Chemical modification of Ce(IV)/EDTA-based artificial restriction DNA cutter for versatile manipulation of double-stranded DNA

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ABSTRACT

A monophosphate group was attached to the terminus of pseudo-complementary peptide nucleic acid (pcPNA), and two of thus modified pcPNAs were combined with Ce(IV)/EDTA for site-selective hydrolysis of double-stranded DNA. The site-selective DNA scission was notably accelerated by this chemical modification of pcPNAs. These second-generation artificial restriction DNA cutters (ARCUTs) differentiated the target sequence so strictly that no scission occurred even when only one DNA base-pair was altered to another. By using two of the activated ARCUTs simultaneously, DNA substrate was selectively cut at two predetermined sites, and the desired fragment was clipped and cloned. The DNA scission by ARCUT was also successful even when the target site was methylated by methyltransferase and protected from the corresponding restriction enzyme. Furthermore, potentiality of ARCUT for manipulation of huge DNA has been substantiated by site-selective scission of genomic DNA of *Escherichia coli* (composed of 4,600,000 bp) at the target site. All these results indicate promising applications of ARCUTs for versatile purposes.

INTRODUCTION

Preparation of artificial restriction enzymes has been attracting significant interests of chemists and biochemists (1–11). One of the promising applications of these tools is to manipulate genomic DNAs which are too huge to treat with naturally occurring restriction enzymes. Progress in this field has been remarkable and several man-made tools for site-selective scission of single-stranded DNA were already prepared (12–14). Double-stranded DNA was cut by (i) conjugation of DNA binders with restriction enzymes (15,16), (ii) use of metal complexes of sequence-recognizing proteins (17,18), and (iii) protection of target site from methylation followed by restriction enzyme digestion (19–22). However, there have been few feasible tools to hydrolyze DNA site selectively at desired site.

Recently (23), we combined Ce(IV)/EDTA complex (24–26) (molecular scissors for hydrolysis of phosphodiester linkages in single-stranded DNA) with two pseudo-complimentary peptide nucleic acid (pcPNA) additives (site-selective DNA activators), and hydrolyzed double-stranded DNA at the target site. In pcPNAs, thymine and adenine in conventional peptide nucleic acids (PNAs) (with poly[\(N-(aminoethyl)glycine\)] backbone) are replaced with pseudo-complementary bases 2-thiouracil (U) and 2,6-diaminopurine (D), respectively (27). Accordingly, their invasion into double-stranded DNA is facilitated by the following two factors: (i) destabilization of pcPNA/pcPNA duplex by steric repulsion between 2-S atom of U and 2-NH\(_2\) of D, and (ii) stabilization of pcPNA/DNA duplex by three hydrogen bonds between D and T. In the Ce(IV)/EDTA-based DNA cutters (23), predetermined sites in both DNA strands are kept single-stranded through invasion of two pcPNAs of appropriate sequences (see the top of Figure 1a). These single-stranded portions are ‘hot spots’ for the scission by Ce(IV)/EDTA and selectively cleaved. Both the scission-site and scission-specificity can be easily altered according to our needs, since the lengths and sequences of pcPNA additives are freely chosen. Furthermore, completely hydrolytic character of the scission was confirmed by ligation of the scission fragments with foreign DNA to recombinant DNA (28).

Here we show that the scission efficiency of ARCUTs is further promoted by attaching monophosphate groups (or iminodiacetate groups) to the termini of pcPNA...
additives and placing them near the target scission-sites (the bottom of Figure 1a). As a result, these modified ARCUTs are applicable for still more versatile purposes. For example, DNA substrate is cut at two predetermined sites and the desired fragment is clipped. Furthermore, it has been confirmed that the site-selective scission occurs only when both strands of DNA and two pcPNA additives are completely complementary with each other, and thus alteration of even one DNA base-pair to another totally inhibits the scission. Significant features of ARCUTs are further evidenced by (1) site-selective scission of genomic DNA of *Escherichia coli* (4.6 Mb) and (2) site-selective scission of enzymatically methylated DNA, both of which are hardly achievable by naturally occurring restriction enzymes.

