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Published on Web 07/30/2004

Site-Selective DNA Hydrolysis by Combining Ce(IV)/EDTA with Monophosphate-Bearing Oligonucleotides and Enzymatic Ligation of the Scission Fragments

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Abstract: By using two oligonucleotide additives that bear a monophosphate group at the termini through various linkers, gap structures were formed at predetermined positions in substrate DNA, and the monophosphate groups were placed at both edges of these gaps. At pH 7.0 and 37 °C, the phosphodiester linkages in the gap sites were efficiently and selectively hydrolyzed by Ce(IV)/EDTA complex (EDTA = ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetate). The linkages in the middle of the gaps were predominantly hydrolyzed. Compared with DNA scission using oligonucleotide additives that bear no terminal monophosphate, the present scission was much faster (22-fold for a 3-base gap and 14-fold for a 5-base gap) and more site selective. Introduction of one monophosphate group to either edge of the gaps was also effective for promotion of both site selectivity and scission rate. The monophosphate group(s) at the gap site recruits the Ce(IV) to the target site and magnifies the difference in intrinsic reactivity between the target site and the others. Even at higher reaction temperatures, the site selectivity remained satisfactorily high. Furthermore, the fragments formed by the site-selective scission were connected with various oligonucleotides by using DNA ligase, producing desired recombinant DNAs.

Introduction

Molecular design of artificial restriction enzymes for site-selective scission of DNA is one of the key issues today, mainly because their high site specificity is essential for precise manipulation of huge DNAs of higher animals and plants.[1−5] Much effort has been already devoted to the development of catalysts which hydrolyze phosphodiester linkages in DNA (these linkages are enormously stable, and their half-life under physiological conditions is estimated to be 200 million years).[6] Among the catalysts previously reported, Ce(IV) ion and its complexes are characterized by the remarkable catalytic activity.[7] The DNA scission proceeds totally via a hydrolytic pathway without the contribution of oxidative scission. Furthermore, site-selective DNA hydrolysis was achieved by attaching these catalysts to sequence-recognizing oligonucleotides.[8−10] However, both site selectivity and scission efficiency, dictated by their "proximity effect", were not satisfactorily high for practical applications. A new strategy should be required for further progress.

Recently, it was shown that Ce(IV)/EDTA complex preferentially hydrolyzes gap sites in substrate DNA even when the complex is not covalently linked to any sequence-recognizing moiety.[11,12] Apparently, the phosphodiester linkages in gap sites are more susceptible to the catalysis of this complex than the others in the substrate.[13] These findings have indicated that site-selective DNA scission should be improved if appropriate metal-binding groups are introduced to the gap site. As shown in this paper, monophosphate groups are very effective for this purpose and greatly magnify the difference in intrinsic reactivity between the target site and the others.[14] As a result, unprecedented clear-cut site-selective DNA hydrolysis has been accomplished at the gap sites. The structures of termini of the scission fragments are characterized, and the effects of gap length and linker structure on the scission are systematically investigated. Furthermore, the scission fragments are ligated to various oligo-

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(9) Artificial restriction enzymes that cut DNA through oxidative cleavage of the ribose at the target site showed higher scission efficiency. However, site-selective scission of DNA through a hydrolytic pathway is advantageous in that the fragments are susceptible to enzymatic transformation and can be directly susceptible to enzymatic manipulation.

Substrate DNA

DNA(S5): 5'-CAATTAGAATCAGGAATGGC**TTATG**GTGCAGACTGTCGACCTAAG-3'
DNA(S3): 5'-CAATTAGAATCAGGAATGGC**TTA**GTGCAGACTGTCGACCTAAG-3'
DNA(S2): 5'-CAATTAGAATCAGGAATGGC**TT**GTGCAGACTGTCGACCTAAG-3'
DNA(S1): 5'-CAATTAGAATCAGGAATGGC**T**GTGCAGACTGTCGACCTAAG-3'

Gap-forming Additives

DNA(L)-Ln-P: 3'-GTTAATCTTAGTCCTTACCG-Ln-P-5'
P-Ln-DNA(R): 3'-P-Ln-CACGTCTGACAGCTGGATTC-5'
DNA(L)-H: 3'-GTTAATCTTAGTCCTTACCG-5'
H-DNA(R): 3'-CACGTCTGACAGCTGGATTC-5'

n = 0, 3, 9, 12, 18

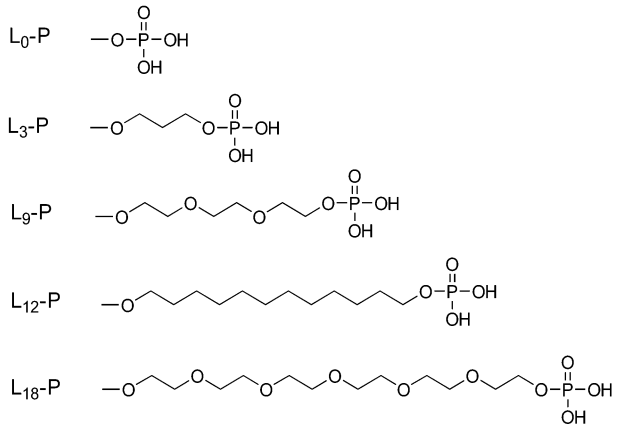

Figure 1. DNA substrates and oligonucleotide additives used in the present study. The linkers L_n are bound to the oligonucleotides by phosphodiester linkages.

