Artificial Restriction DNA Cutters (ARCUT) as New Tools to Manipulate Human Genome

JUN SUMAOKA AND MAKOTO KOMIYAMA*
Research Center for Advance Science and Technology
The University of Tokyo
4-6-1 Komaba, Meguro-ku, Tokyo 153-8904
JAPAN
komiyama@mkomi.rcast.u-tokyo.ac.jp http://www.mkomi.rcast.u-tokyo.ac.jp/index_e_link_e.html

Abstract: We developed artificial restriction DNA cutter (ARCUT) which cuts double-stranded DNA at one desired site. These tools are composed of (i) Ce(IV)/EDTA complex as molecular scissors and (ii) a pair of pseudo-complementary PNAs, and their scission-site is determined by simple Watson-Crick rule. Accordingly, the scission-site and site-specificity are freely chosen by changing the sequences and the lengths of PNA strands. With the use of ARCUT, even huge DNAs (e.g., the whole genome of human beings) are selectively cut at one target site. Furthermore, site-selective scission by ARCUT notably stimulates targeted homologous recombination in human cells.

Key-Words: DNA, Cerium(IV), Hydrolysis, Site-selective scission, Artificial Enzyme, PNA, Gene manipulation, Homologous recombination

1 Introduction
Conventional molecular biology and biotechnology are based on the following two processes: (i) site-selective scission of DNA by restriction enzymes and (ii) recombination of DNA fragments by ligase. As far as small DNAs such as plasmid DNA of bacteria are treated, these two processes satisfactorily work. In the near future, however, we need to manipulate genomic DNAs directly. However, naturally occurring restriction enzymes cannot be used to cut huge DNAs, since these enzymes usually recognize a specific sequence composed of 4-6 DNA bases and cleave there (the scission site should appear in average at every 4^4-4^6 base-pairs). These site-specificities are sufficient for site-selective scission of plasmid DNAs, but too low to cut huge DNAs of higher organisms at desired site. For example, if the genomic DNA of human beings, which is composed of 3x10^9 base-pairs, is treated with a 6-nucleobase recognizing restriction enzyme, the scission should occur at more than 10^5 sites. Precise gene manipulation is impossible. Only with new artificial restriction DNA cutters which recognize 16-nucleobase sequence, we can cut human genome at one site to manipulate this DNA appropriately (note that 4^{16} > 3x10^9). Thus, the preparation of such DNA cutters is crucially important for further development of molecular biology and biotechnology.

Recently we developed artificial restriction DNA cutters (ARCUT) which can selectively cut huge DNA at desired site. With these completely chemistry-based new tools, genomic DNAs of E. coli and human beings are cut at one desired site. Furthermore, homologous recombination in human cells is promoted by these artificial DNA cutters (the applications of ARCUT to DNA manipulation have been already reported [1-3]).

2 Design of artificial restriction DNA cutter (ARCUT)
The artificial restriction DNA cutter (ARCUT) developed in our laboratory is composed of two components [2]. One is Ce(IV)/EDTA complex as a catalyst for DNA hydrolysis and another is a pair of pseudo-complementary peptide nucleic acid (pcPNA, Fig.1) for recognition of DNA sequences. In 2002, we found that Ce(IV)/EDTA complex hydrolyzes single-stranded DNA far more efficiently than double-stranded DNA [4]. This finding indicated us that target phosphodiester linkages in double-stranded DNA substrate should be selectively hydrolyzed, if only these linkages are made single-stranded and all other linkages are kept double-stranded.

The site-selective scission of DNA by ARCUT proceeds as schematically depicted in Fig. 2. In order to form single-stranded portions at desired site of the substrate DNA, we use double-duplex invasion of two
Fig. 1 Chemical structures of PNA and DNA. In pcPNA, adenine and thymine groups are chemically modified into 2,6-diaminopurine (D) and 2-thiouracil (U) to suppress the formation of pcPNA duplexes.