**MATERIALS AND METHODS**

**Materials**

For the preparation of the pcPNA additives, Boc-protected monomers of 2-thiouracil and 2,6-diaminopurine [synthesized according to the literatures (27,29)] were used together with commercially available Boc-protected monomers and (4-methylbenzhydryl)amine resin (both from ABI) (30). In order to introduce L-phosphoserine to N-terminus, N-Fmoc-O-benzyl-L-phosphoserine (from Novabiochem) was attached after the final deprotection of Boc group of pcPNA. Fmoc groups were removed by 20% piperidine/\(N\)-methyl-2-pyrrolidinone for 15 min, and the resultant phosphate-modified pcPNA was removed from the resin by ‘low–high TFMSA method’. In order to introduce iminodiacetate groups to N-terminus or C-terminus, appropriately protected monomers (synthesized according to the Supplementary Scheme) were used. All these pcPNAs were purified by reversed-phase HPLC and characterized by MALDI-TOFMS (Bruker, AutoFLEX).

The substrate DNA for the site-selective scission was prepared by linearizing pBR322 plasmid DNA with EcoRI (both from Takara). To introduce a mismatched site into this DNA substrate, mutated double-stranded DNA was amplified from pBR322 by overlapping PCR using mutated primers. The resultant DNA was inserted into the corresponding site of pBR322 by a conventional method using restriction enzymes and a ligase. After cloning in JM109, the plasmid was purified by QiAprep Spin Miniprep Kit (from Qiagen) and digested with EcoRI to convert form I into form III. The 130-mer double-stranded DNA having the same sequence as T1776-G1905 of the 4361-mer DNA was prepared by PCR using the following two primers; 5'-FAM-TACCGCCAGTTTACCTCCCTCCATAC-3' and 5'-GTAAGGGTTTCTGTT-3', and purified by QiAquick PCR Purification Kit (from Qiagen). DNA oligomers were prepared on an ABI 394 DNA/RNA synthesizer using the phosphoroamidite monomers (from Glen Research), purified by reversed-phase HPLC and characterized by MALDI-TOFMS. The Ce(IV)/EDTA complex was prepared by mixing 20 mM solution of Ce(NH₄)₂(NO₃)₆ (from Nacalai Tesque) in water and 20 mM EDTA/\(C\)₄Na (from Tokyo Kasei Kogyo) in HEPES buffer, and then the pH was adjusted to 7.0 with small amounts of NaOH (23).
Site-selective scission of double-stranded 4361-mer DNA by the second-generation ARCUT

At pH 7.0 (5 mM HEPES buffer without addition of NaCl), double-stranded 4361-mer DNA (linearized pBR322) was incubated with a combination of two pcPNAs 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. Typical cleavage conditions: [4361-mer DNA] = 8 nM, [each of pcPNAs] = 200 nM, [Ce(IV)/EDTA] = 50 μM, [NaCl] = 100 mM, and [HEPES] = 5 mM at pH 7.0. After a predetermined reaction time, ethylenediaminetetramethylene phosphonic acid (aqueous solution adjusted to pH 7.0) was added to a final concentration of 500 μM to stop the reaction. The mixture was further incubated for 1 h at 50°C, and then subjected to 0.8% agarose gel electrophoresis. The bands were detected by staining with GelStar (from Cambrex), and quantified by assuming that the band intensity is proportional to the length of fragment.

Double digestion of DNA for clipping of desired fragment and its characterization

By using four pcPNA additives simultaneously, substrate DNA was cut at two sites and the fragment was purified by agarose gel electrophoresis. The cloning vector was prepared by treating pUC18 with HindIII, BamHI, and then calf intestinal alkaline phosphatase (all from Takara). Two ligation joints (5'-pAGCTATCATGATACCC-3' and 5'-pGATCATGACAGTAGAATTC-3') were attached to the ends of linearized pUC18 vector by DNA Ligation Kit (from Takara), and the termini of the vector were modified complementary with the termini of the fragment clipped by the ARCUT. Then the double-digested fragment was ligated with the vector, and transfected into JM109 and cultured on LB-agar media. Colonies containing the recombinant plasmid were picked up by colony PCR, and cultured in 2× YT media. The plasmid DNA was purified with QIAprep Spin Miniprep Kit, and its sequence was determined on an ABI PRISM 310 Genetic Analyzer. The sequencing results showed that the corresponding scission fragment was selectively combined with the modified vector (see the Results and Discussion section for details). Although the scission was not restricted to one phosphodiester linkage for each strand, the desired fragment was picked up in the ligation step, as shown in ref. (23).

