Activation of Double-stranded DNA by One pcPNA Strand for Its Site-selective Scission with Ce^{IV}/EDTA

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In the presence of only one strand of pseudo-complementary PNA (pcPNA), double-stranded DNA was selectively hydrolyzed by Ce^{IV}/EDTA (EDTA = ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetate) at target site. Compared with previously reported artificial restriction DNA cutters using two pcPNA strands, the present cutters are simple and promising for various applications.

Chemical tools for site-selective scission of double-stranded DNA are necessary for further developments of molecular biology and biotechnology.[1] They should be useful, for example, for manipulation of genomic DNA which is too huge to treat with naturally occurring restriction enzymes. Recently,[2] we hydrolyzed target phosphodiester linkages in double-stranded DNA by combining Ce^{IV}/EDTA complex (molecular scissors hydrolyzing the phosphodiester linkages) with two pseudo-complementary PNA strands[3] (pcPNAs; site-selective DNA-activators). In pcPNA, 2-thiouracil (U) and 2,6-diaminopurine (D) are employed in place of conventional T and A (together with C and G), and invasion of two pcPNA strands to double-stranded DNA has been well documented.[3] In the new artificial restriction DNA cutters, two pcPNAs were designed so that they should invade the substrate DNA at predetermined site and form single-stranded portions in both strands of the DNA (see Figure 1a). These portions are far more susceptible to the catalysis by Ce^{IV}/EDTA because of the substrate-specificity of this complex[4] and thus were selectively hydrolyzed by the complex. The DNA scissions are completely hydrolytic so that the resultant fragments were easily reconnected with various foreign DNA fragments by using ligase. These attractive features of the DNA cutters have prompted us to study on the systems more in detail. In the course of these studies, we have found that site-selective scission of double-stranded DNA can be achieved even with the use of only one pcPNA strand (Figure 1b). The pcPNA additive activates the target site for site-selective scission by Ce^{IV}/EDTA.

The substrate DNA (4361-mer) was obtained by linearizing pBR322 plasmid DNA with *Eco*RI (both from Takara), and purified by QIAquick PCR Purification Kit (QIAGEN). The target sites for site-selective scission are located in 1820–1855 base-pair region (Figure 2a). The pcPNA additives were synthesized from Boc-protected monomers, and purified by the reversed-phase HPLC (the structures of U and D are presented in Figure 2b).

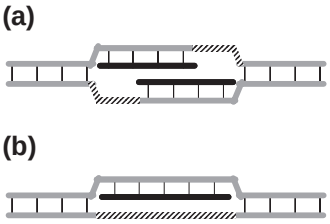

Figure 1. (a) Invasion complex formed by a pair of pcPNAs. (b) Invasion complex formed by only one pcPNA strand. The single-stranded portions for site-selective scission are hatched.

First, 15-mer pcPNA additives (15-1, 15-2, 15-3, and 15-4 in Figure 2a) were used. The double-stranded DNA was incubated with one of these additives at 50 °C for 1 h ([NaCl] = 0 mM). And then NaCl was added to final concentration of 100 mM, where substrate DNA was treated with Ce^{IV}/EDTA complex (100 μM). After 17.5 h, the mixtures were analyzed by 0.8% agarose gel electrophoresis. As shown in lanes 1–4 of Figure 3, two scission bands were observed. The sizes of these two scission fragments (1800 and 2500 bp) are consistent with the site-selective scission at the target site (1830 and 2530 bp scission bands should be obtained if the scission occurs at the midpoints of PNA 15-1). The scission efficiency is considerably dependent on the sequence, and the scission in lane 3 was especially evident. The site-selective scission was still more efficient when 20-mer pcPNA (PNA 20-1) was used (lane 5 in Figure 3). It has been concluded that the double-stranded DNA is selectively cut at the target site even with the use of only one pcPNA probe.[5]

When one pcPNA strand invades double-stranded DNA, the noncomplementary DNA strand remains single-stranded at the

