Enhanced Strand Invasion by Peptide Nucleic Acid−Peptide Conjugates†

Kunihiro Kaihatsu, Dwaine A. Braasch, Ahmet Cansizoglu, and David R. Corey*

Departments of Pharmacology and Biochemistry, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, Texas 75390-9041

Received June 27, 2002; Revised Manuscript Received July 18, 2002

ABSTRACT: Efficient and selective recognition of DNA by proteins is due to sequence-specific interactions with a target site and nonselective electrostatic interactions that promote the target's rapid location. If synthetic molecules could mimic these functions, they would render a wide range of chromosome sequences accessible to rationally designed probes. Here we describe conjugates between bispeptide nucleic acids (bisPNAs) designed to specifically recognize duplex DNA and peptides that have been designed to promote rapid sequence recognition. Peptide design was based on the surface of staphylococcal nuclease, a cationic DNA binding protein with low sequence selectivity. We observe that attachment of the designed peptide increases rates of strand invasion by 100-fold relative to unmodified bisPNA. The peptide can contain D-amino acids, increasing the likelihood that it will be stable in cell extract and inside cells. Binding of the conjugate containing the D-amino acid peptide occurred over a broad range of experimental conditions and was sensitive to a single mismatch. Strand invasion was efficient at neutral to basic pH, a wide range of temperatures (0−65 °C), and in the presence of up to 7 mM Mg^{2+} and 100 mM Na^+ or K^+. Our data suggest that attachment of peptides that mimic cationic protein surfaces to PNAs can afford conjugates that mimic the rapid and selective binding that characterizes native DNA binding proteins. Rapid strand invasion over a wide range of experimental conditions should further expand the utility of strand invasion by PNAs.

Synthetic molecules that bind duplex DNA have the potential to selectively control gene expression or induce mutations at specific sites. To succeed, such agents must mimic natural DNA binding proteins that combine a high affinity for their "correct" target sites with a lower affinity for similar "incorrect" sites. This combination allows proteins to scan duplex DNA and rapidly locate and bind to critical control sequences (*1*, *2*). A synthetic option for duplex recognition is peptide nucleic acid (PNA)[1] (Figure 1A), an RNA/DNA mimic in which the phosphate deoxyribose backbone has been replaced by a neutral amide backbone composed of *N*-(2-aminoethyl)glycine linkages (*3*−*6*).

PNA offers many advantages for molecular recognition, including high-affinity binding (*3*−*6*), good mismatch discrimination (*7*), resistance to digestion by nucleases or proteases (*8*), low affinity for proteins (*9*), and a high propensity for strand invasion of duplex DNA (*3*, *4*, *10*, *11*). PNA is well suited for strand invasion because there is no electrostatic repulsion between an incoming neutral PNA and the negatively charged strands of the duplex DNA target sequence, facilitating the initiation and subsequent maintenance of the invasion complex. Applications of strand invasion by PNAs include purification of genomic DNA (*12*, *13*), cleavage of DNA (*14*), creation of artificial primosomes (*15*), inhibition of transcription (*16*, *17*), activation of transcription (*18*, *19*), site-directed mutagenesis of chromosomal DNA (*20*), noncovalent labeling of plasmids with fluorophores (*21*), recruitment of transcription factors to artificial promoters (*22*), and recognition of double-stranded DNA by molecular beacons (*23*).

Strand invasion of relaxed DNA by PNAs occurs at polypurine−polypyrimidine sequences through formation of a four-stranded complex in which one PNA strand binds by Watson−Crick base pairing while a second PNA strand binds to the PNA−DNA hybrid by Hoogsteen pairing (*3*, *4*, *10*, *11*, *24*−*30*). The entropic price of binding can be reduced and duplex recognition made more rapid by tethering the two PNA strands to form a bisPNA (*30*). Even when bisPNAs are used, however, strand invasion requires relatively long incubation times and high concentrations of PNA. Efficient strand invasion is hindered at high pH, which weakens Hoogsteen interactions by the PNA. Duplex recognition is also almost completely eliminated by the presence of moderate concentrations of mono- and divalent cations that reduce electrostatic repulsion between the DNA strands, stabilize the parent duplex, and prevent the initiation of strand invasion.

Griffith (*30*), Nielsen (*31*), Frank-Kamenetskii (*32*), and Glazer (*20*) have appended three or four lysines to bisPNA strands and have observed that the recognition of linear DNA was significantly accelerated. Nielsen and Frank-Kamenetskii also demonstrated that PNAs with three lysines at the carboxyl termini accelerated hybridization while preserving a high level of kinetic sequence discrimination (*32*). Hairpin polyamides containing pyrrole, imidazole, and hydroxypyrrole amino acids have also been shown to bind with higher

† This work was supported by grants from the National Institutes of Health (GM 60624) and the Robert A. Welch Foundation (I-1244).

* To whom correspondence should be addressed. E-mail: david.corey@utsouthwestern.edu.

[1] Abbreviations: PNA, peptide nucleic acid; AEEA, 2-(aminoethoxy)-2-ethoxyacetic acid.

