

End invasion of peptide nucleic acids (PNAs) with mixed-base composition into linear DNA duplexes

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ABSTRACT

Peptide nucleic acid (PNA) is a synthetic DNA mimic with valuable properties and a rapidly growing scope of applications. With the exception of recently introduced pseudocomplementary PNAs, binding of common PNA oligomers to target sites located inside linear double-stranded DNAs (dsDNAs) is essentially restricted to homopurine–homopyrimidine sequence motifs, which significantly hampers some of the PNA applications. Here, we suggest an approach to bypass this limitation of common PNAs. We demonstrate that PNA with mixed composition of ordinary nucleobases is capable of sequence-specific targeting of complementary dsDNA sites if they are located at the very termini of DNA duplex. We then show that such targeting makes it possible to perform capturing of designated dsDNA fragments via the DNA-bound biotinylated PNA as well as to signal the presence of a specific dsDNA sequence, in the case a PNA beacon is employed. We also examine the PNA–DNA conjugate and prove that it can initiate the primer-extension reaction starting from the duplex DNA termini when a DNA polymerase with the strand-displacement ability is used. We thus conclude that recognition of duplex DNA by mixed-base PNAs via the end invasion has a promising potential for site-specific and sequence-unrestricted DNA manipulation and detection.

INTRODUCTION

Recognition of specific sequences within double-stranded (ds) DNA via ligand binding is a general and potent strategy for

probing DNA functions, and for the control of gene expression (1,2). Synthetic oligonucleotide analogs and mimics, which can bind to DNA more strongly than regular oligonucleotides, and without the loss of specificity, are of major interest in this respect (3). The well-known nucleic acid mimics of this kind are peptide nucleic acids (PNAs) (4–7).

Owing to the uncharged PNA backbone, PNA–DNA duplexes display higher melting temperatures than the corresponding DNA–DNA duplexes, particularly at low salt concentrations (8). Consequently, at low ionic strength, PNA oligomers have the capacity to invade a DNA duplex in a highly sequence-specific manner (9,10). Together with the fact that PNA is resistant to proteases and nucleases (11), this has made the PNA oligomers attractive tools in both molecular biotechnology and biomedicine (5,12). Some of the suggested applications of strand-invading PNAs include the antigene and gene-activation strategies (13–18). PNAs have also been used as sequence-specific probes in clinical diagnostics (19,20), and as strand-specific dsDNA ‘openers’ (21–26), and as sequence-specific DNA binding anchors for biomarkers and other biologically active molecules (27–29).

Though a variety of strand-invasion modes were described for different PNAs (9,30–33), for effective binding to linear DNA duplexes homopyrimidine PNAs with the capacity to form high-stability PNA–DNA complexes have mostly been used. The remarkable feature of homopyrimidine PNAs is their ability to displace one of the two dsDNA strands via formation of the triplex strand-invasion complex (10,34,35). This particular mode of PNA binding has made it possible to develop numerous tools for DNA analysis [reviewed in (5,26)]. In order to increase the hybridization efficiency, bis-PNAs have been extensively used, in which two pyrimidine PNA strands merge in to a single molecule via a flexible linker (34,36–38).

Along with its many advantages, the triplex-invasion mode of PNA–dsDNA binding has serious sequence limitations due

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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the fact that predominantly homopurine–homopyrimidine sites can be targeted by triplex-forming homopyrimidine PNAs. High-affinity homopurine and TG-rich PNAs can also invade the DNA duplexes (9,31–33), but essentially the same sequence limitations remain. ‘Tail-clamp’ PNAs consisting of a short pyrimidine bis-PNA appended to a PNA oligomer with mixed-base composition (mixed-base PNA) have recently been tested to reduce sequence limitations (39,40). However, the sequence specificity of these PNA constructs was rather low, and the tail interfered with helix invasion reducing the PNA binding rate.

In this study, we concentrate on the binding of mixed-base PNAs to linear dsDNA fragments. Mixed-base PNAs, which cannot form stable triplexes or high-affinity duplexes, are evidently incapable by themselves of invasion inside linear dsDNA even at low ionic strengths, when DNA duplexes become very unstable with respect to strand separation. Apparently, for short (20mer or less) PNA oligomers, which are normally used, the energetic cost of formation of two helix boundaries at the edges of PNA–DNA strand-invasion complexes proves to be unacceptably high virtually at any conditions [with the exception of supercoiled DNA, into which mixed-base PNAs can stably invade, similarly to regular oligonucleotides (30,41,42)]. Pseudocomplementary (pc) PNA modifications can help with this, but two mixed-base PNA oligomers are required here (43,44) and pcPNAs are not readily available.

