

Brief Communication

PNA Beacons for Duplex DNA[†]

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ABSTRACT

We report here on the hybridization of peptide nucleic acid (PNA)-based molecular beacons (MB) directly to duplex DNA sites locally exposed by PNA openers. Two stemless PNA beacons were tested, both featuring the same recognition sequence and fluorophore-quencher pair (Fluorescein and DABCYL, respectively) but differing in arrangement of these groups and net electrostatic charge. It was found that one PNA beacon rapidly hybridized, with the aid of openers, to its complementary target within duplex DNA at ambient conditions via formation of a PD-like loop. In contrast, the other PNA beacon bound more slowly to preopened duplex DNA target and only at elevated temperatures, although it readily hybridized to single-stranded (ss) DNA target. Besides a higher selectivity of hybridization provided by site-specific PNA openers, we expect this approach to be very useful in those MB applications when denaturation of the duplex DNA analytes is unfavorable or undesirable. Furthermore, we show that PNA beacons are advantageous over DNA beacons for analyzing unpurified/nondeproteinized DNA samples. This feature of PNA beacons and our innovative hybridization strategy may find applications in emerging fluorescent DNA diagnostics.

INTRODUCTION

MOLECULAR BEACONS (MB) REPRESENT robust hybridization probes carrying a fluorophore and a quencher at their termini (Tyagi and Kramer, 1996; Tan et al., 2000; Tyagi et al., 2000). These probes are ingeniously designed to exhibit a fluorescence signal on binding to complementary targets, thus allowing the real-time quantitative monitoring of hybridization. MB and other fluorescent probes have become very useful tools for DNA diagnostics (Kostrikis et al., 1998; Piatek et al., 1998; Tyagi et al., 1998; Vet et al., 1999; Vogelstein and Kinzler, 1999; Fang et al., 1999; Whitcombe et al., 1999; Svanvik et al., 2000). To use them, however, DNA must be in a denatured single-stranded (ss) form to allow Watson-Crick pairing of the MB to the target site. (There is an abstract reporting on the Hoogsteen-type hybridization of a triplex-forming molecular beacon to a GC-rich site on dsDNA [see Antony et al., 2000]). This requirement limits applications of MB.

We have developed an approach that allows probe hybridization to double-stranded (ds) DNA (Bukanov et al., 1998). Our approach is based on local opening of dsDNA by a pair of pyrimidine bis-peptide nucleic acid (PNA) openers (Nielsen et al., 1991; Egholm et al., 1995), yielding an extended strand displacement complex (see Fig. 1). This structure is able to accommodate DNA and PNA probes by formation of PD-loop or PP-loop complexes, respectively (PD-loop and PP-loop, looped complexes formed inside duplex DNA by PNA openers in concert with oligonucleotide or PNA probes, respectively). Thus, formed PD-like loops comprise both PNA openers and a probe oligomer, which can carry reporter groups or be further processed within these composite PNA-DNA nanoconstructs. As a result, highly selective detection of specific sequences within linear DNA duplexes (Broude et al., 1999; Demidov et al., 2001a) and topologic labeling of dsDNA (Kuhn et al., 1999, 2000; Demidov et al., 2001b) have become possible.

Here, we show that a dsDNA target, when locally exposed by

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[†]This paper is dedicated to the forthcoming 10 years of use of PNA, which was first introduced in December 1991.

PNA openers, may effectively hybridize with a PNA-based MB under appropriate conditions. We demonstrate that the new molecular-diagnostic approach works by the selective fluorescent detection of HIV-1 marker in the form of dsDNA that exemplifies an integrated provirus. Moreover, we show that PNA MB are able to detect selectively target DNA in the presence of DNA-binding proteins. Our findings open the way for application of the MB methodology to nondeproteinized and nondenatured duplex DNA analytes.