10.1021/bi0263659 CCC: $22.00
Published on Web 08/21/2002

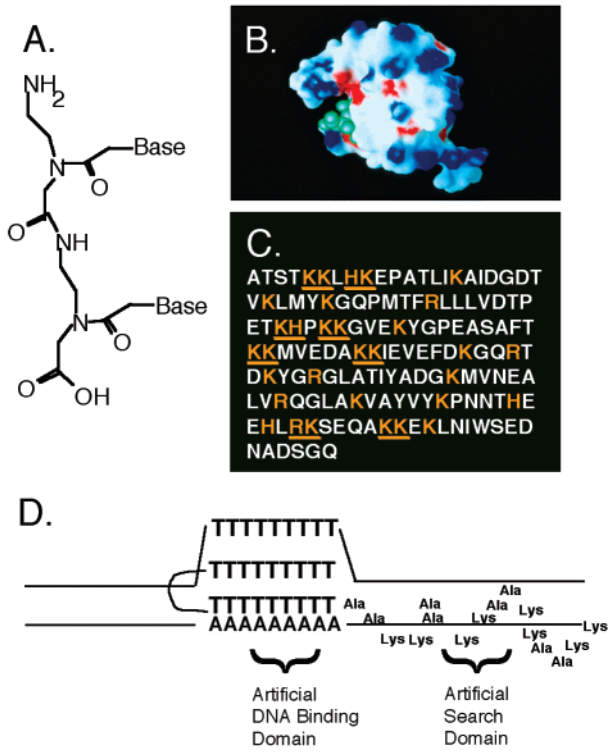

FIGURE 1: (A) Structure of PNA. (B) Electrostatic surface of staphylococcal nuclease. Positive residues are in blue, negative residues are in red, and the bound inhibitor 3′,5′-diphosphothymidine is in green and marks the active site. (C) Sequence of staphylococcal nuclease with residues that are positively charged at neutral pH highlighted. (D) Scheme for recognition of a duplex DNA target by a bisPNA−peptide chimera.

affinity when attached to positively charged dimethylamino substituents (*33*).

Here we test the hypothesis that a peptide sequence that mimics the charge distribution on the protein surface can further enhance strand invasion of linear DNA by bisPNAs. We demonstrate that attachment of cationic peptides based on the surface of staphylococcal nuclease enhances the hybridization of bisPNAs to polypurine−polypyrimidine sequences with linear DNA. Strand invasion is more rapid and occurs over a much broader the range of pH, ionic strength, and temperature. Efficient hybridization of PNA−peptide conjugates should lead to improved applications for PNA.

MATERIALS AND METHODS

Synthesis of PNAs and PNA−Peptides. PNA monomers, Fmoc-T-OH, Fmoc-C(Bhoc)-OH, and Fmoc-A(Bhoc)-OH were obtained from Applied Biosystems (Foster City, CA). Linker molecule Fmoc-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 Novabiochem (Laufelfingen, Switzerland). Amino acid monomers Fmoc-Ala-OH, Fmoc-Lys(Boc)-OH, Fmoc-D-Ala-OH, and Fmoc-D-Lys(Boc)-OH, Fmoc-His(Trt)-OH, and Fmoc-Arg(Pbf)-OH were from Novabiochem.

PNAs were synthesized on an Expedite 8909 synthesizer (Applied Biosystems, Foster City, CA) using standard fluorenylmethoxycarbonyl (Fmoc) chemistry (*34*, *35*). The Expedite synthesizer has three reagent ports that are not used for routine PNA synthesis, and these were used to directly add amino acid residues alanine and lysine at the N-terminus of the completed PNA. The conjugate between the bisPNA and peptide RHGEKWLDDFTNNQMDQDY contains more than three different amino acids. In this case the resin containing the completed PNA was transferred from the Expedite synthesizer to a Symphony peptide synthesizer (Rainin, Waltham, MA) for peptide addition. After completion of synthesis, the resin was taken off the synthesizer, and PNA−peptide conjugates were cleaved by trifluoroacetic acid (TFA) (*34*, *35*). PNAs were purified by reversed-phase HPLC on a C18 column and analyzed by time-of-flight mass spectrometry (MALDI-TOF) as described (*34*, *35*). PNA concentrations were quantified on the basis of spectrometric A_{260} values using the conversion factor of molar extinction coefficients: 8800 (T), 6600 (C), and 13700 (A) M^{-1} cm^{-1}.

Preparation of Duplex DNA Targets. 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 and in bold face) were obtained from Dr. Bo Liu and Dr. Thomas Kodadek (UT Southwestern) (*22*). Another pair of synthetic oligonucleotides encoding one pyrimidine at the center of the polypurine PNA binding site (5′ gcgcggcagt cacgacgttg taaaacgacg gccagtgcca agcttga***aag gatgaga***gtc gacctcgagg catgtgctct gtatcgcgcc 3′ and 5′ ggcgcgatac agagcacatg cctcgaggtc gactctcatc ctttcaagct tggcactggc cgtcgtttta caacgtcgtg actgccgcgc 3′; target site underlined and in bold face) were obtained from Sigma Genosys.

Each pair of complementary oligonucleotides was annealed and 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 Inc.) using standard procedures. Double-stranded DNA concentrations were quantified on the basis of spectrometric A_{260} values and the conversion factor of 50 μg/mL 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).

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 and 1 mM EDTA (adjusted to the described pH) 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 in solutions containing varying concentrations of mono- or divalent cations, the required concentrations of NaCl, KCl, or $MgCl_2$ were added to γ^{32}-P-labeled dsDNA in 10 mM sodium phosphate (pH 6.9 or 7.4). Incubations were performed at 37 °C for 30 min prior to addition of PNA or PNA−peptide conjugates. 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% 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.

RESULTS AND DISCUSSION

Design and Synthesis of BisPNA−Peptide Chimera. BisPNAs were designed to be complementary to a polypurine−polypyrimidine site within linear DNA. BisPNAs were used rather than single PNA strands because tethering the two PNAs reduces the entropic penalty paid during formation of the four-stranded invasion complex and increases the rate of hybridization (*30*).

Peptide sequences were added to the N-termini of bisPNAs after completion of PNA synthesis. The peptide sequence L(AAKK)$_4$ was chosen after examining the surface of staphylococcal nuclease (*36*) and noting the repeated pairing of positively charged residues (Figure 1B,C). We had previously shown that this peptide could promote strand invasion of supercoiled DNA by mixed sequence PNAs, but these conjugates were unable to detectably bind linear DNA (*34*). von Hippel (*37*) and Record (*38*) have noted that the binding to DNA can induce α-helix formation by very similar peptides, suggesting that secondary structure may play a role in recognition. The peptide D(AAKK)$_4$ was selected to test the effect of amino acid stereochemistry. Use of D-amino acids also makes the peptide less susceptible to protease degradation and would facilitate eventual use of the conjugates in cell extracts and inside cells.

