Artificial Site-Specific DNA-Nicking System Based on Common Restriction Enzymes Assisted by PNA Openers†

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ABSTRACT: We report on the peptide nucleic acid (PNA)-directed design of a DNA-nicking system that enables selective and quantitative cleavage of one strand of duplex DNA at a designated site, thus mimicking natural nickases and significantly extending their potential. This system exploits the ability of pyrimidine PNAs to serve as openers for specific DNA sites by invading the DNA duplex and exposing one DNA strand for oligonucleotide hybridization. The resultant secondary duplex can act as a substrate for a restriction enzyme, which ultimately creates a nick in the parent DNA. We demonstrate that several restriction enzymes of different types could be successfully used in the PNA-assisted system we developed. Importantly, the enzyme cleavage efficiency is basically not impaired on such artificially generated substrates, compared with the efficiency on regular DNA duplexes. Our design originates a vast class of semisynthetic rare-cleaving DNA nickases, which are essentially absent at present. In addition, we show that the site-specific PNA-assisted nicking of duplex DNA can be engaged in a rolling-circle DNA amplification (RCA) reaction. This new RCA format demonstrates the practical potential of the novel biomolecular tool we propose for DNA technology and DNA diagnostics.

Site-specific nicking of DNA duplexes, i.e., cleavage of designated sequences in only one strand of double-stranded DNA (dsDNA),[1] is a key step in a number of biochemical and molecular biology research protocols. Important examples include site-directed mutagenesis (*1*−*3*), strand displacement amplification (*4*, *5*), and DNA labeling (*6*, *7*). Selective nicking of dsDNA might also be employed in solving certain DNA nanotechnology and DNA computing problems (*8*, *9*). Site-specific dsDNA-nicking enzymes (DNA nickases) (*10*−*13*), which are closely related to restriction endonucleases, can be used for these purposes. However, only a very limited number of DNA nickases are available at present, all recognizing short sequences of ≤7 bp, therefore cleaving DNA rather frequently (*13*). Other DNA-nicking proteins are also rare, representing a few enzymes involved in DNA replication, transcription, recombination, and reparation, which bind and cleave a small number of DNA sequences ≥10 bp (*11*, *14*−*18*).

Thus, it is highly desirable to extend the repertoire of DNA nickases. Accordingly, several approaches have been developed for converting common restriction endonucleases into DNA nickases by either modifying these enzymes themselves (*19*−*22*) or using stable chemical (*2*, *4*) and noncovalent (*6*, *23*−*25*) modifications of their DNA recognition sites. In addition, sequence-nonselective DNA-cleaving proteins can be transformed into site-specific DNA nickases by cooperating them with the conjugates of triplex-forming oligonucleotides or minor groove-binding polyamides and certain ligands (*26*−*28*). Still, the progress in these directions is limited, justifying the elaboration of alternative site-specific dsDNA nicking systems, or artificial nickases.

Chemical sequence-selective nucleases can partially resolve the problem by serving as effective reagents for footprinting of DNA-binding ligands (*29*−*31*). However, the DNA nicks produced by them are not as strictly localized as with the use of natural enzymes, which may be a serious disadvantage in some applications. Semisynthetic nucleases can provide more precisely positioned single- and double-stranded DNA breaks (*32*−*35*), but this elegant and promising approach has not been adapted to produce artificial DNA nickases. Here we present a PNA-based approach to the biomolecular design of high-yield artificial nickases that confers the DNA nicking activity to common restriction enzymes. Furthermore, the exemplary application of the PNA-based artificial nickase for the nick-induced rolling-circle DNA amplification (RCA) demonstrates the robust practical potential of our design for DNA technology and DNA diagnostics.

EXPERIMENTAL PROCEDURES

Materials. Plasmids pN-BglII, pN-BbsI, and pHIV, carrying targets for *Bgl*II, *Bbs*I, and *Alu*I restriction enzymes, respectively, were constructed by cloning the appropriate complementary oligonucleotides into the *Sal*I site (pN-BglII), into the *Bam*HI site (pHIV), or between the *Eco*RI and *Hin*dIII sites (pN-BbsI) of pUC19. The corresponding inserts

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[1] Abbreviations: dsDNA, double-stranded DNA; nt, nucleotide; ssDNA, single-stranded DNA; scDNA, supercoiled DNA; PNA, peptide nucleic acid; bisPNA, "clamp" of two pyrimidine PNA oligomers connected by a flexible linker; PD-loop, looped complex formed inside dsDNA by bisPNAs and an oligonucleotide; RCA, rolling-circle amplification; ↓, position of the DNA nicking within a certain sequence.

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contained the following sequences (only one of the two complementary oligonucleotides is shown for each plasmid): 5′-TCGAT**AAGGAGAGAA**↓GATCT**AAGAAGAAAA**T (pN-BglII, artificially designed sequence), 5′-AGCT**AAG**↓**GAAAG**TGTCTTC**AGGGGAAG**CGTTAAC (pN-BbsI, artificially designed sequence), and 5′-GATC**AGAGGAAG**↓CTACT**GGAGGAGA**C (pHIV, natural sequence from the HIV-1 *nef* gene). In these sequences, the PNA-binding sites are in boldface and the restriction enzyme recognition sequences are underlined (*Bgl*II, *Bbs*I, and *Alu*I, respectively). Linear fragments of pN-BglII were obtained by digestion of the circular plasmid with the *Pvu*II restriction enzyme, resulting in two fragments ~2370 and 350 bp in length (the latter being the target fragment), or by PCR (see below). *Pvu*II-linearized samples of pHIV were used in the nick-induced RCA experiment (note that *Alu*I, which was employed in this assay, additionally cuts pHIV at several sites).

