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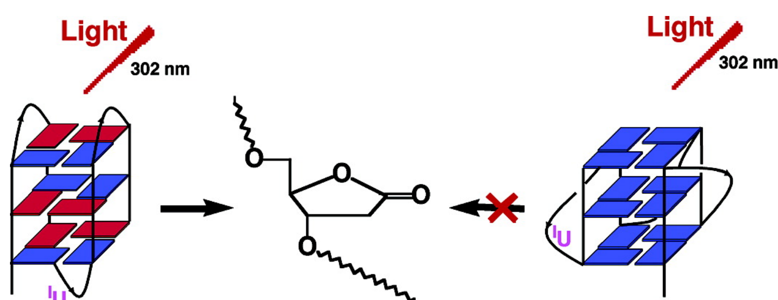

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Highly Efficient Photochemical 2′-Deoxyribonolactone Formation at the Diagonal Loop of a 5-Iodouracil-Containing Antiparallel G-Quartet

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Abstract: To explore the structure-dependent hydrogen abstraction in antiparallel and parallel G-quartet DNA structures, the photochemical reactivity of 5-iodouracil (^{I}U)-containing human telomeric DNA 22-mers was investigated under the 302 nm UV irradiation conditions. We discovered that only antiparallel ODN 4, in which the second T residue in the diagonal loop of the antiparallel G-quartet is substituted with ^{I}U, was rapidly consumed as compared with parallel ODN 4 and the other ^{I}U-containing 22-mers under the irradiation conditions. Product analysis of the photolyzate of antiparallel ODN 4 indicated that a 2′-deoxyribonolactone residue was effectively produced at the 5′ side of the ^{I}U residue in the diagonal loop. Photochemical 2′-deoxyribonolactone formation was also found in the ^{I}U-containing diagonal loop of antiparallel G-quartets d(GGGGTTT^{I}UGGGG)$_2$ and d(GGGGTT^{I}UTGGGG)$_2$, whereas the reaction did not occur at other DNA structures, including the single-stranded form, the loop region of the hairpin, and linear four-stranded G-quartets. The results clearly indicate that this type of 2′-deoxyribonolactone formation efficiently occurrs only in the diagonal loop of the antiparallel G-quartet. Furthermore, we found that 2′-deoxyribonolactone was formed at the ^{I}U-containing G-rich sequence of the IgG switch regions and the 5′ termini of the Rb gene, suggesting the formation of an antiparallel G-quartet with a diagonal loop in these sequences. These results suggest that the present photochemical method can be used as a specific probe for the antiparallel G-quartet with the diagonal loop.

Introduction

DNA tetraplexes, otherwise known as DNA quadruplexes or G-quartets, are four-stranded DNA structures formed by G-rich sequences.[1−3] Although G-quartets have thus far been studied only in vitro, they are attracting increasing attention because of their postulated involvement in a variety of biological processes. For example, telomeric DNA is fundamental in protecting the cell from recombination and degradation.[4,5] Disruption of telomere maintenance leads to eventual cell death, which can be exploited for therapeutic intervention in cancer treatment.[6−12] The DNA of human telomeres consists of repeats of the nucleotide sequence TTAGGG, ending in a single-stranded segment that overhangs at the end of the double-stranded DNA helix. The single-stranded repeats can form four-stranded G-quartet structures.[13−16] The solution structure of d[AGGG(TTAGGG)$_3$] in the presence of Na^+ ions has been elucidated by NMR analysis.[17] This showed an antiparallel G-quartet structure in which the opposing GGG columns are antiparallel with one diagonal and two lateral TTA loops (Figure 1). On the other hand, the same four-repeat human telomere sequence adopts a completely different G-quartet architecture in a crystal grown in the presence of K^+ ions.[18] In the crystal structure, four core GGGs are parallel, with the three linking external loops positioned on the exterior of the G-quartet core. These results suggest that d[AGGG(TTAGGG)$_3$] can form both antiparallel and parallel G-quartets and these two conformations can be modulated by the concentration of Na^+ and K^+ ions.[19,20]

† Tokyo Medical and Dental University.
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Figure 1. Schematic representations of the folded structures of d[AG_3-(T_2AG_3)$_3$]. (a) The K^+-stabilized crystal structure, with TTA external loops abutting the sides of the G-quartet and parallel GGG columns. (b) The Na^+-stabilized solution structure, with one diagonal and two lateral TTA. The blue and red boxes represent guanine base in the anti and syn conformations, respectively.