The role of the bisPNA domain was to achieve sequence-specific binding to duplex DNA, while the cationic peptide was intended to increase the rate of hybridization through non-sequence-specific electrostatic interactions (Figure 1D). BisPNAs and analogous bisPNA−peptide conjguates were synthesized by automated methods, and their identity was confirmed by mass spectral analysis (Table 1). The PNA strands were connected by three 2-(aminoethoxy)-2-ethoxy-acetic acid (AEEA) linker molecules, and all bisPNA and bisPNA−peptide conjugates contained a single lysine residue at the C-terminus of the PNA.

Strand Invasion by BisPNAs and BisPNA−Peptide Chimera. We compared strand invasion of bisPNAs modified with L(AAKK)$_4$ and D(AAKK)$_4$ with a bisPNA labeled with a peptide with no overall charge and an unmodified bisPNA

Table 1: Composition of the PNA and PNA−Peptides Used in These Studies with Their Predicted and Observed Molecular Weights[a]

PNA	mol wt calcd	mol wt found
LysTCTCCTCCTT-(AEEA)$_3$-TTCCTCCTCT	5755	5755
LysTCTCCTCCTT-(AEEA)$_3$-TTCCTCCTCT-(AEEA)$_3$-RHGEKWFLDDFTNNQMDQDY	8731	8729
LysTCTCCTCCTT-(AEEA)$_3$-TTCCTCCTCT-L(AAKK)$_4$	7349	7344
LysTCTCCTCCTT-(AEEA)$_3$-TTCCTCCTCT-D(AAKK)$_4$	7349	7347
LysTCTCATCCTT-(AEEA)$_3$-TTCCTACTCT	5803	5814
LysTCTCATCCTT-(AEEA)$_3$-TTCCTACTCT-D(AAKK)$_4$	7398	7403
LysTCTCCTCCTT-(AEEA)$_3$-TTCCTCCTCT-L(KKK)	6140	6142
LysTCTCCTCCTT-(AEEA)$_3$-TTCCTCCTCT-L(AAKK)	6154	6162
LysTCTCCTCCTT-(AEEA)$_3$-TTCCTCCTCT-L(AAKK)$_2$	6552	6558
LysTCTCCTCCTT-(AEEA)$_3$-TTCCTCCTCT-L(AAKK)$_3$	6951	6955
LysTCTCCTCCTT-(AEEA)$_3$-TTCCTCCTCT-L(AAHH)$_4$	7421	7425
LysTCTCCTCCTT-(AEEA)$_3$-TTCCTCCTCT-L(AARR)$_4$	7573	7577

[a] PNA−peptide conjugate sequences are listed from C- to N-termini.

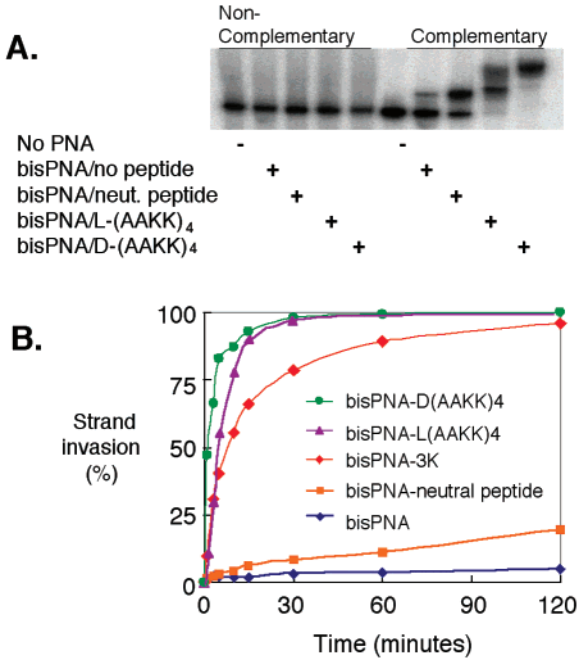

FIGURE 2: (A) Recognition of partially and fully complementary duplex DNA (12.5 nM) by unmodified bisPNA, bisPNA−neutral peptide conjugate, bisPNA-L(AAKK)$_4$, and bisPNA-D(AAKK)$_4$ (125 nM). Duplex DNA was labeled with ^{32}P. (B) Recognition as a function of time of fully complementary DNA (12.5 nM) by a 5-fold excess of unmodified bisPNA, a PNA−neutral peptide conjugate, bisPNA-3K, PNA-L(AAKK)$_4$, and PNA-D(AAKK)$_4$ (62.5 nM each). Complexes were formed at 37 °C in pH 6.9 10 mM sodium phosphate and 1 mM EDTA buffer. Bound and unbound complexes were separated by a nondenaturing polyacrylamide gel shift assay.

(Figure 2A). The neutral peptide was RHGEKWLDDFTNNQMDQDY, which also contained eight charged residues and had been identified through a selection for the ability to bind the transcription factor Gal 4 (*22*). PNAs were present in 10-fold excess over the radiolabeled DNA target, and incubations were allowed to proceed for 2 h at 37 °C and pH 6.9. Binding was evaluated using a nondenaturing polyacrylamide gel shift assay, with bound PNA causing duplex DNA to migrate more slowly.