nucleotides using DNA ligase, showing strong potential of the present site-selective scission for further applications.

Results and Discussion

Gap-Selective DNA Hydrolysis with the Use of Oligonucleotide Additives Bearing Monophosphate Group at the Terminal Position. In the oligonucleotide additives used, a monophosphate group was attached to either the 3′- or 5′-end through various linkers (see Figure 1). With these additives a gap structure of predetermined length was formed at the target site in substrate DNA, and monophosphate group(s) was introduced to the edge of this gap.[15] In lane 4 of Figure 2A, DNA(L)-L_{12}-P and H-DNA(R) were combined to form a 5-base gap in the 45-mer substrate DNA(S5). A monophosphate group was placed at the 5′-side edge of this 5-base gap through a $(CH_2)_{12}$ linker. Under these conditions, the scission by Ce(IV)/EDTA complex at pH 7.0 and 37 °C was strictly restricted to the gap region (from T21 to G25, see the marker lane M). With the use of the DNA(L)-H/P-L_{12}-DNA(R) combination, which provides a monophosphate group to the 3′-side edge of the gap, site-selective DNA scission was also successful (lane 5). The DNA(L)-L_{12}-P/P-L_{12}-DNA(R) combination was still more effective for the site-selective scission (lane 6). When two oligonucleotide additives without the terminal monophosphate (DNA(L)-H and H-DNA(R)) were combined (lane 3 in Figure 2A), however, DNA scission by Ce(IV)/EDTA was much slower than the scissions presented above (this scission is 14-fold slower than that in lane 6, vide infra).

The main parts in lanes 4−6 of Figure 2A are enlarged in Figure 2B. All the scission fragments are clearly separated from each other. In Figure 2C, the relative efficiency of the scission at each linkage is presented in terms of the length of the arrow

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(13) In ref 11, the greater reactivity at the gap site was ascribed to the larger flexibility of the DNA backbone which facilitates the binding of Ce(IV).

(14) Ethylenediamine-*N*,*N*,*N*′-triacetate groups were previously introduced into oligonucleotide additives, and they accelerated the Ce(IV)-induced DNA hydrolysis at the gap site (Arishima, H.; Yokoyama, M.; Komiyama, M. *Nucleic Acids Res. Suppl.* **2003**, *3*, 137−138). However, the monophosphate systems presented here are far superior in that the DNA scission is strictly restricted to the gap site and hardly occurs in the flanking double-stranded region. Furthermore, the scission by these systems is considerably faster than that by the ethylenediaminetriacetate systems.

(15) The melting temperatures (T_m) of duplexes between the additive oligonucleotide and the complementary portion in substrate DNA are sufficiently higher than the reaction temperature (37 °C). For example, T_m of the duplex between DNA(S5) and DNA(L)−L_{12}−P is 57.6 °C. Therefore, these duplexes are almost completely formed under the reaction conditions.