Several other G-rich sequences have also been proposed as showing biological functions through the formation of a G-quartet. For instance, G-quartets have been associated with site-specific genetic recombination in immunoglobulin switch regions (IgG) and the insulin gene-linked polymorphic region (ILPR).[21,22] Furthermore, it is also suggested that G-quartets form in the promoter regions of the retinoblastoma susceptibility genes (Rb) and the c-MYC oncogene,[23−25] raising intriguing possibilities for controlling gene expression by G-quartet formation. In most of these studies, the inaccessibility of the N-7 position of guanines to dimethyl sulfate (DMS) and anomalous electrophoretic mobility nondenaturing gels were used as diagnostics for the G-quartet formation.[26,27] Although there have been many reports on the formation of various G-quartets, a method of chemical reaction that can distinguish antiparallel and parallel G-quartets has not been reported. For several years, we have investigated the photoreaction of 5-halouracil-containing DNA as a probe of DNA local conformation. It has been found that hydrogen abstraction of the deoxyribose backbone by 2′-deoxyuridin-5-yl generated from 5-halouracil under 302 nm irradiation is atom specific and highly conformation dependent. For example, competitive C1′- and C2′α-hydrogen abstractions are observed in B-DNA,[28−30] whereas selective C1′-hydrogen abstraction occurs in the A-like structure of DNA−RNA hybrids.[31] In Z-form DNA, stereospecific C2′β-hydrogen abstraction gives rise to C2′α-hydroxylation.[32,33] In protein-induced DNA kinks, photoirradiation causes intrastrand hydrogen abstraction from the 5-methyl group of thymine at the 5′ side.[34] In the present study, the photoreactivity of 5-iodouracil (^{I}U)-containing telomeric DNA was investigated to explore the structure-dependent hydrogen abstraction in antiparallel and parallel G-quartets. The results demonstrate that ^{I}U in the diagonal loop of antiparallel G-quartets undergoes an extremely facile photoreaction to produce a 2′-deoxyribonolactone residue with the release of a nucleic acid base. This study has determined the detailed relationship between the G-quartet structure and photoproduct so that the potential of this photochemical method can be realized for detecting the G-quartets.

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Experimental Section

Synthesis of Oligonucleotides. ^{I}U-containing oligonucleotides were prepared by the phosphoramidite method on controlled pore glass supports (1 μmol) by using a Beckman OLIGO1000 DNA synthesizer. After automated synthesis, the oligomers were detached from the support, deprotected, and purified by HPLC. The oligomers were identified by electrospray ionization mass spectrometry (ESI-MS) on a Perkin-Elmer SCIEX API 165 mass spectrometer (negative mode). The purity and concentrations of these oligomers were determined by complete digestion of the oligomers with alkaline phosphatase and P1 nuclease to 2′-deoxymononucleosides.

Quantitative HPLC Analysis of Photoirradiated Mixtures. The reaction mixture (total volume 100 μL) contained ^{I}U-modified oligonucleotides (0.3 mM total base concentration) in 2 mM sodium cacodylate buffer (pH 7.0) in the presence of 100 mM NaCl or 100 mM KCl in a microcentrifuge tube (0.6 mL). After irradiation with monochromatic 302 nm UV light (HM-5 hypermonochromator, Jasco) at 0 °C for 10 min, 10 μL of the aliquot was analyzed by HPLC. HPLC analysis was carried out with a Cosmosil 5C18-MS column; for ODNs 1−6, elution was done with 0.05 M TEAA containing 0−11% acetonitrile, in a linear gradient at a flow rate of 1.0 mL/min for 50 min, at 40 °C; for ODNs 7 and 8, elution was done with 0.05 M ammonium formate containing 0−11% acetonitrile, in a linear gradient (30 min) at a flow rate of 1.0 mL/min, at 40 °C. Quantum yield measurements were carried out at 0 °C on the monochromator (302 nm). The photopower of this photoreaction at 302 nm was 2.3×10^{-3} J/s. Each reaction mixture (100 μL) was monitored by UV absorption at 302 nm assuming extinction coefficients of $\epsilon_{302} = 1200$ M^{-1} cm^{-1} (Na^+-form), 1060 M^{-1} cm^{-1} (K^+-form), and $\epsilon_{302} = 910$ M^{-1} cm^{-1} (^{I}U modified the double-stranded telomeric DNA, Table 1). The reaction mixture (100 μL) was irradiated at 302 nm at 0 °C for 10 min, and 10 μL of the aliquot was analyzed by HPLC, as described above.

