Site-specific Scission of Lambda Phage Genomic DNA by Ce(IV)/EDTA-based Artificial Restriction DNA Cutter

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Lambda phage genomic DNA (48502 base pairs) was site-selectively hydrolyzed by artificial restriction DNA cutter (ARCUT), which we have developed by combining Ce(IV)/EDTA and pseudo-complementary PNA. PNA satisfactorily invaded lambda DNA and activated the target site for the scission by Ce(IV)/EDTA.

Site-selective DNA hydrolysis is one of the most important processes in current molecular biology and biotechnology, and natural restriction enzymes are being used for this purpose. Manipulation of small DNAs such as plasmid DNA is easily achievable by using them. However, their site-specificity is too low to manipulate genomic DNAs of viruses or phages, which are widely used for gene therapy, construction of DNA library, and so on.

Many chemists have been trying to prepare man-made DNA cutter through chemical approaches.[1] However, there are few feasible tools for site-selective DNA hydrolysis. Recently, we have developed a novel artificial restriction DNA cutter (ARCUT) using Ce(IV)/EDTA complex as molecular scissors and pseudo-complementary PNA (pcPNA)[2] as sequence recognizing moiety.[3] In these works, several nucleotides in both strands of substrate DNA form gap-like structures through invasion of two pseudo-complementary PNAs, and these sites are selectively hydrolyzed by Ce(IV)/EDTA complex since this complex hydrolyzes single-stranded DNA far faster than double-stranded DNA.[4] Furthermore, we successfully manipulated plasmid DNA using this man-made tool and constructed fusion protein.[5] Next important step for us is to apply this tool to manipulation of much longer DNA, which cannot be easily dealt with by natural restriction enzymes. In this study, lambda phage genomic DNA whose length is more than 48 kbp (about 10 fold greater than plasmid DNA) is cleaved by ARCUT, and its applicability to site-selective scission of longer DNA is substantiated.

In order to investigate invasion efficiency of pcPNA, 30-base DNA sequence around 39170 base pair region involving *Eco*RI site was targeted by ARCUT (Figure 1a; *Eco*RI site is *GAATTC* from 39168 to 39173). Two pcPNA additives (PNA1 and PNA2) were prepared to cut the DNA strands at the corresponding position. PNA1 and PNA2 bear 2-thiouracil (U) and 2,6-diaminopurine (D) in place of thymine and adenine (Figure 1b). This chemical modification destabilizes PNA/PNA duplex and promotes their invasion into double-stranded DNA.[2] PNA1 is complementary with A39167–T39186 of the upper strand of lambda DNA, and PNA2 is complementary with A39157–C39176 of the lower strand. Accordingly, single-stranded portions, which should be hydrolyzed by Ce(IV)/EDTA, are formed at T39157–G39166 of the upper strand and G39177–A39186 of the lower strand (underlined bases in Figure 1a; see Figure 1c for the structure of invasion complex).

In order to confirm invasion of PNA additives into lambda DNA, inhibitory effect of PNA on *Eco*RI reaction was investigated (Figure 2a).[6,7] Invasion complex was formed by incubating lambda DNA[8] in the presence of PNA1 and PNA2 at 50 °C for 1 h. The resultant complex was treated with *Eco*RI and subjected to agarose gel electrophoresis. In lane 2, two bands corresponding to 7.4 and 5.8 kbp, which were formed in the absence of the PNAs (lane 1; indicated by arrows B and C in the left hand side), almost disappeared, and a new band of 13.2 kbp (indicated by an arrow A) appeared. Apparently, PNA invade lambda DNA site-specifically, and inhibit *Eco*RI digestion. Invasion efficiency is as high as 90%. Quite importantly, PNA efficiently invades very long DNA such as lambda phage genomic DNA.[9]

In Figure 2b, stability of invasion complex under the conditions for cleavage reaction (i.e. [NaCl] = 100 mM) was investi-