Fig. 2 Site-selective scission of double-stranded DNA by ARCUT. Two pcPNA strands (the blue lines) invade DNA substrate to form single-stranded portions (the red lines) in both DNA strands (step 1). The phosphodiester linkages in the single-stranded portions are hydrolyzed by Ce(IV)/EDTA (step 2).

pcPNA strands (the blue lines) [5, 6]. Since the main chain of pcPNA has no negative charges, in contrast with DNA, this DNA analog forms more stable duplex with its complementary DNA than does the corresponding DNA. Accordingly, a pair of pcPNAs easily invades double-stranded DNA by Watson-Crick base-pairings. The important point is that the binding sites of two pcPNA strands in double-duplex invasion complexes are laterally shifted to one another by several nucleobases so that single-stranded-like portions (the red parts) are formed at predetermined site in the target DNA (step 1 of Fig. 2). In step 2, these portions are preferentially hydrolyzed by Ce(IV)/EDTA due to the single-strand specificity of this Ce(IV) complex (vide ante). The scission-site and site-specificity of ARCUT can be tuned simply by changing the sequences and lengths of the pcPNAs.

3 Sequence specific scission of huge DNA using ARCUT

One of the biggest advantages of ARCUT over naturally occurring restriction enzymes is its extraordinarily high aptitude to recognize long sequence. Thus, even huge genomic DNA that is too large to manipulate by conventional restriction enzymes can be satisfactorily cut at one target site.

3.1 Scission of lambda phage DNA (48.5 kbp) by ARCUT [7]

Each of two pcPNA additives (pcPNA1 and pcPNA2) involves 15 nucleobases. They invade the lambda phage DNA at the target position, and form single-stranded-like portions in both sides of the invasion site (see Fig. 3a). Exactly as designed, 9.3 kbp fragment was selectively formed by the ARCUT treatment, confirming the selective scission at the target site (lane 2 of Fig. 3b; another longer fragment was not separated from the genomic DNA in this gel). In the scission by a restriction enzyme HindIII, however, several scission fragments were formed as shown in lane M. The superiority of ARCUT over naturally occurring restriction enzymes is evident.

3.2 Scission of genomic DNA of E. coli (4.6 Mbp) by ARCUT [8]

Genomic DNA of E. coli was also selectively cut at the predetermined site. As presented in Fig. 4, the
downstream of dam (a gene coding DNA adenine methyltransferase) was chosen as the target site for the scission (this choice is arbitrary). The ARCUT scission was achieved by incubating the genomic DNA with Ce(IV)/EDTA in the presence of two pcPNA strands (pcPNA3/pcPNA4). Then, the product was further treated with a restriction enzyme PstI to prepare an easily analyzable fragment (PstI cuts the DNA at the upstream of dam; see Fig. 4a). The resultant product was subjected to agarose gel electrophoresis, and detected by Southern blotting using digoxigenin-labeled probe1 which binds to the dam gene. A scission band of 4.0 kbp size was clearly observed, confirming the site-selective scission of E. coli genome at the target site (lanes 2–4 of Fig. 4b). As expected, the amount of this 4.0 kbp fragment monotonously increased with increasing concentration of Ce(IV)/EDTA.

3.3 Site-selective scission of the whole genome of human beings (3 Gbp) by ARCUT [9]

The target site of the site-selective scission exists in the gene of FMR1 (Fragile X Mental Retardation 1) in the X chromosome, which is related to fragile X syndrome (Fig. 5). The whole human genomic DNA as substrate was isolated from cultured Flp-in 293 cells, and a 1:1 combination of pcPNA5 and pcPNA6 was added to form a double-duplex inversion complex. The mixture was treated with the Ce(IV)/EDTA complex, and then with EcoRI (to prepare easily analyzable fragments as described in 3.2). Finally, the products were analyzed by Southern blotting using Probe 2 and Probe 3, which bind to the upstream and the downstream of the ARCUT scission site, respectively. In the Southern blotting, two bands of the expected sizes (2.9 kbp for the upstream fragment and 2.3 kbp for the downstream fragment), which correspond to the dual scission by the ARCUT and EcoRI, were explicitly observed. It has been concretely confirmed that the human genomic DNA was site-selectively cleaved at one target site by the ARCUT.
Control experiments showed that other similar sequences in the human genome were never cut by the ARCUT. Apparently, subtle difference in sequence between the target site and these non-target sites is satisfactorily distinguished by the ARCUT and only the target site is hydrolyzed.