The following bisPNAs (either obtained commercially from PE Biosystems or given to us by collaborating groups) were used as openers for corresponding dsDNA sites: PNA 522, H-$T_2JT_2JT_4$-$(eg1)_3$-$T_4CT_2CT_2$-Lys-NH_2; PNA 669, H-Lys_2-TJ_4T_2J-$(eg1)_3$-CT_2C_4T-Lys-NH_2; PNA 1719, H-Lys_2-$T_2J_2TJTJT_2$-$(eg1)_3$-$T_2CTCTC_2T_2$-Lys-NH_2; PNA 6812, H-Lys_2-$T_2J_2T_3J$-$(eg1)_3$-$CT_3C_2T_2$-Lys-NH_2; PNA 7260, H-Lys_2-$TCTC_2$-TC_2-$(eg1)_3$-J_2TJ_2TJT-Lys-NH_2; PNA 7280, H-Lys_2-$TJTJ_2T_2J$-$(eg1)_3$-CT_2C_2TCT-Lys-NH_2. The PNAs are written from the N-terminus to the C-terminus using the following normal peptide conventions: H is a free amino group, NH_2 a terminal carboxamide, Lys a lysine amino acid residue, eg1 a linker unit (3,6-dioxaoctanoic acid), and J pseudoisocytosine (*36*). All PNAs were synthesized as previously described (*36*, *37*), and were purified by reversed-phase HPLC. Their identity was confirmed by MALDI-TOF mass spectrometry analysis.

Oligonucleotides that were employed as restriction enzyme templates for DNA nicking included the following: 5′-AGAGAAGATCTA (ODN12-NBgl), 5′-AGAAGATCTAAGA (ODN13-NBgl), 5′-GAGAGAAGATCTAA (ODN14-NBgl), 5′-AGGAGAGAAGATCTAA (ODN16-NBgl), 5′-AAGGAGAGAAGATCTAAGAAGAAAA (ODN25-NBgl), 5′-AAGGAAAGTGTCTTCAGGGGAAG (ODN23-NBbs), and 5′-AGAGGAAGCTACTGG (ODN15-NAlu). The following forward and reverse primers were used for PCR amplification of target DNA fragments in the nick mapping experiment: 5′-CAGGGTTTTCCCAGTCACGA (pUC19 locus, 355−374 bp) and 5′-TTGTGTGGAATTGTGAGCGGATAAC (pUC19 locus, 491−515 bp). A 95-nucleotide oligonucleotide was used as an annealing template for ODN14-NBgl and ODN16-NBgl in the comparative kinetic experiments with *Bgl*II [5′-GGCTGAAGTGGCAACAGAGTGTATCATCCTCGCACCTGGATTTTCTTCTTAGATCTTCTCTCCTTATCGACCCATCAGAATACTGCCATTTGTAC (ODN95-NBgl)]. These three oligonucleotides were used in the RCA experiment: 5′-GTGTATCATCC TCGCATCCGTAAGAAGAAAATCACAAGTCGTTCTCGTACACACTACTGGAGGAGACTATATTGTATTCATCACACTCAGTATCAATC (98 nucleotide circularized probe), 5′-GAGGATGATACACGATTGATACTGAG (splint oligonucleotide), and 5′-ATCAATCGTGTATCATCCTCGCATC (RCA primer).

PNA-Directed Artificial DNA-Nicking System. DNA (typically 1−10 μg) was first incubated with a pair of PNA openers (final concentration of 1−2 μM) in 10 mM sodium phosphate buffer (pH 6.8) for 1−2 h at 37 °C. The PNA binding was checked by gel electrophoresis, and unbound PNAs were removed by gel filtration. The resulting DNA−PNA complexes were precipitated with ethanol and redissolved in 10 μL of TPE buffer [10 mM Tris-phosphate and 0.1 mM EDTA (pH 6.8)]. To form PD-loops, 5 μL of H_2O, 1 μL of DNA−PNA complexes (0.15−0.3 μg), 1 μL of corresponding 10× NE buffer (New England Biolabs) for the restriction enzyme used next, and 2 μL of a 10 μM solution of oligonucleotide complementary to the displaced DNA strand were mixed and incubated for 10−15 min at 37 °C. For subsequent DNA nicking, typically 1 μL with 5−10 units of the required restriction enzyme was added to the PD loop-containing samples, followed by incubation for 2−3 h at 37 °C. Then, PNA openers were dissociated from DNA (except the RCA experiment; see below) by high-temperature incubation (typically 70 °C for 0.5−1 h). Finally, the samples were desalted by gel filtration and analyzed by nondenaturing electrophoresis in either 10% polyacrylamide gels run at 50 °C (*38*) or 1% agarose gels at room temperature. Gels were stained with ethidium bromide, illuminated at 302 nm, and scanned with a CCD camera using the IS-1000 digital imaging system (Alpha Innotech Corp.).

