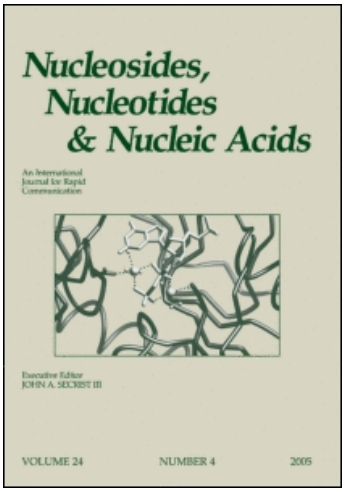

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DEVELOPMENT OF ARTIFICIAL RESTRICTION DNA CUTTER COMPOSED OF Ce(IV)/EDTA AND PNA

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□ *An artificial restriction enzyme, which we developed recently by combining Ce(IV)/EDTA and peptide nucleic acids, was used for PCR-free construction of a fusion protein. The fusion protein was successfully expressed in mammalian cells. This artificial DNA cutter can be also applied to site-selective scission of huge DNAs. Promising features of this novel tool were concretely evidenced.*

Keywords PNA; cerium; hydrolysis; restriction enzyme; DNA

INTRODUCTION

Site-selective scission of double-stranded DNA is quite important for current biotechnology and molecular biology. We recently developed novel artificial system to cleave double-stranded DNA at the desired site, in which Ce(IV)/EDTA complex[1] (as molecular DNA scissors) and peptide nucleic acids[2,3] (PNAs; as sequence recognizing moieties) were combined. As shown in Figure 1, sequences of PNA additives are designed so that several nucleotides in the substrate DNA are kept unpaired through the invasion of PNAs. When this invasion complex is treated with Ce(IV)/EDTA complex, single-stranded portions in both strands of double-stranded DNA are site-selectively cleaved[4] (Ce(IV)/EDTA complex hydrolyzes single-stranded DNA far faster than double-stranded DNA[5]). This man-made DNA cutter, ARCUT (Artificial Restriction DNA CUTter), should be a powerful tool in the relevant field since its recognition sequence and site-specificity can be freely changed according to our requests. This paper describes our recent studies on construction of a fusion protein[6] and site-selective cleavage of

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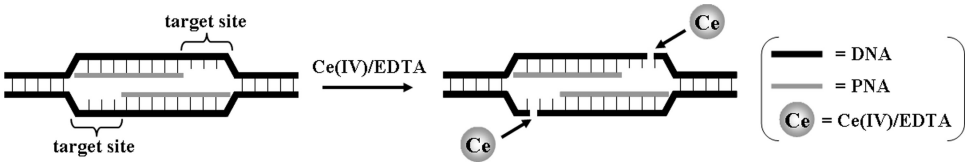

FIGURE 1 Site-selective hydrolysis of double-stranded DNA by ARCUT composed of Ce(IV)/EDTA and two PNA additives.

lambda phage genomic DNA[7] by ARCUT in order to show its promising features for future application to biotechnology and molecular biology.

PCR-FREE CONSTRUCTION OF A FUSION PROTEIN BY ARCUT

Procedure for construction of a fusion protein by using ARCUT is shown in Figure 2a. Here, WWOX (WW-domain-containing oxidoreductase; a proposed tumor suppressor) and EGFP (enhanced green fluorescent protein) were selected as target proteins. In order to construct the fusion gene by recombination of pGEM-WWOX and pEGFP-N1, the former gene, *WWOX*, should be clipped from pGEM-WWOX just before its stop codon. Otherwise, translation of the fusion protein stops at the stop codon of *WWOX*, or incomplete and truncated WWOX is conjugated with EGFP. However, there

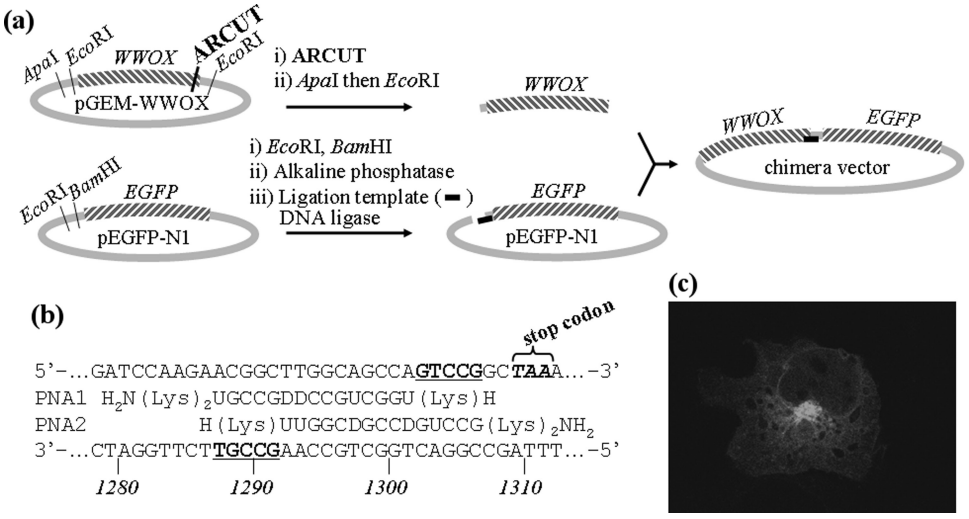

FIGURE 2 a) Procedure for PCR-free construction of a fusion protein by the use of ARCUT. In order to cut pGEM-WWOX just before the stop codon of *WWOX*, two PNA additives in b) were used. In place of conventional bases, U and D in these PNAs bear 2-thiouracil and 2,6-diaminopurine residues, respectively. Through invasion of these two PNAs, underlined nucleotides are kept unpaired and hydrolyzed by Ce(IV)/EDTA. c) Expression of the present fusion protein in Cos-7 cells.

are no restriction enzyme sites near this stop codon. ARCUT, whose recognition sequence can be freely changed, is essential to cut there.