Obviously, no new helix boundaries need to be formed in the case of invasion into DNA duplex at the very end of linear dsDNA. Grounding on this simple reasoning, we assumed that mixed-base PNAs would invade the DNA double helix under appropriate conditions, if targeted at the duplex terminus. Indeed, with decreasing salt concentration, PNA–DNA duplex is known to become energetically much more favorable than DNA–DNA duplex (8). Here, we present experimental data, which support our assumption.

Besides PNA oligomers, we study the possibility of the dsDNA end invasion for PNA conjugates with very short DNA oligomers (PNA/DNA chimeras) (45–47). These constructs are recognized as functional substrates by various nucleic acid processing enzymes and consequently can assume biochemical functions, e.g. serving as a primer for DNA polymerases (46,47).

Using various assays we show that linear dsDNA can be effectively and sequence-specifically targeted by mixed-base PNAs and PNA/DNA chimeras via the end invasion process. We demonstrate a diverse potential of this new mode of PNA binding to dsDNA for site-specific DNA manipulation and detection. Our findings are promising for prospective use of mixed-base PNAs and their biomolecular constructs in DNA biotechnology and DNA diagnostics.

MATERIALS AND METHODS

PNA oligomers and PNA/DNA chimera

The mixed-base biotinylated PNA was gifted by Dr P. E. Nielsen. The stemless PNA beacon was supplied by Boston Probes/Applied Biosystems (Bedford, MA). PNA oligomers were high-performance liquid chromatography purified and characterized by matrix-assisted laser desorption

ionization time-of-flight mass spectrometry. PNA/DNA chimera was received as a gift from Dr V. N. Pandey. The following PNA oligomers and PNA/DNA chimera are used in this study (the DNA part of chimera is given in small letters):

Biotinylated PNAs: PNA 1720 (13mer), Biotin-linker-Lys₂-AAAATATCATTGG-Lys₂-NH₂; PNA 6976 (13mer), Biotin-linker-Lys₂-GAAGGCCTATGCA-Lys₂-NH₂; PNA 6977 (13mer), Biotin-linker-Lys₂-AGGCCTATGCACC-Lys₂-NH₂; PNA/DNA chimera ([19+2]mer), 5'-N-GTCCC-TGTTCGGGGCGCCAC-5' *tg*^{3'}; PNA beacon (13mer), Fl-Glu-GAAGCTACTGGAG-Lys₂(dabcyI)-NH₂.

As usual, the normally neutral PNA oligomers were equipped with the cationic Lys residues to increase the PNA solubility and binding efficacy (48). PNA concentration in stock solutions was determined spectrophotometrically. The PNA/DNA chimeric molecule comprises 19 PNA monomers followed by a normal dinucleotide TpG with G having a free 3'OH terminus (46). PNA beacon contains fluorescein (Flu) as the fluorophore and DABCYL (4-(4'-dimethylamino-phenylazo)benzoic acid) as the quencher. Flu was conjugated to the probe oligomer via an amide link.

DNA targets

The 100 bp long oligonucleotides carrying correct or singly mismatched PNA-binding sites (and also sequence-complements of these oligomers) were purchased from MWG Biotech and used without further purification. Complementary oligos were annealed to each other by mixing them at the 1:1.25 target-to-no target-strand ratios to safely avoid the target strand in the single-stranded form (see Figure 1A; blue letters indicate mismatched nucleotides). The same oligonucleotides carrying PNA-binding sites for two PNAs: on one end for PNA 1720 (shown in red) and for PNA 6976 (shown in blue) on the other end. The third PNA 6977 (shown in green) has recognition sequences (underlined) that shifted on 2 nt from the end.

PNA-targeted oligonucleotide: 5'-AAAATATC(A/t)TTGG-(N)₇₂-GGTGCATAGGCCTTC-3'; non-targeted oligo complement: 5'-GAAGGCCTATGCACC-(M)₇₂-CCAA(T/a)GATATTTT-3', where the arbitrary chosen sequences, (N)₇₂ and (M)₇₂, were complementary to each other.

Recombinant plasmids carrying correct binding sites for PNA 1720, PNA/DNA chimera and PNA beacon were obtained by cloning of corresponding inserts into EcoRI–BamHI and BamHI–HindIII sites of the pUC19 polylinker. Plasmids used in the PNA-binding studies were cloned in the high-efficiency competent DH5 *Escherichia coli* cells (Gibco BRL). For end-invasion experiments, DNA fragments carrying a PNA-binding site at the end of duplex were prepared via cutting the plasmid DNA with suitable restriction enzymes (DraI and EaeI for PNA; AatII for PNA/DNA-chimera; HinfI for PNA beacon; see Figure 1). For nick-invasion experiments, 192 bp long DNA fragments carrying the PNA-binding site near the DNA nickase recognition/cleavage sites (see Figure 1E) were prepared by PCR amplification of the plasmid DNA and then by N.AIwI nicking enzyme single-stranded nick was introduced inside of duplex DNA. All restriction enzymes were obtained from New England Biolabs and were used in their reaction buffers.