Nick-Mapping Assay. To determine the exact DNA nicking position, ~190 bp Cy5-labeled fragments of pN-BglII were obtained by PCR. The PCRs were performed with one unlabeled and one 5′-Cy5-labeled primer in 50 μL reaction volumes containing 1× *Pfu* buffer (Stratagene), 100 ng of pN-BglII, dNTPs (200 μM each), both primers (0.5 μM), and 2.5 units of *Pfu* DNA polymerase. PCR conditions included initial denaturation at 94 °C for 60 s, followed by 30 cycles of denaturation at 94 °C for 45 s, primer annealing at 62 °C for 45 s, and primer extension at 72 °C for 45 s. The last cycle was followed by an extension step at 72 °C for 10 min. The intact, nicked, and restriction-digested PCR products were analyzed concurrently with the dideoxy sequencing ladders in 6% denaturing (8 M urea) polyacrylamide gels run at 50 °C on the ALF Express DNA sequencer (Amersham Pharmacia Biotech). Sequencing ladders were obtained with the same PCR primers by using the AutoRead 200 sequencing kit (Amersham Pharmacia Biotech). The postrun data analysis was performed with the ALFwin Sequence Analyzer and DNA Fragment Analyzer software (Amersham Pharmacia Biotech).

Kinetic Analysis of DNA Cleavage. One part of this study was performed with the ~350 bp target fragment of plasmid pN-BglII. The corresponding DNA substrate (15 nM regular dsDNA fragments or those carrying PD loops) was incubated at 37 °C with 40 units of *Bgl*II. Aliquots (10 μL) were taken at the desired time and immediately mixed with 2 μL of a 0.5 M EDTA solution to quench the enzymatic reaction. Cleavage of normal DNA duplexes was analyzed by nondenaturing 10% polyacrylamide gel electrophoresis at ambient temperature with ethidium bromide staining. Samples with nicked DNA were processed and analyzed generally as described above (see the section about the PNA-directed artificial DNA-nicking system). Quantitative analysis of DNA cleavage in all cases was carried out by calculating the normalized intensities of corresponding bands using the

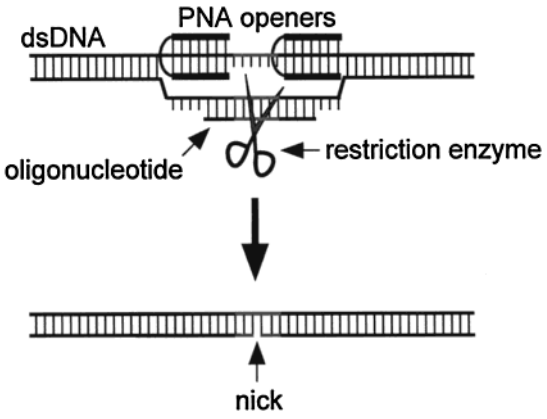

FIGURE 1: PNA-assisted design of artificial DNA nickases. A key element here is the PD-loop consisting of a pair of bisPNA "openers", the selectively exposed dsDNA segment, and a hybridized oligonucleotide (*40*). The resulting secondary DNA duplex is cut with a common restriction enzyme symbolized by scissors, yielding, after removal of PNA openers and enzyme-digested oligonucleotide, the site- and strand-specific dsDNA nick. For some applications, the PNA openers can be retained (see Figure 6 as an example). The gray lines indicate a recognition sequence for the restriction enzyme.

transilluminator/CCD camera/IS-1000 digital imaging equipment.

Another part of this study was performed with mostly ssDNA fragments carrying in the middle a short duplex with the recognition sequence for *Bgl*II. These substrates were prepared by annealing of 14- and 16-mer oligonucleotides (ODN14-NBgl and ODN16-NBgl, respectively) with a 95-mer oligonucleotide (ODN95-NBgl). Then, 40 nM oligonucleotides were incubated at 37 °C with 40 units of *Bgl*II and processed as described above. An excessive amount of these substrates, as compared with the dsDNA fragments, was taken given the single-stranded nature of samples to be stained and analyzed after gel electrophoresis. The samples were resolved in 10% regular polyacrylamide gels run at 25 °C. In this case, gels were stained with SYBR Green II.

Nick-Induced RCA Experiment. Preparation of a circular DNA probe from the circularized linear precursor was done with the use of a splint oligonucleotide (digested after assembly with *Exo*VII exonuclease), as previously described (*39*). For the RCA protocol, the desired amount of prior nicked PNA-bound target DNA (nicking performed using ODN15-NAlu and *Alu*I), 0.1 μM circular probe, and 0.5 μM primer were preincubated for 30 min at 37 °C in 20 μL of Sequenase reaction buffer. Then, 3.5 μL of H_2O, 1.0 μL of 100 mM DTT, 1.0 μL of a mixture containing all four dNTPs (25 mM each), 0.5 μL of SSB protein (2.2 mg/mL), and 1.0 μL of Sequenase 2.0 DNA polymerase (1.6 units/μL) were added, and the reaction mixture was incubated overnight at 37 °C. Aliquots of the samples were then analyzed by electrophoresis on a 2% agarose gel.

