Oligonucleotide Bearing Ethylenediamine-*N,N,N′*-Triacetates for Gap-Selective DNA Hydrolysis by Ce^{4+}/EDTA

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With the use of two oligonucleotides bearing ethylenediamine-N,N,N′-triacetate groups as additives, gap sites were formed at predetermined sites in substrate DNA. Upon treating these systems with a Ce^{4+}/EDTA complex at pH 7.0 and 37 °C, the phosphodiester linkages at the gap site were selectively hydrolyzed. The DNA scission was greatly promoted by the introduction of ethylenediaminetriacetate groups, and the scission efficiency increased as the number of these groups increased. Even a one-base gap was successfully hydrolyzed when three ethylenediaminetriacetate groups were placed consecutively at both edges of the gap, although the scission was minimal in the absence of these groups. The site-selective scission could be also achieved at higher temperatures without any significant loss of site-selectivity.

Introduction

Preparation of man-made restriction enzymes is attracting interest, mainly because their high site-specificity should be useful for manipulating the huge DNAs of higher animals and higher plants.[1–7] Several catalysts for DNA hydrolysis have already been reported.[8–24] The activities of the Ce^{4+} ion and its complexes are especially remarkable.[8–19] Furthermore, these catalysts were covalently attached to oligonucleotides that are bound to substrate DNA near the target site and serve as sequence-recognizing moieties.[25,26] However, both site selectivity and scission efficiency, dictated simply by "proximity-effect", were not satisfactorily high, and a new strategy would be required to fulfill both of these requirements.

Recently, it has been shown that the phosphodiester linkages in gap sites are preferentially hydrolyzed by a Ce^{4+}/EDTA complex (EDTA = ethylenediamine-*N,N,N′,N′*-tetraacetate).[27–30] Even though the complex is not covalently bound to any sequence-recognizing moiety, the DNA scission selectively occurs at the gap sites, since the linkages therein are more susceptible to catalysis by the Ce^{4+} complex than are those in double-stranded portions.[31] Furthermore, this gap-selective DNA hydrolysis was greatly promoted by introducing monophosphate groups at the gap sites and recruiting the Ce^{4+} complex to the gap site.[32] In this paper, we introduce ethylenediamine-*N,N,N′*-triacetate groups to gap sites and promote gap-selective DNA scission by the Ce^{4+}/EDTA complex. The effects of gap size and number of ethylenediaminetriacetate groups on site selectivity and reaction rate are systematically studied. Furthermore, the reaction mechanism is proposed on the basis of the results obtained with a fluorescent probe.

Results and Discussion

Promotion of gap-selective DNA scission by introducing two ethylenediamine-*N,N,N′*-triacetate groups to the gap site

With the use of the oligonucleotides in Figure 1, gap structures were formed at predetermined positions in DNA substrates. As can be seen from lane 4 of Figure 2, when $DNA^{(L)}$-X_1 and $DNA^{(R)}$-X_1 were combined as the additives, a five-base gap was formed in the middle of 45-mer substrate $DNA^{(S5)}$. The $DNA^{(L)}$-X_1 is complementary to the 5′-side of $DNA^{(S5)}$ (G1–A20), whereas $DNA^{(R)}$-X_1 is complementary to A26–C45 of $DNA^{(S5)}$. These two oligonucleotides bear ethylenediamine-*N,N,N′*-triacetate groups on the X residues at the 5′ and the 3′ termini, respectively. Note that X is a thymidine derivative and forms a Watson–Crick base pair with adenosine in $DNA^{(S5)}$. As the result, the five-base gap ranged from T21 to T25, and two ethylenediamine-*N,N,N′*-triacetate groups were placed at the 5′ and 3′ edges of this gap. Under these conditions, the scission of $DNA^{(S5)}$ (^{32}P-labelled at the 5′ end) by the Ce^{4+}/EDTA complex (0.5 mM) at pH 7.0 and 37 °C preferentially occurred in the gap region (the two markers in the gel are 21- and 25-mers of the corresponding sequences). With two unmodified oligonucleotides combined ($DNA^{(L)}$-X_0/$DNA^{(R)}$-X_0), however, the scission was only marginal under the same conditions (lane 3). According to quantitative analysis, the scission by $DNA^{(L)}$-X_1/$DNA^{(R)}$-X_1