MATERIALS AND METHODS

PNA openers and PNA MB

Bis-PNA openers, HLys₂-TCTC₂TC₂-(eg1)₃-J₂TJ₂TJT-LysNH₂ (**I**) and HLys₂-TJTJ₂T₂J-(eg1)₃-CT₂C₂TCT-LysNH₂ (**II**) (Lys denotes lysine, eg1 denotes the linker unit 8-amino-3,6-dioxaoctanoic acid, and J is pseudoisocytosine), were obtained commercially from PE Biosystems (Norwalk, CT). PNA MB **1**, HLys₂-Lys(Flu)-AAGCTACTGGA-Lys(DABCYL)-Lys₂NH₂, and PNA MB **2**, Flu-Glu-AAGCTACTGGA-Lys-Lys(DABCYL)NH₂ (Flu denotes Fluorescein and Glu glutamic acid, respectively), were synthesized using Fmoc PNA chemistry as described by Thomson et al. (1995). (PNA beacons are commercially available from Boston Probes Inc., www.boston-probes.com.) The Fluorescein and DABCYL [4-(4'-dimethylaminophenylazo)benzoic acid] residues were conjugated to the parent PNA oligomers either via the ϵ -amino group of Lys or linked to the N-terminal amino group of Glu. The synthesized PNA MB were purified by HPLC, and their identity was confirmed by MALDI-TOF mass spectrometry: PNA beacon **1**, calculated mass 4407.7, found mass 4406.3; PNA beacon **2**, calculated mass 4022.9, found mass 4022.7.

DNA targets

Duplex DNA target, the recombinant plasmid (pUC19 derivative) that carries a part of the HIV-1 sequence (pHIV), was constructed by cloning the appropriate oligonucleotides into the *Bam*HI site of pUC19. It carries a 21-bp-long sequence, 5'-AGAGGAAGCTACTGGAGGAGA-3' (the binding sites for PNA openers are underlined), from the HIV-1 *nef* gene coding region (strain 92ug037 of HIV-1 subtype A). Note that PNA beacon binds to the complement of this sequence. As ssDNA target, the 21-mer oligonucleotide 5'-TCTCCTCCAGTAGCTTCCGTT-3' was employed (the sequence complementary to the PNA beacons is underlined). pUC19 (New England BioLabs, Beverly, MA) was used as the unrelated dsDNA control. To check the binding of PNA openers and PNA beacons by gel-shift assay, pHIV was digested with *Pvu*II. Plasmids were targeted by PNA openers, gel-filtrated through a Sephadex G-50 spin column, ethanol precipitated, and redissolved, as previously described (Kuhn et al., 2000). Concentrations of all samples were determined spectrophotometrically at 260 nm following standard procedures (Sambrook et al., 1989; Nielsen and Egholm, 1999).

Fluorescence measurements

Fluorescence was monitored in a standard microcuvette on a TimeMaster spectrofluorometer (Photon Technology Interna-

tional, Lawrenceville, NJ) equipped with an external waterbath. For fluorescent monitoring of hybridization kinetics, 180 μ l of a PNA MB solution in 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM MgCl₂ was preequilibrated until no change in fluorescence with time was detected. After that, 20 μ l control oligonucleotide or DNA plasmid solutions was added to a PNA MB sample (\sim 200 nM final concentrations of both target DNA and PNA MB), followed by vigorous mixing for 5–10 seconds. The fluorescent response was recorded every 5 seconds until the detected signal reached a plateau. The samples were excited at 493 nm, and emission was measured at 520 nm. Steady-state fluorescence spectra were measured in the emission range 500–650 nm (excitation at 493 nm).

Gel electrophoresis

Electrophoresis was performed in a 10% native polyacrylamide gel at 25–30°C for 4 hours using TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0). The gel was subsequently stained by ethidium bromide, visualized by transillumination at 302 nm, and scanned with a CCD camera. A commercial 100-bp ladder (GIBCO-BRL, Gaithersburg, MD) was used as a size marker.

RESULTS AND DISCUSSION

As a model dsDNA target, the recombinant plasmid pHIV was constructed that carries a part of a conservative coding region of the HIV-1 *nef* gene capable of PD-loop formation. Instead of the commonly used DNA MB, PNA MB **1** and **2**, each consisting of a PNA probe oligomer end-tagged by a fluorophore (Fluorescein) and a quencher (DABCYL), were employed herein to provide stronger binding of the probe (Egholm et al., 1993; Jenssen et al., 1997; Ortiz et al., 1998). Both PNA MB have the same recognition sequence but differ in arrangement of reporter groups and net electrostatic charge (see Materials and Methods for their sequence and construction).