RESULTS

Figure 1 schematically shows our experimental design of artificial DNA nickases. It is based on the PNA-assisted assembly of a so-called PD-loop structure (*40*, *41*), featuring a secondary DNA duplex formed by an oligodeoxyribonucleotide within the locally opened dsDNA. We assumed that if the PD-loop were assembled on a DNA target site, which embodies a restriction enzyme recognition sequence, the corresponding enzyme might act on the PD-loop by catalyzing a complete cleavage of the secondary DNA duplex. Importantly, the restriction enzyme should not cleave the other strand of the target DNA inside the PD-loop since (i) this strand is partially in a single-stranded (ss) form, which is a poor substrate for the vast majority of restriction enzymes (*13*, *42*, *43*) and (ii) it is mostly occupied by PNA openers that hamper the enzymatic action (*44*, *45*). Therefore, the postcleavage dissociation of the split oligonucleotide and removal of PNA openers would result in site-directed nicking of dsDNA.

FIGURE 2: Site-specific nicking of linear dsDNA using the *Bgl*II-based artificial nickase: effect of oligonucleotide length on the yield of centrally nicked ~350 bp DNA fragments analyzed by the mobility shift assay (nondenaturing polyacrylamide gel electrophoresis). Target fragments of pN-BglII and PNAs 522 and 1719 were employed in these experiments. Lanes 1 and 2 correspond to the control, in which a dsDNA fragment of the pUC19 derivative, pHIV (*47*, *48*), similar in length (lane 1) was treated with the natural DNA nickase N.*Bst*NBI (lane 2) to introduce a nick in the middle. Lanes 4−7 represent the experiments in which the pN-BglII dsDNA fragment was treated with the artificial nickase applying 12-, 14-, 16-, and 25-mer oligonucleotides, respectively (lane 3 is control without oligonucleotide). Lane M is a 50-bp dsDNA ladder.

Although we have previously demonstrated that certain DNA-processing enzymes and other proteins, like DNA ligase, DNA polymerase, and streptavidin, could successfully function on PD-loops (*39*, *46*−*51*), it was unclear whether restriction enzymes would effectively work on the PD-loop structure, which differs significantly from the regular DNA duplex. First, the PD-loop is rather small (~20 bp), being at the lower limit of continuous duplex length normally required for the effective performance of restriction enzymes (*40*, *42*). Second, two bulky PNA_2−DNA triplexes are located close to the restriction site (see Figure 1), which may interfere with enzyme binding and functioning.

Despite the aforementioned concerns, the results of our experiments with the restriction enzyme *Bgl*II, (10+10)-mer bisPNA openers, and linear dsDNA presented in Figures 2 and 3 were encouraging. We observed highly efficient site-specific DNA nicking, when a sufficiently long oligonucleotide with the enzyme's recognition sequence was employed to transform the restriction enzyme into an artificial nickase according to the strategy of Figure 1.

First, using the gel electrophoretic mobility shift assay (*38*), we monitored, as shown in Figure 2, the site-specific nicking of a DNA fragment carrying in the middle the PNA-binding and enzyme recognition sites (lane 3). One can see that in the case of a 16-nt oligonucleotide this DNA fragment was completely converted, when treated as in Figure 1, into a product with a lower electrophoretic mobility (lane 6). On the basis of our previous observations (*25*, *38*), we considered this product the anticipated centrally nicked dsDNA fragment. Control experiments with the treatment of another DNA fragment similar in length with a natural nickase (lanes 1 and 2 of Figure 2) support this conclusion: it clearly shows that formation of a site-specific DNA nick results in the

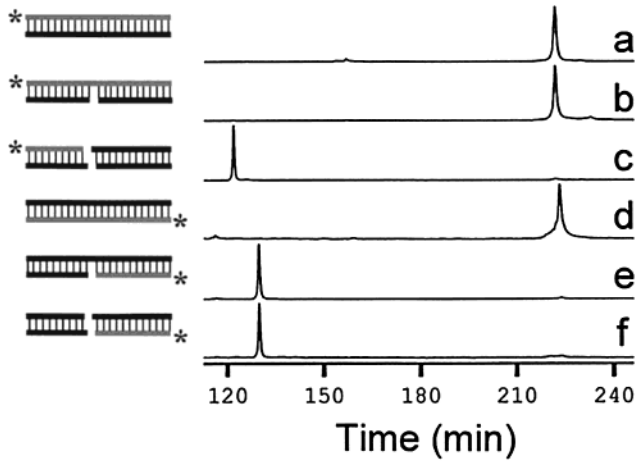

FIGURE 3: Site-specific nicking of linear dsDNA using the *Bgl*II-based artificial nickase: location of a nick introduced by artificial nickase in the center of fluorescently labeled (*) 190 bp DNA target fragments. The nick position (corresponds to 130 min) was assessed by the time of the label movement through the fluorescence detector of the DNA sequencer during denaturing polyacrylamide gel electrophoresis: lanes a and d, intact DNA fragments; lanes b and e, DNA fragments nicked according to Figure 1; lanes c and f, DNA fragments directly cut by the restriction enzyme. The schematics at the left represent the corresponding analytes before denaturing; peaks of fluorescence on electrophoregrams at the right correspond to different lengths of the labeled DNA strands (gray). The 16-nt oligonucleotide and PNAs 522 and 1719 were used for the artificial nickase design.

appearance of a low-mobility band. Yet, a sufficiently long, ≥15-nt oligonucleotide is evidently required to make the PD loop susceptible to the restriction enzyme, as no or incomplete nicking activity was observed when shorter oligonucleotides were used (see lanes 4 and 5 of Figure 2).

