were used for the gel shift assay, were end-labeled by standard procedures using [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Sigma, St. Louis MO). The incorporated free [γ-^{32}P] ATP was removed using Bio-Spin 6 chromatography columns (Biorad, Hercules CA)

Strand Invasion by PNAs and Gel Shift Assay

Hybridization of duplex DNA and bisPNA was accomplished by mixing 5 nM γ-^{32}P labeled dsDNA with 10–20 equivalents of PNA in 10 mM Tris-Cl (adjust pH with HCl to 6.9 or 7.4) and 5 mM NaCl for 2 h at 37°C. PNAs were heated at 75°C for 3 min and then cooled down to 37°C gradually before adding to the DNA solution to prevent any possible aggregation of PNA molecules during storage. Experiments were performed in siliconized tubes to reduce nonspecific binding of PNAs and increase the reproducibility of experiments.

A gel shift assay was used to separate double-stranded DNA duplexes that were bound to PNA from unbound duplex DNAs. After incubation, 5 μL 80% glycerol was added to each hybridization reaction, and the mixture of DNA and DNA/PNA complex were loaded onto the gel and electrophoresed at 250 volts for 2 h on a 8% nondenaturing polyacrylamide gel using 1 × TBE (Ameresco, Solon OH) as a running buffer. The products were visualized by autoradiography and quantified by using a Molecular Dynamics (Sunnyvale, CA) model 425F phosphorimager.

Strand Invasion by PNAs and In Vitro Transcription Experiments

Hybridization of duplex DNA and bisPNA was accomplished by the same protocol used for the gel shift assay except that unlabeled duplex DNA 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 [α-^{32}P]CTP were added and the in vitro transcription was initiated by adding 40 units T7 polymerase (Ambion). After incubation at 37°C for 1 h, the reaction was stopped by adding 1 μL 0.5 M EDTA. The samples were mixed with equal volume of formamide, heated at 95°C for 3 min and loaded on 5.5% polyacrylamide gels containing 8 M urea

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(Ameresco), and electrophoresed at 250 volts for 2 h using 1 × TBE as running buffer. The RNA transcripts were visualized by autoradiography and quantified by using a Molecular Dynamics model 425F phosphorimager

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