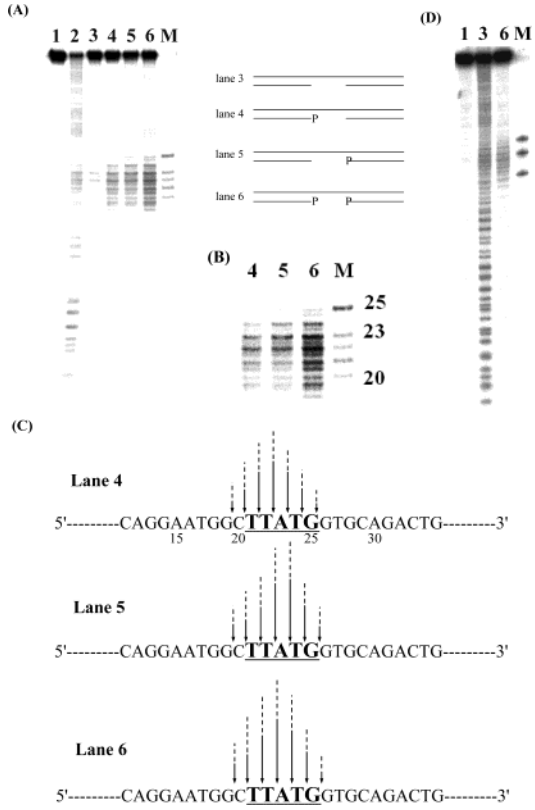

Figure 2. (A) Polyacrylamide gel electrophoresis (PAGE) patterns for the hydrolysis of $DNA^{(S5)}$ (^{32}P-labeled at the 5′-end) at a 5-base gap by Ce(IV)/EDTA complex at pH 7.0 and 37 °C. Lane 1, control without both additive DNAs and Ce(IV)/EDTA; lane 2, control in the absence of the additives (only with Ce(IV)/EDTA); lane 3, $DNA^{(L)}$-H/H-$DNA^{(R)}$ with Ce(IV)/EDTA; lane 4, $DNA^{(L)}$-L_{12}-P/H-$DNA^{(R)}$ with Ce(IV)/EDTA; lane 5, $DNA^{(L)}$-H/P-L_{12}-$DNA^{(R)}$ with Ce(IV)/EDTA; lane 6, $DNA^{(L)}$-L_{12}-P/P-L_{12}-$DNA^{(R)}$ with Ce(IV)/EDTA; M, the markers (authentic oligonucleotides having 3′-OH termini). Reaction conditions: $[DNA^{(S5)}]_0 = 1.0\ \mu M$, [each of the additive DNAs]$_0 = 2.0\ \mu M$, $[NaCl]_0 = 100$ mM, and [Ce(IV)/EDTA] = 1.0 mM at pH 7.0 (7.5 mM Hepes buffer) for 95 h. (B) Magnified versions of the main part in lanes 4−6, and (C) results of quantitative analysis. Here, the length of the arrow corresponds to scission efficiency (the solid part is for the formation of the 3′-OH terminus, and the broken one is for the formation of the 3′-phosphate terminus). (D) Reactions of lanes 1, 3, and 6 in A were achieved at 50 °C for 2.5 h.

(the solid parts and the broken parts correspond to the formation of the 3′-OH termini and 3′-phosphate termini, respectively, as described below). When the monophosphate group of the additive oligonucleotide was placed at the 5′-side edge of the gap (lane 4), the scission was most efficient at the 5′-side of A23 that is located in the middle of the gap. The 5′-sides of T22 and T24 were also notably hydrolyzed. More than 60% of the total scission occurred at these three linkages (the total conversion of scission in the gap region was 15%). In lane 5, the monophosphate group was placed at the 3′-edge of the gap. Here, the linkages in the 5′-sides of A23 and T24 were most vigorously hydrolyzed. Compared with lane 4, the positions of scission slightly shift toward the 3′-side of substrate DNA, as expected from the accumulation of Ce(IV) by the monophosphate bound to the 3′-edge of the gap. When two monophosphate groups were placed at both 3′- and 5′-edges of the gap by using the $DNA^{(L)}$-L_{12}-P/P-L_{12}-$DNA^{(R)}$ combination (lane 6), the site-selective scission was still faster than those by the one-monophosphate systems in lanes 4 and 5 (1.7- and 1.3-fold, respectively).[16] The site selectivity remained satisfactorily high.

The completely hydrolytic character of the present site-selective DNA scission has been confirmed by successful ligation of the scission fragments with the use of DNA ligase (vide infra). Furthermore, hydrolytic scission of DNA by Ce(IV)/EDTA[17] (as well as by Ce(IV) ion)[18] has been previously substantiated by the fact that only hydrolytic products are detected by the HPLC analysis. Furthermore, the scission fragments were successfully transformed to the expected forms using various naturally occurring enzymes that should strictly differentiate between the products of hydrolytic scission and others.

Structures of the Termini of Scission Fragments. The bands of the scission fragments in Figure 2 are classified into two categories. The bands in one group comigrate with authentic samples of oligonucleotides that have 3′-OH termini (in lane M). On the other hand, the bands in another group are found between the bands of authentic samples. Apparently, the first group has 3′-OH termini, and the second group has 3′-phosphate termini. In most cases, the 3′-OH termini (shown by the solid parts of the arrows in Figure 2C) are preferentially formed to the 3′-phosphate termini (the broken parts). For example, the 3′-OH/3′-phosphate ratio is around 2:1 for the strongest scission in lane 6 of Figure 2A (the 5′-side of A23 which is located in the middle of gap).