Characterization of the Photoproduct of 1. The reaction mixture (total volume 100 μL), which contained 0.3 mM (base concentration) ODN 4 in 2 mM sodium cacodylate buffer pH 7.0 in 100 mM NaCl, was irradiated at 0 °C for 10 min. The HPLC fractions of **1**, eluted at 30.1 min, were collected and concentrated. The residue was dissolved in water (10 mM sodium cacodylate buffer pH 7.0), and then the mixture was heated at 90 °C for 10 min. Aliquots of 10 μL each, before (Figure 4b) and after heating (Figure 4c), were analyzed by HPLC. HPLC analysis was carried out with a Cosmosil 5C18-MS column: 0.05 M ammonium formate containing 0−15% acetonitrile, linear gradient (30 min) at a flow rate of 1.0 mL/min, at 40 °C. The formation of products **2** and **3** as major peaks together with a minor peak eluted at 10 min was observed. The minor peak is assumed to be 5-methylenefuranone which comigrated with the product generated from ribonolactone containing the hexamer, d(GCLUGC) (L = 2′-deoxyribonolactone).[28] The HPLC fractions of **2** and **3** (retention times 18.5 and 19.1 min) were collected and concentrated. The mixture of **2** and

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Table 1. Product Analysis in the Photoreaction of 5-Iodouracil-Containing Oligonucleotides for Different Structures[a]

Structure							
Sequence	d(GTGCT^{I}UACG)	d(AG$_3$T$_2$AG$_3$T^{I}UAG$_3$T$_2$AG$_3$)/ d(TC$_3$A$_2$TC$_3$A$_2$TC$_3$A$_2$TC$_3$)	^{I}U T CGTCGT-5' A GCAGCA-3'	[d(T^{I}UG$_4$T)]$_4$	[d(G$_4$TTT^{I}UG$_4$)]$_2$	[d(G$_4$TT^{I}UTG$_4$)]$_2$	d(AG$_3$T$_2$AG$_3$T^{I}UAG$_3$T$_2$AG$_3$)
2'-deoxyribonolactone in Na^+ ions (%)	0 (2%)	0 (1%)	0 (2%)	0 (1%)	90 (50%)	89 (35%)	95 (60%)
2'-deoxyribonolactone in K^+ ions (%)	0 (1%)	0 (1%)	0 (1%)	0 (2%)	90 (51%)	90 (35%)	0 (2%)

[a] The reaction mixture that contained ^{I}U-modified ODNs (0.3 mM total base concentration) in 2 mM sodium cacodylate buffer (pH 7.0) in the presence of 100 mM NaCl or 100 mM KCl was irradiated at 0 °C with a monochromator (302 nm) for 10 min. The numbers in parentheses are the consumption of ODNs.

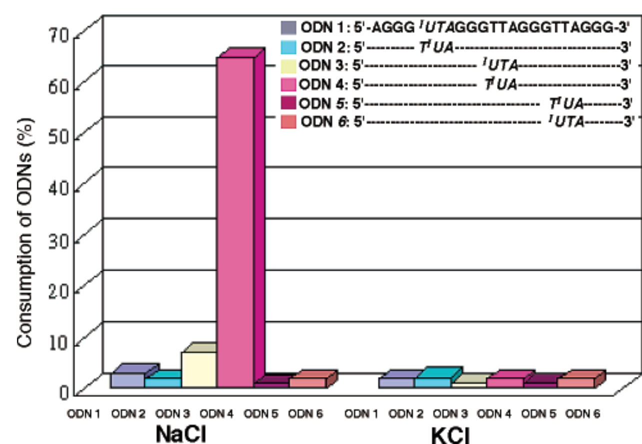

Figure 2. HPLC analysis of the degree of consumed ODNs 1−6 (0.3 mM base concentration) in 2 mM sodium cacodylate buffer (pH 7.0) in the presence of 100 mM NaCl or 100 mM KCl after 10 min irradiation by UV light (302 nm) at 0 °C. The inset indicates the position of ^{I}U.