Note that the MB we used did not have the classic stem-loop structure because this structure had been found unnecessary for their functioning (Coull et al., 1999; Gildea et al., 1999; Seitz, 2000). We believe that such a stemless PNA MB forms a closed loop, as fluorophore and quencher fasten it as a result of hydrophobic or electrostatic interactions (or both) between them. The high flexibility of the PNA backbone (Egholm et al., 1993; Brown et al., 1994; Chen et al., 1994; Leijon et al., 1994; Püschel et al., 2000) warrants these interactions.

The procedure we followed is schematically summarized in Figure 1. First, an intermediate strand-invasion complex is formed within pHIV. In it, a pair of bis-PNA openers **I** and **II** is bound to one of the two DNA strands at closely located octapurine dsDNA sites, leaving the opposite strand locally displaced and hence available for Watson-Crick hybridization. Next, binding of the PNA beacon, which is complementary to the displaced strand of the preformed strand-invasion complex, is performed, resulting in an intense fluorescence signal due to PP-loop formation. Note that in the absence of a target, both PNA beacons show very weak background fluorescence and are, therefore, predominantly in the closed conformation. Thus, it was by no means obvious that our procedure would yield PP-

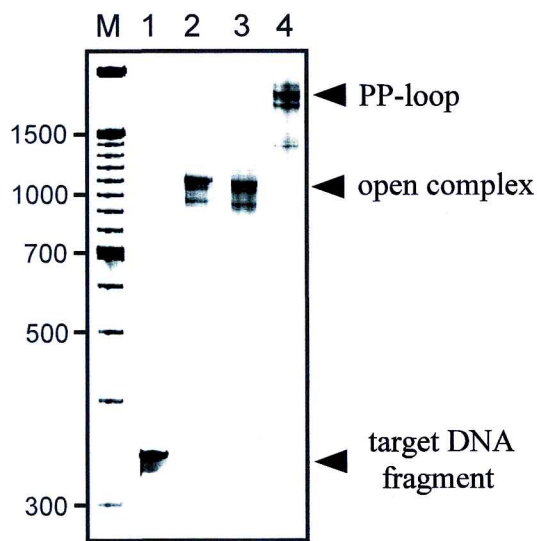
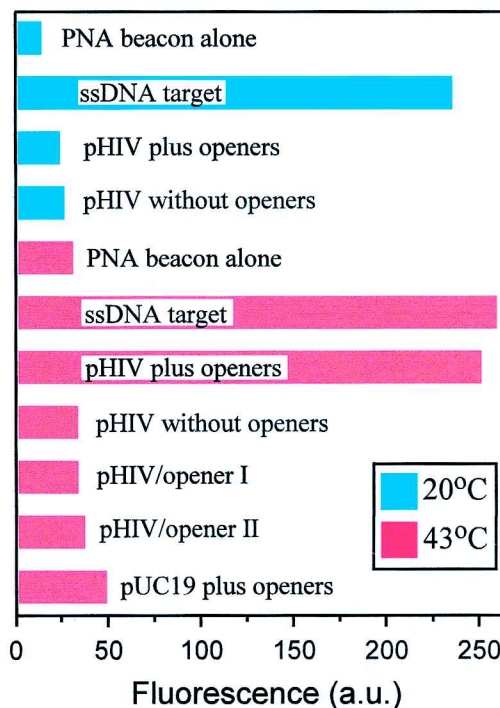
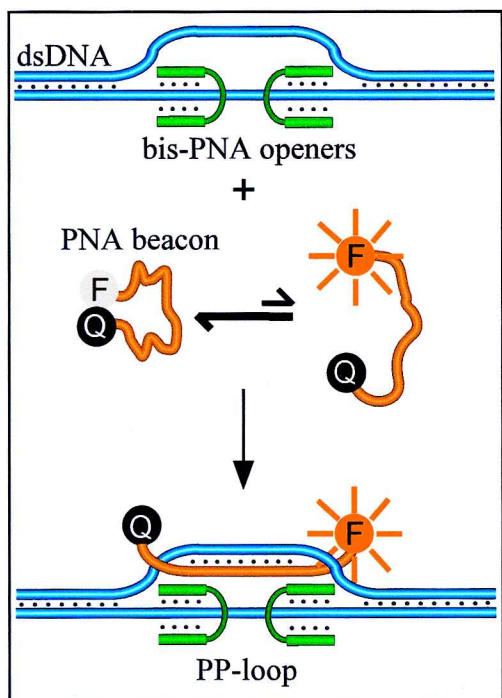