In the next series of experiments (Figure 3), we verified that the low-mobility products observed in the previous tests were indeed the singly nicked dsDNA fragments. To this end, we used dsDNA fragments, in which either the PNA-targeted strand or its complement was fluorescently labeled at the 5′-end. Such labeling allowed us to analyze, after denaturation, the treated and control DNA samples on a DNA sequencer concurrently with the Sanger dideoxy sequencing ladders. Figure 3 shows that following the treatment depicted in Figure 1, the DNA strand hybridized with the oligonucleotide was cleaved at a single position (lane e), while the complementary strand containing the PNA-binding sites remained intact (lane b). Moreover, the length of the cleaved DNA strand in lane e, as assessed by its electrophoretic migration time in a denaturing gel, corresponded exactly to the length of a similarly labeled DNA strand from the sample directly digested with the restriction enzyme (lane f). Comparison of the DNA sequencing ladders with both peaks proved that the PNA-assisted DNA nicking, indicated below as ↓, took place exactly at the expected unique position on the DNA duplex (5′-A↓GATCT, data not shown).

The efficiency of enzymatic DNA cleavage in our artificial system has been comparatively analyzed in quantitative kinetic experiments involving different natural and nonnatural substrates (Figure 4). These data show that the apparent DNA cutting rate of *Bgl*II is only 2−3 times slower with PD-loops than with a regular DNA duplex, if oligonucleotides of optimal length are taken for the PD-loop formation (see Figure 4C and Table 1). For the PD-loop with the 14-mer

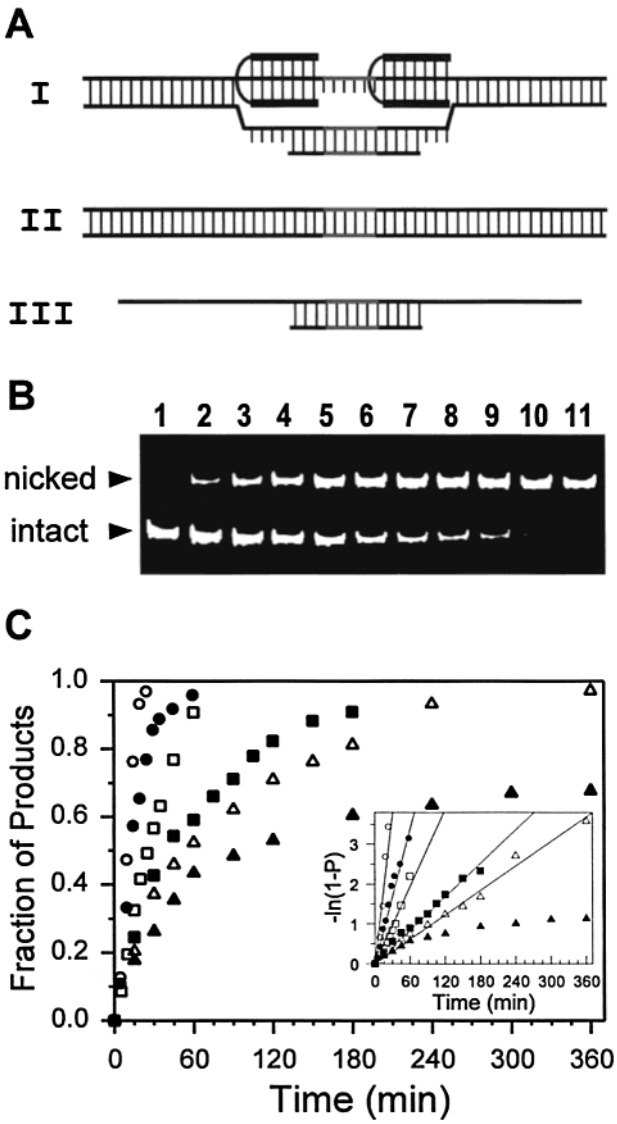

FIGURE 4: Comparative kinetic analysis of the *Bgl*II cleavage efficiency on different DNA substrates shown schematically in panel A. Long single-stranded overhangs in substrates III were used to increase the total amount of DNA in the sample to enhance the efficacy of DNA staining and, as a result, to improve the visualization of reaction products. (B) Representative gel showing the accumulation with time of site-specific nicks within the target DNA fragment generated by the *Bgl*II-based artificial nickase using ODN16-NBgl. Lane 1 is a control in the absence of enzyme; lanes 2−11 show digestion with *Bgl*II over the course of 15, 30, and 45 min and 1, 1.5, 2, 2.5, 3, 4, and 6 h, respectively. (C) Time course for the cleavage of different DNA substrates with *Bgl*II. DNA nicks have been detected with substrates I from panel A formed by oligonucleotides of different lengths: 25-mer (■), 16-mer (△), and 14-mer (▲). Double-stranded breaks have been detected with the regular dsDNA fragment [substrate II (□)] or substrates III (○ and ●, respectively, 14- and 16-mer duplex with single-stranded overhangs). The inset is a semilogarithmic plot of the kinetic data, where *P* denotes the fraction of cleavage products: 15 nM substrates I and II, 40 nM substrate III, and 40 units of *Bgl*II (see Experimental Procedures for other details).