3 was dissolved in water, and then the solution was subjected to enzymatic dephosphorylation with alkaline phosphatase (100 units/ml). HPLC analysis of the hydrolysate indicated the formation of d(AGGGTTAGGG) and d(UAGGGTTAGGG) by comparison with the authentic oligomers. Structures of **1**, d(AGGGTTAGGG), and d(UAGGGTTAGGG) were further confirmed by ESI-MS. ESI-MS (negative) for **1**, calcd 6684.2, found 6684.1; for d(AGGGTTAGGG), calcd 3148.1, found 3148.0; for d(UAGGGTTAGGG), calcd 3438.2, found 3438.0.

Results and Discussion

To explore the structure-dependent hydrogen abstraction in antiparallel and parallel G-quartets, one of the six thymine (T) residues in 22-mer human telomeric DNA 5′-d(AGGGT$_1$T$_2$AGGGT$_3$T$_4$AGGGT$_5$T$_6$AGGG)-3′ was substituted with ^{I}U to generate six kinds of oligodeoxynucleotides, ODNs 1−6 (Figure 2). The CD spectra of ODNs 1−6 and unsubstituted 22-mer exhibit a positive band at 295 nm and a negative band around 265 nm in the presence of 100 mM Na^+ ions, which is characteristic of an antiparallel G-quartet structure.[17] In the presence of 100 mM K^+ ions, these showed a negative band around 240 nm and a remarkable increase in the 260 nm CD band as compared with the CD spectra in 100 mM NaCl, indicating a parallel G-quartet structure (Figure 1S, Supporting Information). Similar CD spectra have been reported in other studies.[35,36] Although different monovalent cations dramatically alter the G-quartet topology,[37−40] the reasons for the remarkable difference are yet to be fully elucidated.[41] However, it is assumed that K^+ ions (ionic radius of 1.51 Å) are invariably sandwiched between adjacent guanine tetrads, whereas Na^+ ions (1.18 Å) can sometimes be coordinated within a tetrad.[18] We also investigated the photoreactivities of antiparallel and parallel ODNs 1−6 under the 302 nm irradiation. HPLC analysis of photolyzate indicated that the amount of consumed ODNs 1−6 after 10 min photoirradiation varied significantly with the orientation of the G-quartet and the incorporated position of ^{I}U (Figure 2). Surprisingly, more than 60% of antiparallel ODN 4 was consumed when T$_4$ in the middle of the diagonal loop was substituted with ^{I}U. Antiparallel ODN 3 in which T$_3$ at the 5′ side of the diagonal loop was substituted with ^{I}U was slightly consumed (7%). Other antiparallel ODNs with ^{I}U in a lateral loop were not consumed (<3%), indicating that ^{I}U residues in the diagonal loop are photoreactive; in particular, the ^{I}U in the middle of the diagonal loop has significant photoreactivity. In marked contrast to the photoreactivity of the antiparallel ODN, the parallel ODNs 1−6 were not consumed (<2%) under the same irradiation conditions. Under 30 min irradiation, most of the antiparallel ODN4 was consumed, whereas consumption of parallel ODN4 and other ODN possessing ^{I}U at different places was found to be less than 10% (data not shown). The observations indicated that such a highly efficient photoreaction uniquely occurs in the diagonal loop. The results suggest that the photoreactivity of ^{I}U-containing G-quartets highly depends on differences in the loop structures of the G-quartet conformations.

To understand the molecular basis of the high photoreactivity of the antiparallel ODN 4, product analysis of photoirradiated ODN 4 in the presence of Na^+ or K^+ ions was investigated in