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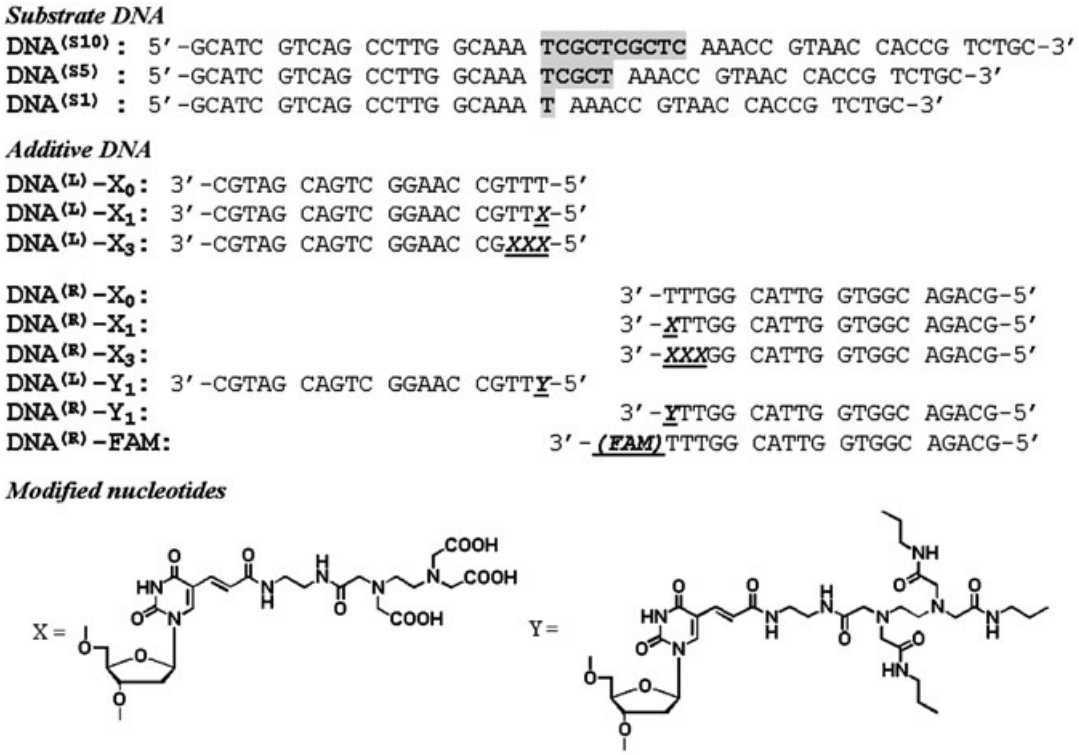

Figure 1. *Sequences of substrate and additive DNAs. The nucleotides at the gap site are indicated by shadowed rectangles. The structures of modified nucleotides X and Y are also shown.*