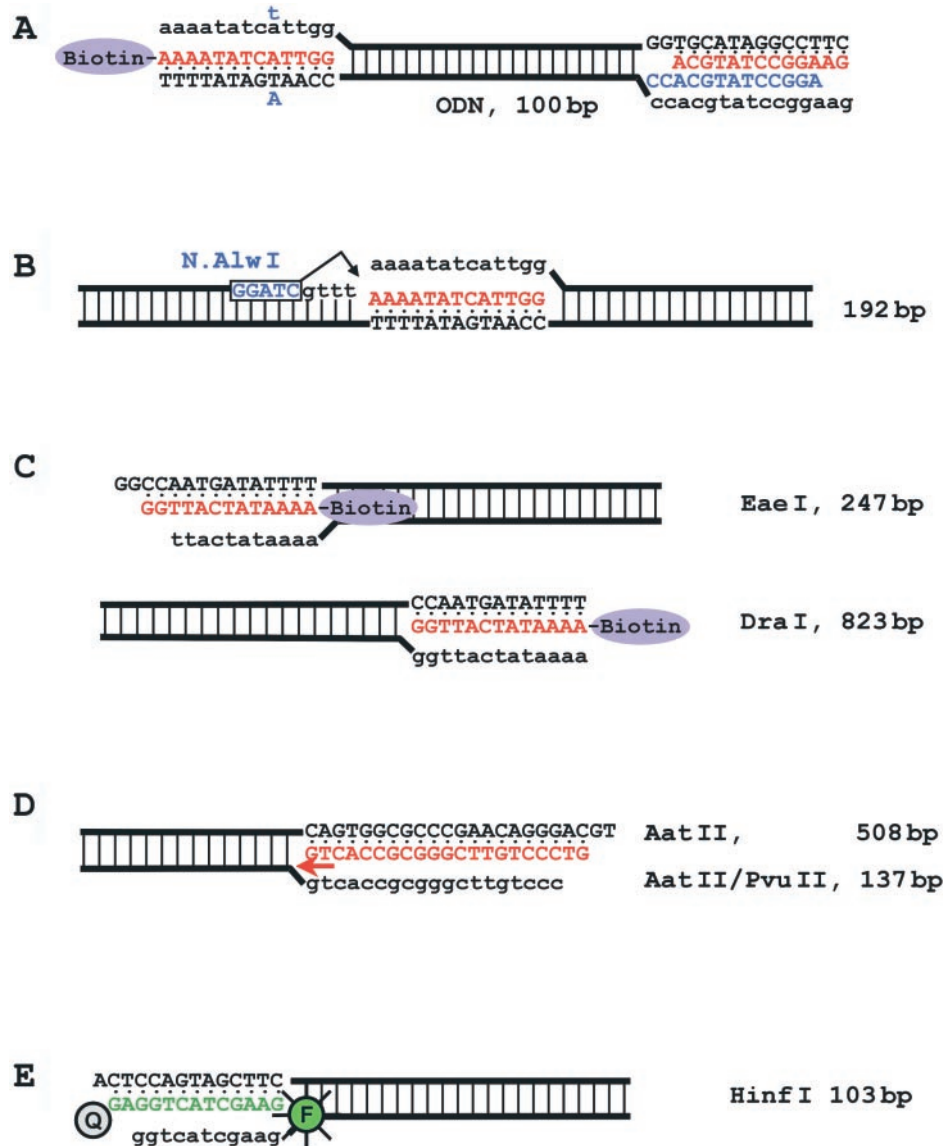


Figure 1. Schematics of DNA targets used in this study. (A) Pairs oligonucleotides carrying correct and singly mismatched (shown blue) PNA-binding sites on the end of one of them are annealed to each other and are used for PNA end invasion. (B) Longer DNA fragments prepared by their cutting out of plasmid with EaeI and DraI restriction enzymes. Note that the EaeI fragment carries an overhang, while the DraI fragments has the blunt end. For end-invasion and capturing experiments EaeI fragments was prepared by cutting the plasmid DNA to obtain the 247 bp long fragment carrying a PNA-binding site at the end of duplex. For AFM analysis the plasmid DNA was first PCR-amplified using corresponding primers and then digested by EaeI to obtain the 230 bp long fragment. (C) The construct used in primer-extension experiments, which were obtained using the AatII and PvuII restriction enzymes. The red arrow underlying the DNA part of PNA–DNA chimera and the arrow shows the direct of the chain elongation. (D) Schematics of the construct used for fluorescence measurements. (E) Duplex DNA fragments with single stranded break prepared by N.AlwI nicking enzymes.