In order to cut pGEM-WWOX, PNA1 and PNA2 shown in Figure 2b were combined with Ce(IV)/EDTA complex. When the invasion complex was formed from them, G1303-G1307 in the upper strand and T1288-G1292 in the lower strand were kept single-stranded (underlined parts in Figures 2b). This scission site is located just before the stop codon of *WWOX* (TAA at 1310–1312). After cutting pGEM-WWOX by Ce(IV)/EDTA and PNA1/PNA2 combination, the desired *WWOX*-containing fragment was obtained by treatment with appropriate natural enzymes. Independently, pEGFP-N1 was linearized by using two natural restriction enzymes to insert *WWOX* therein.

Since the scission termini of ARCUT and restriction enzyme are not complementary with each other, a short synthetic DNA oligomer was used as a template to ligate them as shown in the lower side of Figure 2a. Sequence of this DNA template was designed so that its 5′- and 3′-side are completely complementary with *Bam*HI-scission terminus of *EGFP*-containing fragment and ARCUT-scission terminus of *WWOX*-containing fragment, respectively. These two fragments were successfully ligated with each other to fuse *WWOX* and *EGFP* by using this template.

When the present fusion vector was transfected into mammalian cells (Cos-7), the fluorescent protein was successfully expressed (Figure 2c). The fusion protein localized at the Golgi apparatus, which was different from nuclei localization of nonconjugated EGFP. The function of WWOX moiety was also studied, and several interactive proteins were obtained by coimmunoprecipitation. Both of WWOX and EGFP moieties of the present fusion protein exhibit their normal functions, indicating applicability of ARCUT to gene manipulation.

SITE-SELECTIVE SCISSION OF LAMBDA PHAGE GENOMIC DNA BY ARCUT

ARCUT can be applied to site-selective scission of not only plasmid DNA but also much larger DNAs such as virus genome and phage genome. As shown in Figure 3a, two PNA additives (PNA3 and PNA4) were prepared in order to cut around 39,170 base pair region of lambda phage genomic DNA. After the scission by ARCUT using these PNAs and Ce(IV)/EDTA, one scission band whose mobility was comparable with that of 9.4 kbp marker was clearly observed (Figure 3b, lane 2). This size is consistent with that obtained after the site-selective scission by ARCUT (9.3 kbp and 39.2 kbp fragments should be obtained).

Total length of lambda phage genomic DNA is 48,502 bp, and it is comparable with those of viruses and phages which are widely used for gene

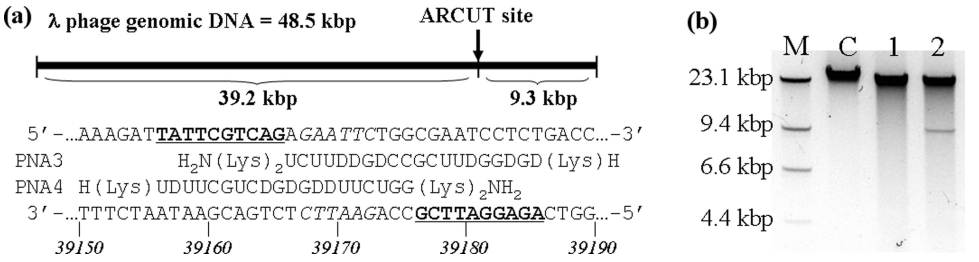

FIGURE 3 a) Sequences of PNA additives used for site-selective hydrolysis of lambda phage genomic DNA. b) Agarose gel electrophoresis patterns for site-selective scission of lambda DNA by ARCUT. Lane 1, Ce(IV)/EDTA only; lane 2, after ARCUT treatment; C, without PNA and Ce(IV)/EDTA; M, lambda/*Hind*III marker.

therapy, construction of genomic library, and so on. Free gene manipulation of these useful virus and phage vectors is quite difficult with the use of natural restriction enzymes. ARCUT should be a strong tool for these purposes.

CONCLUSION

A novel man-made tool, ARCUT, was applied to PCR-free gene recombination and site-selective scission of huge DNA. The fragment obtained by ARCUT scission was correctly connected with another fragment to provide the expression vector, and the resultant recombinant protein was also successfully expressed in mammalian cells. Furthermore, ARCUT can site-selectively cleave lambda phage genomic DNA, which is too long to be dealt with by natural restriction enzymes. These results show promising applicability of ARCUT to manipulation of huge genomic DNAs of higher animals and plants. These attempts are currently under way in our laboratory.

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