Site-selective scission of genomic DNA of E. coli

Using Genomic-tip 100 G (from Qiagen), E. coli genomic DNA was prepared from K12 strain (MG1655), and was dissolved in 1 mM Tris buffer (pH 8.5). This stock solution of genomic DNA was diluted with 5 mM HEPES buffer containing 10 mM NaCl. The invasion complex was formed by incubating the mixture at 50°C for 3 h in the presence of pcPNA additives. NaCl was added to a final concentration of 100 mM, and then the DNA hydrolysis was started by the addition of Ce(IV)/EDTA. Cleavage conditions: [E. coli genomic DNA] = 20 ng/μl, [Ce(IV)/EDTA] = 0–40 μM, [each of pcPNAs] = 400 nM, [HEPES] = 5 mM, [Tris] = 0.5 mM, [NaCl] = 100 mM at pH 7.0 and 37°C for 64 h. After stopping the reaction with ethylenediaminetetramethylene phosphonic acid, the product was treated with PstI in the presence of buffer M (both from Takara) at 37°C for 4 h.

Disgoxinigen-labeled probe was prepared by using PCR DIG Probe Synthesis Kit (from Roche) from E. coli genomic DNA. Southern blotting and hybridization were performed according to the recommended protocol, and the probe on the membrane was detected with anti-digoxigenin-AP and AttoPhos Substrate Set (both from Roche).

Gel-shift assay for the formation of invasion complex

Invasion complex composed of the FAM-labeled 130-mer double-stranded DNA (T1776-G1905 in the 4361-mer DNA) and pcPNA additives was prepared as described above. Invasion conditions: [130-mer double-stranded DNA] = 50 nM, [each of pcPNAs] = 300 nM, and [HEPES] = 5 mM at pH 7.0 and 50°C for 1 h. Loading buffer containing bromophenol blue (0.05%) and glycerol (30%) in 0.5× TBE buffer was added, and the mixture was subjected to 10% non-denaturing polyacrylamide gel electrophoresis (PAGE). The bands were detected by staining with GelStar.

RESULTS AND DISCUSSION

Invasion complex formation of pcPNA additives bearing terminal monophosphates

In order to facilitate the gel-shift assay, a 130-mer double-stranded DNA having exactly the same sequence as T1776-G1905 of the 4361-mer DNA substrate was used. The invasion site exists almost in the middle of this DNA. An N-terminus of pcPNA, and two of these modified pcPNAs were incubated with the DNA. One of the pcPNA additives (PNA1-P) is complementary with C1823-C1835 of the upper strand of the DNA, and has a monophosphate on its N-terminal serine (Figure 1b). Another additive PNA2-P is complementary with G1826-C1838 of the lower strand of the DNA, and has a monophosphate at the edges of these single-stranded portions. These single-stranded portions are highly susceptible to the hydrolysis by Ce(IV)/EDTA (23). The PNA3-P/PNA4-P combination also invaded the double-stranded DNA almost completely (lane 5 of Figure 1c). In this invasion complex, three nucleotides in both strands remained unpaired and negative charges on the monophosphates. Invasion was nearly completed under the conditions employed. In this invasion complex, three nucleotides in both strands of the DNA (C1836-G1838 of the upper strand and G1823-A1825 of the lower strand) remained unpaired and the monophosphate groups on the pcPNAs were placed at the edges of these single-stranded portions. These single-stranded portions are highly susceptible to the hydrolysis by Ce(IV)/EDTA (23). The PNA3-P/PNA4-P combination also invaded the double-stranded DNA almost completely (lane 5 of Figure 1c). The two monophosphates on the pcPNAs were placed at the edges of the single-stranded portions composed of unpaired five nucleotides.
Terminal monophosphates on the pcPNA additives for the promotion of site-selective scission of double-stranded DNA

