# Strand Invasion of Mixed-Sequence, Double-Helical B-DNA by γ-Peptide Nucleic Acids Containing G-Clamp Nucleobases under Physiological Conditions

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ABSTRACT: Peptide nucleic acids (PNAs) make up the only class of nucleic acid mimics developed to date that has been shown to be capable of invading double-helical B-form DNA. Recently, we showed that sequence limitation associated with PNA recognition can be relaxed by utilizing conformationally preorganized γ-peptide nucleic acids (γPNAs). However, like all the previous studies, with the exception of triplex binding, DNA strand invasion was performed at relatively low salt concentrations. When physiological ionic strengths were used, little to no binding was observed. On the basis of this finding, it was not clear whether the lack of binding is due to the lack of base pair opening or the lack of binding free energy, either of which would result in no productive binding. In this work, we show that it is the latter. Under simulated physiological conditions, the DNA double helix is sufficiently dynamic to permit strand invasion by the designer oligonucleotide molecules provided that the required binding free energy can be met. This finding has important implications for the design oligonucleotides for recognition of B-DNA via direct Watson—Crick base pairing.
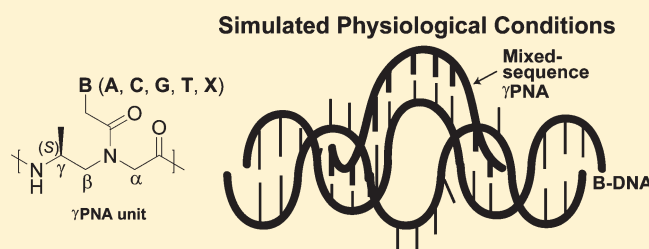
Double-helical DNA has traditionally been considered as a relatively static molecular entity, specifically designed for the storage and safeguard of genetic information.[1] Early theoretical estimations[2] and nuclear magnetic resonance (NMR) imino proton exchange studies[3,4] placed the probability of internal base pair opening right around 10^{-5}, the open base pair lifetime around 10^{-7} s, and the base pair activation energy in the range of 43—65 kJ/mol at ambient temperature. These initial estimates cast a grim outlook on the prospect of being able to access the Watson—Crick (W—C) hydrogen bond donors and acceptors of nucleobases for recognition. As a consequence, efforts to establish sequence-specific interactions with double-stranded DNA (dsDNA) in the past have generally been focused on chemical groups that reside in the minor[5] and major[6,7] grooves, in large part, because of the ease of accessibility and the precedence for their recognition in nature. However, over the past two decades, peptide nucleic acids (PNAs),[8] a particular class of nucleic acid mimics comprising a pseudopeptide backbone (Chart 1a, top), have been shown to be capable of invading dsDNA.[8—10] This finding was significant because, contrary to the traditional belief, it demonstrates that W—C base pairing interactions can be established with intact dsDNA at physiological temperature. Though they are promising as antigene reagents, because of the generality and specificity of recognition, the originally designed achiral PNAs can recognize only homopurine[11] and homopyrimidine targets.[12] Mixed-sequence PNAs can invade topologically constrained supercoiled plasmid DNA[13,14] and structurally perturbed regions of genomic DNA;[15] however, they are unable to invade linear, double-helical B-form DNA (B-DNA).

Recently, two approaches, tail clamp[16,17] and double-duplex invasion,[18] have been developed, allowing mixed-sequence PNAs to invade B-DNA. However, they are not without limitations. The first approach still requires a homopyrimidine tract for anchoring the triplex binding, while the second approach, although more relaxed in sequence selection, is complicated by the need to use two separate strands of pseudocomplementary PNA to invade dsDNA. More recently, we showed that sequence restriction can be relaxed by utilizing conformationally preorganized γPNAs as molecular reagents (Chart 1a, bottom).[19] However, like all the other studies, with the exception of triplex binding by homopyrimidine PNAs,[20] strand invasion of linear double-helical B-DNA was performed in buffers containing relatively low salt concentrations.[11,18,19,21,22] When physiological ionic strengths were used, little to no binding was observed.[23] On the basis of this result, it was not clear whether the lack of binding is due to the lack of base pair opening (accessibility) or the lack of binding free energy, because the kinetics of base pair breathing and the thermodynamic stability of the DNA duplex are tightly coupled. Knowing the answer to this question is important because it will tell us whether W—C recognition is a viable option for targeting mixed-sequence B-DNA under physiological conditions. If the former is true, there is little that can be done from a design standpoint. However, if the latter is true, this issue could be resolved through molecular design. Herein, we show that it is the latter. Under simulated