FIG. 2B

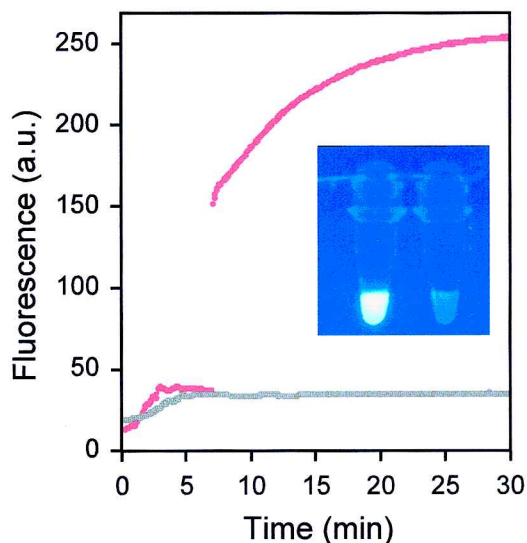


FIG. 2C

FIG. 1. Schematics of the hybridization/detection assay. Duplex DNA target is locally exposed by a pair of bis-PNA openers via strand displacement. The displaced DNA strand contains the complementary binding site for a PNA MB. The formation of the resulting PP-loop manifests itself in the form of a fluorescence response due to spatial separation of fluorophore (F) and quencher (Q) in the open PNA MB. Note that an equilibrium between closed and open conformations of the PNA MB is required for the PP-loop formation (see Results and Discussion).

FIG. 2. Hybridization of PNA MB 1 with dsDNA target. (A) Bar diagram showing a steady-state fluorescent emission of PNA MB 1 alone and after addition of preopened dsDNA target or control samples. (B) Gel-shift assay of PP-loop formation by PNA MB 1. The 344-bp-long *PvuII*-*PvuII* target fragment of pHIV (lane 1) was first incubated with both PNA openers, yielding the locally exposed pHIV (lane 2). Then, PNA MB 1, preincubated for 20 minutes at 20°C (lane 3) or 43°C (lane 4), was added, and the mixture was incubated at the corresponding temperature for an additional 30 minutes before electrophoretic analysis. (C) Kinetics of PNA MB 1 hybridization to preopened pHIV target (red curve) and pHIV in the native state (gray curve) at 43°C. The dsDNA samples were added after a steady level of background fluorescence of PNA MB was reached (7 minutes). (Inset) Photograph of two microtubes each with 2 μM PNA MB 1 in 50 mM Tris-HCl buffer, pH 8.0, with 1 mM MgCl₂ added. The left tube contains 2 μM preopened pHIV, and the right tube contains the same amount of the native plasmid. After addition of the DNA targets to the PNA MB, the two samples were incubated for 30 minutes at 43°C in the dark. They were then illuminated with a broad-wavelength UV light source and photographed using ASA 200 film.

loop complex and the corresponding fluorescence enhancement.

Indeed, to form the final PP-loop structure, the PNA MB must transiently open, as shown schematically in Figure 1, and thread itself in the open form through the needle's eyelike strand-invasion complex, making about one turn around the DNA target strand (Eriksson and Nielsen, 1996). For that, the PNA MB should breathe extensively to perform the operation without serious problems. This situation differs radically from the MB hybridization to ssDNA target, which may readily thread a closed MB form and then open it in the process of complementary binding.

In fact, in our first experiments, we failed to observe binding of PNA MB **1** to the preopened pHIV target at ambient temperature. At the same time, this probe readily fluoresced under the same conditions on addition of the complementary (but not unrelated) oligonucleotide (Fig. 2A). The gel-mobility shift assay additionally proved that PNA MB **1** did not bind at ambient temperature to the exposed pHIV target even during a long incubation time (compare lanes 2 and 3 in Fig. 2B). However, the corresponding PP-loop complex was readily formed at 43°C (lane 4 of Fig. 2B; see also Fig. 2A).