oligonucleotide, only partial cleavage is observed, even after incubation for 6 h with the enzyme. The gel-shift assay has proven that PNA openers do not dissociate during the prolonged incubations (data not shown), thus indicating that the PD-loop stability is not an issue here. Further incubation of the sample supplemented by addition of fresh portions of

Table 1: Apparent Rate Constants (k_{ps}) for the Cleavage of Different DNA Substrates by *Bgl*II[a]

substrates[b]	$k_{ps} \times 10^2$ (min^{-1})	substrates[b]	$k_{ps} \times 10^2$ (min^{-1})
I/14-mer oligo	0.9[c]	II	3.1
I/16-mer oligo	1.0	III/14-mer oligo	14.4
I/25-mer oligo	1.4	III/16-mer oligo	7.1

[a] The values of the pseudo-first-order rate constants for the overall processes were obtained from the slopes of linearized plots, $-\ln(1 - P)$ vs time t (see Figure 4C). The error in k_{ps} determination is less than 20%. [b] See Figure 4A for the substrate notations. [c] Estimated from the initial slope.

the restriction enzyme finally yields the complete digestion in this case as well.

Significantly for our design, we have observed a quite moderate decrease in the efficiency of DNA cleavage, which makes it possible to quantitatively nick specific sites on linear DNA duplexes in ~2 h at 37 °C. Also worth mentioning is the fact that the efficiency decrease is not due to the small size of the continual duplex substrates involved in the enzymatic reaction with PD-loops but is intrinsic in its unusual structure, which somehow affects the performance of the enzyme. Indeed, *Bgl*II did not exhibit any decrease in efficiency with shorter regular duplexes we have studied (Figure 4C and Table 1). Though larger amounts of substrates III have been used to compare their cleavage rates with those of substrates I, this conclusion is quite justified given that an excess of DNA may only decrease the rate of DNA digestion (*52*). Also note that the observed differences for the efficiency of *Bgl*II cleavage of different substrates are not unusual (*53*, *54*) and may reflect the electrostatic effects of ssDNA overhangs in substrates III and those of the displaced DNA strand and/or cationic residue of PNA openers in substrates I, which are evidently absent in regular DNA duplexes (substrate II).

Hence, the feasibility study performed with the restriction enzyme *Bgl*II and linear dsDNA demonstrates that the design shown in Figure 1 does efficiently operate as an artificial nickase. To verify the generality of our approach, we extended our study by using another restriction enzyme, *Bbs*I, which has a different mode of dsDNA cleavage. *Bgl*II represents the most common type II restriction endonucleases that recognize and cleave particular palindromic DNA sequences, while *Bbs*I belongs to a smaller type IIs subclass of these enzymes that recognize asymmetric DNA sequences and cleave at defined positions outside them (see Figure 5). We have employed shorter bisPNA openers [i.e., (8+8)-mer] to convert the *Bbs*I enzyme into a nickase. In this series of experiments, we have used a supercoiled (sc) form of dsDNA to subject the selectivity of our artificial nickase system to a more stringent test. Figure 5 shows that the treatment of target scDNAs following our PNA-assisted design results in their complete conversion into the open circular form (lanes 5), a clear indication of dsDNA nicking.[2] At the same time, exclusion of any assembly and/or reaction components [PNA openers (lanes 2), auxiliary oligonucleotide (lanes 3), or restriction enzyme (lanes 4)] either does not affect the target scDNAs during the procedure (lanes 3 and 4) or yields linear DNA (lanes 2), as expected. Also important is the fact that none of the components by itself introduces random DNA nicks (lanes 3 and 4).

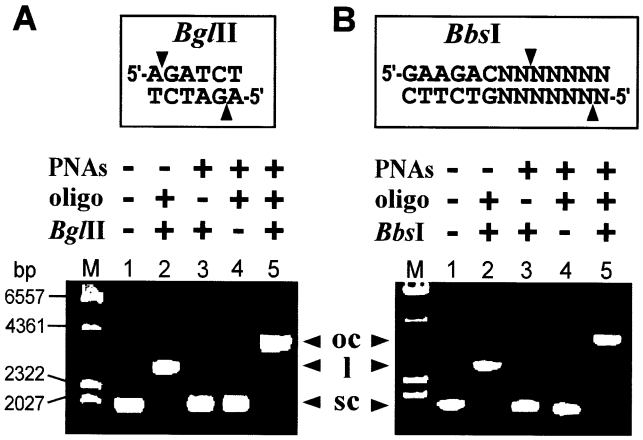

FIGURE 5: Site-specific nicking of scDNAs with artificial nickases monitored by regular agarose gel electrophoresis. Target plasmids, pN-BglII and pN-BbsI, with native negative supercoiling were employed in these experiments. Both plasmids contain a single recognition sequence for the corresponding restriction enzyme (shown within gray boxes) flanked by 8−10-mer oligopurine stretches. (A) pN-BglII was targeted by PNAs 522 and 1719 and oligonucleotide ODN13-NBgl, and digested with *Bgl*II. (B) pN-BbsI was targeted by PNAs 669 and 6812 and oligonucleotide ODN23-NBbs, and digested with *Bbs*I. Arrowheads indicate three different forms of plasmid DNA: supercoiled (sc), linear (l), and open circular (oc). M is a λ DNA/*Hin*dIII marker.