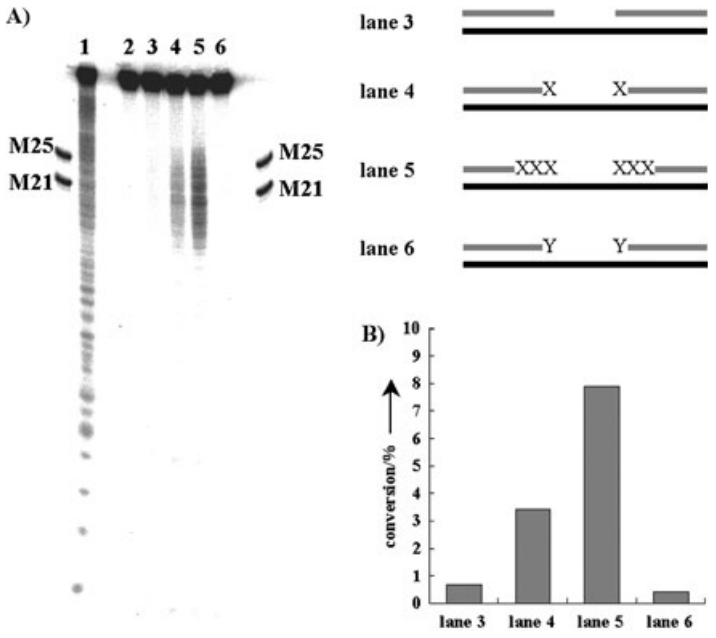

Figure 2. *Gel-electrophoresis patterns for the hydrolysis of DNA$^{(S5)}$ (^{32}P-labelled at the 5′ end) at five-base gap by combining the Ce^{4+}/EDTA complex with various modified oligonucleotides. A) Lane 1: Ce^{4+}/EDTA complex only; lane 2: control without Ce^{4+}/EDTA; lane 3: DNA$^{(L)}$-X_0/DNA$^{(R)}$-X_0; lane 4: DNA$^{(L)}$-X_1/DNA$^{(R)}$-X_1; lane 5: DNA$^{(L)}$-X_3/DNA$^{(R)}$-X_3; lane 6: DNA$^{(L)}$-Y_1/DNA$^{(R)}$-Y_1. The five-base gap is formed between the two markers. The structures in lanes 3–6 are depicted schematically on the right-hand side. B) Percentage conversion for the scission between T21 and T25. Reaction conditions: [DNA$^{(S5)}$]$_0$ = 1.0 μM, [each of the additive DNAs]$_0$ = 1.5 μM, [NaCl]$_0$ = 100 mM, and [Ce^{4+}/EDTA complex] = 0.5 mM at pH 7.0 (10 mM Hepes buffer); 37°C for 18 h.*

was more than fivefold faster than that by DNA$^{(L)}$-X_0/DNA$^{(R)}$-X_0 (the conversions for the DNA scission between T21 and T25 are presented in Figure 2B). By using a similar two-ethylenediaminetriacetate strategy, a ten-base gap was also selectively hydrolyzed (lane 5 in Figure 3A; the gap site is between T21 and C30 in DNA$^{(S10)}$). About 15% of DNA$^{(S10)}$ was hydrolyzed at the gap site (see Figure 3B). Without introduction of these groups, however, the scission was less efficient (lane 2). Thus, the replacement of conventional T nucleotides in DNA$^{(L)}$-X_0 and DNA$^{(R)}$-X_0 with X residues drastically promotes selective scission at the gap site. The crucial roles of the ethylenediamine-*N,N,N′*-triacetate groups are evident.

The scission at the one-base gap in DNA$^{(S1)}$ was also promoted by this strategy, although the scission was slow (lane 7 in Figure 3). With the use of unmodified oligonucleotides as the additives, however, DNA scission was almost completely nil (lane 4). Thus, the promotion effects by ethylenediamine-*N,N,N′*-triacetate groups are also evident here. Even when only one of the two oligonucleotide additives bore an ethylenediamine-*N,N,N′*-triacetate group at the terminus and the other additive was an unmodified oligonucleotide, the gap-selective scission was promoted to some extent (DNA$^{(L)}$-X_0/DNA$^{(R)}$-X_1 and DNA$^{(L)}$-X_1/DNA$^{(R)}$-X_0 combinations). However, the two-ethylenediaminetriacetate system (e.g., DNA$^{(L)}$-X_1/