Figure 2C shows the hybridization kinetics of PNA MB **1** to the pHIV target as monitored by fluorescence at elevated temperature. One can see that, in agreement with the gel-shift data, this MB gradually hybridizes to the locally opened pHIV target at 43°C, developing a bright fluorescence (Fig. 2C, Inset). The fluorescent response of the observed hybridization is sequence specific, as no significant rise in fluorescence was detected when PNA MB **1** was added to the unrelated plasmid, pUC19, preincubated with both PNA openers (Fig. 2A). Similarly, no fluorescence enhancement was observed when unopened pHIV

target or pHIV opened by only one of the two PNA openers was added to the MB (Fig. 2A).

The lack of binding of PNA MB **1** to the preopened pHIV target at ambient temperature can be explained by the apparent inability of its closed conformation, which is predominant under normal conditions, to thread the local opening within the dsDNA target. Presumably, at elevated temperature, the equilibrium between closed and open states of this MB is somewhat shifted to the open species, as indicated by a 2–3-fold increase in its background fluorescence (Fig. 2C, the first 5 minutes), thus accelerating the PP-loop formation.

In contrast, PNA MB **2** yielded, after mixing with either complementary oligonucleotide or the pHIV target exposed by PNA openers, a rapid increase in fluorescence at ambient temperature (Fig. 3A), which leveled off within a few minutes. At present, we cannot explain why the two MB we used behaved differently. Answering this question requires further, more systematic studies. Figures 2A and 3B demonstrate that fluorescent responses observed as a result of the formation of sequence-specific PP-loop complexes of pHIV with both PNA MB are significantly higher than for control samples. The signal/background ratios were close to 8 and 11 for PNA MB **1** and PNA MB **2**, respectively (measured at fluorescence emission maximum of 520–530 nm) (Fig. 3A, Inset) Because the intensities of steady-state fluorescence developed by PNA MB with dsDNA and ssDNA targets were virtually identical, we conclude that the yield of hybridization in our experiments was close to 100%.

The fluorescence of DNA MB is known to be strongly enhanced in the presence of certain proteins, such as SSB protein, and some enzymes (Li et al., 2000a,b; Fang et al., 2000). Whereas this feature of DNA MB is beneficial for developing