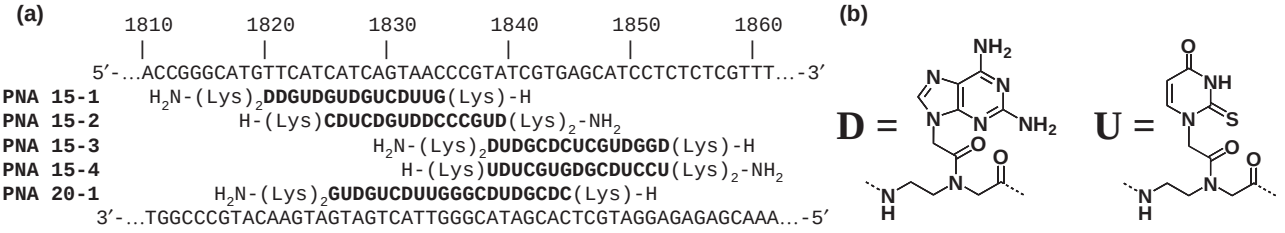

Figure 2. (a) The sequences of substrate DNA (linearized pBR322: 4361 bp) and pcPNA additives. (b) Structures of 2,6-diaminopurine (D) and 2-thiouracil (U) used in the pcPNA additives.

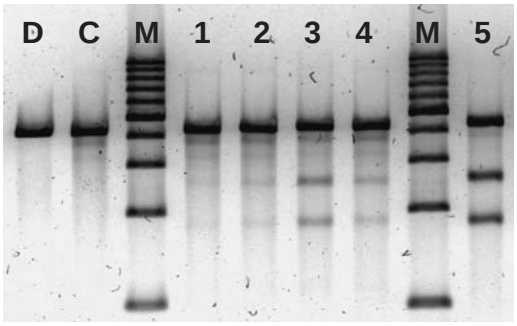

Figure 3. Agarose gel electrophoresis patterns for site-selective hydrolysis of linearized pBR322 DNA by Ce^{IV}/EDTA in the presence of only one pcPNA additive. Lane 1, PNA 15-1 + Ce^{IV}/EDTA; lane 2, PNA 15-2 + Ce^{IV}/EDTA; lane 3, PNA 15-3 + Ce^{IV}/EDTA; lane 4, PNA 15-4 + Ce^{IV}/EDTA; lane 5, PNA 20-1 + Ce^{IV}/EDTA; M, 1000 bp ladder. Lanes D and C are control lanes. D, DNA only; C, Ce^{IV}/EDTA only (without pcPNA). Scission conditions: [linearized pBR322] = 8 nM, [pcPNA] = 200 nM, [Ce^{IV}/EDTA] = 100 μM, [NaCl] = 100 mM, and [HEPES] = 5 mM at 50 °C and pH 7.0 for 17.5 h (these conditions are similar to those employed for the scission by two-pcPNA-based cutters in Ref. 2). The bands were detected by staining with GelStar (from Cambrex).

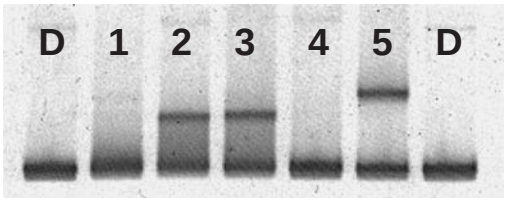

Figure 4. Gel-shift assay for invasion of one pcPNA strand to double-stranded DNA. The 130-mer double-stranded DNA was incubated with one pcPNA strand for 1 h, and subjected to PAGE. The bands were stained with GelStar. Lane 1, PNA 15-1; lane 2, PNA 15-2; lane 3, PNA 15-3; lane 4, PNA 15-4; lane 5, PNA 20-1; D, DNA only (without PNA). Invasion conditions: [130-mer DNA] = 50 nM, [pcPNA] = 200 nM, and [HEPES] = 5 mM at 50 °C and pH 7.0 for 1 h.

invasion site (see Figure 1b) and thus Ce^{IV}/EDTA preferentially hydrolyzes there. Then, the pcPNA-binding DNA-strand is hydrolyzed, probably through the breathing motion near the nick which is formed by the first scission. The scission occurs near the invasion site, although it is not clear which phosphodiester linkages are hydrolyzed (Figure 3). Detailed analyses of the scission products are currently under way.