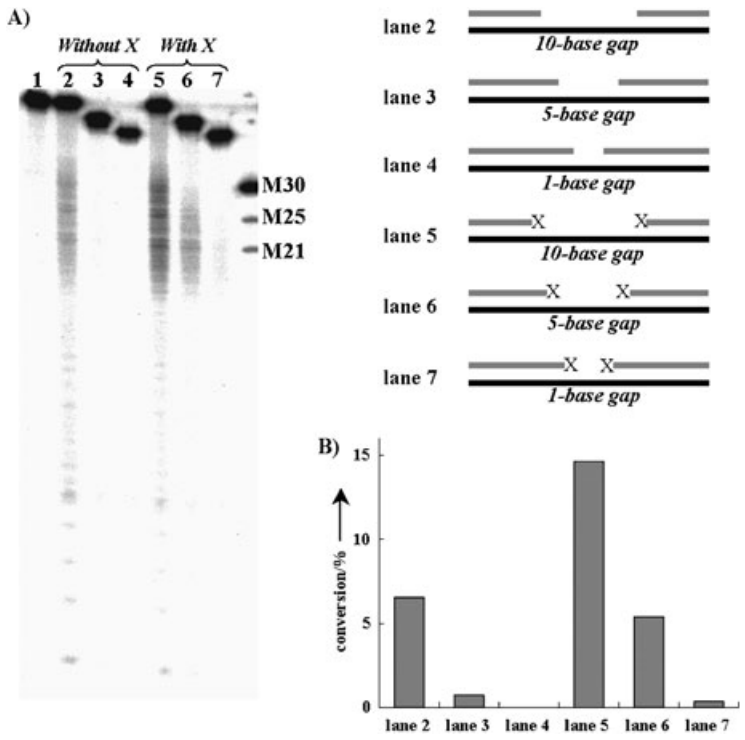

Figure 3. *Effects of gap length on hydrolysis by the Ce^{4+}/EDTA complex. Lane 1: control ($DNA^{(S10)}/DNA^{(L)}$-$X_0/DNA^{(R)}$-X_0 in the absence of the Ce^{4+} complex); lane 2: ten-base gap without X ($DNA^{(S10)}/DNA^{(L)}$-$X_0/DNA^{(R)}$-X_0); lane 3: five-base gap without X ($DNA^{(S5)}/DNA^{(L)}$-$X_0/DNA^{(R)}$-X_0); lane 4: one-base gap without X ($DNA^{(S1)}/DNA^{(L)}$-$X_0/DNA^{(R)}$-X_0); lane 5: ten-base gap with two X groups ($DNA^{(S10)}/DNA^{(L)}$-$X_1/DNA^{(R)}$-X_1); lane 6: five-base gap with two X groups ($DNA^{(S5)}/DNA^{(L)}$-$X_1/DNA^{(R)}$-X_1); lane 7: one-base gap with two X groups ($DNA^{(S1)}/DNA^{(L)}$-$X_1/DNA^{(R)}$-X_1). Reaction conditions: pH 7.0 and 37°C for 70 h. B) Percentage conversion for the scission in the corresponding gap region.*

$DNA^{(R)}$-X_1 combination) is far better with respect to both site selectivity and scission rate.

DNA scission by Ce^{4+}/EDTA proceeds completely by hydrolysis of the phosphodiester linkages, as confirmed by previous HPLC analysis that showed that the only reaction products are oligonucleotides, nucleotides, and nucleosides.[16] No release of nucleobases from the ribose was detected, although they would be formed if the reaction were proceeding by oxidative scission of ribose residues. The hydrolytic character of the scission by Ce^{4+} ion (without EDTA) was also confirmed by HPLC analysis.[13] Furthermore, the scission fragments were susceptible to various enzymatic reactions (alkaline phosphatase, polynucleotide kinase, and terminal deoxynucleotidyl transferase) and were transformed to the expected forms.[33] Attempts to separate the scission fragments in the electrophoresis gels more explicitly have been unsuccessful, partially because the products are mixtures of fragments bearing 3′-OH termini or 3′-phosphate termini.

Introduction of more than two ethylenediamine-*N,N,N′*-triacetate group to the gap site

The gap-selective scission was still more efficient when $DNA^{(L)}$-X_3 was combined with $DNA^{(R)}$-X_3 and three ethylenediamine-*N,N,N′*-triacetate groups were placed at each edge of the five-base gap (lane 5 in Figure 2A). This six-ethylenediaminetriacetate system was more than ten times more active for DNA scission than was the $DNA^{(L)}$-X_0/$DNA^{(R)}$-X_0 combination (see Figure 2B). The scission-efficiency of this system is comparable with that of DNA scission accomplished by using monophosphate-bearing oligonucleotides under the same conditions.[32] It is noteworthy that even one-base gaps were efficiently hydrolyzed when three ethylenediamine-*N,N,N′*-triacetate groups were bound consecutively to the termini of the additives and six ethylenediaminetriacetate groups were introduced to the gap site (see Figure 4). The scission was also successful when two ethylenediaminetriacetate groups were consecutively bound to each of the additives. Without the introduction of the ethylenediamine-*N,N,N′*-triacetate groups, however, the scission of the one-base gap by Ce^{4+}/EDTA was almost nil. This result is completely consistent with the previous finding.[27] The significant roles of ethylenediamine-*N,N,N′*-triacetate groups for gap-selective scission have been vividly substantiated again. Some of the present DNA scissions by Ce^{4+}/EDTA occurred at the linkages outside the gap region. They are probably associated with the breathing motion near the edges of the gap. Suppression of this breathing motion by chemical modification of oligonucleotide additives (e.g., attachment of intercalator) is being attempted.