Chart 1. Chemical Structures of (a) PNA and γPNA Building Blocks, (b) C-G and X-G Base Pairs, and (c) Sequences of γPNA Oligomers and (d) an Illustration of the DNA Targets

MATERIALS AND METHODS

Monomer and Oligomer Synthesis. Boc-protected PNA monomers were obtained from ASM Research Chemicals. Methyl-containing γPNA monomers were prepared according to published procedures.[24] The G-clamp nucleobase and the corresponding γPNA monomer were prepared according to the protocol reported by Chenna and co-workers.[23] The oligomers were synthesized on MBHA (4-methylbenzhydrylamine) resin according to standard procedures.[25] The oligomers were cleaved from the resin using an *m*-cresol/thioanisole/TFMSA/TFA (1:1:2:6) cocktail, and the resulting mixtures were precipitated with ethyl ether, purified by RP-HPLC, and characterized with a MALDI-TOF mass spectrometer.

Cloning. Two DNA inserts, containing a perfectly matched (**PM**) and single-base mismatched (**MM**) binding site, were cloned into the *Bam*HI and *Eco*RI restriction sites of the pSuper plasmid vector.[26] The recombinant vectors were transformed into *Escherichia coli* (X-Gold 10). Following selection, clones were expanded and the plasmids were extracted using the Qiagen plasmid extraction kit. The identities of the inserts were verified by sequencing: insert 1 (**PM**), p-gatcgatatctatgagaccacagatc-taagctagc-3′/p-aattgctagcttagatctgtggtctcatagatatc-3′; insert 2 (**MM**), p-gatcgatatctatgagacctcagatctaagctagc-3′/p-aattgctagctta-gatctgaggtctcatagatatc-3′.

Polymerase Chain Reaction (PCR). The 171 bp DNA fragments containing **PM** and **MM** binding sites were PCR-amplified from the corresponding plasmid vectors using the following primer set: 5′-gctactcaagctttcactatagggcgaattgga-3′/5′-gtgttcccgcctagtgaca-3′. The PCR was performed as followed: (i) 95 °C for 2 min, (ii) 95 °C for 30 s, (iii) 55 °C for 30 s, (iv) 72 °C for 1 min, and (v) repeated steps ii—iv for 32 cycles. The reaction mixtures were pooled and then quenched with 0.2 volume of 10 mM EDTA. The mixtures were extracted twice with 1 volume of a phenol/chloroform/IAA mixture (24:5:1), and the aqueous portions were combined and precipitated via addition of 1 μL of 5 mg/mL

glycogen, 0.1 volume of 3 M sodium acetate, and 3 volumes of cold ethanol and placed in dry ice for 30 min. Following centrifugation and removal of the liquid layers, the remaining DNA pellets were washed three times with 70% ethanol, air-dried, and then reconstituted with Nanopure water. The concentrations of the PCR products (171 bp linear DNA fragments) were estimated on the basis of the following parameters: 1 OD_{260} = 50 μg/mL and molecular mass = 649 Da/bp.

Gel Shift Assay. Following incubation of the DNA targets with the appropriate oligomers at the indicated concentrations, time points, and temperatures, the samples were separated on precast, 10% nondenaturing polyacrylamide gels using 0.5× TBE buffer. The gels were run at 10 V/cm for 3 h in a water bath at 37 °C. After electrophoresis, the gels were stained with 1× SYBR-Gold for 5 min, washed twice with 0.5× TBE buffer, and then imaged using a gel documentation system (BioDoc-It System). The images were then inverted using Adobe Photoshop version 6.0.