PNA–DNA complex formation

Target DNA (annealed ds-oligonucleotides or plasmid restriction fragments) was incubated for required time at 37°C in a siliconized microtube with 20–200 pmol of mix-PNAs in 40 µl of 10 mM sodium phosphate buffer (pH 7.0). Typically, the DNA concentration in the reaction mixtures was <10 nM, while the PNA concentration was always >100 nM providing a significant excess of PNA over DNA targets. Unbound PNA was then removed by gel filtration through a Sephadex-G50 spin column (Pharmacia Biotech) equilibrated with sodium phosphate buffer. Formation of mixPNA–dsDNA complexes was monitored by gel-shift assay in nondenaturing 8%

polyacrylamide gels using 1× TBE as a running buffer. DNA was visualized by ethidium bromide staining and detected by the CCD camera coupled with the IS-1000 digital imaging system/software (Alpha Innotech Corporation).

Affinity capturing procedure

Affinity capture of plasmid DNA fragments was performed using BioMag Streptavidin beads (1 µl binds 1 pmol, Polysciences, Inc.). An aliquot of 15 µl of BioMag Streptavidin pre-washed beads was added to the formed PNA–DNA complexes and incubated at room temperature for 1 h. Beads were

washed twice in 100 μ l of TE buffer containing 0.5 M NaCl to remove unbound DNA. The bound fragment was eluted from the beads by incubation at 65°C for 30 min in 20 μ l of TE buffer containing 0.05 M NaCl. Supernatant and captured DNAs were analyzed by 1% agarose gel electrophoresis.

Extension of PNA/DNA chimera

The PNA/DNA chimera complex formed with a plasmid cleaved with the AatII restriction enzyme was used for the primer-extension reaction. An aliquot of 10 μ l of the pre-formed complex was purified by gel filtration through a Sephadex-G50 and used for multiple fluorescent labeling. Aliquots containing 2 μ l of 5 \times Sequenase buffer, 2 μ l of dNTPs mixture (5 mM each dCTP, dTTP and dGTP; Promega) and 1 μ l of 10 mM fluorescent Cy5-dATP were added. The primer-extension reaction was started by the addition 0.5 μ l of Sequenase 2.0 (8 U/ μ l, Amersham Pharmacia Biotech). After incubation at 37°C for 15 min, the reaction (15 μ l total volume) was terminated by the addition of Stop Solution (Pharmacia Biotech). The samples were resolved, along with the sequencing ladders by 6% denaturing polyacrylamide gel filled with 7 M Urea in TBE buffer, \sim 6 h at 55°C, using ALF Express DNA Sequencer (Pharmacia Biotech).

Atomic force microscopy (AFM) analysis

Recombinant plasmids carrying correct PNA 1720 binding sites were PCR-amplified using corresponding primers and digested by EaeI to obtain the 230 bp long fragment with 4 bp overhang end, then was isolated and purified by PCR-columns (Quagene). PNA-dsDNA complex was formed via strand invasion with PNA and the final PNA-DNA complex was purified by gel filtration through a Sephadex-G50. The isolated 230 bp long fragments or their complexes with PNAs in the Mg⁺⁺-containing buffer (49) were deposited on an atomically flat mica surface, allowed to adsorb for 1 min, rinsed with deionized water and dried in a gentle nitrogen flow. The AFM images were acquired in air using a NanoScope III system equipped with E-scanner (Digital Instruments) operating in tapping mode. The tapping frequency of the 125 μ m silicon cantilever was 300–400 kHz and the nominal scanning rate was set at 1–2 Hz. At least 50 uncomplexed DNA molecules and no less than 25 PNA-DNA complexes were analyzed.

Fluorescent measurements with PNA beacon

Fluorescence was monitored on a Hitachi Fluorescence Spectrophotometer F-2500 equipped with an external water-bath, using a semimicrofluorometer cell with a Teflon stopper. Kinetic responses of the stemless PNA beacon upon end invasion to duplex DNA target was measured at 37 and 45°C in 20 mM Tris-HCl buffer (pH 8.0). For fluorescent monitoring of hybridization kinetics, 20 pmol of PNA beacon was pre-equilibrated in 200 μ l of TE buffer (pH 7.2) until no change in fluorescence with time was detected. After that, the \sim 40 pmol of DNA target was added followed by vigorous mixing for 5–10 s. The fluorescent response was then recorded every 5 s until the detected signal reached a plateau, then oligonucleotide complementary to the PNA beacon was added to account fluorescence on 1:1 hybridization with molecular

beacon. Samples were excited at 468 nm, and the emission was measured at 517 nm.

RESULTS AND DISCUSSION

End invasion of mixed-base PNA

We first used the gel-shift assay to detect and to study the formation of PNA-DNA complexes. Figure 2 shows typical patterns, which demonstrate \geq 50% binding of the mixed-base 13mer PNA 1720 to the correct dsDNA target fragment, which results in its significant retardation (lanes 2 and 3). We failed to make any quantitative kinetic measurements with this assay since the PNA-DNA complexes are very unstable in more than 50 mM Na⁺ while the binding was too fast (within just a few minutes) at low ionic conditions to be accurately detected by the slow-responding gel-electrophoretic approach. The PNA end invasion was efficient up to 20 mM Na⁺, after which it was strongly inhibited by salt (data not shown).