We observed that PNAs conjugated to L(AAKK)$_4$ or D(AAKK)$_4$ bound duplex DNA much more efficiently than the unmodified bisPNA or the bisPNA−neutral peptide

Table 2: Rate Association Constants (M^{-1} s^{-1}) for Strand Invasion by BisPNA and BisPNA−Peptide Conjugates at pH 6.9 or 7.4 in the Presence of 0 or 3 mM $MgCl_2$[a]

	pH 6.9				pH 7.4			
	0 mM $MgCl_2$		3 mM $MgCl_2$		0 mM $MgCl_2$		3 mM $MgCl_2$	
PNA	K_{obs}	k_{rel}	K_{obs}	k_{rel}	K_{obs}	k_{rel}	K_{obs}	k_{rel}
bisPNA	3.4×10^2	1	nd	−	nd	−		
bisPNA neutral peptide	1.3×10^3	3.9	nd	−				
bisPNA-3K	1.2×10^4	35	nd	−	3.5×10^3	10	nd	−
bisPNA-L(AAKK)$_4$	3.7×10^4	110	4.7×10^3	14	1.6×10^4	47	7.2×10^2	2.1
bisPNA-D(AAKK)$_4$	9.0×10^4	268	1.8×10^4	53	3.6×10^4	107	1.5×10^3	5.6
bisPNA-L(AAKK)	9.8×10^3	29						
bisPNA-L(AAKK)$_2$	2.4×10^4	71						
bisPNA-L(AAKK)$_3$	3.1×10^4	92						
bisPNA-L(AAHH)$_4$	1.4×10	43	nd	−	nd	−		
bisPNA-L(AARR)$_4$	2.5×10^4	74	1.7×10^3	5	1.7×10^4	50	nd	−

[a] nd: k_{obs} too low, not detected. k_{rel} values are calculated relative to k_{obs} for unmodified bisPNA at pH 6.9. Blank spaces indicate that assays were not performed. All PNAs have the sequence LysTCTCCTCCTT-(AEEA)$_3$-TTCCTCCTCT. The sequence of the neutral peptide is RHGEKWFLDDFTNNQMDQDY.

conjugate (Figure 2A). In these and subsequent data it is apparent from inspection of the mobility of the PNA−DNA complexes that more than one species is present. The ability of bisPNAs to produce multiple bands upon gel shift analysis has been noted by Nielsen and co-workers, and these have been attributed to multiple binding geometries and the presence of more than one bisPNA per complex (*29*).

Neither the bisPNA nor the bisPNA−peptide conjugates showed any association with a duplex DNA that lacked a fully complementary binding site (Figure 2A). Similarly, a bisPNA-D(AAKK)$_4$ conjugate that contains a single purine base is also not able to associate detectably with the duplex DNA (results not shown). These data demonstrate a high degree of mismatch discrimination for binding to our DNA target sequence under conditions (pH 6.9, low ionic strength) that are optimal for binding and in the presence of high concentrations of PNA.

Effect of Peptide Composition on Rate Constant Strand Invasion. Improved binding by conjugates containing PNAs attached to designed peptides L- or D(AAKK)$_4$ relative to the unmodified bisPNA or the neutral peptide−PNA conjugate encouraged us to explore binding by PNA−peptide conjugates in detail. We first examined the time dependence of strand invasion at pH 6.9, a pH that previous studies had identified as optimal (*24*). To put binding by PNA−peptide conjugates into proper context with prior work in the field, we also monitored strand invasion by a bisPNA with three L-lysines attached to the N-terminus (bisPNA-3K). Positively charged PNAs with three consecutive lysines had been noted to possess improved properties in numerous prior studies (*20*, *30*−*32*) and represent a standard against which attempts to improve strand invasion by bisPNAs should be judged.

We observed that attachment of the designed cationic peptides D- or L(AAKK)$_4$ significantly increases the observed rates of PNA association with duplex DNA relative to the unmodified bisPNA, the bisPNA−neutral peptide conjugate, or bisPNA-3K (Figure 2B). The rank order of hybridization efficiency and observed rate association constants (k_{obs}, M^{-1} s^{-1}) were bisPNA-D(AAKK)$_4$, 9.0×10^4, > bisPNA-L(AAKK)$_4$, 3.7×10^4, > bisPNA-3K, 1.2×10^4, ≫ bisPNA−neutral peptide, 1.3×10^3, > bisPNA, 3.4×10^2 (Table 2).

To further understand the specificity of amino acid determinants that contribute to enhanced strand invasion, we also obtained rate constants for PNAs attached to two L(AAKK), four L(AAKK)$_2$, or six lysines L(AAKK)$_3$. We observed that the rate constants varied with charge, with k_{obs} decreasing as the number of lysines was reduced (Table 2). We also examined strand invasion by PNAs attached to peptides containing eight arginine L(AARR)$_4$ or histidine L(AAHH)$_4$ residues. We observed a slight decrease in k_{obs} for strand invasion by these conjugates relative to the analogous conjugate containing lysine (Table 2).

The observation that bisPNA-D(AAKK)$_4$ hybridized 2.5-fold better than bisPNA-L(AAKK)$_4$ suggests that the natural stereochemical configuration is not necessary for enhanced strand invasion. Likewise, the similar effects on strand invasion produced by attachment of peptides L(AAKK)$_4$, L(AAHH)$_4$, and L(AARR)$_4$ at pH 6.9 support the conclusion that the number of charged residues, rather than their identity, is the primary determinant of the efficiency of strand invasion by PNA−peptide conjugates.

These initial studies were performed under conditions that are optimal for strand invasion by unmodified bisPNAs. To investigate the performance of PNA−peptide conjugates under less favorable and more physiologically relevant conditions, we assayed strand invasion at varied pH, ionic strength, and temperature. We focused on bisPNA-D(AAKK)$_4$ for further study because its greater resistance to proteolysis is likely make it a better candidate for use in cell extracts and inside cells.

Stand Invasion as a Function of pH. Stand invasion of duplex DNA by bisPNAs is dependent on one of the PNA strands binding by triple helix formation. One general limitation of triple helix formation is that its efficiency decreases as pH is raised, and the efficiency of strand invasion by bisPNAs has been observed to fall off sharply above pH 6.9 as Hoogsteen base pairing is disrupted (*21*). Consistent with these previous results, we observed almost no association of the unmodified bisPNA with duplex DNA above pH 6.9, while bisPNA-3K bound the duplex at 50% efficiency at pH 7.4 (Figure 3). The PNA-D(AAKK)$_4$ conjugate, by contrast, was able to efficiently hybridize with 100% efficiency at pH 7.4 and 80% efficiency at pH 7.8 (Figure 3). Improved binding by bisPNA-D(AAKK)$_4$ demonstrates that attachment of a designed cationic peptide significantly widens the range of pH values that permit the recognition of duplex DNA.