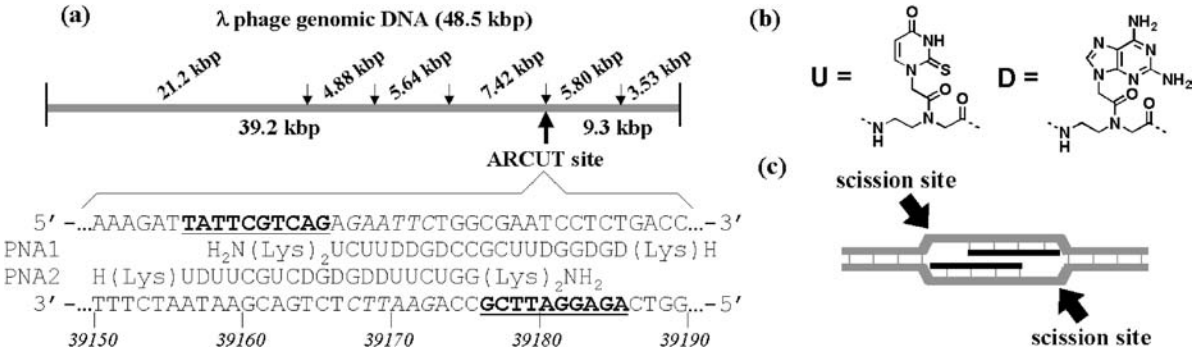

Figure 1. a) Sequences of PNAs used for site-selective hydrolysis of lambda phage genomic DNA. Downward and upward arrows indicate *Eco*RI sites and ARCUT site, respectively. The lengths of fragments obtained by treatment with *Eco*RI or ARCUT are also shown. In order to promote invasion of PNA into double-stranded DNA, U and D monomers in b) are used.[2] In invasion complex composed of lambda phage genomic DNA, PNA1 and PNA2, underlined nucleotides are kept unpaired as shown in c). GAATTC in italic in DNA sequence is *Eco*RI scission site.

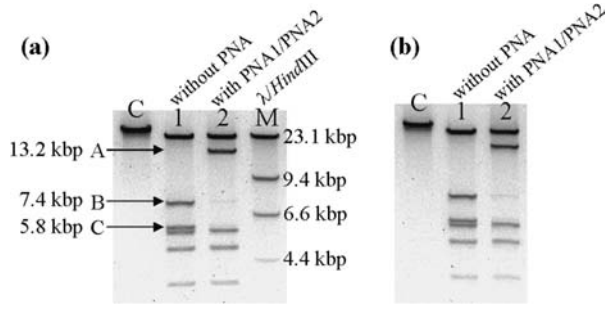

Figure 2. a) Inhibition of *Eco*RI digestion by invasion of PNA1 and PNA2. Lane 1, without PNA; lane 2, with PNA1/PNA2; C, without *Eco*RI treatment; M, lambda/*Hind*III marker. Invasion conditions: [lambda DNA] = 20 ng/μL, [PNA] = 300 nM, [HEPES] = 5 mM, [TRIS] = 1 mM, [NaCl] = 10 mM, pH 7.0, 50 °C, 1 h. The bands were stained with GelStar. In b), stability of invasion complex under the conditions for ARCUT cleavage reaction was investigated. Lane 1, without PNA; lane 2, with PNA1/PNA2; C, without *Eco*RI treatment. Invasion complex was first formed in the presence of 10 mM NaCl, and then further incubated for 24 h at 45 °C with 100 mM NaCl before *Eco*RI digestion.

gated.[10] In lane 2, invasion complex was first formed in the presence of 10 mM of NaCl at 50 °C for 1 h. Then, NaCl was added to a final concentration of 100 mM and further incubated at 45 °C for 24 h. Finally, *Eco*RI was added to this mixture and the scission pattern was analyzed. Two bands of 7.4 and 5.8 kbp were still very weak in lane 2, and the band of 13.2 kbp, which was formed in the absence of *Eco*RI digestion, was also observed even after the incubation in the presence of 100 mM NaCl. In the presence of high concentration of NaCl, pcPNA never invades double-stranded DNA.[7] However, invasion complex is sufficiently stable under ARCUT cleavage conditions ([NaCl] = 100 mM) once it was formed at low NaCl concentration.