DNase-I Footprinting Assay. The 5′-gctactcaagctttcacta-tagggcgaattgga-3′/5′-gtgttcccgcctagtgaca-3′ primer set was used to PCR-amplify the DNA fragments containing **PM** and **MM** binding sites from the corresponding vectors. The PCR products were digested with *Hin*dIII, heat-inactivated, and then 3′-labeled with P-32 using the Klenow fragment of *E. coli* DNA polymerase. Notice that in this case the 3′-end of the target DNA strand was labeled with P-32. The DNA targets were gel-purified and ethanol-precipitated. The radioactively labeled targets were then redissolved in water and divided into aliquots containing approximately 10000 cpm each. The invasion complex was initiated by incubation of 10000 cpm of the labeled and 0.2 μM of the cold (unlabeled) DNA target with the indicated oligomers and concentrations in a simulated physiological buffer [10 mM sodium phosphate, 2 mM $MgCl_2$, and 150 mM KCl (pH 7.4)] at 37 °C. Footprinting of the invasion complex was performed by incubating the mixtures with 1 unit of DNase-I for 30 s. The reactions were then immediately quenched via addition of 20 μL of stop buffer (1.5 M sodium acetate, 10 mM EDTA, 1 M β-mercaptoethanol, and 250 μg/mL calf thymus) and 400 μL of chilled ethanol. The samples were briefly vortexed and placed in dry ice for 30 min, followed by centrifugation and washing three times with 70% ethanol. The samples were air-dried and then
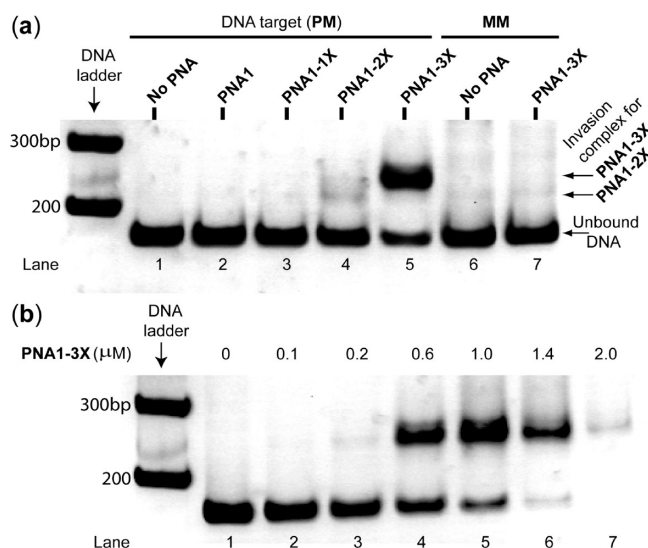
Figure 1. Result of a gel shift assay following incubation of a 171 bp DNA fragment at 0.2 μM containing (a) a perfectly matched (**PM**, lanes 1−5) and single-base mismatched (**MM**, lanes 6 and 7) binding site with the indicated γPNA oligomers at 1.0 μM and (b) a **PM** binding site with the indicated concentrations of the **PNA1-3X** oligomer in a simulated physiological buffer at 37 °C for 16 h, followed by separation on nondenaturing PAGE and staining with SYBR-Gold.

separated on 10% urea−denaturing polyacrylamide gels. The cleavage patterns were visualized by autoradiography.

DEPC Chemical Probing Assay. The 5′-tcactataggggcgaattgga-3′/5′-gctactcaagcttgtgttcccgcctagtgaca-3′ PCR primer set was used to PCR-amplify the DNA targets containing **PM** and **MM** binding sites from the corresponding plasmid vectors. The PCR products were digested with *Hin*dIII and 3′-labeled with P-32 under conditions identical to those described above. The invasion complex was prepared the same way as that for DNase-I footprinting. DEPC treatment was initiated via addition of a 10% volume of DEPC to the invasion samples. The mixtures were incubated at room temperature for 5 min, followed by precipitation and piperidine treatment. The samples were then separated on 10% urea−denaturing polyacrylamide gels, and the cleavage patterns were visualized by autoradiography.