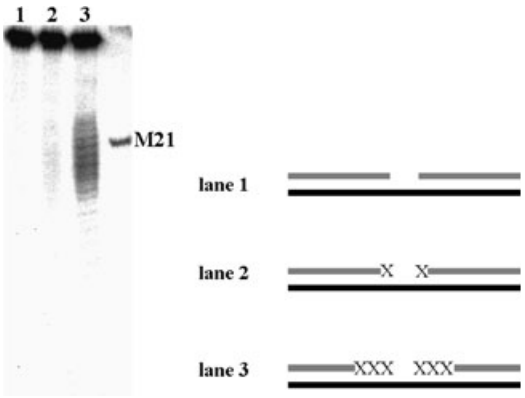

Figure 4. *Gel-electrophoresis patterns for the hydrolysis of $DNA^{(S1)}$ at a one-base gap. Lane 1: $DNA^{(L)}$-$X_0/DNA^{(R)}$-X_0; lane 2: $DNA^{(L)}$-$X_1/DNA^{(R)}$-X_1; lane 3: $DNA^{(L)}$-$X_3/DNA^{(R)}$-X_3. The gap position is shown by the marker (21-mer). The reaction conditions are the same as described in Figure 3.*

Site-selective DNA scission at higher temperatures

When necessary, to accelerate the reactions, the site-selective scissions could be achieved at higher temperatures. The temperature dependency of the reaction rate was significant. For example, gap-selective hydrolysis by the $DNA^{(L)}$-X_1/$DNA^{(R)}$-X_1 combination was accelerated fivefold when the reaction temperature was increased from 37°C to 47°C. The site-selectivity was kept satisfactorily high. The temperature can be further elevated as long as it is lower than the melting temperatures of duplexes between the substrate DNA and the DNA additives.

Requisites for the site-selective scission of DNA

In the present gap-selective scissions, both the site-selectivity and the scission-rate were hardly dependent on the sequences in either the gap region or the double-stranded portion. Accordingly, the scission site could be freely chosen.

However, DNA scission was only marginal when all the nucleotides in the substrate DNA formed Watson–Crick base pairs with the two oligonucleotide additives. Even when ethylenediaminetriacetate groups were introduced to these nick-sites, DNA scission was inefficient. Thus, gap structures are essential for the present site-selective DNA scission. When either $DNA^{(R)}$-X_1 or $DNA^{(L)}$-X_1 was used alone as the additive (without another oligonucleotide additive), no selective scission took place. Instead, the single-stranded portion in the substrate DNA was hydrolyzed almost randomly, whereas the double-stranded portion was kept intact.

Recruitment of Ce^{4+}/EDTA by the ethylenediamine-*N,N,N′*-triacetate groups

In order to shed light on the roles of the ethylenediamine-*N,N,N′*-triacetate groups in the present gap-selective hydrolysis, a fluorescein was introduced to a one-base gap by using $DNA^{(S1)}$/$DNA^{(R)}$-FAM/$DNA^{(L)}$-X_n systems (see Figure 5A). Even when $DNA^{(L)}$-X_0 was used as the additive, the fluorescence intensity was decreased about by 40% upon addition of the Ce^{4+}/EDTA complex (see Figure 5B). Here, the Ce^{4+} complex is bound near to the fluorescein due to the interactions with the phosphodiester linkages of $DNA^{(S1)}$, and quenches the fluorescence from the fluorescein. Significantly, quenching of the fluorescence was more efficient when $DNA^{(L)}$-X_1 was used in place of $DNA^{(L)}$-X_0 and one ethylenediaminetriacetate group was introduced to the gap site. With three ethylenediaminetriacetate groups ($DNA^{(L)}$-X_3), the quenching by Ce^{4+}/EDTA was still more efficient, and more than 80% of the fluorescence was quenched. Apparently, the Ce^{4+} complex is accumulated near the gap site by the ethylenediaminetriacetates of the X residues. It is noteworthy that the order in the efficiency of DNA scission ($X_3 > X_1 > X_0$) is exactly the same as that in the quenching efficiency (compare Figure 5B with C). Both analyses were made under the same conditions ([Ce^{4+}/EDTA] = 0.5 mM at pH 7.0 and 37 °C).