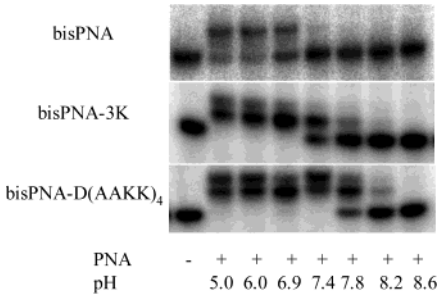

FIGURE 3: Strand invasion of duplex DNA (12.5 nM) by bisPNA (250 nM), bisPNA-3K (125 nM), and bisPNA-D(AAKK)$_4$ (125 nM) at pH 5, 6, 6.9, 7.4, 7.8, 8.2, and 8.6. PNAs were incubated with duplex DNA for 2 h at 37 °C at the indicated pH in 10 mM sodium phosphate and 1 mM EDTA buffer.

We also examined the time dependence of strand invasion at pH 7.4. k_{obs} values were reduced 3.6-, 2.3-, 2.5-, and 1.5-fold for conjugates bisPNA-3K, bisPNA-L(AAKK)$_4$, bisPNA-D(AAKK)$_4$, and bisPNA-L(AARR)$_4$, respectively (Table 2), relative to the analogous values at pH 6.9. Since the modest change in pH is unlikely to significantly alter the protonation state of lysine or arginine, reduced strand invasion by the bisPNAs that contain these amino acids is probably due to loss of protonation for Hoogsteen base pairing. By contrast, the change in pH is likely to affect the protonation of histidine, which possesses a pK_a of 7, and strand invasion of the bisPNA conjugate containing the peptide (AAHH)$_4$ was reduced to undetectable levels. The lack of strand invasion by bisPNA-L(AAHH)$_4$ at pH 7.4 provides further support for the conclusion that peptide charge is an critical determinant for enhanced binding.

Frank-Kamenetskii and colleagues have proposed that strand invasion by bisPNAs proceeds by a route that requires Hoogsteen base pairing prior to Watson−Crick base pairing (*28*). This mechanism suggests that the initiation of strand invasion should be sensitive to experimental conditions that affect triplex formation and is consistent with the observation that binding by the unmodified bisPNA does not occur above pH 6.9. Our finding that attachment of peptide D(AAKK)$_4$ permits efficient strand invasion at pH 7.8 suggests that positive charge can stabilize the initial triple helical complex. If attachment of the peptide were promoting Watson−Crick base pairing, we would not expect to see an improvement in strand invasion, since Watson−Crick pairing should be unaffected by pH changes between 6.9 and 7.8. Pseudoisocytosine has been shown to enhance Hoogsteen base pairing strand invasion by bisPNAs (*31*). This base was not available to us, and had it been incorporated into our PNAs, it is likely that even more substantial improvements would have been observed.

Persistence of Strand Invasion at Elevated pH. We also tested the influence of high pH on dissociation of bound bisPNA−DNA complexes. Strand invasion complexes consisting of duplex DNA and the unmodified bisPNA, bisPNA-3K, or bisPNA-D(AAKK)$_4$ that were formed at pH 6.9 were only partially dissociated after 2 h at pH 8.2 (results not shown). This observation suggests that low pH is more important for the initiation of strand invasion than for its maintenance and that attachment of peptide D(AAKK)$_4$ does not affect the rate of dissociation at high pH.

Strand Invasion as a Function of the Concentration of Mono- and Divalent Cations. To be useful in vivo or in cell extracts, strand invasion will need to occur in solutions containing physiologic concentrations of mono- and divalent cations. Such cations stabilize duplex DNA by masking phosphate−phosphate repulsion, slow the initiation of base pairing, and hinder strand invasion by PNAs (*10*).

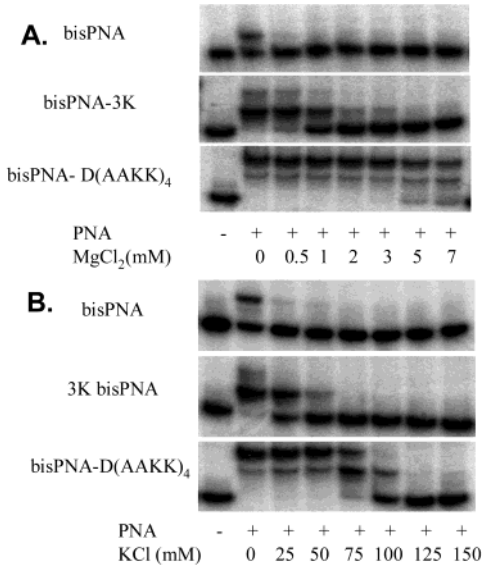

FIGURE 4: Stand invasion of duplex DNA (12.5 nM) by bisPNA (250 nM), bisPNA-3K (125 nM), and conjugate bisPNA-D(AAKK)$_4$ (125 nM) as a function of the concentration of (A) 0, 0.5, 1, 2, 3, 5, and 7 mM $MgCl_2$ and (B) 0, 25, 50, 75, 100, 125, and 150 mM KCl. PNAs were incubated with duplex DNA for 2 h at the indicated ionic strength.