■ RESULTS AND DISCUSSION

To address the question of accessibility versus thermodynamic stability, we synthesized a series of decameric γPNA oligomers containing the same nucleobase sequence content but with a different number of cytosine (C) to G-clamp (X) nucleobase substitutions (Chart 1b,c). We then assessed their DNA strand invasion capability using a combination of gel shift, enzymatic footprinting, and chemical probing assays. The C → X nucleobase substitutions were made in an attempt to systematically improve the binding free energy of γPNAs without resorting to changing the nucleobase sequence or chain length. This particular cytosine analogue was chosen because prior studies showed that C → X substitution can significantly enhance the thermodynamic stability of the PNA−DNA duplex.[27] Given the already strong binding affinity of γPNAs,[22] making such nucleobase substitutions should significantly further enhance their thermodynamic stability. Implementing such a design would allow us to survey the invasion capability of γPNAs over a range of binding
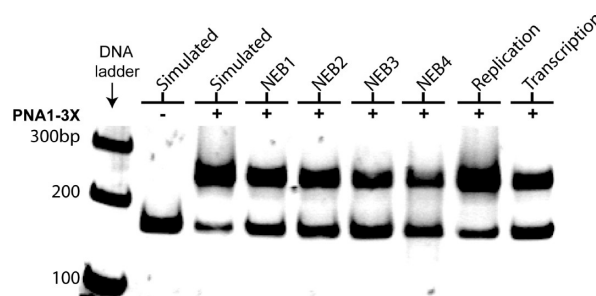
Figure 2. Effects of biological buffers on DNA strand invasion by **PNA1-3X**. A gel shift assay was performed by incubating 0.2 μM DNA containing a **PM** binding site with 1.0 μM **PNA1-3X** in the indicated buffers at 37 °C for 16 h. Simulated: 10 mM NaP$_i$, 2 mM MgCl$_2$, and 150 mM KCl. NEB1 (New England Biolabs buffer 1, 1×): 10 mM Bis Tris propane-HCl, 10 mM MgCl$_2$, and 1 mM dithiothreitol. NEB2: 10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl$_2$, and 1 mM dithiothreitol. NEB3: 10 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl$_2$, and 1 mM dithiothreitol. NEB4: 20 mM Tris-OAc, 50 mM KOAc, 10 mM MgCl$_2$, and 1 mM dithiothreitol. Replication: 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl$_2$, and 0.1% Triton X-100. Transcription: 40 mM Tris-HCl, 6 mM MgCl$_2$, 10 mM dithiothreitol, and 2 mM spermidine.

free energies. Our working hypothesis is that if DNA strand invasion under simulated physiological conditions is predominated by thermodynamics, we would expect a gradual increase in the invasion efficiency with binding free energy, or in this case the number of C → X nucleobase substitutions. On the other hand, if base pair accessibility is the determining factor in DNA strand invasion, we do not expect the correlation described above but instead expect a steep dependence on temperature, which has a direct effect on the rate of base pair opening.