These arguments are concretely supported by the fact that the fluorescence from fluorescein is hardly quenched by Ce^{4+}/EDTA when the fluorescein is not conjugated to DNA oligomers (see Figure S1 in the Supporting Information). On the other hand, efficient quenching is accomplished by FAM-$EDTA_2$, which bears two ethylenediamine-*N,N,N′,N′*-tetraacetate groups. In order to quench the fluorescence efficiently, the Ce^{4+}/EDTA must be concentrated near the fluorescein. Consistently, the attachment of two glycine groups to fluorescein (FAM-Gly_2), in place of two EDTA groups, hardly affects the quenching efficiency, as also shown in Figure S1.

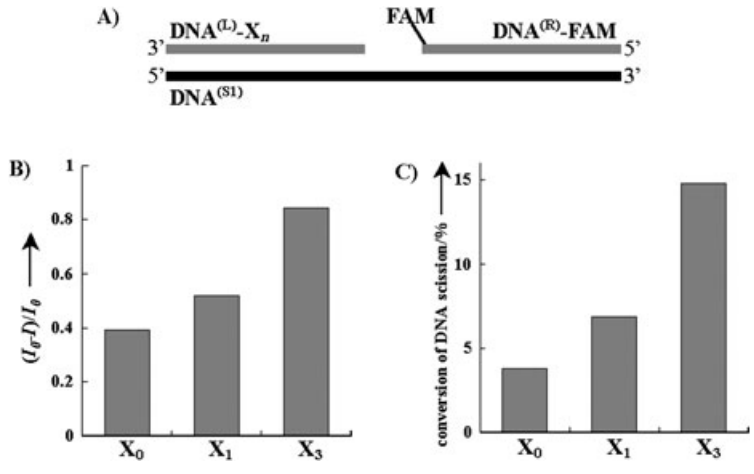

Figure 5. *A) Attachment of FAM to a one-base gap as a fluorescent probe for the interactions between Ce^{4+}/EDTA and X residues. B) The changes in fluorescence intensity upon the addition of 0.5 mM Ce^{4+}/EDTA at pH 7.0 and 37 °C. I_0 and I are the fluorescence intensity in the absence and the presence of the complex, respectively. C) The efficiency of DNA scission by Ce^{4+}/EDTA at the gap site in the systems used for the fluorescent analysis (reaction time = 63 h).*

Proposed mechanism

The phosphodiester linkages in the gap region are intrinsically more susceptible to hydrolysis by the Ce^{4+}/EDTA complex than are those in double-stranded portions.[27] In the present strategy, the catalyst for DNA hydrolysis is accumulated in the vicinity of these linkages by the ethylenediamine-*N,N,N′*-triacetate groups, as clearly evidenced by the fluorescence-quenching experiments described above. As the result, the linkages in the gap site are still more preferentially hydrolyzed, and the difference in intrinsic reactivity (the gap site ≫ the others) has been further magnified. The structural flexibility of gap sites is favorable for this catalysis. It is also plausible that the conformation of the DNA substrate is altered upon its binding to the additives, and this factor contributes to the gap-selective DNA scission. These arguments are supported by the result in lane 6 of Figure 2, in which all the carboxylate groups in the X residues of $DNA^{(L)}$-X_1 and $DNA^{(R)}$-X_1 were converted to their amides (Y residues in Figure 1). With the combination of these modified oligonucleotides as the additives, gap-selective scission by Ce^{4+}/EDTA was hardly detectable. The carboxylate residues in ethylenediamine-*N,N,N′*-triacetate groups are necessary for the present site-selective scission.