Terminal structures of the other fragments of scission (3′-side portions of substrates) were analyzed by labeling the substrates with ^{32}P at the 3′-end. As shown in Figure 1 in the Supporting Information, the scission was restricted to the gap site, confirming the results of the 5′-labeled experiments. The fragments having 5′-phosphate termini were formed in greater amounts than those having 5′-OH termini. These results indicate that the hydrolysis of phosphodiester linkages in DNA substrates preferentially proceeds via the P−O(3′) scission over the P−O(5′) scission. The possibility that phosphate termini formed in the primary scission were subsequently converted to OH termini is unlikely since Ce(IV)/EDTA does not hydrolyze phosphomonoesters much under the conditions used here (see Figure 2 in the Supporting Information).

Effects of Gap Length on the Site-Selective Scission. When a 3-base gap was formed in $DNA^{(S3)}$ (from T21 to A23) by using the monophosphate-bearing oligonucleotide additives, this gap site was also selectively hydrolyzed by Ce(IV)/EDTA (Figure 3A). With the use of the $DNA^{(L)}$-H/P-L_{12}-$DNA^{(R)}$ and the $DNA^{(L)}$-L_{12}-P/H-$DNA^{(R)}$ combinations, the scission occurred primarily at the 5′-sides of T22 and A23 (lanes 4 and 5; also see the scission patterns in Figure 3B). The scission by the $DNA^{(L)}$-H/P-L_{12}-$DNA^{(R)}$ combination, which has a monophosphate at the 3′-edge of the gap, was slightly faster.[19] The $DNA^{(L)}$-L_{12}-P/P-L_{12}-$DNA^{(R)}$ combination promoted the reaction still more effectively than did these one-monophosphate systems (compare lane 6 with lanes 4 and 5), as also found in the 5-base gap scission. Furthermore, the site-selective scission was

(16) The conversion of the scission in lane 6, Figure 2A, was 30% after 6 days (the longest reaction investigated). According to the corresponding time-conversion curve, still higher conversions should be achieved in longer reactions.
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(18) (a) Reference 7d. (b) Sumaoka, J.; Azuma, Y.; Komiyama, M. *Chem. Eur. J.* **1998**, *4*, 205.
(19) In the DNA scission by the oligonucleotide additives employing other linkers in Figure 1, a monophosphate introduced to the 3′-side edge of gaps was also slightly more efficient than that introduced to the 5′-side edge (data not presented).

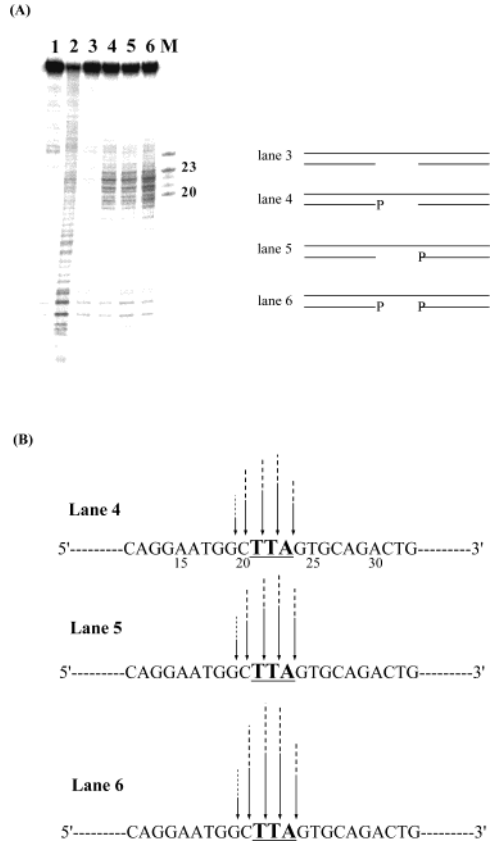

Figure 3. (A) PAGE patterns for the hydrolysis of DNA$^{(S3)}$ at a 3-base gap by Ce(IV)/EDTA complex at pH 7.0 and 37 °C. (B) Results of quantitative analysis on lanes 4−6. Lanes and the reaction conditions are the same as those described for Figure 2.

successful at either a 2-base gap (formed in DNA$^{(S2)}$) or a 1-base gap (formed in DNA$^{(S1)}$). When DNA$^{(L)}$-H and H-DNA$^{(R)}$ were combined and thus no monophosphate groups were introduced to these gaps, however, DNA scission was marginal (the scission at 3-base gap by the nonmonophosphate system is presented in lane 3 of Figure 3A).

In Figure 4, the site selectivity and the scission efficiency for the gaps of various lengths are quantitatively compared. In lanes 1−4, the scissions are strictly restricted to the gap site, so that the site selectivity monotonically increases with decreasing gap length. With a 1-base gap, the scission primarily takes place at both sides of the unpaired nucleotide T21. The scission at the 2-base gap (as well as at the 3- and 5-gaps) is vigorous in the middle of gap. The total conversion in the gap region notably decreases with decreasing gap length. However, the efficiency for the scission of each linkage in the gap site is not very dependent on the gap length.