A gel shift assay was performed by incubating a 171 bp, linear doubled-stranded B-DNA containing an internal, perfectly matched (**PM**) binding site (Chart 1d) with various γPNA oligomers in a buffer containing physiological salt concentrations[28] [10 mM sodium phosphate, 2 mM MgCl$_2$, and 150 mM KCl (pH 7.4)] at 37 °C. The mixtures were separated on nondenaturing polyacrylamide gels and stained with SYBR-Gold for visualization. Inspection of Figure 1a reveals no binding for **PNA1** or **PNA1-1X**, but a small amount for **PNA1-2X**, as evidenced by the formation of a shifted band following a 16 h incubation. This result is consistent with an earlier finding.[23] With a further increase in the number of C → X nucleobase substitutions from two to three, we noticed a dramatic increase in the intensity of the shifted band, from ∼3% with **PNA1-2X** (lane 4) to ∼80% with **PNA1-3X** (lane 5), indicating that more of the bound complex was formed. Formation of this complex was sequence-specific, because no trace of the shifted band was observed with DNA containing an inverted, single-base mismatched (**MM**) binding site (compare lane 7 to lane 6). Nucleobase inversion is expected to have a minimal impact on the thermodynamic stability (or base pair breathing rate) of the DNA double helix. Thus, the difference in binding efficiency is predominantly due to sequence mismatch discrimination. We attribute the high level of sequence specificity to the competitive binding of the native cDNA strand. Our attempt to drive the reaction to completion by increasing the concentration of **PNA1-3X** was unsuccessful because beyond a 5:1 **PNA1-3X**:DNA ratio, the DNA bands gradually disappeared from the gel (in Figure 1b, compare lanes 6 and 7 to lane 5). This is probably due to aggregation and nonspecific binding of
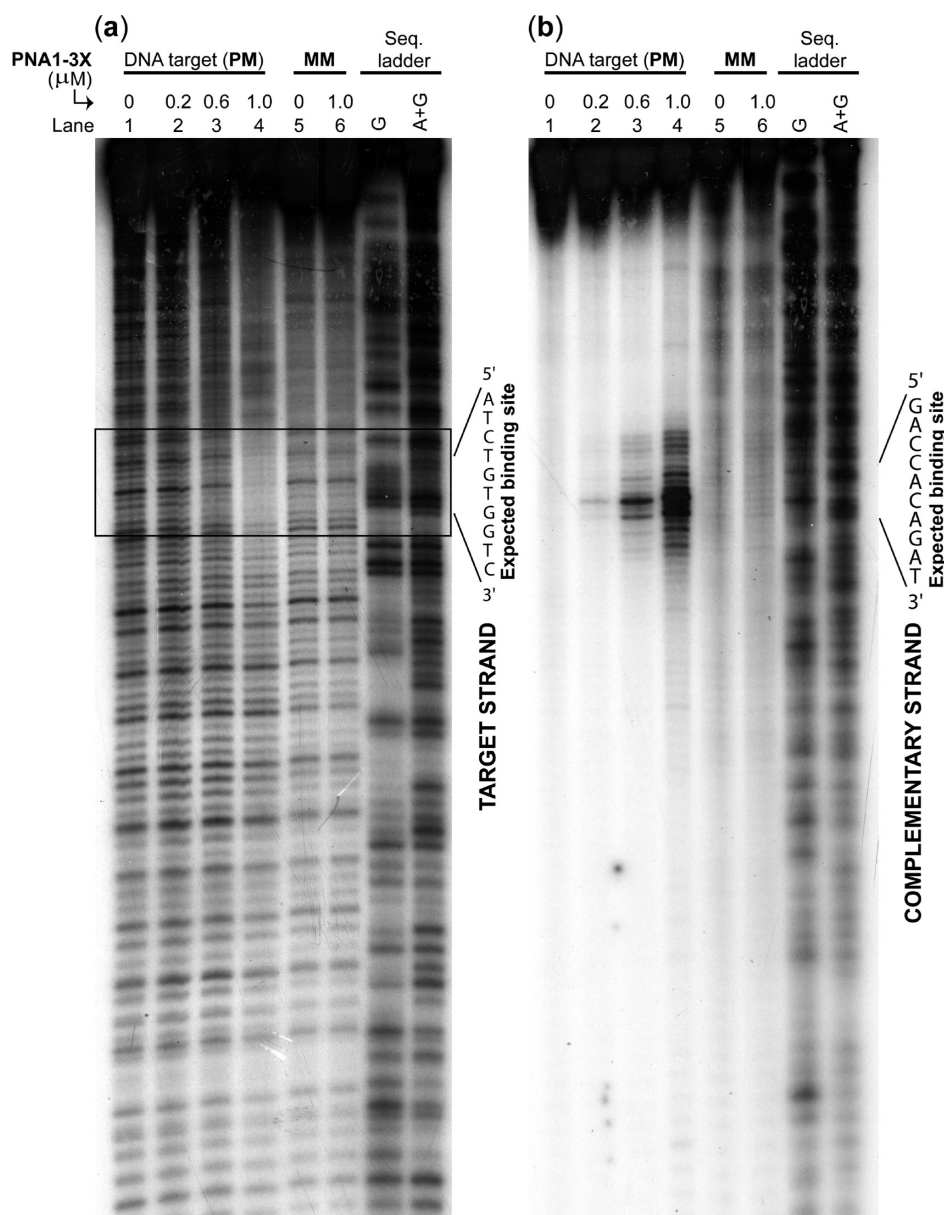
Figure 3. (a) DNase-I footprinting and (b) DEPC chemical probing assay following incubation with 10000 cpm of the 3′-labeled DNA target containing a **PM** (lanes 1–4) and **MM** (lanes 5 and 6) binding site with the indicated concentrations of **PNA1-3X** in a simulated physiological buffer at 37 °C for 16 h. In panel a, the 3′-end of the target strand was labeled with P-32, and in panel b, the homologous strand was labeled with P-32.

PNA1-3X to DNA as the result of the charge-neutral polyamide backbone and hydrophobic character of the methyl group at the γ-backbone. In addition to the simulated physiological buffer, we have examined a number of other biologically relevant buffers, including those employed in restriction digestion, transcription, and replication to assess the generality of **PNA1-3X** binding. In all of these buffers, some of which contained concentrations of $MgCl_2$ as high as 10 mM, binding efficiencies of >60% were achieved within a 16 h incubation period (Figure 2). Longer incubation times did not produce additional binding, indicating that equilibrium had been reached. Taken together, these results show that **PNA1-3X** can invade dsDNA under physiologically relevant conditions. Strand invasion is unique to γPNA, because no evidence of binding for the unmodified PNA containing the same nucleobase sequence as **PNA1-3X** has been found.

To confirm that binding of **PNA1-3X** to DNA occurred at the expected site and through a strand invasion mechanism, we performed DNase footprinting and DEPC (diethyl pyrocarbonate) chemical probing assays. DNase is an endonuclease enzyme that indiscriminately cleaves dsDNA. Selective binding of **PNA1-3X** to dsDNA is expected to block DNase digestion, resulting in a footprinting pattern following electrophoretic separation and detection of the DNA targets. DEPC, on the other hand, is a chemical reagent known to react selectively with adenine and to a lesser extent with the guanine nucleobase of single-stranded or perturbed regions of double-stranded DNA.[29] Strand invasion of DNA by **PNA1-3X** is expected to result in local displacement of the homologous DNA strand. The looped-out strand can be revealed in the form of strand cleavage following DEPC and subsequent piperidine treatments.
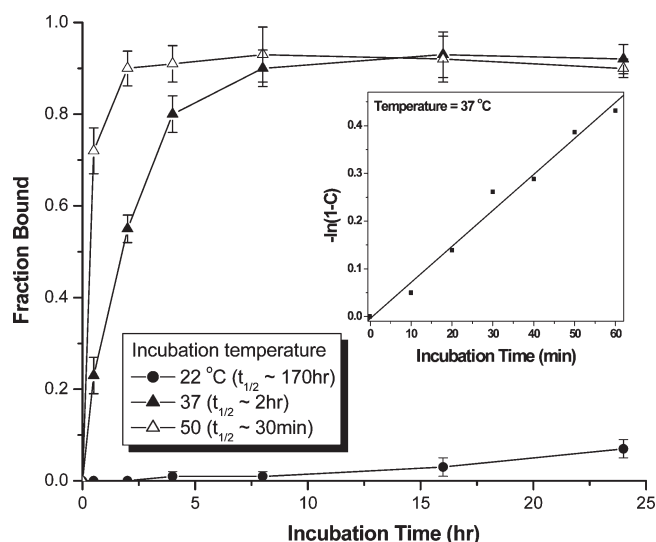
Figure 4. Effects of temperature and incubation time on DNA strand invasion by **PNA1-3X**. The fraction bound was determined by gel shift assays following incubation of 0.2 μM DNA containing a **PM** binding site with 1.0 μM **PNA1-3X** in a simulated physiological buffer at the indicated temperatures and time points. $t_{1/2}$ is defined as the time it took to reach 50% binding. The inset shows a plot of $-\ln(1 - C)$ vs incubation time; the data show that strand invasion of B-DNA by **PNA1-3X** at 37 °C obeys pseudo-first-order kinetics: $C = 1 - \exp(-k_{ps}t)$, where C is the fraction bound at time t and k_{ps} is the pseudo-first-order rate constant ($k_{ps,37 °C} = 0.0075$ min^{-1}).