The invasion complex of the substrate 4361-mer DNA (prepared by cutting pBR322 with EcoRI) with PNA1-P/PNA2-P was treated with Ce(IV)/EDTA at pH 7.0 and 50 °C for 17 h. The result of agarose gel electrophoresis (stained with GelStar) is presented in lane 3 of Figure 2a. Only two scission bands were observed at around 1800 and 2500 bp, exactly as expected from the site-selective scission by this ARCUT (1830 and 2530 bp fragments should be formed). The yield for each of the two scission-fragments was 35% under the conditions employed. It is noteworthy that this scission was much faster than that in lane 4 where unmodified pcPNAs were used (the yields of the fragments by PNA1/PNA2 combination were less than 13%). Notable promotion by the two terminal monophosphates on the pcPNA additives is clearly evidenced. When there existed five unpaired nucleotides at the target site and pcPNA additives had the terminal monophosphates (PNA3-P/PNA4-P), the scission was still faster and the yields of scission fragments were 43% (lane 3 of Figure 2b). The site-selective scission at all other DNA sequences investigated was also accelerated by phosphate modification of pcPNA additives (see Supplementary Figure 1). These promotions of scission efficiencies are crucially important for various applications, and the advantages are especially explicit when substrate DNA is cut at two sites to clip a desired fragment (vide infra).

In Figure 3, the lengths of single-stranded DNA portions in the invasion complex were systematically changed. In all the cases, the promotion by the terminal monophosphates of pcPNA additives was evident (compare lanes 3 versus 4, 5 versus 6, 7 versus 8 and 9 versus 10, respectively). The magnitude of promotion by the monophosphates increased as the number of unpaired nucleotides at the target site decreased. Thus, the second-generation ARCUT showed notable site-selective scission in lane 7 where only one nucleotide (for each strand) remained unpaired at the target site. The site-selective scission was also successful even when there existed no unpaired nucleotide at the target site (lane 9). In either case, however, the corresponding first-generation ARCUT was almost completely inactive (lanes 8 and 10). In the first-generation ARCUT, the DNA scission requires rather long single-stranded portions, since otherwise steric repulsion by either the nucleobases or the main-chains of DNA and pcPNA is dominant. In the second-generation ARCUT, however, these factors are satisfactorily compensated by the accumulation of the Ce(IV) complex by the monophosphates at the target site.

![Figure 2](image-url)  
**Figure 2.** Site-selective hydrolysis of the double-stranded 4361-mer DNA using monophosphate-bearing pcPNA additives: (a) three unpaired nucleotides and (b) five unpaired nucleotides for each strand of the substrate DNA. Lane 1, without PNA and Ce(IV)/EDTA; lane 2, Ce(IV)/EDTA only; lane 3, PNA1-P/PNA2-P (or PNA3-P/PNA4-P) in (b) + Ce(IV)/EDTA; lane 4, PNA1/PNA2 (or PNA3/PNA4) + Ce(IV)/EDTA; M, 1000 bp ladder. Reaction conditions: [4361-mer DNA (linearized pBR322)] = 8 nM, [each of pcPNAs] = 200 nM, [Ce(IV)/EDTA] = 50 μM, [NaCl] = 100 mM, and [HEPES] = 5 mM at pH 7.0 and 50 °C for 17 h.