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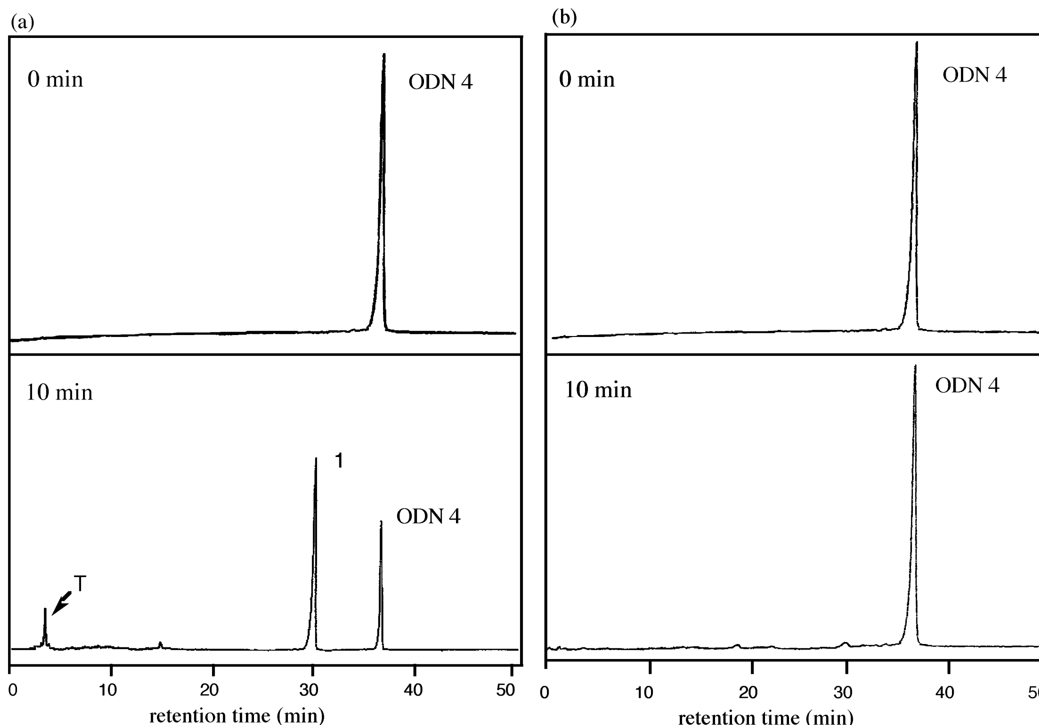


Figure 3. HPLC analysis of UV-irradiated ODN 4 in 2 mM sodium cacodylate buffer (pH 7.0) in the presence of (a) 100 mM NaCl (antiparallel) or (b) 100 mM KCl (parallel). (top) Before irradiation; (bottom) after 10 min of irradiation (302 nm) at 0 °C.

detail. Before irradiation, a single peak of ODN 4 was observed at a retention time of 37.1 min in both cases (Figure 3). In the antiparallel G-quartet, 10 min irradiation resulted in the formation of a new peak at 30.1 min (product **1**) with concomitant release of free thymine, which eluted at 2.7 min (Figure 3a). Quantitative analysis indicates that the yield of **1** from photoirradiated ODN 4 in the antiparallel structure was >95% based on the consumed ODN 4 with a quantum yield of 2.9×10^{-3} relative to the double-stranded telomeric DNA with a lower quantum yield of 1.3×10^{-5} M^{-1} cm^{-1}.[33] On the other hand, the consumption of parallel ODN 4 in the presence of K^+ ions was low (2%), with almost no generation of such product (Figure 3b). To elucidate the structure of product **1**, the HPLC fractions containing product **1** were collected and lyophilized. Upon heating at 90 °C for 10 min at neutral pH 7.0, 70% of **1** was found to decompose to **2** and **3** with retention times of 18.5 and 19.1 min, respectively (Figure 4b). Treatment of **2** and **3** with alkaline phosphatase produced d(AGGGTTAGGG) and d(UAGGGTTAGGG), indicating that **2** and **3** are their phosphorylated DNA fragments (Figure 4c). The structure of **1** was further confirmed by ESI-MS analysis (Figure 2S, Supporting Information). These results indicate that **1** is a 2′-deoxyribonolactone-containing 22-mer produced from the C1′-hydrogen abstraction of adjacent T_3 by the 2′-deoxyuridin-5-yl radical (Scheme 1). The high photoreactivity of ODN 4 in antiparallel G-quartet and the poor photoreactivity of ODN 4 in parallel G-quartet can be explained by comparison of the two structures (Figure 5).[17,18] In the parallel G-quartet, the adenine in TTA linking the external loop is swung back so that it intercalates between the two thymines. It is reasonable to assume that the intercalated adenine base prevents hydrogen abstraction. In contrast, there is no such intercalation of adenine base in the diagonal loop structure of the antiparallel G-quartet,[18] thereby allowing the C1′-hydrogen abstraction by the 2′-deoxyuridin-5-yl radical in the loop.[42,43] Furthermore, the NMR structure suggests that the 2′-deoxyuridin-5-yl is close to the C1′-hydrogen