Invasion of one pcPNA additive to double-stranded DNA was little evidenced previously.[6,7] Accordingly, gel-shift assay was investigated in Figure 4. In order to facilitate the assay by 10% nondenaturing polyacrylamide gel electrophoresis (PAGE), 130-mer double-stranded DNA involving scission site was used instead of the linearized plasmid DNA. Lanes 1–5 in this Figure correspond to the invasion complex formation for the DNA scission in lanes 1–5 in Figure 3, respectively. In lanes 2, 3, and 5, a new band of smaller mobility was clearly observed, showing that one pcPNA strand invaded the DNA. The site-selective scission by Ce^{IV}/EDTA was notable as shown by the corresponding lanes in Figure 3. Interestingly, no invasion complex was detected in lanes 1 and 4, although the scission explicitly occurred. Assumedly, these invasion complexes are too unstable to be detected by the present gel-shift assay.[8] As described in Ref. 6, invasion of one conventional PNA strand to homopurine–homopyrimidine sequences was never detected by gel-shift assay. These arguments are further supported by the fact that the DNA scission by Ce^{IV}/EDTA did not occur to a measurable extent when one 10-mer or 13-mer pcPNA additive was used. These short pcPNAs did not invade the double-stranded DNA sufficiently, and thus could not form hot-spots for the scission.

Unnatural nucleobase 2,6-diaminopurine (D) has 2-amino group and thus forms three hydrogen bonds with T, whereas a D–U pair is destabilized by the steric repulsion between this 2-NH_2 and the 2-S of U.[3] These factors facilitate single-strand invasion, since it accompanies the addition of one hydrogen bond for one D–T pair (upon the invasion, two hydrogen bonds of A–T pair in DNA duplex are cleaved, but three hydrogen bonds are instead formed in D–T pair in pcPNA/DNA duplex). The use of pcPNA is essential for the present strategy. Simple structures of one-pcPNA-based cutters would be advantageous for versatile applications, although scission efficiency is rather strongly dependent on the length and sequence of pcPNA additive.[9]

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References and Notes

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5 The sequence-dependency was also notable when 20-mer pcPNA was used (data not shown).

6 One conventional PNA (bearing A and T, in place of D and U) invaded homopurine–homopyrimidine sequences in double-stranded DNA (P. E. Nielsen, L. Christensen, *J. Am. Chem. Soc.* **1996**, *118*, 2287. Conditions: [Tris-HCl] = 10 mM at room temperature and pH 7.2). These specific sequences were absolutely necessary for the invasion. However, gel-shift assay gave no new bands. Accordingly, these authors concluded the formation of "single invasion complex" in terms of titration experiments.

7 Invasion of one conventional mixed-base PNA to supercoiled DNA was also proposed. However, the invasion never occurred to nonsupercoiled DNA (X. Zhang, T. Ishihara, D. R. Corey, *Nucleic Acids Res.* **2000**, *28*, 3332. Conditions: [Tris-HCl] = 10 mM at 37 or 50 °C and pH 7.5).

8 Melting temperatures (T_m) of the duplexes of PNA 15-1, 15-2, 15-3, 15-4, and 20-1 with the corresponding complementary single-stranded DNA were 82.0, 87.5, 88.0, 84.5, and 92.0 °C, respectively (Conditions: [pcPNA] = [DNA] = 2 μM, [HEPES] = 5 mM, and [NaCl] = 100 mM).

9 According to rough estimation, the scission efficiency obtained by using one 15-mer pcPNA is about 1/5–1/10 of the two-pcPNA-based cutters reported in Ref. 2. When 20-mer pcPNA is used, however, the one-pcPNA-based cutter is almost as active as the two-pcPNA-based cutter. Precise comparison is difficult because of high sequence-dependence for the scission by the one-pcPNA system.

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