![Figure 3](image-url)  
**Figure 3.** (a) Sequences of pcPNA additives used to investigate the effect of length of single-stranded portions in DNA substrate. By the PNA3/PNA4, PNA1/PNA2, PNA5/PNA6, and PNA7/PNA8 combinations, five, three, one and zero nucleotides in the DNA are kept unpaired, respectively. (b) Effect of the length of unpaired portions on the promotion by monophosphate groups. Lane 1, without PNA and Ce(IV)/EDTA; lane 2, Ce(IV)/EDTA only; lane 3, PNA3-P/PNA4-P + Ce(IV)/EDTA; lane 4, PNA3/PNA4 + Ce(IV)/EDTA; lane 5, PNA1-P/PNA2-P + Ce(IV)/EDTA; lane 6, PNA1/PNA2 + Ce(IV)/EDTA; lane 7, PNA5-P/PNA6-P + Ce(IV)/EDTA; lane 8, PNA5/PNA6 + Ce(IV)/EDTA; lane 9, PNA7-P/PNA8-P + Ce(IV)/EDTA; lane 10, PNA7/PNA8 + Ce(IV)/EDTA; M, 1000 bp ladder. Reaction conditions: [4361-mer DNA] = 8 nM, [each of pcPNAs] = 200 nM, [Ce(IV)/EDTA] = 50 μM, [NaCl] = 100 mM, and [HEPES] = 5 mM at pH 7.0 and 50 °C for 17 h. All the pairs of pcPNAs satisfactorily invaded the substrate DNA, as was completely substantiated by the gel-shift assay (data not shown).
Absolute necessity of the phosphate modification of pcPNA additives for the efficient DNA scission is further evidenced by the following results. (1) With the use of non-phosphorylated pcPNAs, sufficiently high scission efficiency was never achieved even when these additives were employed at high concentrations. The efficiency obtained when \([\text{PNA1}] = \text{PNA2} = 500 \text{nM}\), for example, was almost the same as the value at \([\text{PNAs}] = 200 \text{nM}\) (in Figure 2a). (2) The scission efficiency with non-phosphorylated pcPNAs was not improved even when these additives were incubated with the DNA for a prolonged time (e.g. 6 h) in the invasion process (in Figure 2, the incubation time was 1 h). (3) The conversion of DNA scission by Ce(IV)/EDTA with non-phosphorylated pcPNAs (PNA1/PNA2) was only 15% when the reaction time was prolonged to 27 h (with the use of phosphorylated pcPNAs, much higher conversions were accomplished at the reaction time 17 h, as shown in Figure 2a). Apparently, the phosphate groups directly accelerate the scission of phosphodiester linkages, rather than they promote the invasion process.

Recognition of mismatch for the site-selective DNA scission

The C–G pair at 1829 of the linearized pBR322 (see Figure 1b) was systematically changed to another base-pair, and the phosphorylated puDNAs were treated with Ce(IV)/EDTA in the presence of PNA3-P/PNA4-P combination. When this C–G pair was exchanged with an A–T pair, the DNA was never cleaved by the ARCUT (Supplementary Figure 2). Other mismatched DNA substrates involving 1-bp alteration were also never hydrolyzed. The change of only 1-bp was strictly recognized by the ARCUT. These results indicate that only the target site can be selectively cut by an ARCUT, even when substrate DNA is huge and there exist many similar sequences therein.

Clipping of desired fragments by using two of the second-generation ARCUTs

Clipping of a desired fragment from huge DNA is crucially important in molecular biology and biotechnology. In order to obtain the fragment in reasonable yield, substrate DNA must be cut at two predetermined sites with sufficiently high conversions. The present second-generation ARCUT is active enough to fulfill this requirement. In the following example, 1010-mer DNA was clipped in situ (not in a stepwise manner) from the 4361-mer double-stranded DNA (2840–1830 = 1010). In the presence of two pairs of pcPNA additives (PNA3-P/PNA4-P in Figure 1b and PNA9-P/PNA10-P in Figure 4a), the 4361-mer DNA was treated with Ce(IV)/EDTA. The former pcPNA combination promotes the scission at around 1830bp (the target site 1) as described above, whereas the selective scission with the PNA9-P/PNA10-P combination occurs at around 2840bp (the target site 2).

The electrophoresis patterns for the clipping experiments are presented in Figure 4b. By combining these two pairs of chemically modified pcPNA additives, the scissions at the two sites were satisfactorily efficient and the desired fragment was obtained in 10% yield (the bottom band in lane 4). This fragment was cloned and its sequence was completely characterized, as described in the Materials and methods section. When two combinations of unmodified pcPNAs (PNA3/PNA4 and PNA9/PNA10) were used, however, the corresponding band was too weak because of low scission efficiencies at both of the target sites, and the clipping was unsuccessful (lane 3). It is concluded that the monophosphate groups on the pcPNA additives are absolutely necessary for the efficient clipping of the desired fragment. Other scission bands in lanes 3 and 4 (around 2800, 2500, 1800 and 1500 bp) came from the scission of the 4361-mer DNA at either one of the two target sites. As expected, all the bands in lane 4 were stronger than the corresponding bands in lane 3. Consistently, when either of the two ARCUTs was first-generation and the other was second-generation (lanes 5 and 6), the scission was efficient only at the site of the second-generation ARCUT and the clipping was unsatisfactory.

