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Sequence-Universal Recognition of Duplex DNA by Oligonucleotides via Pseudocomplementarity and Helix Invasion

Brief Communication

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Summary

The well-known Watson-Crick complementarity rules, which were discovered 50 years ago, elegantly direct the specific pairing of two DNA single strands. On the contrary, once formed, the double-stranded (ds) DNA lacks such a simple and sequence-universal recognition principle, since most of the characteristic chemical groups of nucleobases are now buried deep inside the double helix, the major DNA form. We report a promising versatile approach for highly selective recognition of designated sites within dsDNA featuring considerable practical potential for a variety of molecular-biological, biotechnological, gene-therapeutic, and diagnostic applications. It may also have implications for prebiotic evolution of genetic machinery at the primordial stages of the origin of life. Our design synergistically employs the robust helix-invasion ability of recently developed DNA mimics and analogs, pseudocomplementary peptide nucleic acids and pseudocomplementary oligonucleotides, thus enabling the sequence-unrestricted recognition of chosen DNA duplexes by nucleobase oligomers. Using this basically general approach, we selectively tagged a unique mixed-base site on the target dsDNA fragment with streptavidin and/or multiply labeled this site with fluorophores via the primer-extension reaction.

Introduction

The recognition of a specific DNA strand by the second (complementary) one proceeds easily via Watson-Crick base pairing [1–3]. Yet, special techniques are required to enable a third DNA single strand (usually in the form of an oligonucleotide) to recognize the designated sequence within linear DNA duplexes. Note that although various DNA binding ligands can be used for this purpose [4], targeting of dsDNA with oligonucleotides is advantageous for many applications. Accordingly, several approaches have been developed to this end.

One of them utilizes triplexes formed via Hoogsteen and Hoogsteen-like pairing of purine or pyrimidine oligonucleotides with a corresponding dsDNA site [4–6]. Another approach employs RecA protein as sequence-universal helper for binding of ordinary mixed-base oligonucleotides to dsDNAs [7, 8]. Pyrimidine peptide nucleic acids (PNAs) [9] have been used as site-directed "openers" for DNA duplexes [10–12], thus enabling hybridization of regular oligonucleotides and related probes with dsDNA via formation of so-called PD-loops [13–15]. One more approach, originally called "selective complementarity," is based on the pseudocomplementary strategy (Figure 1) that makes it possible for a pair of mixed-base modified oligonucleotides to hybridize to dsDNA by strand invasion [16, 17]. Pseudocomplementarity means that two special derivatives of initially paired normal purine and pyrimidine are structurally adjusted in such a way that they (1) do not match each other but (2) are capable of a stable Watson-Crick-type pairing with the natural nucleobase complements (see Figure 1A for pseudocomplementary modified nucleobases we used).

Though robust, these approaches did not provide a fully satisfactory solution of the problem of dsDNA targeting by oligonucleotides. Indeed, mostly long oligopurine tracts could presently be recognized via triplex formation [6]. Although more general, the PD-loop-based approach is still limited by purine-rich sequences as well [11, 13]. On the other hand, the RecA-assisted sequence-unrestricted DNA recognition has much lower specificity as compared to "pure" DNA-DNA (or DNA-RNA) interactions [18]. As for pseudocomplementary oligodeoxynucleotides (pcODNs), they can bind dsDNA only at the end of DNA duplexes [16, 17] (see structure I in Figure 1B), which significantly limits their application. We demonstrate here a protein-free sequence-unrestricted system for binding oligonucleotide probes to internally located dsDNA sites. This approach suggests a general principle for sequence-specific binding of oligonucleotides to duplex DNA, thus offering numerous subsequent uses.

Results and Discussion

Our design employs pseudocomplementary (pc) PNAs [19–23] as sequence-specific mixed-base openers for DNA duplexes to selectively generate duplex edges (structure II in Figure 1B) near the designated oligonucleotide binding site within a targeted dsDNA fragment. We assume that such dsDNA edges will then facilitate the proximal strand invasion of corresponding pcODNs (structure III in Figure 1B), similar to their binding at the very end of the dsDNA (see structure I in the same figure). Consequently, the internal dsDNA sites of an arbitrary sequence could be sequence specifically targeted by oligonucleotide probes. While this design is rather simple and straightforward, its workability is not obvious at the outset.