Conclusion

By using two oligonucleotide additives that bear ethylenediamine-*N,N,N′*-triacetate groups at their termini, DNA has been selectively hydrolyzed by a Ce^{4+}/EDTA complex at a gap site. With an increasing number of ethylenediaminetriacetate groups introduced, the scission activity increases, since the Ce^{4+}/EDTA complex is more efficiently recruited to the gap site. When three ethylenediaminetriacetate groups are introduced, the scission efficiency is comparable with that accomplished by using monophosphate-bearing oligonucleotides.[32] It is expected that

the scission efficiency can be further improved by more precise design of the linker that connects the ethylenediamine-*N,N,N′*-triacetate groups to the oligonucleotides. The positions of these groups in the oligonucleotide additives should be also optimized. These attempts are currently under way in our laboratory.

Experimental Section

Materials: The phosphoramidite monomer for the triethyl ester of X was purchased from Glen Research Co (Virginia, USA). The oligonucleotides, prepared on an automated synthesizer, were treated with aqueous NaOH solution (or propylamine), and the triethyl esters in them were converted to X (or Y). All the oligonucleotides were characterized by MALDI-TOF MS. Water was deionized by the Millipore water-purification system and sterilized in an autoclave immediately before use. Commercially obtainable $Ce(NH_4)_2(NO_3)_6$ (from Nakalai Tesque, Inc) and EDTA·4Na (from Tokyo Kasei Kogyo Co., Ltd) were used without further purification. Homogeneous Ce^{4+}/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.

The CPG column for introduction of fluorescein to the 3′ terminus of the oligonucleotide additives was obtained from Glen Research Co. FAM-$EDTA_2$ and FAM-Gly_2 used to produce Figure S1 were synthesized according to Scheme S1 (see Supporting Information). The amino acid derivatives were prepared on NovaSynTGR resin (Novabiochem) by using Fmoc-protected EDTA monomer (synthesized according to Scheme S1 A) or Fmoc-protected Gly monomer, and then fluorescein was attached to their N termini by treating the resin with 5(6)-carboxyfluorescein *N*-succinimidyl ester (see Scheme S1 B). After deprotection, the products were removed from the resin, purified by RP-HPLC, and characterized by MALDI-TOF MS.

MALDI-TOF MS data for newly synthesized oligonucleotides and fluorescent probes. $DNA^{(L)}$-Y_1 calcd: 6626.5 $[M+H]^+$, found 6627.7; $DNA^{(R)}$-Y_1 calcd: 6697.5 $[M+H]^+$, found 6697.7; FAM-$EDTA_2$ calcd: 1268.4 $[M+H]^+$, found 1269.1; FAM-Gly_2 calcd: 490.1 $[M+H]^+$, found 489.2.

DNA hydrolysis: The hydrolysis of DNA substrate (^{32}P-labelled at the 5′ end) was initiated by adding the solution of Ce^{4+}/EDTA complex to reaction mixtures, and carried out at pH 7.0 (10 mM Hepes buffer) and 37 °C unless noted otherwise; $[DNA^{(S)}]_0 = 1.0\ \mu M$, [each of oligonucleotide additives]$_0 = 1.5\ \mu M$, and $[NaCl]_0 = 100$ mM. After a predetermined time, the reactions were stopped by adding water containing EDTA (10 mM) and inorganic phosphate (70 mM, in 1/2 volume of the reaction mixture). 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.

Spectroscopy: Fluorescence spectra were measured on an FP-750 spectrometer (JASCO). The conditions (pH 7.0 and 37 °C) were exactly the same as those employed for the DNA scission: $[DNA^{(S1)}] = 1.2\ \mu M$, $[DNA^{(R)}\text{-FAM}] = 1.0\ \mu M$, $[DNA^{(L)}\ \text{additive}] = 1.5\ \mu M$, [Hepes] = 10 mM, [NaCl] = 100 mM, and $[Ce^{4+}/EDTA] = 0.5$ mM, $\lambda_{ex} = 470$ nm and $\lambda_{em} = 520$ nm.

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