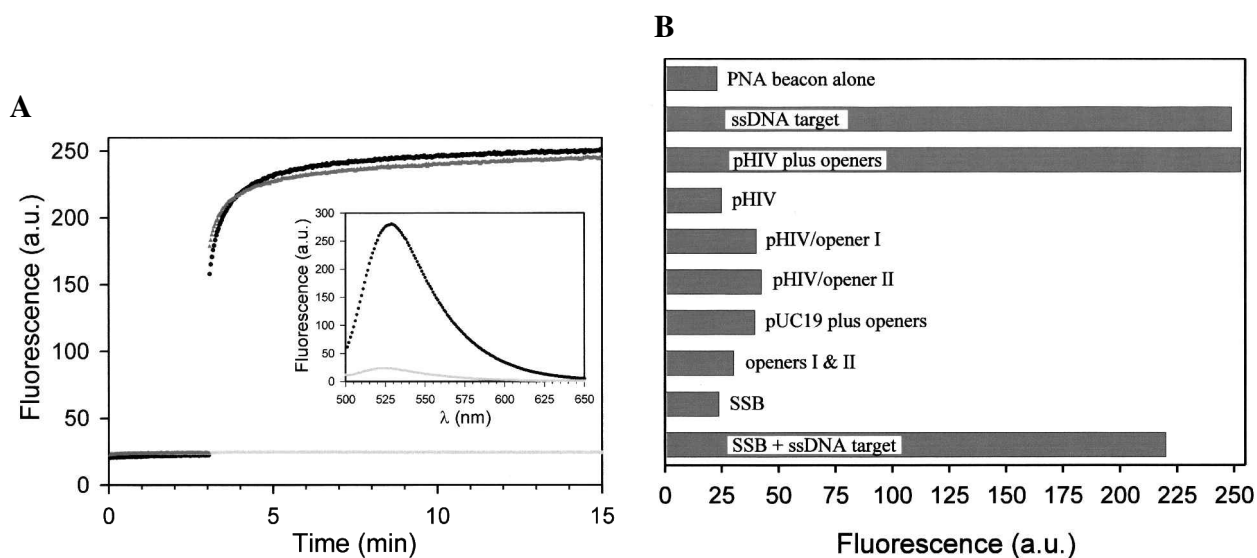


FIG. 3. Hybridization of PNA MB **2** with dsDNA target. (A) Kinetics of PNA MB **2** hybridization to preopened pHIV (black curve), native pHIV (light gray curve), or complementary oligonucleotide (dark gray curve) at 25°C. All DNA samples were added after 3 minutes preincubation of the MB alone. (Inset) Steady-state fluorescence spectra of PNA MB **2** in the presence of preopened pHIV (black curve) or native pHIV (light gray curve). (B) Bar diagram showing a steady-state fluorescent emission of PNA MB **2** alone and after the addition of PNA openers, preopened dsDNA target, or control samples. The lower two bars show the fluorescent response obtained after the addition of 1 equivalent of *Escherichia coli* ssDNA-binding protein (SSB) (Promega, Madison, WI), followed by the addition of 1.5 equivalent of complementary oligonucleotide. Note that PNA openers do not interfere with the MB.

sensitive protein assays, it is disadvantageous for those DNA analytes in which DNA-binding proteins are present. It is well known that PNA is not a substrate for DNA/RNA-processing enzymes (Hanvey et al., 1992; Demidov et al., 1994; Lutz et al., 1997) and, most probably, should not bind them or change its conformation in the presence of the majority of proteins.

In accord with this expectation, we found that PNA MB **2** remained quenched in the presence of SSB protein (Fig. 3B), whereas DNA MB substantially responded to this protein (Li et al., 2000a; unpublished observations). However, despite the presence of SSB, the fluorescence of PNA MB **2** reaches almost a maximum level by further addition of ssDNA target (Fig. 3B, last bar). Therefore, PNA MB are well suited for real-time fluorescent detection of specific DNA markers in unpurified/nondeproteinized analytes.

We have demonstrated here that under some conditions, PNA MB can efficiently hybridize to a specific target within dsDNA locally exposed by PNA openers. We expect this approach to be useful in diagnostic applications in which denaturation of DNA analytes is unfavorable or undesirable. Moreover, even thorough purification of target DNA most probably will not be necessary allowing to analyze crude DNA extracts. It is reasonable to assume that other PNA-based fluorescent probes (Svanvik et al., 2000) could be of similar use.

Affinity capture and labeling of dsDNA via PD-like loops have already demonstrated a remarkable, single-nucleotide sensitivity of the PD-loop technology (Bukanov et al., 1998; Broude et al., 1999; Kuhn et al., 1999, 2000; Demidov et al., 2000, 2001b). As in the case of other applications of PD-loop and PP-loop strategy, a major advantage of targeting duplex DNA with MB may be a higher selectivity of binding because of the probe's inability to hybridize with most of the mismatched binding sites because they are not accessible. Together with the immunity of PNA MB to the presence of proteins, this important feature of PD-like loops may yield robust DNA diagnostics capable of detecting specific DNA markers in raw samples contaminated not only by proteins but also by an excessive background of unrelated DNA. A study on mutation discrimination with PNA and DNA MB of different forms (stem-containing and stemless) via PP-loop/PD-loop hybridization is currently in progress.

The use of MB for nondenaturing DNA diagnostics via PD-like loops is limited by the need for two short, closely located oligopurine sequences as binding sites for PNA openers within a corresponding DNA marker site. However, these limitations are rather insignificant for those diagnostic applications when detection of foreign DNA, for example, DNA of pathogenic microorganisms (as we demonstrated here with the HIV-1 marker), is required. Indeed, such sites should occur quite frequently, statistically in every 400–500 base pairs of a random DNA sequence, on average (Bukanov et al., 1998; Demidov et al., 2000, 2001a,b). Normally, each gene/genome of interest must contain more than one site capable of forming PD-like loops. Still, a typical PD-loop and similar structures include more than 20 bp, and, therefore, the corresponding site is normally unique as a marker for a specific genome. An analysis of numerous genomic sequences of various species currently available has confirmed this expectation.

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