One important physiologic divalent cation is magnesium, and we found that the presence of >0.5 mM $MgCl_2$ blocked strand invasion by the unmodified bisPNA (Figure 4A). BisPNA-3K performed only slightly better, with little hybridization observed in the presence of >1 mM $MgCl_2$. Hybridization by the PNA-D(AAKK)$_4$ conjugate, by contrast, continued to be efficient in the presence of $MgCl_2$ concentrations as high as 7 mM (Figure 4A) and was only reduced to 30% efficiency at 10 mM $MgCl_2$ (data not shown). We then monitored strand invasion in the presence of 3 mM Mg^{2+} as a function of time to determine k_{obs} values (Table 2). Even after 2 h incubations, strand invasion by the unmodified bisPNA, the bisPNA−neutral peptide conjugate, or bisPNA-3K was too low to allow calculation of rate constants. By contrast, strand invasion by bisPNA-L(AAKK)$_4$ and bisPNA-D(AAKK)$_4$ could be readily measured, with k_{obs} values (M^{-1} s^{-1}) of 4.7×10^3 and 1.8×10^4, respectively. Strand invasion by bisPNA-L(AARR)$_4$ and bisPNA-(AAHH)$_4$ was reduced to low or undetectable levels, respectively (Table 2). When strand invasion was tested in the presence of 3 mM $MgCl_2$ and at pH 7.4, binding by bisPNA-3K was not detected, while binding by bisPNA-D(AAKK)$_4$ and bisPNA-L(AAKK)$_4$ occurred with k_{obs} values of 79 and 154 (M^{-1} s^{-1}), respectively (Table 2).

Potassium and sodium are physiologically important monovalent cations. We observed that the unmodified bisPNA did not readily hybridize in the presence of KCl while bisPNA-3K showed less than 25% hybridization at 50 mM KCl. Hybridization by the bisPNA-D(AAKK)$_4$, by contrast, occurred at 90% efficiency at 75 mM KCl and 25% efficiency in the presence of 100 mM KCl (Figure 4B). When binding was performed in the presence of NaCl, attachment

of peptide D(AAKK)$_4$ yielded a similar improvement of invasion relative to the unmodified bisPNA or bisPNA-3K (results not shown).

These data are significant because they demonstrate that strand invasion by the bisPNA-D(AAKK)$_4$ conjugate can overcome the helix stabilization afforded by physiologically relevant concentrations of mono- or divalent cations. This capability should enhance the potential of PNAs for efficient recognition of specific sequences in vivo and in cell extracts. It is worth noting that increased ionic strength has the potential to mask the interactions between the cationic peptide and the phosphate backbone of the duplex, reducing the value of these interactions for strand invasion. Apparently, such masking interactions, if they occur, are not sufficient to prevent improved strand invasion at higher ionic strength.

Persistence of Strand Invasion at High Ionic Strength. One explanation for the increased ability of bisPNA-D(AAKK)$_4$ to hybridize in the presence of mono- or divalent cations is that the peptide promotes the initiation of strand invasion. Alternatively, higher ionic strength might be less able to destabilize the bound complex and promote dissociation of the PNA when peptide D(AAKK)$_4$ is present. To discriminate among these possibilities, we first annealed the unmodified bisPNA, bisPNA-3K, and bisPNA-D(AAKK)$_4$ to duplex DNA at low ionic strength to achieve maximal strand invasion. We then added magnesium, potassium, or sodium cations and measured the stability of the bound PNA−DNA complexes. In all cases, addition of cation after binding had occurred did not alter the efficiency of strand invasion (results not shown). These data support the conclusion peptide D(AAKK)$_4$ promotes formation of the strand invasion complex in the presence of cations but is not necessary for maintenance of the preformed complex. The stability of the bound complex formed by unmodified PNAs in the presence of cations is consistent with previous reports (*10*, *27*, *39*).

Strand Invasion as a Function of Temperature. To elucidate whether an attached peptide can expand the range of temperatures that permit strand invasion, we incubated the unmodified bisPNA, bisPNA-3K, and the bisPNA-D(AAKK)$_4$ conjugate at varied temperatures prior to analysis. We observed striking differences. The bisPNA-D(AAKK)$_4$ conjugate was able to efficiently hybridize at temperatures as high as 65 °C, while hybridization by the unmodified bisPNA or bisPNA-3K peptide was not apparent above 37 and 45 °C, respectively (Figure 5A). We also obtained the k_{obs} value for strand invasion at 55 °C by bisPNA-D(AAKK)$_4$ and found that it was similar to the k_{obs} value at 37 °C. The ability to hybridize by strand invasion at elevated temperatures should assist protocols that use PNA recognition in vitro.

Persistence of Strand Invasion at Elevated Temperatures. The greatly enhanced ability to bind at elevated temperatures by the bisPNA-D(AAKK)$_4$ conjugate may be due to stabilization of the bound complex. To test this hypothesis, we first incubated the unmodified bisPNA, bisPNA-3K, and bisPNA-D(AAKK)$_4$ at 37 °C to achieve binding and then raised the incubation temperature to 65 °C for 2 h to examine whether binding could persist (Figure 5B). In striking contrast to the experiments described above in which we examined dissociation at high pH and ionic strength and observed no effect of peptide D(AAKK)$_4$, at high temperatures we

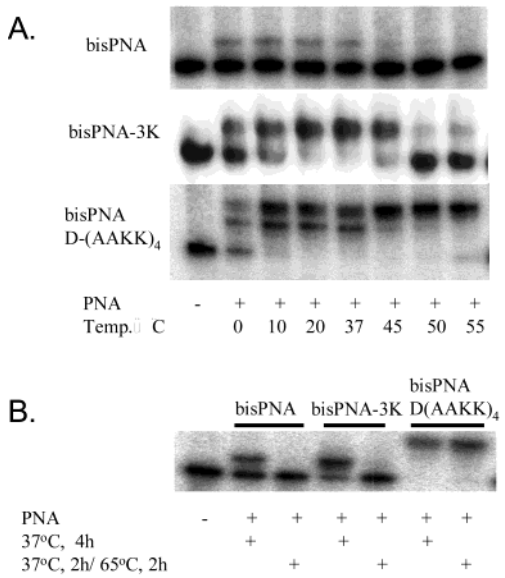

FIGURE 5: Effect of incubation temperature on strand invasion. (A) Strand invasion of duplex DNA (12.5 nM) by bisPNA (250 nM), bisPNA-3K (125 nM), and conjugate bisPNA-D(AAKK)$_4$ (125 nM) at 0, 10, 20, 37, 45, 50, and 55 °C. (B) Effect of 65 °C incubation temperature on a hybridized complex. BisPNA (250 nM), bisPNA-3K (125 nM), and bisPNA-D(AAKK)$_4$ (125 nM) were incubated with duplex DNA (12.5 nM) at 37 °C for 2 h to allow the PNA−DNA complex to form, followed by a subsequent incubation at 65 °C for 2 h.