To demonstrate the practical potential of artificial nickases, we applied our biomolecular design to selectively cut the unique HIV-1 DNA site capable of PD-loop formation (*47*−*50*), followed by a nick-induced rolling-circle DNA amplification (RCA). In this experiment (Figure 6), one more restriction enzyme, the endonuclease *Alu*I, was used to site-specifically generate a nick within the HIV-1 sequence with the assistance of PNA openers. As can be seen from the schematics in Figure 6A, the appearance of a nick at the PD-loop forming site will initiate the RCA reaction in the presence of the RCA-capable DNA polymerase if the appropriate DNA minicircle is hybridized to the displaced and nicked DNA strand. As a result, the generation of characteristic high-molecular-weight RCA products in this system will signal the formation of a site-specific nick within the designated target DNA sequence.

Figure 6B shows the results obtained with different input numbers of nicked target DNAs following the strategy depicted in Figure 6A. One can see that while the intact DNA control revealed no RCA products in the case of the hyperbranched-type RCA reaction (*58*, *59*), the nicked DNA samples yielded, after gel electrophoresis, the distinct, ladder-like bands typical for this RCA format (*60*) if the number of target molecules is $\geq 10^4$. Note here that the primary goal of this experiment was to check the principal workability of a new RCA format but not to optimize its sensitivity, which may be much higher, as another PNA-directed approach to

[2] Note here that the oligonucleotide could be as short as a 13-mer to make the PD loop susceptible to *Bgl*II within scDNA, while a ≥15-nt oligonucleotide is necessary for a linear dsDNA fragment (Figure 2). The reduced length requirement for oligonucleotide in the case of scDNA might reflect the higher efficiency of hybridization between the displaced DNA strand and oligonucleotide relative to that in linear duplexes. Also, the PD-loop, like other looped DNA structures (*55*−*57*), is less intertwined in negatively supercoiled DNA than in linear dsDNA, thus making the secondary duplex more easily accessible to restriction enzymes.