In Figure 3, lambda DNA was treated with ARCUT using PNA1/PNA2 combination and Ce(IV)/EDTA.[11] As shown in lane 2, lambda DNA was site-selectively cleaved by ARCUT and 9.3 kbp fragment was clearly observed in the gel (note that this small fragment is less susceptible to the staining than much larger lambda DNA).[12] Natural restriction enzymes used in current technology usually recognize 6-base DNA sequence. Since their recognition sequence statistically appears at every 4096 base pairs ($4^6 = 4096$), there should be more than ten scission sites in lambda DNA. In the case of *Eco*RI, there are five scission sites as shown in Figure 1a. However, ARCUT can cut the DNA at single site as evidenced here. Although natural restriction site is targeted by ARCUT in this study in order to investigate invasion efficiency, ARCUT can cleave at almost any desired site by tuning PNA sequences.

In conclusion, PNA satisfactorily invades lambda DNA, and lambda DNA can be site-selectively cleaved at the desired site by Ce(IV)/EDTA. Man-made tool ARCUT should be a powerful tool for genomic manipulation of useful viruses such as adenovirus and baculovirus since genome size of lambda phage is comparable with those of these viruses. PNA1 and PNA2 used here recognize 30-base DNA sequence, and thus site-specificity of this ARCUT is high enough to cut various genomic DNAs of higher animals and plants. ARCUT should also open the way to manipulation of genomic DNA of higher livings. Manipulation of virus genomes and *E. coli* genome is under way in our laboratory.

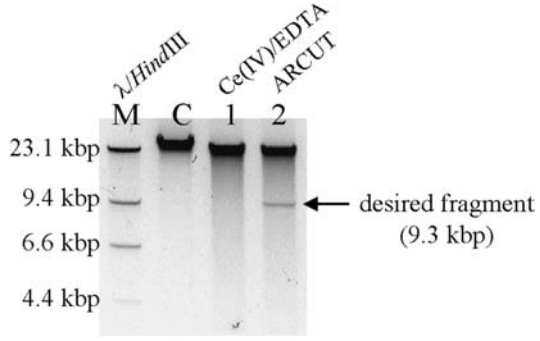

Figure 3. Site-selective hydrolysis of lambda phage genomic DNA by ARCUT. Lane 1, Ce(IV)/EDTA only; lane 2, after ARCUT treatment; C, without PNA and Ce(IV)/EDTA; M, lambda/*Hind*III marker. Reaction conditions: [lambda DNA] = 20 ng/μL, [Ce(IV)/EDTA] = 200 μM, [PNA] = 300 nM, [HEPES] = 5 mM, [TRIS] = 1 mM, [NaCl] = 100 mM, pH 7.0, 45 °C, 24 h. The bands were detected by staining with GelStar.

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References and Notes

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6 Lambda DNA was incubated with PNA1 and PNA2 for 1 h at 50 °C in pH 7 buffer in the presence of 10 mM NaCl. Then, H buffer and *Eco*RI (both from TAKARA) were added and incubated for 1 h at 37 °C.
7 Similar assay was performed to investigate the invasion of PNA to plasmid DNA: K. I. Izvolsky, V. V. Demidov, P. E. Nielsen, M. D. Frank-Kamenetskii, *Biochemistry* **2000**, *39*, 10908.
8 Lambda DNA (from TAKARA) was purified by ethanol precipitation, and dissolved in 10 mM TRIS buffer (pH 8.0) before use.
9 According to the literature, invasion rate decreases with increasing the concentration of non-targeted DNA since PNA somewhat binds DNA nonspecifically by electrostatic interaction: A. Abibi, E. Protozanova, V. V. Demidov, M. D. Frank-Kamenetskii, *Biophys. J.* **2004**, *86*, 3070. However, 1 h incubation was sufficient for the invasion in our experiments.
10 M buffer (from TAKARA) was used for *Eco*RI digestion.
11 At pH 7.0 buffer (containing 10 mM NaCl), lambda DNA was incubated with 1:1 mixture of PNA1 and PNA2 (300 nM each) at 50 °C for 1 h. Then, NaCl was added to a final concentration of 100 mM. The DNA hydrolysis was started by adding aqueous solution of Ce(IV)/EDTA. The reaction was stopped by adding ethylenediaminetetramethylenephosphonic acid (aqueous solution adjusted to pH 7.0) to a final concentration of 1 mM. The mixture was further incubated for 1 h at 50 °C before agarose gel electrophoresis.
12 The other scission fragment (39.2 kbp) is too long and hardly separated from the substrate DNA (48.5 kbp).

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