Kinetic Analysis on the Site-Selective DNA Scission. As described in Figure 3 in the Supporting Information, the rate constants for the hydrolysis of DNA$^{(S5)}$ at the 5-base gap formed by the DNA$^{(L)}$-L_{12}-P/P-L_{12}-DNA$^{(R)}$ and DNA$^{(L)}$-H/H-DNA$^{(R)}$ combinations were evaluated to be 5.4×10^{-3} and 3.8×10^{-4} h^{-1}, respectively ([Ce(IV)/EDTA] = 1.0 mM). The corresponding values for the hydrolysis of DNA$^{(S3)}$ at the 3-base gap were 2.4×10^{-3} and 1.1×10^{-4} h^{-1}. Thus, the DNA$^{(L)}$-L_{12}-P/P-L_{12}-DNA$^{(R)}$ combination is 14-fold more active than DNA$^{(L)}$-H/H-DNA$^{(R)}$ for the hydrolysis of 5-base gap and is 22-fold more active for the hydrolysis of 3-base gap.

By employing higher reaction temperatures, the reaction time could be notably shortened without significant loss in site selectivity (the results for the scission at 50 °C are presented in lane 6 of Figure 2D). With the use of the DNA$^{(L)}$-H/H-DNA$^{(R)}$ combination, however, the scission was hardly site selective (lane 3 in Figure 2D). Only by using the additives bearing monophosphate groups was prompt and site-selective scission achievable.

Requisites for the Present Site-Selective Scission. The target site for the site-selective scission must be placed in gap structures and be differentiated from the other sites in terms of intrinsic reactivity. Accordingly, site-selective scission was unsuccessful when only one oligonucleotide additive bearing a monophosphate (without the second oligonucleotide additive) was used and no gap structure was formed in substrate DNA. There the single-stranded portion of the DNA substrate was hydrolyzed by Ce(IV)/EDTA without any remarkable selectivity. In the present site-selective DNA scission, the nucleobases in the substrates and the derivatized oligonucleotide additives take no critical roles. Thus, both the site selectivity and the scission efficiency were not very dependent on the sequence near the scission site (data not shown). This is certainly one of the advantages of the present method.

When oligonucleotides bearing only the linker groups (without monophosphate groups bound) were used as the additives, the DNA scission at the target site was never promoted (Figure 4, Supporting Information). The essential role of the monophosphate groups (and not of the linker residues) for the present site-selective scission has been further substantiated. These groups are necessary to recruit the Ce(IV) to the target site.[20] For efficient hydrolysis of small gaps in DNA substrates, the choice of linker is rather significant. For example, the scission at a 1-base gap by the DNA$^{(L)}$-L_{12}-P/P-L_{12}-DNA$^{(R)}$ combination was about 3 times as fast as that by the DNA$^{(L)}$-L_0-P/P-L_0-DNA$^{(R)}$ combination. However, the structure of the linker is less important for the scission at a 3-base gap and a 5-base gap (the rates of DNA scission by the oligonucleotide additives using various linkers are presented in Figure 5 in the Supporting Information).

To evaluate the stability of the derivatized oligonucleotide additives during the DNA hydrolysis, the terminal monophosphates of DNA$^{(L)}$-L_0-P and P-L_{12}-DNA$^{(R)}$ were labeled with ^{32}P. By using these additives, site-selective scission of DNA$^{(S5)}$ by Ce(IV)/EDTA was achieved as described above, and the reaction mixtures were analyzed by PAGE. As shown in Figure 2 in the Supporting Information, the intensities of the bands for the additives hardly changed during the course of reactions and no new bands appeared. Apparently, these terminal monophosphate groups were not removed from the additives. The phosphodiester linkages in these additives were not very hydrolyzed either. Similarly, the monophosphates at the 5′-end of DNA substrates, used for ^{32}P-labeling in Figures 2−4, remained in the substrates during the reactions. Thus, only the internal phosphodiester linkages at the target site in DNA substrates are efficiently hydrolyzed by Ce(IV)/EDTA, whereas all other phosphoesters

(20) When the salt $Ce(NH_4)_2(NO_3)_6$ alone (without EDTA) was directly added to the reaction mixtures, DNA scission was far slower than described in the text and occurred almost randomly throughout the DNA strand. Here, the Ce(IV) ions in the reaction mixtures promptly aggregate and form hydroxide gels before they are bound by the monophosphate groups in the oligonucleotide additives and placed at the target site. Thus, nonselective scission by the heterogeneous gels (ref 7d) predominantly proceeds.