Consistent with these predictions, we observed a distinct footprinting pattern on the target strand, localized at the expected binding site following incubation of DNA with **PNA1-3X** (Figure 3a). The extent of the footprint became more pronounced as the **PNA1-3X** concentrations increased, and it was observed only with DNA containing a **PM** binding site (lanes 1−4). Likewise, we observed cleavage of the cDNA strand directly across from the binding site (Figure 3b). Strand cleavage occurred only with DNA containing a **PM** binding site (lanes 1−4). These results are consistent with **PNA1-3X** binding selectively to its target site through a strand invasion mechanism.

Next, we assessed the effects of temperature and incubation time on the efficiency of DNA strand invasion. Our result shows that the efficiency of strand invasion is strongly dependent on temperature in the range of 22−37 °C (Figure 4). This is not surprising because temperature has a direct effect on the rate of base pair opening. Strand invasion was extremely inefficient at ambient temperature, in which case <10% invasion took place after incubation for 24 h. The rate of strand invasion dramatically increased at 37 °C, reaching equilibrium after incubation for ∼6 h. Further increases in temperature resulted in further increases in the rate of strand invasion, but the difference in the rates at 37 and 50 °C is not as dramatic as compared to the difference in the rates at 22 and 37 °C ($t_{1/2,22 °C} \sim$ 170 h; $t_{1/2,37 °C} \sim$ 2 h; $t_{1/2,45 °C} \sim$ 30 min). There is an only 4-fold difference in the $t_{1/2}$ in the higher-temperature regimes as compared to 135-fold in the lower-temperature regimes. Regardless of whether the samples were incubated at 37 or 50 °C, the invasion profiles reached a plateau corresponding to ∼90% binding efficiency. This result indicates that at 37 °C, the DNA double helix is sufficiently dynamic to permit strand invasion by γPNAs. However, whether successful strand invasion takes place

depends on whether γPNA has the required binding free energy to displace the native cDNA strand.

■ CONCLUSION

In summary, we have shown that **PNA1-3X** can invade double-helical B-DNA under physiologically relevant conditions. Improvements in thermodynamic stability significantly enhance the efficiency of DNA strand invasion. We have already demonstrated how additional binding free energy may be achieved, through backbone preorganization and nucleobase substitution in this study, covalent attachment of a DNA intercalating agent, such as acridine, as demonstrated in prior studies,[20,22] and extension of the length of oligonucleotides.[19] Besides cytosine, other nucleobases, including thymine, could also be modified to improve their hydrogen bonding and base stacking capabilities. Important work in this area by Nielsen and co-workers[30,31] has already begun in earnest. Replacement of natural nucleobases with synthetic analogues capable of forming additional hydrogen bonding interactions and larger and geometrically aligned hydrophobic cores would further expand the recognition repertoire of PNA and γPNA. Issues with aggregation and nonspecific binding, as observed at high PNA and γPNA concentrations, could be alleviated by installing a hydrophilic group at the γ-backbone.[24] We have already embarked on this front, the result of which will be reported in due course.

γPNAs are attractive, as compared to other classes of antigene reagents developed to date,[5,32−36] because they are relatively easy to synthesize and they hybridize to their targets (via strand invasion) in a highly sequence-specific and predictable manner in accordance with the Watson−Crick base pairing rules. Molecules that can recognize and bind double-stranded DNA in a sequence-specific manner are of considerable interest in biology, biotechnology, and medicine. They could be used as molecular tools to probe sequence information, manipulate a genome's structure and function, and regulate gene expression at the transcriptional level, which may be more effective than the antisense approach because it shuts down gene expression at the initial step, as well as potential therapeutic and diagnostic reagents for the treatment and detection of genetic diseases.

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■ ABBREVIATIONS

PNA, peptide nucleic acid; γPNA, γ-backbone-modified peptide nucleic acid; A, adenine; C, cytosine; G, guanine; T, thymine; X,

G-clamp; TFA, trifluoroacetic acid; TFMSA, trifluoromethane-sulfonic acid; DEPC, diethyl pyrocarbonate.

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