First, possible steric interference may occur between two complexes formed in close vicinity. Second, the conflicting salt requirements for formation and stability of pcPNA-dsDNA and pcODN-dsDNA complexes have to be taken into account. In fact, low salt is known to facilitate the pcPNA invasion into dsDNA [20]. Moreover, our own experience shows that in some cases stability of

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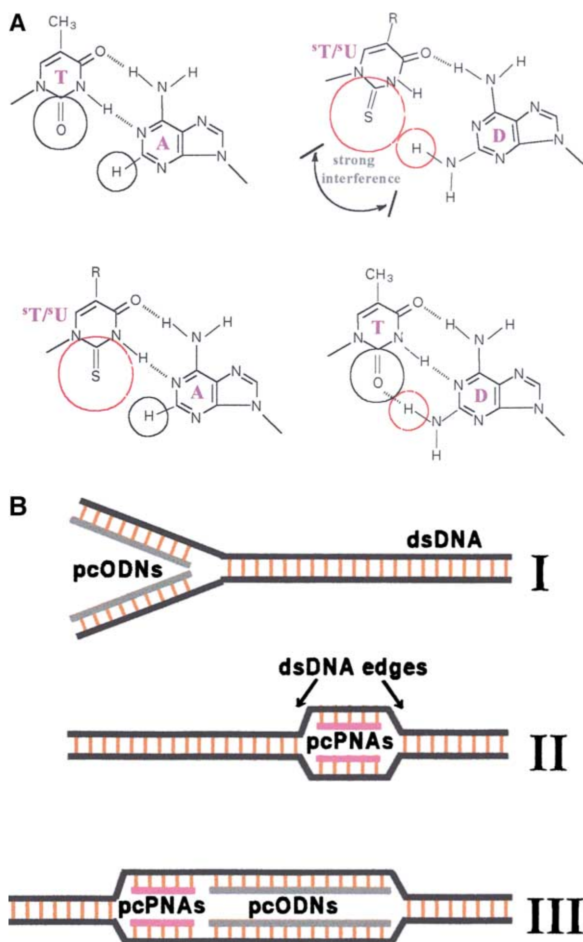

Figure 1. Schematic Representation of the Pseudocomplementary Nucleobase Oligomers' Hybridization with Duplex DNA

(A) Base-pairing schemes explaining the concept of pseudocomplementarity. Bulky groups of modified nucleobases, adenine derivative (D), and thymine derivatives (^{s}T [ODN] or ^{s}U [PNA]) cause a steric clash in D-^{s}T/^{s}U base pair (interfering atoms are shown as red spheres). Nevertheless, a perfect fit is retained in D-T and A-^{s}T/^{s}U pairs (sterically suitable atoms of A and T are shown as black spheres). As a result, only the complementary interactions between thus modified nucleobases are significantly obstructed, but they can form stable pairs with normal DNA counterparts.

(B) Schematics of different modes of dsDNA recognition by pseudocomplementary oligomers via the strand displacement: (I) the Y-shaped complex formed by pcODNs at the end of DNA duplex; (II) the eye-like double-duplex invasion complex formed by pcPNAs inside the DNA duplex; (III) the PNA-assisted internal binding of pcODNs developed in this work.

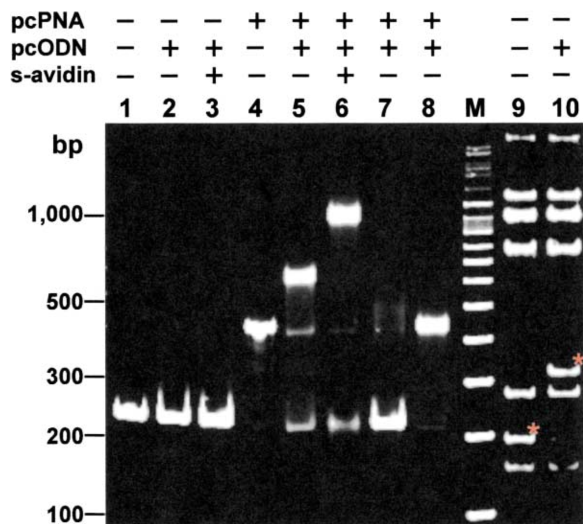

Figure 2. Gel-Shift Assay of pcODN-DNA Complexes Formed in the Middle of a dsDNA Target Fragment with the Aid of pcPNAs