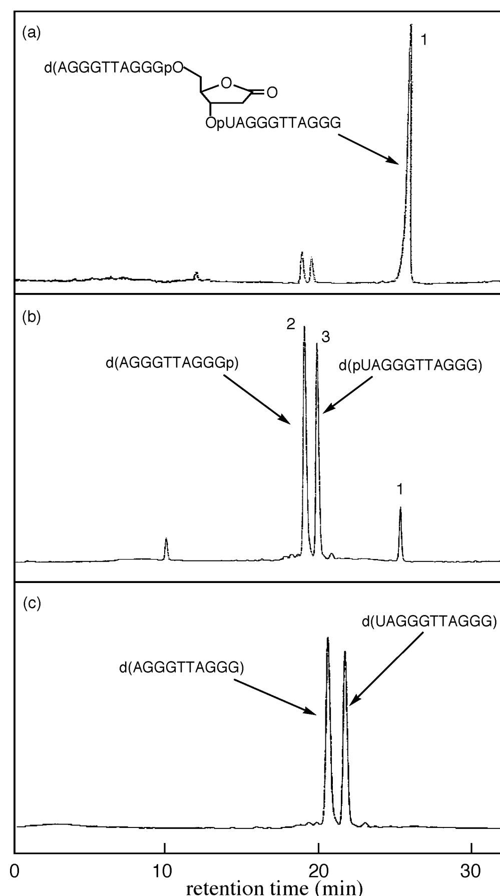


Figure 4. HPLC of (a) isolated **1**, (b) the products (**2**, **3**) of heating treatment **1**, and (c) the dephosphorylation products of **2** and **3** after treatment with alkaline phosphatase.

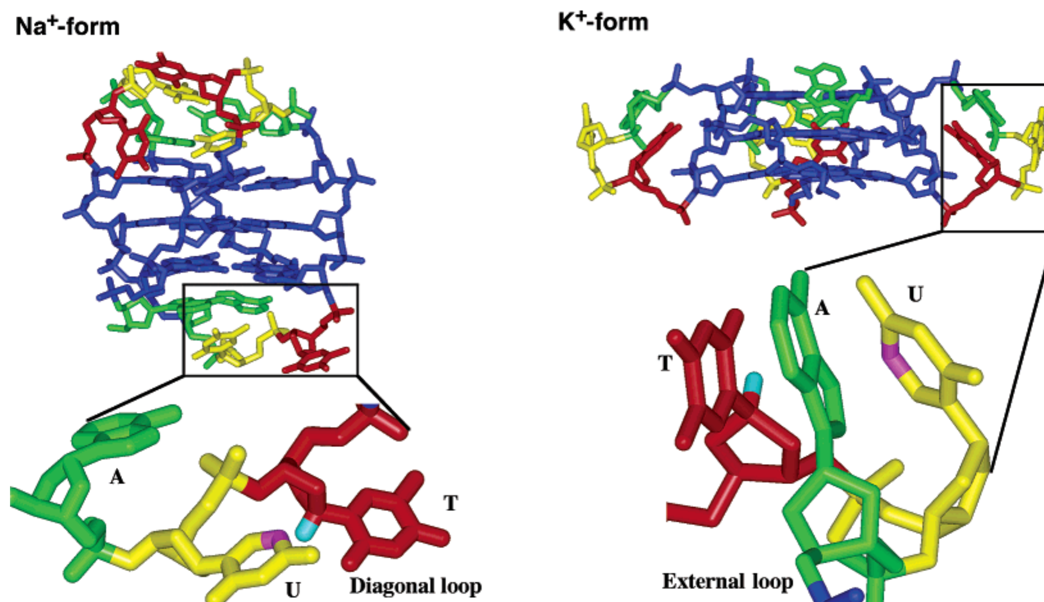
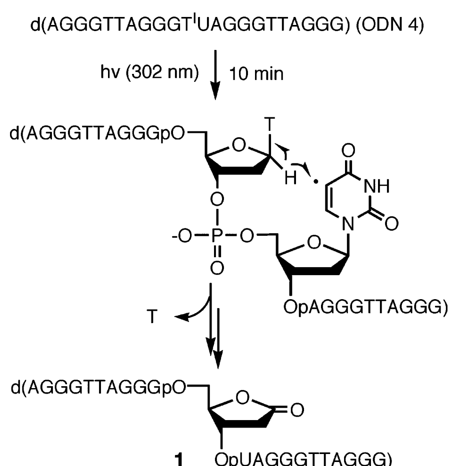


Figure 5. (top) Structure of photoirradiated oligonucleotide d(AGGGTTAGGGT^{I}UAGGGTTAGGG) (ODN 4) based on the X-ray crystal structure (K^+-form) and NMR structure (Na^+-form), and (bottom) a close-up view of the loop region. Guanines are in blue, C5 of U is in pink, abstracted-hydrogen of T is in cyan, and T, U, and A are in red, yellow, and green, respectively.

Scheme 1



of the adjacent T_3 as compared with the other hydrogens in the diagonal loop (Figure 5, Na^+-form). Moreover, under irradiation conditions for the ODNs1−6 in the presence of 100 mM 2-propanol, the photoreduced uracil (U)-containing 22-mer was obtained in 50−80% yield, indicating that the 2′-deoxyuridin-5-yl radical was generated from ^{I}U at all sites. In the case of ODN 4, the 2′-deoxyribonolactone residue was competitively formed with U-containing 22-mer under these conditions. The results clearly indicate that the C1′ hydrogen in the diagonal loop of ODN 4 is located at a favorable position for hydrogen abstraction by the 2′-deoxyuridin-5-yl radical. These results further confirmed that hydrogen abstraction in the antiparallel G-quartets by 2′-deoxyuridin-5-yl generated from ^{I}U is highly conformation dependent.

To elucidate the structural requirement for the efficient formation of 2′-deoxyribonolactone in loop regions, the photoreactivity of various ^{I}U-containing DNA structures was examined (Table 1). These structures included the single-stranded form of d(GTGCT^{I}UACG), the double-stranded telomeric sequenced (AGGTTAGGGT^{I}UAGGGTTAGGG)/d(CCCT-AAC-CCTAACCCTAACCCT), the hairpin duplex of d(TGCT-GCT^{I}UAGCAGCA), and the linear G-quartet $[d(G_4T^{I}UG_4T)]_4$ in the presence of K^+ or Na^+ ions. We found that these oligomers did not efficiently produce 2′-deoxyribonolactone residues, and the consumption of the starting oligomers was very low under the present irradiation conditions (Table 1). In contrast, the dodecamer d(GGGGTTTTGGGG), which is known to form an antiparallel G-quartet in the presence of K^+ or Na^+ ions,[44] efficiently produced the 2′-deoxyribonolactone residues when T_7 or T_8 in the diagonal loop was substituted with ^{I}U (Figure 3S, Supporting Information). As control experiments, the photoreactivities of d(AGGGTTAGGGT^{I}UAGGG) and d(GGG-T^{I}UAGGGTTAGGG) where one loop and strand was deleted from d(AGGGTTAGGGT^{I}UAGGGTTAGGG) were investigated in the presence of K^+ or Na^+ ions. Neither consumption of the starting oligomers (<2%) nor formation of the 2′-deoxyribonolactone residue was observed in both cases. These experiments further confirmed that this type of 2′-deoxyribonolactone formation efficiently and specifically occurred in the diagonal loop of antiparallel G-quartets and did not depend on monovalent cations present in the photoreaction. The above results suggest an intriguing possibility that this type of photoreaction can be used as a specific probe for parallel G-quartets with a diagonal loop.

IgG switch regions participate in a process of regulated DNA deletion, during which one or more constant regions are excised to join the expressed variable to a new constant region.[45] The Rb gene encodes a nuclear phosphoprotein that acts as a tumor suppressor by affecting the cell cycle.[46] These G-rich sequences have been proposed to form G-quartet structures by DMS protection experiments and nondenaturing gel electrophoresis.

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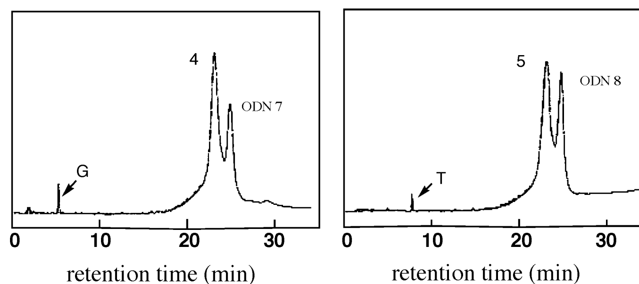

Figure 6. HPLC analysis of UV-irradiated ODN 7 (left) and ODN 8 (right) in 2 mM sodium cacodylate buffer (pH 7.0) in the presence of 100 mM KCl. The reaction was performed at 0 °C for 30 min.

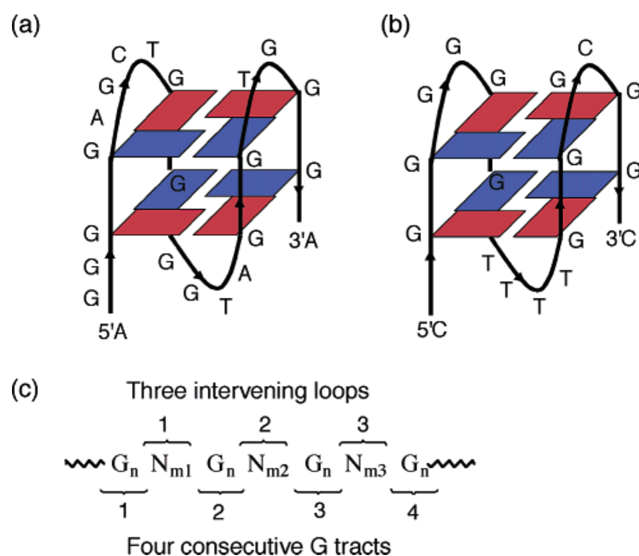

Figure 7. Schematic showing the K^+-induced G-quartet forms of IgG (a), and Rb (b), that are consistent with photochemical probing results. (c) Generic G-quartet-forming sequence, G_n, from four G-rich tracts hydrogen bond to form tetrads, which then stack to form the G-quartet stem; 1, 2, and 3 form the three loops.

However, the detailed structures have not been elucidated. To test the efficacy of the present photochemical method, the photoreactions of the ^{I}U-substituted IgG switch regions and the 5′ termini of the Rb gene were examined. The photoirradiation (302 nm) of 5′-d(AGGGGAGCTGGGG^{I}UAGGTGGGA)-3′ (ODN 7) (IgG) and 5′-d(CGGGGGGTT^{I}UTGGGCGGC)-3′ (ODN 8) (Rb) in the presence of 100 mM KCl in 2 mM sodium cacodylate buffer (pH 7.0) was performed at 0 °C for 30 min. HPLC analysis of the photolyzate of ODN 7 and 8 indicated that photoproducts **4** and **5** (~90% yield) were obtained as the major products with release of free guanine or thymine (Figure 6). The photoproducts **4** and **5** were found to be 2′-deoxyribonolactone-containing oligomers, confirmed by the same method as described in the characterization of **1** (Figure 4S, Supporting Information). This highly efficient 2′-deoxyribonolactone production strongly suggests the formation of an antiparallel G-quartet with a diagonal loop for these G-rich sequences. Figure 7a,b shows the proposed G-quartet structure based on the present photochemical method. It consists of two stacked G-tetrads and a diagonal four-base loop for both IgG and Rb. It is noted that our proposed structures are consistent with the previous results of gel electrophoresis and DMS protection experiments.[21,23] Previously used methods, such as CD spectroscopy, gel electrophoresis, and small molecular probes, can be applied to studying the structure of DNA, but they report the average conformation of the entire sample and cannot be used to pinpoint local conformational differences.[47] Here, the photochemical reactivity depends on an intrinsic property of the G-quartet; the yield of the photoproducts can reflect the conformation of G-quartet during irradiation, even the detailed loop structure in the G-quartet.

It has been pointed out that there are numerous potential G-quartet forming sequences in many important genes.[48] The sequence motif required for intrastrand G-quartets can be written $G_nN_{m1}G_nN_{m2}G_nN_{m3}G_n$, where n is the number of guanine tetrads and $m1$, $m2$, and $m3$ are the loop lengths, where the diagonal loop (N_{m2}) is common in the sequence motif (Figure 7c). We found that the photoreactivity of ^{I}U-containing telomeric DNA depends on the orientation of the G-quartet, in which the 2′-deoxyribonolactone residue is effectively produced only in the diagonal loop of the antiparallel G-quartet. The present photochemical method could be used as a conformational probe to detect G-quartets with the diagonal loop in vitro.

Supporting Information Available: CD measurement (Figure 1S), ESMS analysis (Figure 2S), HPLC analysis of UV-irradiated various ^{I}U-containing DNA structures (Figure 3S), and HPLC profile of **3**−**12** (Figure 4S). This material is available free of charge via the Internet at http://pubs.acs.org.

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