observed that strand invasion by the unmodified bisPNA or by bisPNA-3K was reversed upon incubation at 65 °C, whereas stand invasion by bisPNA-D(AAKK)$_4$ persisted. The observation that strand invasion by the unmodified bisPNA and bisPNA-3K was not stable at elevated temperatures is in contrast to the finding that binding of the unmodified bisPNAs and the bisPNA−peptide conjugates to single-stranded DNA was characterized by melting temperature (T_m) values greater than 80 °C (results not shown). One reason for hybridization of PNAs to duplex DNA being destabilized relative to binding to single-stranded DNA is the potential of the strand invasion complex to re-form the parent duplex, an alternate structure that is irrelevant for single-stranded targets. Dissociation of bisPNAs bound to duplex DNA upon incubation at 65 °C has also been observed by Frank-Kamenetskii and co-workers (*40*).

Contribution of Positive Charge to Recognition of Duplex DNA and Enhanced Strand Invasion. Protein surfaces that interact with DNA are enriched in cationic amino acids by 2−3-fold relative to similarly sized surfaces that interact with proteins, with lysine and arginine accounting for 41% of the interface area of 65 complexes between double-stranded DNA and proteins (*41*). Another study of 129 protein−DNA complexes in the Protein Data Bank revealed that lysine and arginine are the leading sources of hydrogen bonds to the phosphate backbone and the DNA bases and also make more van der Waals contacts and water-mediated bonds than do other amino acids (*42*).

Thus it is clear that positively charged residues from multiple types of interactions with DNA make a major contribution to the affinity and selectivity of recognition. Our results are consistent with the hypothesis that peptide D(AAKK)$_4$ functions similarly by promoting the initiation of recognition and then by stabilizing the bound complex

once it is formed. We also believe that other cationic peptides may facillitate strand invasion as well or better than the ones we describe here and that their discovery will require a deeper understanding of the mechanism of strand invasion by bisPNA−peptide conjugates. Weakly interacting cationic domains can also act to increase the rate at which DNA sequences are searched (*43*), but this effect is probably not critical for the observed acceleration of strand invasion by $_D(AAKK)_4$ because the DNA target site used in these studies is relatively short.

Attachment of positively charged "transport" peptides has been noted to promote the cellular uptake of PNAs (*44*, *45*) and other molecules (*46*), suggesting that cationic peptides could play a dual role by increasing both membrane translocation and strand invasion. Efficient strand invasion has also been achieved by PNAs containing pseudocomplementary bases that are able to bind both strands of the duplex without binding to each other (*47*, *48*). Designed peptides similar to the ones we describe may also be able to improve this promising approach to duplex recognition. We note, however, that the attached peptide may lessen the specificity of binding.

Applications of Enhanced Strand Invasion. PNAs are one of several nucleic acids and nucleic acid mimics that have attracted attention as promising agents for molecular recognition and control of cellular processes. Other approaches including RNAi, locked nucleic acid (LNA), 2′-*O*-alkyl RNA, and morpholino oligomers appear to be useful for antisense gene inhibition (*49*). PNA, however, stands out for its ability to invade duplex DNA. This strand invasion might allow artificial gene activation, silencing, or mutation in vivo or provide powerful options for technical innovation in cell-free systems. To be widely useful, however, strand invasion will need to be rapid and occur under a wide range of conditions. The ability to improve the efficiency of strand invasion over broad ranges of pH, temperature, and ionic strength should make successful application of PNAs more likely and facilitate use of PNAs for specific recognition of chromosomal DNA.

ACKNOWLEDGMENT

We thank Dr. Bo Liu for advice on performing gel shift assays.