The PNA-DNA complex formation proceeds with a high sequence specificity: PNA does form stable complexes with the complementary dsDNA target but does not if there is a single mismatch inside the PNA-binding site (lanes 5 and 6). Figure 2 also presents additional evidence for the site-specific PNA binding by showing the retardation of biotinylated PNA-DNA complex with streptavidin (lane 4) and no retardation with mismatched target. Under optimal conditions (low salt and high PNA concentration), we obtained \geq 50% of PNA-DNA complexes. Similar data were obtained with another mixed-base PNA oligomer, PNA 6976 (Figure 2, lanes 10 and 11). Note that the end invasion of mixed-base PNA is efficient only at the duplex termini: no binding was observed for PNA 6977 with the same target DNA fragment; in this case the PNA-binding site is positioned just 2 nt from the end of dsDNA fragment (Figure 2, lanes 8 and 9).

AFM analysis of PNA-DNA strand-invasion complexes

To visualize the formation of PNA-DNA complexes via strand invasion at nanometer resolution, we analyzed the PNA-DNA

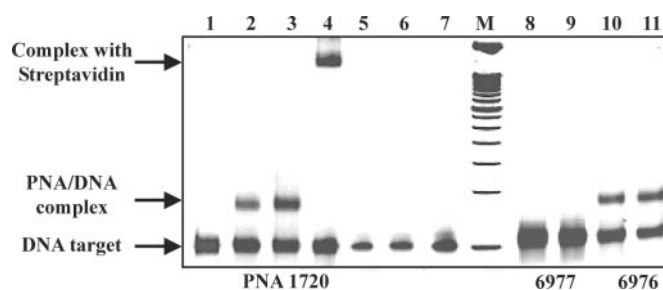


Figure 2. Polyacrylamide gel mobility shift assays. Complex of PNA with 100 bp long ds-oligonucleotides carrying matched and mismatched PNA targets is studied (see Figure 1A). Oligonucleotides with matched (lanes 1–4) and mismatched (lanes 5–7) PNA-binding sites were targeted by 0.5 μ M (lanes 2 and 5) and 5.0 μ M (lanes 3 and 4 and lanes 6 and 7) of PNA 1720 (total PNA concentration). In lanes 4–7 streptavidin was added to the PNA/DNA complex. Lane 1 presents control without PNA (the DNA fragment with the matched PNA-binding site). On the right side of the gel shown PNA-dsDNA complex created via strand invasion exactly on the end of duplex (lanes 10 and 11) and from the 2 nt far from the end (lanes 8 and 9). PNA-binding sites were targeted 0.5 μ M (lanes 8 and 10) and 5.0 μ M (lanes 9 and 11) PNA 6977 and 6976, correspondently. M is a 100 bp ds DNA size marker (Promega).

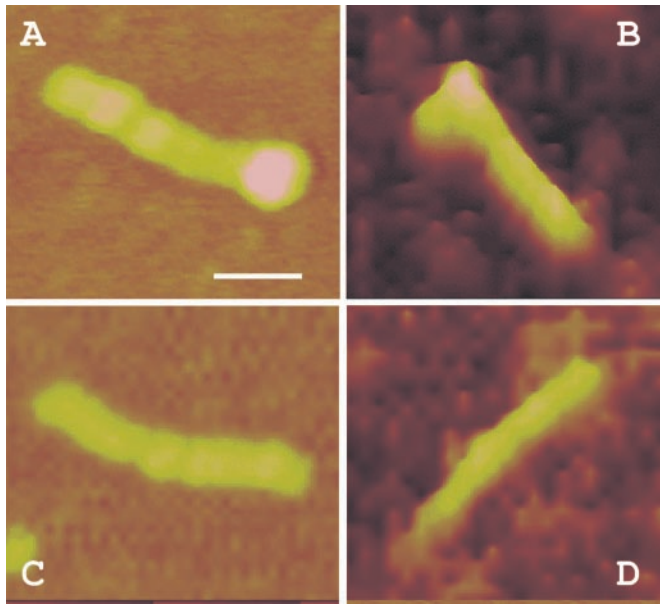


Figure 3. AFM images of the PNA–DNA strand invasion complex. PNA–dsDNA complexes were prepared with PNA 1720 and 230 bp EaeI fragment with 4 bp overhang as described in Materials and Methods and the reaction products were visualized by AFM. Representative AFM images of free DNA molecules (C and D) and the PNA–dsDNA complexes (A and B) are shown. The scale bar represents 25 nm. As predicted, only in the presence of PNA we observed dsDNA molecules with the ‘bump’ or kink at one of DNA ends.

end-invasion complexes by AFM. We prepared the complex of the 230 bp long EaeI-fragment with 13mer PNA 1720 (Figure 1B). We assumed that we could detect a PNA-invasion complex at one of the fragment termini carrying the PNA recognition site.