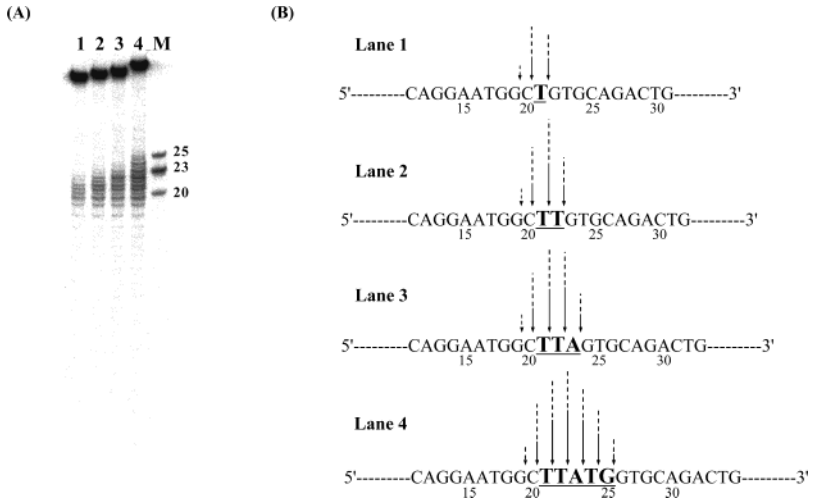

Figure 4. (A) Site-selective scission of gaps of different lengths by Ce(IV)/EDTA in the presence of $DNA^{(L)}$-L_{12}-P/P-L_{12}-$DNA^{(R)}$ at pH 7.0 and 37 °C. Lane 1, 1-base gap; lane 2, 2-base gap; lane 3, 3-base gap; lane 4, 5-base gap; M, the markers (authentic oligonucleotides having 3′-OH termini). These gaps were formed in $DNA^{(S1)}$-$DNA^{(S5)}$ using the $DNA^{(L)}$-L_{12}-P/P-L_{12}-$DNA^{(R)}$ combination. Reaction conditions: $[DNA^{(S)}]_0 = 1.0\ \mu M$, [each of the additive DNAs]$_0$ $= 2.0\ \mu M$, $[NaCl]_0 = 100$ mM, and [Ce(IV)/EDTA] = 1.0 mM at pH 7.0 (7.5 mM Hepes buffer) for 48 h. (B) Quantitative analysis of scission efficiencies (the solid part is for the formation of 3′-OH terminus, and the broken one is for the formation of 3′-phosphate terminus).