4 ARCUT for promotion of homologous recombination in human cells [10]
Homologous recombination is used to manipulate genomic DNA which is too huge to be directly treated by conventional restriction enzyme-based technology. Through this recombination process, a target DNA sequence in a genome can be converted to its homologous sequence of choice. In mammalian cells, however, this recombination occurs with only a limited frequency, and thus must be somehow promoted for practical applications. It is known that a double strand break (DSB) at a specific site in DNA notably activates the endogenous repair system and stimulates homologous recombination [11]. Zinc finger nucleases, conjugates of non-specific nuclease domain of FokI restriction enzyme and tandemly-assembled zinc finger proteins, have been already employed for this purpose [12]. However, the means to promote homologous recombination in mammalian cells is still limited, and a new strategy is demanded to widen the scope of applications of this useful biological process. Accordingly, we employ ARCUT to promote homologous recombination in human cells. If DSBs formed by ARCUT are satisfactorily recognized by the repair system in human cells, the desired homologous recombination should be promoted.

The ARCUT-promoted homologous recombination in human cells was carried out as schematically depicted in Fig. 6. The substrate DNA contains the gene of blue fluorescent protein (BFP), whereas the donor DNA (742 bp) codes enhanced green fluorescent protein (EGFP). The BFP and the EGFP have the same amino acid sequences except for the amino acids in their chromophores. The substrate DNA is cut by ARCUT at the coding site of the BFP chromophore, and then introduced into human cells together with the donor DNA which codes EGFP. If the targeted homologous recombination occurs, the BFP in the substrate DNA is converted to EGFP which is quantified by its green fluorescence emission.

Fig. 6 Outline of ARCUT-promoted homologous recombination in human cells.

Fig. 7 shows the fluorescence microscope images of the transfected cells after 48 h cultivation. The images in the upper and the lower panels refer to the emission from the BFP and the EGFP which were expressed in the human cells, respectively. With the site-selective scission at the chromophore region in the BFP gene by the ARCUT, EGFP was efficiently produced in the human cells and clearly emitted green fluorescence (the lower right). The target homologous recombination successfully occurred between the ARCUT-treated BFP and the donor EGFP fragment (note that the donor EGFP fragment does not contain a promoter operable in the human cells). Without the ARCUT scission prior to the transfection, however, green fluorescence was hardly observable (the lower left). Furthermore, the DSB by a restriction enzyme StuI, which was formed far away from the BFP gene, never stimulated the recombination (data not presented). The ARCUT-induced DSB at the target site is essential to promote the targeted homologous recombination in human cells.

The ARCUT-stimulated homologous recombination in human cells was also successful when adenovirus vector (35 kbp) was used as a repairing target. Apparently, the DSB formed by site-selective scission by ARCUT is satisfactorily recognized by the repair system in human cells, although the scission products of the ARCUT have extraordinary end structures (one strand is protruding from the other by 10-15 nucleotides). ARCUT is promising as a new recombination-stimulator for various applications.
Fig. 7 Fluorescence microscopy images of the 293T cells cultured for 48 h after the transfection with the treated (or untreated) BFP plasmid DNA and the donor EGFP gene fragment. The upper and the lower panels show blue channels (Ex: 360 nm, Em: 470 nm) and green channels (Ex: 480 nm, Em: 520 nm), respectively.

5 Conclusion

With the use of a man-made tool ARCUT, target phosphodiester linkages in substrate DNA can be selectively hydrolyzed regardless of the size of DNA and the position of scission. Even huge genomic DNA can be satisfactorily manipulated. The DNA scission is totally hydrolytic and thus the resultant scission fragments can be directly used for various biological applications. One of the most important features of ARCUT is that both the scission-site and scission-specificity are directly predictable in terms of Watson-Crick base-pairings between the DNA and pcPNA strands. Hence, when one should like to cut DNA at a predetermined position, required ARCUT (the sequences and lengths of the pcPNA strands used) can be straightforwardly designed and synthesized. Accordingly, wide applications to therapy, diagnosis, and others are promising.

Acknowledgements
This work was partially supported by a Grant-in-Aid for Specially Promoted Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (18001001). Support by the Global COE Program for Chemistry Innovation is also acknowledged.

References: