J|A|C|S ARTICLES

Published on Web 02/11/2009

Human Telomeric DNA Sequence-Specific Cleaving by G-Quadruplex Formation

Yan Xu,* Yuta Suzuki, Tuomas Lönnberg, and Makoto Komiyama*

Research Center for Advanced Science and Technology, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8904, Japan

Received September 16, 2008; E-mail: xuyan@mkomi.rcast.u-tokyo.ac.jp; komiyama@mkomi.rcast.u-tokyo.ac.jp

Abstract: Telomere is an emerging target for the treatment of human cancers. Here, we report a structure-based approach to sequence-specific cleaving of human telomeric DNA by G-quadruplex formation. Oligonucleotide with multiphosphonate [DNA−EDTP·Ce(IV)] at the 5′ end binds to human telomere DNA by G-quadruplex formation and causes a sequence-specific strand break. These results provide the first proof of concept for targeting the human telomere DNA based on G-quadruplex formation, and this may serve as a starting point for the design of more efficient telomere sequence-specific cleaving reagents by G-quadruplex formation.

Introduction

Human telomeric DNA consists of a duplex region composed of TTAGGG repeats, ending in a 100−200 nt G-rich single-stranded overhang.[1−6] Telomeric overhang has been implicated as a critical component of telomere structures that is required for telomere end protection.[1,7−9] The telomeric overhang DNA is also a substrate for telomerase, which elongates telomeric sequence by adding G-rich repeats.[1,9,10] Telomerase is activated in 80−90% of human tumors and is low or undetectable in most normal somatic cells.[11] Thus, telomerase or its telomere DNA substrate presents a target with good selectivity for tumor over healthy tissue.[12−27]

Developing various approaches to targeting human telomeric DNA is an area of great interest due to its potential role in discovering anticancer agents.[12−27] A promising method has been to focus on the disruption of the telomere−telomerase interaction via targeting the telomere DNA substrate, thereby preventing telomere elongation by telomerase.[28,29] Human telomeric DNA has been identified to fold into four-stranded G-quadruplex structures.[30,31] Recently, we and others reported a new topology containing the (3 + 1) G-tetrad core for human telomeric G-quadruplex based on CD and NMR studies.[32−35] Patel et al. showed that a dimeric G-quadruplex with the same topology was formed by the three-repeat and single-repeat human telomeric sequences (Figure 1a).[36] This dimeric G-quadruplex structure suggests a way of how a segment of three

(1) Blackburm, E. H. *Cell* **2001**, *106*, 661–673.
(2) Stewart, S. A.; Ben-Porath, I.; Carey, V. J.; O'Connor, B. F.; Hahn, W. C.; Weinberg, R. A. *Nat. Genet.* **2003**, *33*, 492–496.
(3) Cech, T. R. *Cell* **2004**, *116*, 273–279.
(4) Blasco, M. A. *Nat. Rev. Genet.* **2005**, *6*, 611–622.
(5) Karlseder, J.; Verdun, R. E. *Nature* **2007**, *447*, 924–931.
(6) de Lange, T. *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 323–329.
(7) Bhattacharyya, M. K.; Lustig, A. J. *Trends Biochem. Sci.* **2006**, *31*, 114–122.
(8) Lansdorp, P. M. *Trends Biochem. Sci.* **2005**, *30*, 388–395.
(9) Collins, K.; Mitchell, J. R. *Oncogene* **2002**, *21*, 564–579.
(10) Alves, D.; Li, H.; Codrington, R.; Orte, A.; Ren, X.; Klenerman, D.; Balasubramanian, S. *Nat. Chem. Biol.* **2008**, *4*, 287–289.
(11) Kim, N. W.; Piatyszek, M. A.; Prowse, K. R.; Harley, C. B.; West, M. D.; Ho, P. L.; Coviello, G. M.; Wright, W. E.; Weinrich, S. L.; Shay, J. W. *Science* **1994**, *266*, 2011–2015.
(12) Hahn, W. C.; Stewart, S. A.; Brooks, M. W.; York, S. G.; Eaton, E.; Kurachi, A.; Beijersbergen, R. L.; Knoll, J. H.; Meyerson, M.; Weinberg, R. A. *Nat. Med.* **1999**, *5*, 1164–1170.
(13) Neidle, S.; Parkinson, G. *Nat. Drug Discovery* **2002**, *1*, 383–393.
(14) Hurley, L. H. *Nat. Rev. Cancer* **2002**, *2*, 188–200.
(15) Shay, J. W.; Wright, W. E. *Nat. Rev. Drug Discovery* **2006**, *5*, 577–584.
(16) Mergny, J.-L.; Riou, J.-F.; Mailliet, P.; Teulade-Fichou, M. P.; Gilson, E. *Nucleic Acids Res.* **2002**, *30*, 839–865.
(17) De Cian, A.; Cristofari, G.; Reichenbach, P.; De Lemos, E.; Monchaud, D.; Teulade-Fichou, M. P.; Shin-ya, K.; Lacroix, L.; Lingner, J.; Mergny, J.-L. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 17347–17352.
(18) Perry, P. J.; Jenkins, T. C. *Mini. Rev. Med. Chem.* **2001**, *1*, 31–41.
(19) Riou, J. F. *Curr. Med. Chem. Anti-Cancer Agents* **2004**, *4*, 439–443.
(20) Rezler, E. M.; Bearss, D. J.; Hurley, L. H. *Curr. Opin. Pharmacol.* **2002**, *2*, 415–423.
(21) Shay, J. W.; Zou, Y.; Hiyama, E.; Wright, W. E. *Hum. Mol. Genet.* **2001**, *10*, 677–685.
(22) Raymond, E.; Sun, D.; Chen, S. F.; Windle, B.; Von Hoff, D. D. *Curr. Opin. Biotechnol.* **1996**, *7*, 583–591.
(23) Pendino, F.; Tarkanyi, I.; Dudognon, C.; Hillion, J.; Lanotte, M.; Aradi, J.; Segal-Bendirdjian, E. *Curr. Cancer Drug Targets* **2006**, *6*, 147–180.
(24) Cairns, D.; Anderson, R. J.; Perry, P. J.; Jenkins, T. C. *Curr. Pharm. Des.* **2002**, *8*, 2491–2504.
(25) Huard, S.; Autexier, C. *Curr. Med. Chem. Anticancer Agents* **2002**, *2*, 577–587.
(26) Rezler, E. M.; Bearss, D. J. *Annu. Rev. Pharmacol. Toxicol.* **2003**, *43*, 359–379.
(27) Rodriguez, R.; Muller, S.; Yeoman, J. A.; Trentesaux, C.; Riou, J.-F.; Balasubramanian, S. *J. Am. Chem. Soc.* **2006**, *130*, 15758–15759.
(28) Jantos, K.; Rodriguez, R.; Ladame, S.; Shirude, P. S.; Balasubramanian, S. *J. Am. Chem. Soc.* **2006**, *128*, 13662–13663.
(29) De Cian, A.; DeLemos, E.; Mergny, J.-L.; Teulade-Fichou, M.-P.; Monchaud, D. *J. Am. Chem. Soc.* **2007**, *129*, 1856–1857.
(30) Wang, Y.; Patel, D. J. *Structure* **1993**, *1*, 263–282.
(31) Parkinson, G. N.; Lee, M. P.; Neidle, S. *Nature* **2002**, *417*, 876–880.
(32) Xu, Y.; Noguchi, Y.; Sugiyama, H. *Bioorg. Med. Chem.* **2006**, *14*, 5584–5591.
(33) Matsugami, A.; Xu, Y.; Noguchi, Y.; Sugiyama, H.; Katahira, M. *FEBS J.* **2007**, *274*, 3545–3556.
(34) Luu, K. N.; Phan, A. T.; Kuryavyi, V.; Lacroix, L.; Patel, D. J. *J. Am. Chem. Soc.* **2006**, *128*, 9963–9970.
(35) Ambrus, A.; Chen, D.; Dai, J. X.; Bialis, T.; Jones, R. A.; Yang, D. Z. *Nucleic Acids Res.* **2006**, *34*, 2723–2735.

10.1021/ja807313x CCC: $40.75 © 2009 American Chemical Society

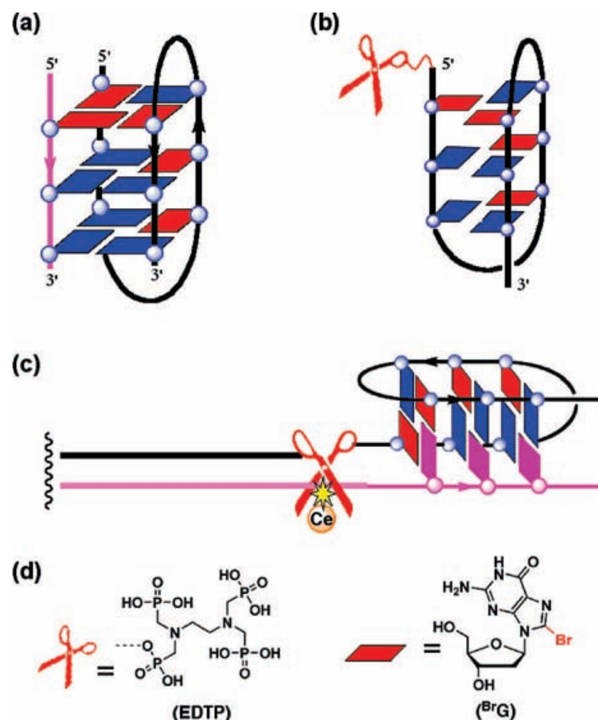

Figure 1. (a) Schematic structure of the (3 + 1) core dimeric G-quadruplex formed by a three-repeat (three G-tracts) and a single-repeat (one G-tract) human telomeric sequence. (b) Three G-tracts oligonucleotide equipped with EDTP at 5′ end. The red boxes showed the substitutions of dG in the syn conformation with 8-bromoguanosine (^{Br}G) in the DNA−EDTP probe, EDTP-5′-^{Br}GGGTTA^{Br}G^{Br}GGTTAGGGT-3. (c) Human telomere sequence-specific cleavage by G-quadruplex-forming DNA−EDTP·Ce(IV) probe complex. (d) The structures of EDTP and ^{Br}G are shown.

G-tracts could bind to a remote G-tract. These pioneering works led us to develop a novel approach toward inhibiting telomerase by targeting its telomere DNA substrate. Here, we report a structure-based approach to sequence-specific cleaving of human telomeric DNA by G-quadruplex formation.

Results and Discussion

Our strategy involves targeting the human telomeric DNA sequence by forming G-quadruplex between three G-tracts probe and target telomeric DNA. A *N*,*N*,*N*′,*N*′- ethylenediaminetetramethylenephosphonic acid (EDTP) as metal binding group was conjugated to the three G-tracts oligonucleotide to yield a strand break at target sequence (Figure 1b,c). Because multiphosphonate-type ligands (EDTP) exhibit high affinity toward lanthanide ions, oligonucleotides bearing this kind of terminal group were efficiently recruiting the catalytic Ce(IV) species to the target site and resulting in an efficient cleavage (Figure 1c).[37−40] The DNA−EDTP probe was prepared by oxime coupling to link the EDTP group to the 5′ end of the oligonucleotide (Supporting Information). Formation of a stable G-quadruplex between the DNA−EDTP probe and the target sequence is a key step in the cleavage reaction. For this purpose,

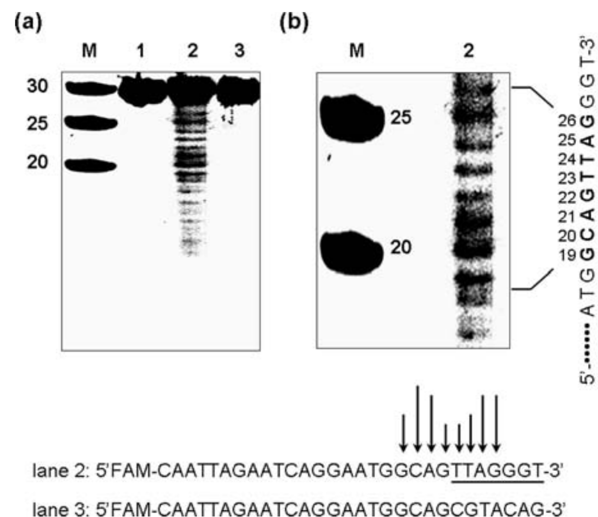

Figure 2. (a) Denaturing polyacrylamide gel electrophoresis (15% PAGE) showing the sequence-specific cleavage of human telomere by G-quadruplex formation. (Lane 1) Control without DNA−EDTP probe with Ce(IV)/EDTA; (lane 2) 5′-end FAM-labeled 29 mer DNA containing a 7 mer human telomere sequence at 3′-end with underline, with both DNA−EDTP probe and Ce(IV)/EDTA; (lane 3) control 5′-end FAM-labeled 29 mer DNA that does not contain telomere sequence, with both DNA−EDTP probe and Ce(IV)/EDTA; (lane M) marker oligonucleotides (the number indicated marker size). Ce(IV)/EDTA = 10 μM, t = 50 h, T = 50 °C, 10 mM HEPES (pH 7.0), [KCl] = 200 mM, [target] = 1 μM, [DNA−EDTP] = 2 μM. (b) Enlargement of cleavage sites of lane 2 and histograms of the DNA cleavage patterns. The numbers indicate the cleavage sites. The heights of the arrows represent the relative cleavage intensities at the indicated bases.

substitutions of dG in the syn conformation with a syn-preferring 8-bromoguanosine (^{Br}G) in the DNA−EDTP probe were performed to form a highly stable G-quadruplex structure between the probe and target sequence (Figure 1). In previous studies, we have demonstrated that the proper substitutions of dG with ^{Br}Gs can stabilize, or "freeze", the G-quadruplex structures.[32,33,41] The CD spectrum of a mixture of the DNA−EDTP probe and a telomere DNA substrate in 200 mM K^+ ion shows a strong positive band at 290 nm, with weak negative peaks near 255 and 235 nm (Figure S1), which is characteristic of a hybrid-type G-quadruplex structure consistent with the results of previous CD and NMR studies.[32,33,36] The ^{Br}G substitution at the syn positions in the DNA−EDTP probe results in a significant increase in thermal stability as compared to no substitution of the ^{Br}G in the DNA−EDTP probe. According to CD melting experiments, the T_m value of G-quadruplexes formed by the DNA−EDTP probe with ^{Br}G was increased 8.7 °C over the DNA−EDTP probe without ^{Br}G (Figure S2). The sequence-specific cleavage by G-quadruplex formation with the DNA−EDTP·Ce(IV) probe complex was examined on a 5′-end FAM-labeled 29 mer DNA substrate, containing a 7 mer human telomeric sequence. The nonradioactive DNA−EDTP probe and 5′-end labeled DNA target were incubated with 10 μM Ce(IV)/EDTA, 200 mM KCl, 10 mM HEPES (pH 7.0) for 50 h. Figure 2 shows the DNA cleavage products analyzed by denaturing PAGE. From the gel-electrophoresis, the cleavage at the sequence of telomere DNA was observed (Figure 2, lane 2). When the DNA−EDTP probe was omitted, no observable cleavage of the target DNA occurred even in the presence of Ce(IV)/EDTA (lane 1). To further confirm that the cleavage results from the formation of a

(36) Zhang, N.; Phan, A. T.; Patel, D. J. *J. Am. Chem. Soc.* **2005**, *127*, 17277–14285.
(37) Pudovik, A. N. *Chemistry of Organophosphorus Compounds*; MIR: Moscow, 1983; Vol. 150.
(38) Chen, W.; Komiyama, M. *ChemBioChem* **2005**, *6*, 1825–1830.
(39) Kitamura, Y.; Sumaoka, J.; Komiyama, M. *Tetrahedron* **2003**, *59*, 10403–10408.
(40) Lönnberg, T.; Suzuki, Y.; Komiyama, M. *Org. Biomol. Chem.* **2008**, *6*, 3580–3587.
(41) Xu, Y.; Sugiyama, H. *Nucleic Acids Res.* **2006**, *34*, 949–954.

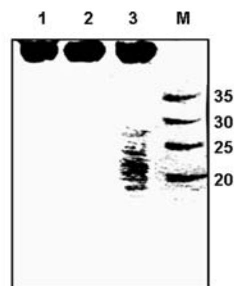

Figure 3. PAGE (15%) showing the sequence-specific cleavage of long strand human telomere by G-qudruplex formation. (Lane 1) Control without both DNA−EDTP probe and Ce(IV)/EDTA; (lane 2) control without DNA−EDTP probe with Ce(IV)/EDTA; (lane 3) 5′-end FAM-labeled 45 mer DNA containing a d(TTAGGG)$_4$ human telomere sequence at 3′-end with underline, with both DNA−EDTP probe and Ce(IV)/EDTA; (lane M) marker oligonucleotides. Ce(IV)/EDTA = 10 μM, t = 50 h, T = 50 °C, 10 mM HEPES (pH 7.0), [KCl] = 200 mM, [target] = 1 μM, [DNA−EDTP] = 2 μM.

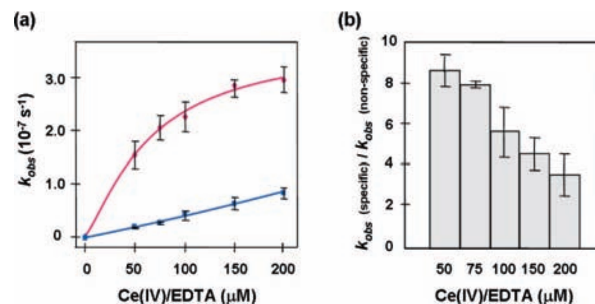

Figure 4. (a) The observed ratio constants of specific cleavage of telomere DNA [k_{obs}(specific)] (purple) and nonspecific cleavage [k_{obs}(nonspecific)] (blue) as a function of Ce(IV)/EDTA concentration. (b) Ratio of k_{obs}(specific) to k_{obs}(nonspecific) as a function of Ce(IV)/EDTA concentration. [Ce(IV)/EDTA] = 0, 50, 75, 100, 150, 200 μM, respectively; 10 mM HEPES (pH 7.0), [KCl] = 200 mM, [target] = 1 μM, [DNA−EDTP] = 2 μM, T = 37 °C, t = 150 h.

G-quadruplex structure in the target DNA, a mutated random template DNA that did not contain the telomere sequence was used in a parallel experiment. No cleavage was observed (lane 3). The cleavage location of target DNA produced by the probe with respect to the position of EDTP group suggested that G-quadruplex formation results in the sequence-specific cleavage in the telomere region of template DNA. Enlargement of cleavage lane corresponding to the scission sites is presented in Figure 2b, together with a histogram showing the relative scission efficiencies at each individual phosphodiester linkage. The cleavage locus extends over five nucleotides, consistent with the results of our previous studies using the Ce(IV)/EDTA complex, in which each cleavage locus extends over four or five base pairs.[37−40,43] We presume that a number of cleavage sites may result from that the Ce(IV) ions are recruited into target sites by DNA−EDTP probe to generate the extended cleavage in the DNA scission sites. These results are consistent with a hydrolytic pathway of DNA scission by a "proximity effect", as indicated in our previous studies.[43]

It is known that human telomeric DNA can form an intramolecular G-quadruplex via a four-repeat telomere sequence. Such secondary structure formed on DNA substrate may hinder the localization of probe to target DNA.[30−35] To investigate the sequence-specific cleavage of the long human telomeric fragment, cleavage reaction was examined on a 5′-end FAM-labeled 45 mer DNA substrate that contained the long sequence d(TTAGGG)$_4$. One cleavage site was observed on the 5′ end of the four-repeat telomeric sequence by comparison with comigrating DNA markers (Figure 3, lane 3). No cleavage occurred when the Ce(IV)/EDTA or the DNA−EDTP probe was not added (lanes 1, 2). We conclude from this result that the ^{Br}Gs-modified DNA−EDTP·Ce (IV) probe complex can form a stable G-quadruplex with the long telomere DNA target by interrupting the self-formed intramolecular G-quadruplex structure in the substrate DNA and induces a telomere sequence-specific cleavage. It is known that the GGG target sequences exist in cellular RNAs.[44−46] We next examined the cleavage efficiency of sequence GGG-containing RNA. It was found that the DNA−EDTP·Ce(IV) probe complex is not capable of specific cleaving of the RNA molecule for 2 h reaction at 37 °C in 50 μM Ce(IV)/EDTA (Figure S3), suggesting such oligonucleotide conjugates are less damaging in RNA molecules to avoid unintended toxic/off-target effects for cellular application.

The kinetics of human telomeric DNA cleavage by the DNA−EDTP·Ce(IV) probe complex was studied in the concentration range from 0 to 200 μM (Figure S4). The concentration dependence of k_{obs} is shown in Figure 4a. The specific cleavage by the DNA−EDTP·Ce(IV) probe complex is significantly more active than nonspecific cleavage. The ratio of rates of site-specific to nonspecific cleavage was calculated and shown in Figure 4b. In lower concentrations, the k_{obs} (specific) is approximately 9 times larger than the k_{obs} (nonspecific). The high specificity in lower concentration will be preferable to such oligonucleotide conjugates for cellular use. A further experiment on cleavage efficiency as a function of reaction time was performed, indicating that the specific cleavage is achieved in a shorter reaction time (Figure S5). Moreover, K_d and k_{max} are determined to be 65.7 ± 17.5 μM and (3.79 ± 0.65) × 10^{-7} s^{-1}. On the basis of these experimental results and together with our previous studies, one possible mechanism for the specific cleavage was proposed (Figure 5).[43,47,48] EDTP, a strong affinity ligand for Ce(IV), recruits the Ce(IV) ions into phosphodiester around target sites to form a complex of EDTP·Ce(IV). As the result, the electrons of the phosphate are strongly withdrawn and lead to activation of the phosphodiester linkage for the nucleophilic attack by the hydroxide ion. Finally, in the breakdown of intermediate, the 5′-OH of the 2′-deoxyribonucleotide is removed from the phosphorus atom through the scission of the P−O bond.

In conclusion, this is the first report demonstrating sequence-specific cleavage of human telomere DNA by G-quadruplex formation. Our approach toward prevention of telomere elongation is conceptually different from previous works in the following aspect: formation of a stable G-quadruplex is used to directly cleave telomere DNA, whereas traditional works were to stabilize telomere G-quadruplex structures by small molecular

(42) Komiyama, M.; Aiba, Y.; Yamamoto, Y.; Sumaoka, J. *Nat. Protocol* **2008**, *3*, 655–662.
(43) Komiyama, M.; Sumaoka, J. *Curr. Opin. Chem. Biol.* **1998**, *2*, 751–757.
(44) Azzalin, C. M.; Reichenbach, P.; Khoriauli, L.; Giulotto, E.; Lingner, J. *Science* **2007**, *318*, 798–801.
(45) Schoefter, S.; Blasco, M. A. *Nat. Cell Biol.* **2008**, *10*, 228–236.
(46) Xu, Y.; Kaminaga, K.; Komiyama, M. *J. Am. Chem. Soc.* **2008**, *130*, 11179–11184.
(47) Komiyama, M.; Takeda, N.; Shigekawa, H. *Chem. Commun.* **1999**, 1443–1451.
(48) Takeda, N.; Shibata, M.; Tajima, N.; Hirao, K.; Komiyama, M. *J. Org. Chem.* **2000**, *65*, 4391–4396.

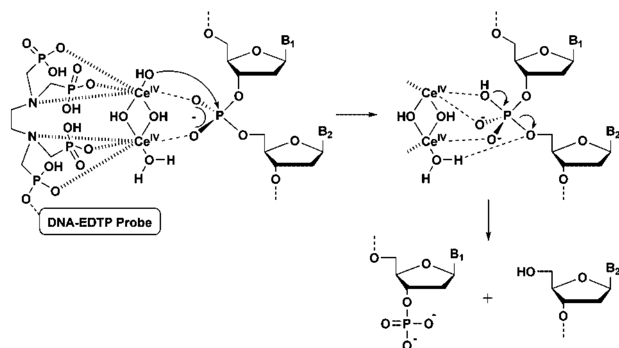

Figure 5. Proposed mechanism of telomere DNA cleavage by DNA−EDTP•Ce(IV) probe complex.

compounds. Our result provides a proof of concept for targeting the human telomere DNA substrate associated with the G-quadruplex formation, and this thus serves as a starting point for the design of more efficient telomere sequence-specific cleaving reagents based on G-quadruplex formation.

Experimental Section

General Method. Nucleoside phosphoramidite monomers were purchased from Glen Research Co. The oligonucleotide multiphosphonate conjugates were prepared on an automated synthesizer, purified by the conventional methods, and characterized by MALDI-TOF MS. The substrate and marker oligonucleotides were commercial products of Sigma Genosys and were purified by the conventional methods before use. 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. 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, as was previously described.[42]

The hydrolysis of the 5′-end FAM-labeled DNA substrate was initiated by adding the solution of Ce(IV)/EDTA complex to the reaction mixtures and carried out at pH 7.0 (10 mM HEPES buffer), 37 or 50 °C; [target DNA] = 1.0 μM, [DNA−EDTP] = 2.0 μM, [KCl] = 200 mM. The reaction mixtures were then analyzed by denaturing 15% polyacrylamide gel electrophoresis, and the scission fragments were quantified with a Fuji Film FLA-3000G imaging analyzer.

Preparation of DNA−EDTP Probe. The 16 mer oligonucleotides with or without ^{Br}G were prepared on an automated synthesizer by the conventional phosphoramidite method. The support-bound oligonucleotides were treated by hydrazine solution (1/32/8, hydrazine/pyridine/AcOH, v/v/v) for 30 min at room temperature and were washed with dry pyridine (2 mL), dry methanol (2 mL), and dry acetonitrile (2 mL). After being dried under vacuum, the supports were transferred to microcentrifuge tubes. By oxime coupling, the EDTP group was linked to the 5′ end of oligonucleotides. The detailed experiments for preparing the DNA−EDTP probe and related synthesis reagents were described in the Supporting Information.

CD Measurements and Analysis of CD Melting Profile. CD spectra were measured using a Jasco model J-725 CD spectrophotometer. The spectra were recorded using a 1 cm path-length cell. Samples were prepared by heating the oligonucleotides at 90 °C for 5 min and gradually cooling to room temperature. In CD melting studies, diluted samples were equilibrated at room temperature for several hours to obtain equilibrium spectra. The melting curves were obtained by monitoring a 290 nm CD band. Solutions for CD spectra were prepared as 0.3 mL samples at 5 μM strand concentration in the presence of 200 mM KCl, 5 mM HEPES buffer, pH 7.0.

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

Supporting Information Available: Method, synthesis (scheme), CD (Figure S1), T_m (Figure S2), PAGE (Figures S3, S4), and chart (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.

JA807313X