REFERENCES

1. von Hippel, P. H., Revzin, A., Gross, C. A., and Wang, A. C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4808−4812.
2. Berg, O. G., and von Hippel, P. H. (1989) *J. Biol. Chem. 264*, 675−678.
3. Nielsen, P. G., Egholm, M., Berg, R. H., and Buchardt, O. (1991) *Science 254*, 1497−1500.
4. Hanvey, J. C., Peffer, N. J., Bisi, J. E., Thomson, S. A., Cadilla, R., Josey, J. A., Ricca, D. J., Hassman, C. F., Bonham, M. A., Au, K. G., Carter, S. G., Bruckenstein, D. A., Boyd, A. L., Nobel, S. A., and Babiss, L. E. (1992) *Science 258*, 1481−1500.
5. Larson, H. J., Bentin, T., and Nielsen, P. E. (1999) *Biochim. Biophys. Acta 1489*, 159−166.
6. Ray, A., and Norden, B. (2000) *FASEB J. 14*, 1041−1060.
7. 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) *Nature 365*, 566−568.
8. Demidov, V. V., Potaman, V. N., Frank-Kamenetskii, M. D., Egholm, M., Buchardt, O., Sonnichsen, S. H., and Nielsen, P. E. (1994) *Biochem. Pharmacol. 48*, 1310−1313.
9. Hamilton, S. E., Iyer, M., Norton, J. C., and Corey, D. R. (1996) *Bioorg. Med. Chem. Lett. 6*, 2897−2900.
10. Cherny, D. Y., Belotserkovskii, B. P., Frank-Kamenetskii, M. D., Egholm, M., Buchardt, O., Berg, R. H., and Nielsen, P. E. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1667−1670.
11. Nielsen, P. E. (2001) *Curr. Med. Chem. 8*, 545−550.
12. Boffa, L. C., Carpaneto, E. M., and Allfrey, V. G. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1901−1905.
13. Seeger, C., Batz, H.-G., and Orum, H. (1997) *BioTechniques 23*, 512−516.
14. Footer, M., Egholm, M., Kron, S., Coull, J. M., and Matusdaira, P. (1996) *Biochemistry 35*, 10673−10679.
15. Demidov, V. V., Broude, N. E., Lavrentieva-Smolina, I. V., Kuhn, H., and Frank-Kamenetskii, M. D. (2001) *ChemBioChem 2*, 133−139.
16. Vickers, T. A., Griffith, M. C., Ramasmay, K., Risen, L. M., and Freier, S. M. (1995) *Nucleic Acids Res. 23*, 3003−3008.
17. Larsen, H. J., and Nielsen, P. E. (1996) *Nucleic Acids Res. 24*, 458−463.
18. Mollegaard, N. E., Buchardt, O., Egholm, M., and Nielsen, P. E. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3892−3895.
19. Wang, G., Jing, K., Balczon, R., and Xu, X. (2001) *J. Mol. Biol. 313*, 933−940.
20. Faruqi, A. F., Egholm, M., and Glazer, P. M. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 1398−1403.
21. Zelphati, O., Liang, X., Nguyen, C., Barlow, S., Sheng, S., Shao, Z., and Felgner, P. L. (2000) *BioTechniques 28*, 304−315.
22. Liu, B., Han, Y., Corey, D. R., and Kodadek, T. K. (2002) *J. Am. Chem. Soc. 124*, 1838−1839.
23. Kuhn, H., Demidov, V. V., Coull, J. M., Fiandaca, M. J., Gildea, B. D., and Frank-Kamenetskii, M. D. (2002) *J. Am. Chem. Soc. 124*, 1097−1103.
24. Kuhn, H., Demidov, V. V., Nielsen, P. E., and Frank-Kamenetskii, M. D. (1999) *J. Mol. Biol. 285*, 1337−1345.
25. Bentin, T., and Nielsen, P. E. (1996) *Biochemistry 35*, 8863−8869.
26. Wittung, P., Nielsen, P., and Norden, B. (1996*) J. Am. Chem. Soc. 118*, 7049−7054.
27. Peffer, N. J., Hanvey, J. C., Bisi, J. E., Thomson, S. A., Hassman, C. F., Noble, S. A., and Babiss, L. E. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10648−10652.
28. Demidov, V. V., Yanilovich, M. V., Belotserkovskii, B. P., Frank-Kamenetskii, M. D., and Neilsen, P. E. (1994) *Proc. Natl. Acad. Sci. U.S.A. 92*, 2637−2641.
29. Hansen, G. I., Bentin, T., Larsen, J. J., and Nielsen, P. E. (2001) *J. Mol. Biol. 307*, 67−74.
30. 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) *J. Am. Chem. Soc. 117*, 831−832.
31. Egholm, M., Christensen, L., Dueholm, K. L., Buchardt, O., Coull, J., and Nielsen, P. E. (1995) *Nucleic Acids Res. 23*, 217−222.
32. Kuhn, H., Demidov, V. V., Frank-Kamenetskii, M. D., and Nielsen, P. E. (1998) *Nucleic Acids Res. 26*, 582−587.
33. Bremer, R. E., Wurtz, N. R., Szewczyk, J. W., and Dervan P. B. (2001) *Bioorg. Med. Chem. 9*, 2093−2103.
34. Zhang, X., Ishihara, T., and Corey, D. R. (2000) *Nucleic Acids Res. 28*, 3332−3338.
35. Mayfield, L. D., and Corey, D. R. (1999) *Anal. Biochem. 268*, 401−404.
36. Loll, P. J., and Lattman, E. E. (1989) *Proteins: Struct., Funct., Genet. 5*, 183−201.
37. Johnson, N. P., Lindstrom, J., Baase, W. A., and von Hippel, P. H. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 4840−4844.
38. Padmanabhan, S., Zhang, W., Capp, M. W., Anderson, C. F., and Record, M. T. (1997) *Biochemistry 36*, 5193−5206.
39. Kurakin, A., Larsen, H. J., and Nielsen, P. E. (1998) *Chem. Biol. 5*, 81−89.
40. Kuhn, K., Demidov, V. V., and Frank-Kamenetskii, M. D. (1999) *Angew. Chem., Int. Ed. 38*, 1446−1449.
41. Nadassy, K., Wodak, S. J., and Janin, J. (1999) *Biochemistry 38*, 1999−2017.
42. Luscombe, N. M., Laskowski, R. A., and Thornton, J. M. (2001) *Nucleic Acids Res. 29*, 2860−2874.
43. Pontius, B. W., and Berg, P. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8237−8241.

44. Simmons, C. G., Pitts, A. E., Mayfield, L. D., Shay, J. W., and Corey, D. R. (1997) *Bioorg. Med. Chem. Lett. 7*, 3001−3007.
45. Pooga, M., Ursel, S., Hallbrink, M., Valkna, A., Saar, K., Rezaei, K., Kahl, U., Hao, J.-K., Xu, X.-J., Wiesenfeld-Hallin, Z., Hokfelt, T., Bartfai, T., and Langel, U. (1998) *Nat. Biotechnol. 16*, 857−861.
46. Wender, P. A., Mitchell, D. J., Pattabiraman, K., Pelkey, E. T., Steinman, L., and Rothbard, J. B. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 13003−13008.
47. Izvolsky, K. I., Demidov, V. V., Nielsen P. E., and Frank-Kamenetskii, M. D. (2000) *Biochemistry 39*, 10908−10913.
48. Demidov, V. V., Protozanova, E., Izvolsky, K. I., Price, C., Nielsen, P. E., and Frank-Kamenetskii, M. D. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 5593−5598.
49. Braasch, D. A., and Corey, D. R. (2002) *Biochemistry 41*, 4503−4510.

BI0263659