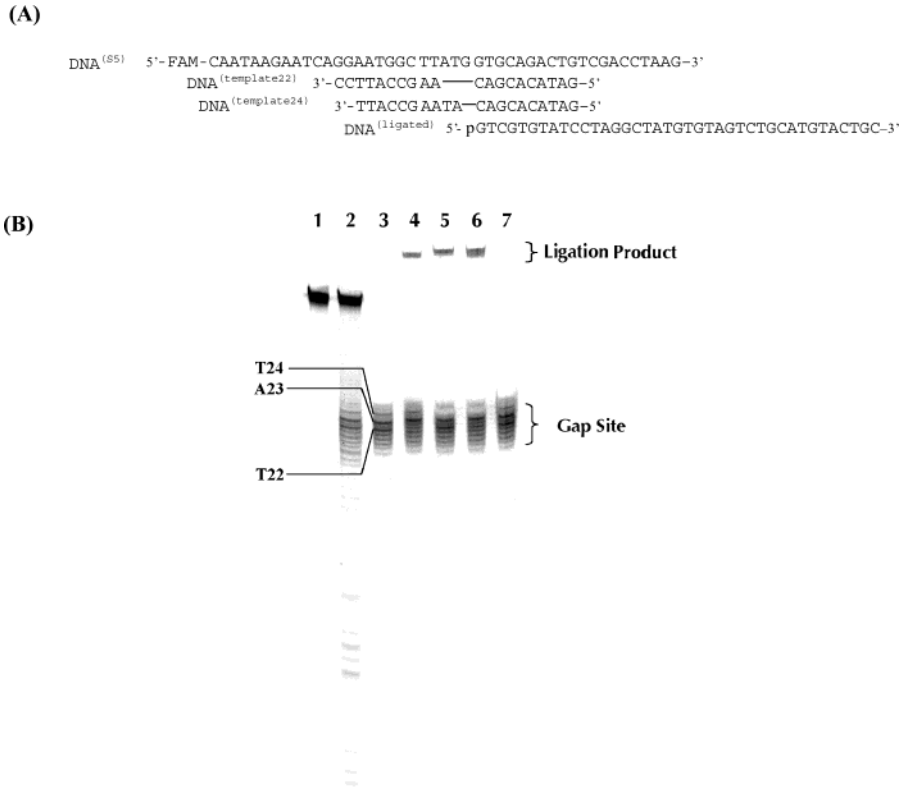

Figure 5. Ligation of the scission fragments by T4 DNA ligase in the presence of various templates. (A) Sequences of the oligonucleotides used. (B) Lane 1, $DNA^{(S5)}$ without treatment; lane 2, the product of site-selective scission of $DNA^{(S5)}$ by Ce(IV)/EDTA complex in the presence of $DNA^{(L)}$-L_0-P/P-L_0-$DNA^{(R)}$ combination; lane 3, the product in lane 2 purified by PAGE (the fragments ranging from C1 to T20-G26 were collected); lane 4, the ligation product in the presence of $DNA^{(template22)}$; lane 5, the ligation product in the presence of $DNA^{(template24)}$; lane 6, the ligation product in the presence of a 1:1 mixture of $DNA^{(template22)}$ and $DNA^{(template24)}$; lane 7, the product obtained by the ligation in the absence of templates. The ligation was carried at 16 °C for 30 min.

in the reaction mixtures (in both the substrates and the additives) are kept rather intact. Consistently, neither site selectivity nor scission efficiency of the present site-selective DNA scission was deteriorated even for prolonged reactions.

Enzymatic Ligation of Fragments Obtained by Site-Selective Scission. The scission fragments can be successfully connected with various oligonucleotides by using DNA ligase. In Figure 5, $DNA^{(S5)}$ (labeled by fluorescein at the 5′-end) was first hydrolyzed by Ce(IV)/EDTA in the presence of the $DNA^{(L)}$-L_0-P/P-L_0-$DNA^{(R)}$ combination (the reaction products were analyzed in lane 3). Then a 39-mer oligonucleotide having a phosphate at the 5′-end ($DNA^{(ligated)}$, arbitrary sequence) was added to the solution, and the mixture was treated with T4 DNA ligase (detailed procedures are presented in the Experimental Section). In lane 4, a 20-mer oligonucleotide $DNA^{(template22)}$ was used as the template. The 3′-side portion of this template is

complementary with G13-T22 of DNA$^{(S5)}$, and the remainder of the template is complementary with the 5′-side of DNA$^{(ligated)}$. After enzymatic treatment, a new band of smaller mobility appeared at the top of gel. The DNA sequencing experiment in Figure 6A in the Supporting Information showed that this new band is for the ligation product (61-mer) of the C1-T22 fragment, formed from DNA$^{(S5)}$ by the site-selective scission, and the DNA$^{(ligated)}$. Among the fragments in solution, only the C1-T22 fragment that is complementary with the template was connected with DNA$^{(ligated)}$. Consistently, the band for the C1-T22 fragment bearing 3′-OH terminus (designated as T22) became weaker on enzymatic reaction, while the intensities of other bands hardly changed (compare lane 3 with lane 4).

The DNA$^{(template24)}$ used in lane 5 is complementary with A15-T24 of DNA$^{(S5)}$ and the 5′-side of DNA$^{(ligated)}$. In its presence, treatment of the scission fragments by DNA ligase also produced a new band in the upper part of the gel. According to the sequencing experiment, this new band corresponds to the ligation product between the C1-T24 fragment and DNA$^{(ligated)}$ (see Figure 6B in the Supporting Information). This product (63-mer) is two bases longer than the one formed in lane 4 and thus has a smaller mobility. The band of the C1-T24 fragment (designated as T24) almost disappeared upon enzymatic treatment (see lane 5). This fragment is complementary with DNA$^{(template24)}$, so that it was picked up from the solution and ligated with DNA$^{(ligated)}$. As expected, two new oligonucleotides (the 61-mer and the 63-mer) were formed when both DNA$^{(template22)}$ and DNA$^{(template24)}$ were added as the templates (lane 6). In the absence of templates, no ligation products were formed (lane 7). These results substantiate that the present site-selective DNA scission by Ce(IV)/EDTA proceeds via hydrolysis of the phosphodiester linkages and is potent for various applications.[21]

Conclusion

When gap structures are formed at a predetermined site in substrate DNA by using monophosphate-bearing oligonucleotides as cofactors and these conjugates are treated with Ce(IV)/EDTA complex, the phosphodiester linkages at the gap site are selectively and efficiently hydrolyzed. The phosphodiester linkages at gap sites are intrinsically more susceptible to catalysis by the Ce(IV) complex, and the monophosphate groups attached to oligonucleotide additives recruit the Ce(IV) complex to these sites. Accordingly, the difference in intrinsic reactivity between the target site and the others is further magnified through the "proximity effect". Consistently, the present site-selective scission is much faster (e.g., >20-fold for 3-base gap) than the scission using unmodified oligonucleotide additives. The scission preferentially proceeds via P−O(3′) scission, forming 3′-OH termini and 5′-phosphate termini.