In the presence of PNA, we observed dsDNA molecules with the terminal bulge and/or kink (Figure 3A and B). No such features were ever observed in control experiments without PNA (Figure 3C and D). Images like in Figure 3 are exactly what one could expect. Indeed, the end-invasion complex must leave the displaced DNA single strand in the coiled state resulting in a bulge on AFM images. In addition, structural disturbances on the junction between dsDNA and PNA/DNA duplex may lead to a kink, which can be apparent or less visible depending on the viewing projection.

Our findings suggest that the PNA–DNA end invasion in sequence-specific fashion may find application in discriminating between free ends of DNA molecule, which could be particularly useful in the mapping of protein binding sites on linear dsDNA fragments in AFM studies.

Affinity capture of dsDNA fragments

To additionally demonstrate that the PNA–DNA complexes we observed were formed via strand invasion, we investigated the affinity capture of a restriction fragment carrying the target site in the presence of the rest of plasmid DNA as a background. To this end we prepared recombinant plasmids carrying correct PNA-binding sites by cloning corresponding inserts into EcoRI–BamHI sites of the pUC19 polylinker. Two different sets of restriction fragments were prepared by using suitable restriction enzymes to create a PNA-binding site

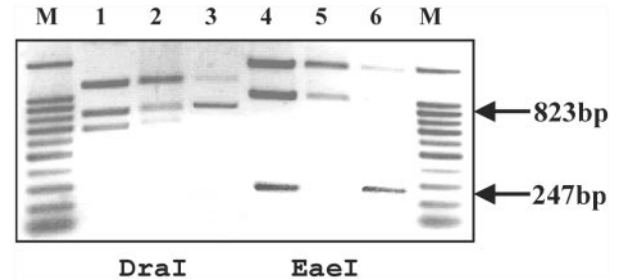


Figure 4. Affinity captures of a specific fragment by biotin-labeled PNA. Lanes 1–3 show fragments with blunt ends obtained by DraI restriction enzyme. Lines 4–6 show fragments with uneven ends obtained by EaeI restriction enzyme. Lanes 1 and 4 show the plasmid fragments after restriction but before capturing. Lanes 2 and 5 and lanes 3 and 6 show non-captured and captured material, respectively. M is a 100 bp dsDNA size marker (Promega).

at the end of the duplex. We used the DraI enzyme to obtain a blunt end and the EaeI restriction enzyme to obtain a 4 nt overhang on the target strand (Figure 1B). The rationale behind using the latter sample was as follows. Grounding on the data of Kushon *et al.* (50) showing that invasion of a mixed-base PNA into short DNA hairpins is facilitated by binding of the part of PNA oligomer to a single-stranded DNA loop, we assumed that the PNA–DNA complex would be stronger (thus featuring an improved capturing affinity) if the target strand carries an overhang, which can transiently bind PNA prior DNA invasion into the adjacent duplex.

We used PNA carrying biotin to allow convenient isolation of dsDNA fragments using streptavidin-coated magnetic beads, with supernatant and captured DNA being analyzed by agarose gel electrophoresis. Figure 4 shows that the corresponding target DNA fragment was indeed selectively captured via the PNA end invasion. Contrary to our expectations, we did not find any substantial differences in capturing fragments with and without overhands.

Extension of PNA/DNA chimera on duplex DNA

With the PNA assistance, an oligonucleotide primer can be hybridized to dsDNA and the primer-extension reaction can be performed on dsDNA with the help of DNA polymerase with strong-strand displacement ability (51–53). Here we show that a PNA/DNA chimera, which binds to dsDNA via terminal strand invasion, can also serve as a primer for DNA polymerase with the strand-displacement potential [modified T7 DNA polymerase or Sequenase (54)]. This allowed us to monitor the products of the primer-extension reaction performed in the presence of fluorescently labeled dNTP using a standard DNA sequencer. We used for detection an ALF Express DNA Sequencer instrument. Denaturing polyacrylamide gel electrophoresis separates the elongation products with a single-base resolution. The full-length product of the primer-extension reaction is expected to be 137 or 508 bp long for AatII/PvuII and AatII restriction fragment, respectively, which contains terminally located PNA/DNA-chimera—binding sites.

In full accordance with our expectation, the data in Figure 5 show the appearance of two characteristic peaks that prove that the primer-extension reaction really occurred in both cases. These data provide an additional evidence of the end invasion of PNA into dsDNA. Moreover, they demonstrate

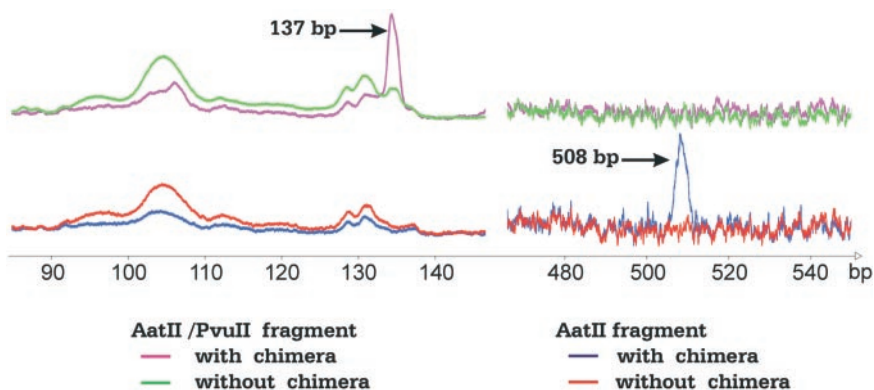


Figure 5. The primer-extension reaction performed on duplex DNA from PNA/DNA chimera as a primer. The primer-extension reaction performed in the presence of fluorescently labeled Cy5-dATP is shown. The elongated products were resolved on 8% polyacrylamide gels containing 7 M Urea using ALF Express DNA Sequencer. The exact location and length of the primer-extension product was determined by sequence mapping with single base-pair resolution. The sequencing ladder was prepared by priming from the identical position on supercoiled plasmid DNA and running in parallel columns on the same gel. As expected, the full-length product of the primer-extension reaction was observed only in a present PNA/DNA chimera: 137 bp in case of AatII/PvuII and 508 bp in case of AatII.

that not only mixed-base PNA but also a PNA/DNA chimera can hybridize to duplex DNA to be isothermally extended and fluorescently detected. Besides, these data clearly prove that all of the bases of this oligomer are paired with the target DNA strand, since only in this case the DNA part of a PNA/DNA chimera can serve as a functional primer for polymerase extension. Note that homologous full-length DNA probes didn't invade the dsDNA termini since no primer extension has been observed in this case (data not shown), in agreement with a prior study (55).

End invasion of PNA beacon into dsDNA

PNA beacons are convenient fluorogenic hybridization probes (21,56). We therefore decided to check whether they could sequence-specifically signal the presence of corresponding dsDNA targets via strand-invasion binding to the complementary sites with arbitrary sequences located at the end of a long linear DNA duplex. Note that we have recently observed the rapid strand-displacement binding of a mixed-base PNA beacon to short DNA duplexes (even at high salt) facilitated by their termini and used this process for DNA detection (57).

Figure 6 demonstrates the normalized kinetic responses of the PNA beacon upon its hybridization to a complementary DNA sequence at the end of the 103 bp long DNA duplex by the end-invasion mechanism at two different temperatures: 37 and 45°C. In both the cases, the PNA beacon rapidly binds to its target. Note that only half of the expected maximal signal response is observed, despite an excess of the DNA target. This is evidently the result of incomplete binding of the PNA beacon, which could be ascribed to additional energetic penalty for the PNA beacon binding due to quencher-fluorophore interactions in the nonbound state (note that regular PNAs may also exhibit the incomplete binding; see Figure 2). Still, the fluorescent response is sufficiently strong to be used for the dsDNA target detection.

Strand invasion of a mixed-base PNA into the nicked dsDNA

Single-stranded DNA break (nick) also creates the duplex boundaries but now within the dsDNA. We thus investigated

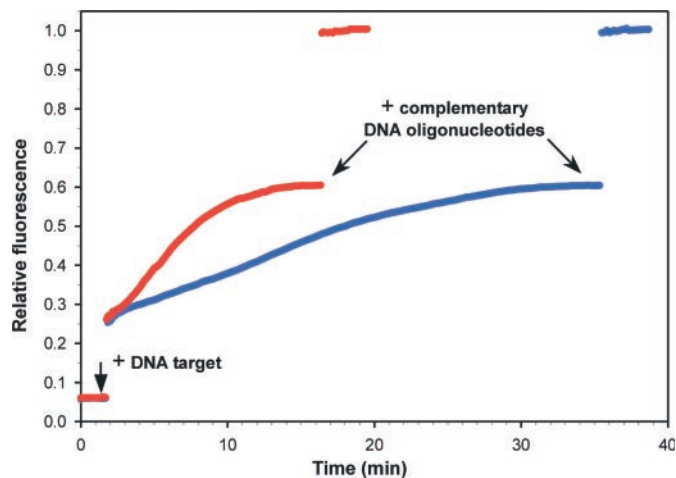


Figure 6. Fluorescence detection of PNA end invasion. Dependence of fluorescence on time of incubation of stemless PNA beacon with target dsDNA fragment (see Figure 1D) at two temperatures: 37°C (blue) and 45°C (red).

if a nick, rather than the double strand break, would be sufficient to allow the mixed-base PNA invasion next to it. For this study, we prepared the DNA fragment that contained a recognition sites for the Nicking Endonuclease N.A1wI and a binding site for the mixed-based PNA 1720 located right near a nick introduced by this enzyme (see Figure 1E). Figure 7 shows the results of the gel-shift assay proving the formation of PNA–DNA complexes within nicked dsDNA. Still, nick is less effective in facilitating the PNA strand invasion: we estimate that only ~30% of nicked DNA can form a complex with mixed-base PNA under conditions where we observed more efficient ($\geq 50\%$) PNA end invasion (compare Figures 2 and 7). Therefore, although these data are promising, the nick-facilitated PNA invasion requires further optimization (the subject of our future studies).

Some mechanistic considerations

The presented data show that mixed-base PNAs are well capable of end-invasion into linear dsDNA in a highly sequence-specific manner. This finding provides us with a strong

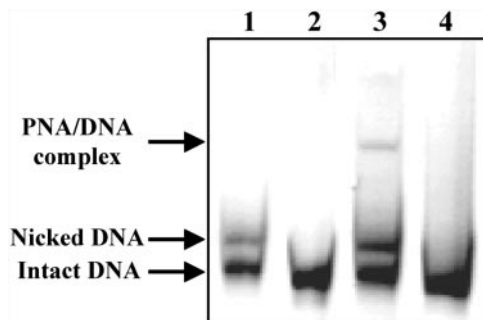


Figure 7. Strand invasion from a single-stranded nick within duplex DNA. Lanes 1 and 3 show the DNA fragments with the nick generated by N.A1wI nicking enzyme. Lines 2 and 4 show intact fragments without a nick. Lanes 3 and 4 show the complex this DNA fragments with PNA 1720. Note that the nicked DNA fragment moves somewhat slower than intact dsDNA (58), thus making it possible to directly monitor further transformations of nicked DNA.

evidence that the inability of a mixed-base PNA to invade internal sites of the double helix is due to the energy cost of the formation of two additional duplex termini flanking the open region within the double helix. Although such cost may eventually be compensated by the favorability of DNA–PNA duplex as compared for DNA–DNA duplex with increasing length of PNA oligomers, in the range of lengths usually dealt with in case of PNA (in the vicinity of 10mers) the unfavorability of the boundaries formation remains uncompensated even at lowest salts normally used. Excellent recognition selectivity exhibited by mixed-base PNAs in the process of their end invasion is a consequence of the stringency-clamping effect (3), i.e. a competition of the perfectly matched complementary DNA strand with the mismatched PNA binding.

Very fast (minutes) PNA invasion at the DNA duplex termini differs dramatically both from slow invasion of PNA oligomers into internal dsDNA sites [dozens of minutes; (10)] and from very slow (hours) kinetics of oligonucleotide replacement in DNA duplexes via strand displacement (59). The first difference can be explained by the known fact that the duplex termini much more readily dissociate than internal base pairs (60–62), thus facilitating the initial ‘nucleation’ event leading to complete PNA–DNA binding (3). A difference of the PNA end invasion from the oligonucleotide replacement is explained by a higher stability of the PNA–DNA duplex, compared with the DNA–DNA duplex (8).

CONCLUSION

In addition to shedding additional light on the mechanism of PNA invasion into the double helix, our data show the direction toward the development of new assays, which would allow using mixed-base PNAs for manipulating with dsDNA in a highly sequence-specific and sequence-unrestricted manner. Indeed, we have demonstrated here that once the DNA duplex boundary is produced, regular mixed-base PNA oligomers become capable of delivering various assays for duplex DNA similar to those which have previously been developed by us and others utilizing the ability of homopyrimidine bis-PNAs or pcPNAs to strand invasion into dsDNA (8,22,24,25,52,63).

Besides the double-strand breaks and nicks, some other means for generating the DNA duplex boundaries next to the PNA-binding site are possible. These boundaries can be created by the invasion of rather short, 6–8mer homopyrimidine PNA inside the DNA double helix. The combined use of mixed-base PNA and homopyrimidine PNA (to facilitate the mixed-base PNA binding) would evidently invoke only mild sequence limitations but it should be more sterically favorable and more sequence specific than tail-clamp PNA constructs (39,40). Furthermore, quinoxaline antibiotics and/or some other DNA-binding drugs that locally open DNA duplex may promote the internal binding of mixed-base PNAs, as it was the case for homopyrimidine bis-PNAs (64). Although all these cases are energetically less favorable compared to free duplex termini, they are worth trying in the future.

Finally note that the PNA sequence limitations are similar to those of triplex-forming oligos (65). Significant advances in sequence-unrestricted triplex-based recognition of dsDNA are recently reported (65,66). Further development of both these strategies would be beneficial for different applications.

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