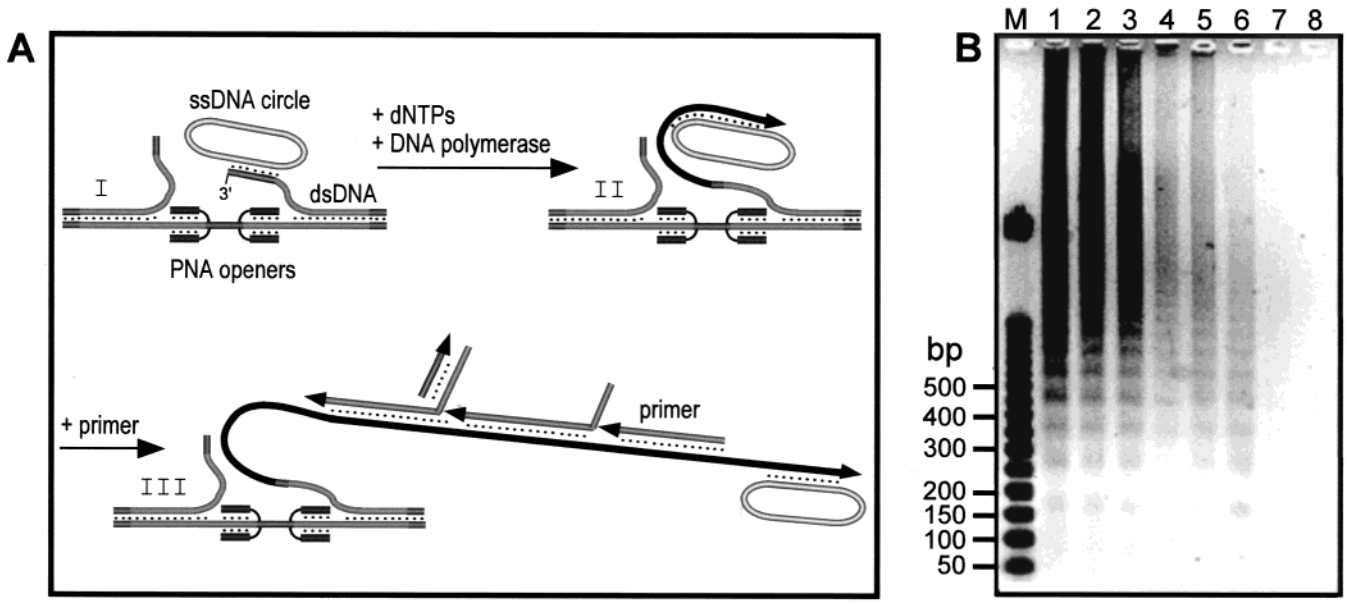

FIGURE 6: Use of the *Alu*I-based artificial DNA nickase in the RCA reaction. (A) Schematics illustrating the assay and its protocol. A DNA nick is introduced following the procedure as in Figure 1, but PNA openers are not removed. The 3′-terminal segment of the nicked PD-loop (I) serves as a primer for the RCA reaction on a DNA minicircle hybridized to this segment. Accordingly, the segment is extended in the presence of all four dNTPs and DNA polymerase (structure II exemplifies an intermediate obtained after initial extension). In the presence of a second primer (oligonucleotide) complementary to the newly synthesized DNA strand, numerous dsDNA fragments are obtained (III). As a result of such a hyperbranched RCA reaction (*58*, *59*), extensive DNA synthesis occurs that serves as a signal amplifier for target DNA detection. (B) Gel-electrophoretic analysis of the RCA products obtained on the nicked HIV-1 DNA site (*nef* gene) within the linearized and fragmented pHIV (image presented as a negative). The 15-nt oligonucleotide and PNAs 7260 and 7280 were used for the artificial nickase design. Input numbers of target nicked DNAs were as follows: 5×10^8 for lane 1, 5×10^7 for lane 2, 5×10^6 for lane 3, 5×10^5 for lane 4, 5×10^4 for lane 5, 10^4 for lane 6, and 5×10^3 for lane 7. Lane 8 is a control with non-nicked DNA (5×10^8 molecules). Lane M is a 50-bp dsDNA ladder.

the RCA detection of dsDNA markers has recently proven (*39*). Also note that in this format the RCA reaction takes place on dsDNA without any restrictions imposed by topology in contrast to a similar design when this reaction proceeds under topological constraints (*39*). In addition, in the case of linear RCA reactions, the RCA products in this format will be linked to the target site, which may be advantageous in some applications. Thus, the PNA-based artificial nickases are promising for the RCA-based DNA diagnostics.

DISCUSSION

The PNA-based model systems we have studied do function as artificial nickases, allowing introduction, with the aid of common restriction enzymes, of a nick into the desired DNA strand at a designated dsDNA site with essentially 100% efficiency. A site of interest should be capable of forming a PD-loop; i.e., it must consist of two closely located short stretches of purines (to bind the all-pyrimidine PNA openers) separated by a mixed sequence of purines and pyrimidines. This site also has to embody a recognition site for a restriction enzyme. Such sites could be deliberately incorporated into the DNA duplex, as we did here in the proof-of-principle experiments, or might be readily found within natural genomic sequences. One example is the HIV-1 site of the *nef* gene we employed in the nick-induced RCA generation. Another example is the *Bacillus anthracis* site (*lef* gene), 5′-AGGAAGAGCA-TTT↓AAAGGAAA, containing a recognition sequence for the *Dra*I restriction enzyme (underlined). Our simple statistical estimations supported by analysis of known DNA sequences show that DNA sites that meet the requisite conditions should occur quite frequently, on average every ~1−2 kb of a random DNA sequence. Hence, virtually every prokaryotic and eukaryotic gene should contain at least one site enabling design of a corresponding artificial nickase. Recently, a communication reporting on the use of a similar, PD-loop based design has been published (*61*), however, with the different aim of analyzing the DNA methylation status by transiently hybridized fluorescent DNA probes (*62*).

Though sites of this type are rather frequent in natural DNAs, each of them will normally be unique in the whole genome as a typical PD-loop spans more than 20 bp. Accordingly, these sites may serve as convenient and selective DNA markers provided that an appropriate detection method, for instance, RCA, is employed. Note that a restriction enzyme will cut dsDNA not only at the PD-loop but also at all other corresponding recognition sites, resulting in fragmentation of sufficiently long DNA duplexes. For the majority of prospective diagnostic assays, this will constitute no problem, as the primary task there is to sequence-specifically introduce a detectable nick within a chosen dsDNA target. Furthermore, the enzymatic DNA methylation (*13*, *61*, *63*) could be employed to avoid the byproduct DNA

[3] Besides *Alu*I, *Bbs*I, and *Bgl*II endonucleases, we were able to generate with high yield the site-specific DNA nicks using restriction enzymes *Kpn*I, *Sbf*I, and *Sph*I (data not shown). Evidently, some exceptional restriction enzymes, such as *Dde*I, *Hha*I, and *Hap*II, which can cut, although less efficiently, ssDNA as well, could be less appropriate for making artificial DNA nickases via our design. Our experience with *Hha*I, which is known to be half-active on ssDNA substrates, proves that. In fact, mostly double-stranded breaks but not nicks were observed by us with several PD-loop systems under different conditions when we tried to employ this enzyme in the artificial nickase design.

fragmentation, if required. Such a treatment, if performed right after the PNA binding and prior to oligonucleotide hybridization and DNA cleavage, will block the DNA digestion by a restriction enzyme at essentially all recognition sites. Besides the locally opened designated dsDNA target, only very few other recognition sites that overlap with binding sites for any PNA opener will be protected from methylation and subsequently cut (*44*, *45*).

To summarize, this study extends the range of artificial DNA substrates available for restriction enzymes (*2*, *4*, *64*). Our scheme for conferring the site- and strand-specific DNA nicking activity on common restriction endonucleases via the PNA-directed opening of dsDNA is quite general so that essentially any restriction enzyme can be converted into a rare nickase with designated sequence specificity.[3] This new design delivers a wide variety of nonnatural high-yield rare-cleaving DNA nickases with ~20 bp recognition specificity, which essentially have been absent until present. We therefore anticipate that thus devised artificial nickases may find applications in genetic engineering, DNA diagnostics, and molecular biological or biochemical studies.

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