Figure 4. (a) Sequences of pcPNA additives used for the double digestion of 4361-mer DNA (linearized pBR322) by second-generation ARCUT. By combining PNA9-P/PNA10-P (or PNA9/PNA10) with Ce(IV)/EDTA, the site 2 around 2840 bp is hydrolyzed. Accordingly, 1010bp fragment should be obtained when this ARCUT is used simultaneously with the other ARCUT (PNA3-P/PNA4-P or PNA3/PNA4). (b) Gel-electrophoresis patterns for the double digestion. Lane 1, without PNA and Ce(IV)/EDTA; lane 2, Ce(IV)/EDTA only; lane 3, PNA3/PNA4/PNA9/PNA10+Ce(IV)/EDTA; lane 4, PNA3-P/PNA4-P/PNA9-P/PNA10-P+Ce(IV)/EDTA; lane 5, PNA3/PNA4/PNA9/PNA10-P+Ce(IV)/EDTA; lane 6, PNA3-P/PNA4-P/PNA9/PNA10+Ce(IV)/EDTA; M, 1000 bp ladder. Reaction conditions: [4361-mer DNA] = 8 \text{nM}, [each of pcPNAs] = 200 \text{nM}, [Ce(IV)/EDTA]=200 \text{mM}, [NaCl]=100 \text{mM}, and [HEPES]=5 \text{mM} at pH 7.0 and 45°C for 40h.
Attachment of other negatively charged groups to pcPNA additives for promotion of the site-selective DNA hydrolysis

Two iminodiacetate groups were bound to the N-terminus of each of PNA3 and PNA4, and these pcPNAs were combined with Ce(IV)/EDTA. The 4361-mer DNA was selectively and efficiently hydrolyzed at the target site, and the scission efficiency was comparable with that obtained with the use of two pcPNAs bearing a monophosphate (see Supplementary Figure 3). Attachment of iminodiacetate groups to the C-terminus of pcPNA was also effective (Supplementary Figure 4).

Still more negative charges were placed at the target site by introducing two iminodiacetate groups to both the N-termini and the C-termini of PNA3 and PNA4 (four iminodiacetate groups for each additive). However, the site-selective scission by Ce(IV)/EDTA was inefficient (data not shown). According to gel-shift assay, these additives only poorly invaded the double-stranded DNA. Many negative charges on the additives repel the negative charges of the DNA substrate. Consistently, the DNA scission was hardly promoted when two EDTA groups were introduced to the N-terminus of each of the pcPNAs. The invasion of two pcPNA additives is essential for the present site-selective DNA scission.

Site-selective hydrolysis of methylated target-site by ARCUT

Some of the naturally occurring restriction enzymes cannot digest DNA when the DNA nucleobases at the scission sites are methylated by methyltransferase. In Figure 5, the effects of this methylation on theARCUT reactions are investigated. The two cytosine groups in BamHI site of pET28b (GGATC*C/CC*TAGG) were first methylated by BamHI methyltransferase, and the methylated plasmid was then treated with ARCUT. These pcPNA additives (PNA11 and PNA12 in Figure 5a) were designed so that the target scission site involves the BamHI site. The methylated plasmid was promptly converted to its linearized form III (lane 3 of Figure 5b). Upon further digestion of the product with EcoRV, two bands were formed as indicated by the arrows (lane 5). Their sizes are consistent with the double digestion at the methylated site by the ARCUT and then by EcoRV (1380 and 3990 bp). The efficiency of the ARCUT scission was almost the same as that for the scission of non-methylated DNA (compare lane 2 with lane 3, and lane 4 with lane 5). Site-selective scission of plasmid DNA by ARCUTs was also notably promoted by introducing monophosphate groups to the pcPNA additives, and the magnitudes of acceleration were similar to those observed for the scission of non-supercoiled 4361-mer DNA (data not shown).