Lanes 1–6 show the results obtained with the correct target site, whereas lanes 7 and 8 correspond to controls in which either PNA or ODN binding sites contain a single mismatch (218 bp dsDNA fragments were obtained by PCR amplification from the pUC19 vector with the cloned EcoR I-BamH I inserts). Lanes 9 and 10 represent the experiments in which the ODN binding site was located at the end of another dsDNA fragment (203 bp; marked by red asterisk) taken in the mixture with other DNA fragments (obtained as the BsmA I-Pvu II digest of the corresponding pUC19 recombinant). M is a 100 bp dsDNA size marker; s-avidin, streptavidin. Concentrations: DNA, 5 nM; PNAs, 10 nM; ODNs, 5 μM. 30 bp DNA target sites: 5′-GTAGATC(G)ACTG*TACA(G)ATTCGAGCTCGGTAC* (AT content [correct site] = 53%; PNA binding site is underlined; ODN binding site is italicized; single mismatches in these sites are given in parentheses; in the case of the end-located ODN binding, the TCTCGTA sequence was added right before the ODN binding site to create the BsmA I recognition/cleavage site). ODNs, 5′-^{s}TDC DD^{s}T^{s}TCGDGC^{s}TCGG^{s}TDC and 5′-G^{s}TDCCGDGC^{s}TCGDD^{s}T^{s}TG^{s}TD-biotin (ordered from MWG-Biotech); PNAs, HLys-G^{s}UDGD^{s}UCDC^{s}U-LysNH$_2$ and HLys-DG^{s}UGD^{s}UC^{s}UDC-LysNH$_2$ (a gift from P.E. Nielsen). PNA-DNA complex formation, 2 hr, 45°C, 10 mM TE buffer (pH 7.4); ODN-DNA complex formation, ~10 hr, 45°C, same buffer with addition of 200 mM NaCl and 10 mM MgCl$_2$; gel electrophoresis, nondenaturing 8% polyacrylamide gel filled with TBM buffer (TBM = TBE + 5 mM MgCl$_2$), 200 V, ~5 hr with ice cooling.

the pcPNA-dsDNA complexes severely decreases with increasing salt. Conversely, the pcODN-dsDNA complexes require high salt for their formation [16, 17]. Nevertheless, despite these complications, we were able to find conditions that are suitable for both pcPNA and pcODN binding to duplex DNA and where the multicomponent PNA-ODN-DNA complex can be stably formed at the internal sequence-unique dsDNA site by sequential pcPNA and pcODN strand invasion.

Figure 2 proves that this is really the case when 2-aminoadenine (or 2,6-diaminopurine, D) and 2-thiouracil (^{s}U) or 2-thiothymine (^{s}T) nucleobases are used in PNA [19] and ODN [16] contexts as the pseudocomplementary substitutes for adenine and thymine/uracil, respectively (Figure 1A). The results of the gel-shift assay we used to analyze the oligonucleotide-dsDNA binding clearly show that the D/^{s}T-substituted mixed-base pcODNs do quantitatively bind the complementary inner dsDNA site, provided the DNA fragment carrying this site was pretargeted with D/^{s}U-substituted mixed-base pcPNAs at a bordering site. Indeed, an additional retardation of the pcPNA-bound DNA fragment is observed upon addition of pcODNs (compare lanes 1, 4, and 5 in Figure 2), which is indicative of the formation of a stable complex between oligonucleotides and target dsDNA. On the contrary, these pcODNs are not able to bind to the internally located sequence-matched dsDNA site alone (lane 2, Figure 2), though they are capable of selective binding to the same site if it is located at the terminus of another dsDNA fragment (compare lanes 9