Furthermore, the resultant DNA fragments can be successfully connected with various oligonucleotides by using T4 DNA ligase. Only the scission fragment that is complementary with the template oligonucleotide used is connected with the counterpart oligonucleotide, and thus the desired recombinant DNA can be prepared by choosing the appropriate template. These results confirm the hydrolytic character of the present site-selective scission and also indicate the strong potential of this method for molecular biology.

Experimental Section

Materials. All phosphoramidite monomers were purchased from Glen Research Co. The oligonucleotides were prepared on an automated synthesizer, purified by usual methods, and completely characterized by MALDI-TOF MS. Water was deionized by a Millipore water purification system and sterilized by an autoclave immediately before use. Commercially obtainable $Ce(NH_4)_2(NO_3)_6$ (from NACALAI TESQUE) and EDTA·4Na (from Tokyo Kasei Kogyo) were used without further purification. According to back-titration with the $FeSO_4$/$Ce(NH_4)_4(SO_4)_4$ system,[7d] the purity of this $Ce(NH_4)_2(NO_3)_6$ agent was greater than 97%. Homogeneous Ce(IV)/EDTA complex was prepared immediately before use by mixing equimolar amounts of $Ce(NH_4)_2(NO_3)_6$ and EDTA (4Na salt) in Hepes buffer.

DNA Hydrolysis. The hydrolysis of DNA substrate (^{32}P-labeled at the 5′-end using kinase) was initiated by adding the solution of Ce(IV)/EDTA complex to the reaction mixtures and carried out at pH 7.0 (7.5 mM Hepes buffer) and 37 °C unless noted otherwise; $[DNA^{(S)}]_0 = 1.0$ and [each derivatized oligonucleotide additives]$_0 = 2.0$ μM, and $[NaCl]_0 = 100$ mM. After a predetermined time, the reactions were stopped by adding EDTA and inorganic phosphate to final concentrations of 10 and 70 mM, respectively. The reaction mixtures were then analyzed by denaturing 20% polyacrylamide gel electrophoresis, and the scission fragments were quantified with a Fuji Film FLA-3000G imaging analyzer. The DNA markers of the corresponding sequences were prepared by using a synthesizer and ^{32}P-labeled at the 5′-end. The method for kinetic analysis of the reactions is described in Figure 3 in the Supporting Information.

To analyze the structures of the 5′-termini of scission fragments, the DNA substrates were ^{32}P-labeled at the 3′-end by use of α-^{32}P ddATP (from Amersham) and terminal deoxynucleotidyl transferase (from Takara). The site-selective DNA scission of these DNAs was carried out exactly as described above. The marker DNAs having 5′-OH were chemically synthesized and ^{32}P-labeled at the 3′-end.

Ligation of Scission Fragments by DNA Ligase. The 39-mer oligonucleotide DNA$^{(ligated)}$ having 5′-terminal monophosphate and the template DNA were synthesized on an automated synthesizer. The DNA substrate DNA$^{(S5)}$ was labeled with fluorescein at the 5′-end and treated with Ce(IV)/EDTA in the presence of DNA$^{(L)}$-L_0-P/P-L_0-DNA$^{(R)}$ combination. The reaction conditions were the same as those described above, except for the reaction time (80 h) and the concentration of HEPES (2.5 mM). The reaction mixtures were subjected to PAGE, and the fragments ranging from C1 to T20-G26 were collected. The products were purified by ethanol precipitation and dissolved in water (the total concentration of DNA was estimated to be ca. 2.5 μM). To this solution, T4 DNA ligase and ligation buffer (both are from DNA Ligation Kit of Takara) as well as DNA$^{(ligated)}$ (17 μM) and DNA template (37.5 μM) were added. The enzymatic reaction was accomplished at 16 °C for 30 min, and the products were directly analyzed by PAGE.

The sequences of ligation products were determined on an ABI PRISM 310 Genetic Analyzer using the dye terminator labeling method. Prior to analysis, the products were amplified by PCR, inserted into pGEM(R)*-T Easy vector (Promega, Tokyo), and screened.

Acknowledgment. This work was partially supported by PROBRAIN. The support by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, Culture, and Technology, Japan, is also acknowledged.

Supporting Information Available: Analysis on the structures of 5′-end termini of scission fragments, stability of monophosphates in the reaction mixtures, determination of the rate constants of site-selective scission, introduction of only the linker

(21) According to the gel electrophoresis in Figure 5, the efficiency of ligation between the scission fragment (selected by the ligation template) and DNA$^{(ligated)}$ was 80−90%.

L_{12} to the oligonucleotide additives, effect of linker on the site-selective scission, and sequencing experiments of ligation products. This material is available free of charge via the Internet at http://pubs.acs.org. See any current masthead page for ordering information and Web access instructions.

JA048953A