When the methylated DNA was treated with BamHI and then with EcoRV, however, only the form III DNA was produced (lane 7). As already reported by many workers, BamHI hardly digested the methylated DNA (data not shown). The double digestion occurred only when the substrate was not methylated (lane 6). In the ARCUT reactions, the methylation does not affect much the Watson–Crick hydrogen bondings between the DNA and the pcPNA additives, and thus the invasion complex is successfully formed even with the methylated DNA. Thus, the hydrolysis of the phosphodiester linkages by Ce(IV)/EDTA is little affected by the methylation of cytosine.

Site-selective scission of genomic DNA (4.6 Mb) using ARCUT

One of the most important features of ARCUT is the potentiality for site-selective hydrolysis of huge DNA. In order to confirm this argument, genomic DNA of E. coli was cut at the predetermined site in Figure 6. This DNA is composed of 4.6 Mb and about 1000 times as large as plasmid DNA. Naturally occurring restriction enzymes are not very useful for its manipulation, since their scission sites should appear too often in this large DNA. For widely employed restriction enzymes...
recognizing a 6-base sequence, for example, the scission sites should appear at every $4^6$ (=4096) bps according to statistical calculation.

As presented in Figure 6a, downstream of *dam* (a gene coding DNA adenine methyltransferase) was chosen as the target site for the ARCUT scission (the choice of this site is arbitrary). The genomic DNA of *E. coli* was first incubated with Ce(IV)/EDTA in the presence of both PNA13 and PNA14. Then, the product was further treated with a restriction enzyme PstI to cut the DNA at the PstI site in the upstream of *dam* and prepare an easily analyzable fragment. The resultant product was subjected to agarose gel electrophoresis, and detected by Southern blotting and hybridization with digoxigenin-labeled probe corresponding to *dam* (Figure 6b). As shown in lanes 2–4, a new band was clearly observed at the size 4.0 kb, which is consistent with the ARCUT/PstI double digestion of the genomic DNA (the length of this DNA was confirmed by using digoxigenin-labeled lambda/HindIII marker). Consistently, this 4.0 kb fragment was not formed in the absence of Ce(IV)/EDTA (lane 1) and its amount increased with increasing concentration of Ce(IV)/EDTA (lane 4 > lane 3 > lane 2).

When the genomic DNA was directly treated with PstI (without the foregoing ARCUT treatment), however, only one fragment of much larger size was detected (the band near the top of the gel). This fragment was formed by the PstI scission at both the upstream and downstream side of the *dam* (the latter PstI site is located at ~20 kb downstream of *dam* as shown in Figure 6a). The site-selective scission of genomic DNA by the ARCUT at the target site has been confirmed.

**CONCLUSIONS**

By attaching either monophosphate or iminodiacetate groups to N-termini of pcPNA additives, second-generation artificial restriction DNA cutters (ARCUTs) have been developed. These groups on the additives are placed near the target site to accumulate the Ce(IV)/EDTA thereto and promote the scission efficiency. These arguments are consistent with the previous findings that selective scission of single-stranded DNA at gap-sites by Ce(IV)/EDTA was promoted by attaching these groups to oligonucleotide additives (13, 31). The promotion of scission efficiency by the chemical modification of pcPNAs is crucial for various applications. For example, the desired fragment is clipped from substrate DNA by using two of these second-generation ARCUTs simultaneously (without the modification, the clipping is unsuccessful). Furthermore, the scission by ARCUTs is achievable even at enzymatically methylated sites.

One of the most significant advantages of ARCUTs is the free tunability of its scission-site and site-specificity. For example, the ARCUT in Figure 2a recognizes 16-base DNA sequence (two 13-mer pcPNAs are shifting each other by 3 bases). Statistically, this scission site appears only once in every $4^{16}$-base sequence which is longer than the whole genome of human beings ($4^{16} > 3 \times 10^5$). Furthermore, DNA scission by ARCUTs occurs only when both strands of this DNA and both of the pcPNA additives are completely complementary with each other (see Supplementary Figure 2). Even when only 1bp in the DNA is converted to another, the scission by ARCUT never occurs. This remarkable sequence differentiation is critical for site-selective scission of huge DNA, since many similar sequences should appear in various parts of this DNA. All these properties of ARCUTs presented here indicate promising applications in the cloning of genomic DNA, DNA shuttling from cloning vector to destination vector, and others.

**SUPPLEMENTARY DATA**

Supplementary Data is available at NAR online.

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