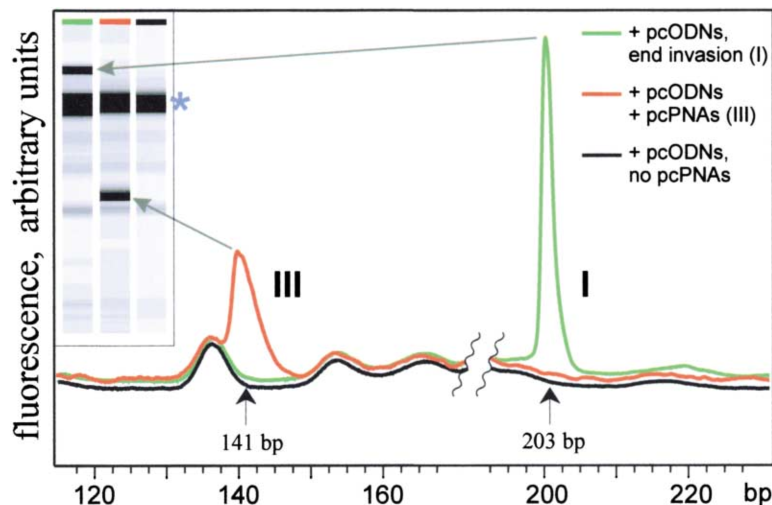

Figure 3. Extension of a Primer on Duplex DNA via the pcODN Hybridization

Primer-extension reactions, as recorded on an automated DNA sequencer, were performed by Sequenase enzyme on two dsDNA fragments targeted by pcODNs either terminally (green line; complex I in Figure 1B) or internally (red line; complex III in Figure 1B). In the latter case, a "no PNA" control has been made as well to detect the background fluorescence (black line). Only one of the two pcODNs had the free 3′ end and served as a primer, since the other oligonucleotide was chemically incapable of extension due to its 3′ capping with biotin (see the ODN structures in the Figure 2 legend). Peaks correspond to the length of extension products, starting from the hybridization site and extending to the end of DNA fragments. Multiple fluorescent labeling of these products was achieved by adding fluorogenic Cy5-dATP to a normal dNTP mixture. The inset shows computer-simulated "bar code" patterns obtained with the DNA sequencing software to imitate the gel-derived autoradiograms commonly used in the primer-extension analysis. The blue asterisk indicates an intense band in the background fluorescence intrinsic to the Cy5-dATP sample. Primer-extension reactions were run for 15 min with 2 μg of ODN-DNA or PNA-ODN-DNA complexes using 2 units of Sequenase and 10 nmol of dNTPs. Gel electrophoresis: 6% denaturing polyacrylamide gel filled with 7 M urea/TBE buffer, ~5 hr at 50°C, A.L.F. Express DNA Sequencer (Pharmacia Biotech).

and 10 of the same figure), in agreement with previous observations [16].

As can be expected, the effect of pcPNA pretargeting on the pcODN binding to DNA duplexes rapidly diminishes with the distance between their binding sites on dsDNA. In the experiments presented here, this distance is merely 1 bp, and we noticed only a slight increase in efficiency of the pcODN binding when the pcODN and pcPNA binding sites were immediately adjacent to each other. However, a significant drop in the pcODN binding to the pcPNA-bound DNA was observed if these sites were separated by 2 bp (data not shown). Importantly, the PNA-assisted binding of pcODNs inside of linear dsDNA exhibits high sequence specificity: a single mismatch in the ODN binding site obstructs its binding despite the PNA-DNA complex formation (lane 8, Figure 2). A mismatch in the PNA binding site also interferes with the pcODN internal invasion (lane 7), thus further enhancing the ODN recognition specificity. Lane 6 of Figure 2, featuring an extra retardation of DNA target fragment in the presence of streptavidin, demonstrates that DNA can be selectively tagged, via the PNA-mediated binding of oligonucleotide, with a protein when biotinylated pcODN is used (see Figure 2, lane 3 as a negative control without pcPNA).

Figure 3 shows the results of the primer-extension assay in the presence of fluorescently labeled nucleotide when one of the two pcODNs hybridized to the dsDNA target fragments acted as a primer for DNA polymerase with the strand-displacement potential. The full-length product of the primer-extension reaction is expected to be 141 or 203 bp long for internally and terminally located ODN binding sites, respectively. In fact, one can see in Figure 3 the appearance of two characteristic peaks that clearly validate the primer extension in both cases and additionally prove the ability of PNA to facilitate pcODN binding to the internal mixed-base dsDNA site. Thus, these data demonstrate that an oligonucleotide primer can be bound to and be isothermally extended on duplex DNA directly and that this reaction can be fluorescently detected.

Although this is not demonstrated here, oligonucleotides with the pseudocomplementary analogs of G and C nucleobases can also be used [17]. They may prove to be more efficient for PNA-directed targeting to the internally located GC-rich dsDNA sequences. Still, highly GC-enriched protracted DNA sites might require the G/C-modified PNAs as well for accomplishment of our approach. Therefore, the prospective synthesis of such pcPNAs and/or entirely modified pcODNs and pcPNAs carrying both A/T and G/C pseudocomplementary replacements should provide the complete range of sequence-universal tools for dsDNA recognition by oligonucleotides.

Significance

The results with protein tagging and primer extension illustrate that our approach can be used for sequence-specific manipulation with basically intact duplex DNA. We anticipate that it may be employed for nondenaturing dsDNA sequencing, labeling, and isolation, hence offering numerous subsequent applications for DNA diagnostics and DNA biotechnology. For instance, the DNA sequence analysis of unique sites in native chromatin and their complexes with different ligands can now be performed if the fluorescently labeled ODN primer and Sanger dideoxy sequencing protocol is used [24, 25]. The multicomponent complex we assembled on duplex DNA may also serve as a convenient polyfunctional building block for fabrication of composite DNA-based supramolecular constructs in the emerging field of DNA nanotechnology [26–29].

The PNA-based approach we developed for recognition and labeling of duplex DNA with oligonucleotides is more general, compared with oligonucleotide uptake

by supercoiled DNA [30–32]. Being also substantially less sequence restricted than another PNA-based approach for hybridizing standard oligonucleotides with dsDNA proposed previously [13], our methodology seems to be essentially sequence universal. As a protein-free system, it is significantly more sequence-specific than the RecA-based approach, which tolerates several nucleobase mismatches [18].

A higher sequence specificity of our design is additionally warranted by the two-step/multiple-coincidence recognition scheme: two closely located but independent DNA sites for simultaneous binding of two PNA oligomers and two oligonucleotides are involved in the complex formation. Note in this connection that even pseudocomplementary oligomers can still bind, although transiently, their pseudocomplementary counterparts by forming weak pcPNA-pcPNA or pcODN-pcODN pairs [16, 17, 21]. This process will further contribute to the superior selectivity of pcPNA-assisted dsDNA recognition by pcODNs via the "stringency clamping" effect of structurally constrained probes [33, 34]. Thus, practically zero tolerance of the complex formation to single-base mismatches is expected for our approach, which is strongly supported by negative results obtained with the mismatched dsDNA targets (Figure 2).

Given the possible role of PNA-based oligomers, including the oligonucleotide-PNA chimeras, in prebiotic chemistry of nucleic acids [35–44], the easily assembled protein-free biomolecular system we developed for PNA-directed dsDNA recognition by oligonucleotides may also have implications for primordial evolution of genetic machinery at the earliest stages of the origin of life. Here, the ability of pcPNA and pcODN pairs to bind conjointly to a specific site on dsDNA without serious sequence limitations, and the capacity of this dual complex to efficiently act as a priming point for the DNA-templated synthesis could be most significant. Importantly, the primer extension initiated by pcODN binding at the very end of the DNA duplex does not require PNA assistance and will evidently provide the complete replica of complementary DNA strand (see the green-colored peak in Figure 3). Note to this end that the alternatively possible triplex-directed scheme proposed for nonenzymatic replication of nucleic acids looks rather complicated [45] and that DNA polymerases do not run the triplex-type primer-extension reaction on continual DNA duplex [46].

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