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One-Electron Photooxidation and Site-Selective Strand Cleavage at 5-Methylcytosine in DNA by Sensitization with 2-Methyl-1,4-naphthoquinone-Tethered Oligonucleotides

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Abstract: Photosensitized one-electron oxidation was applied to discriminate a specific base site of 5-methylcytosine (^{m}C) generated in DNA possessing a partial sequence of naturally occurring p53 gene, using a sensitizing 2-methyl-1,4-naphthoquinone (NQ) chromophore tethered to an interior of oligodeoxynucleotide (ODN) strands. Photoirradiation and subsequent hot piperidine treatment of the duplex consisting of ^{m}C-containing DNA and NQ-tethered complementary ODN led to oxidative strand cleavage selectively at the ^{m}C site, when the NQ chromophore was arranged so as to be in close contact with the target ^{m}C. The target ^{m}C is most likely to be one-electron oxidized into the radical cation intermediate by the sensitization of NQ. The resulting ^{m}C radical cation may undergo rapid deprotonation and subsequent addition of molecular oxygen, thereby leading to its degradation followed by strand cleavage at the target ^{m}C site. In contrast to ^{m}C-containing ODN, ODN analogs with replacement of normal cytosine, thymine, adenine, or guanine at the ^{m}C site underwent less amount of such an oxidative strand cleavage at the target base site, presumably due to occurrence of charge transfer and charge recombination processes between the base radical cation and the NQ radical anion. Furthermore, well designed incorporation of the NQ chromophore into an interior of ODN could suppress a competitive strand cleavage at consecutive guanines, which occurred as a result of positive charge transfer. Thus, photosensitization by an NQ-tethered ODN led to one-electron oxidative strand cleavage exclusively at the target ^{m}C site, providing a convenient method of discriminating ^{m}C in naturally occurring DNA such as human p53 gene as a positive band on a sequencing gel.

Introduction

Cytosine methylation at the 5-position in DNA has been implicated in an epigenetic regulation of genetic information and diseases such as cancer as well.[1,2] Thus, there is increasing importance in identification of the methylation status of a specified cytosine residue in DNA to obtain a molecular insight into the biological function of cytosine methylation. For discrimination between cytosine (C) and 5-methylcytosine (^{m}C), various protocols using their difference in chemical and biological reactivity have been proposed;[3] e.g. Maxam−Gilbert chemical modification,[4] methylation specific PCR amplification involving bisulfite modification,[5] enzymatic digestion of the DNA duplex[3] or PNA−DNA complex,[6] and dihydroxylation of ^{m}C by osmium tetroxide in a bulged DNA duplex.[7]

Selective strand cleavage at ^{m}C in DNA has been recognized to be a useful reaction that is applicable to identification of the methylation site by way of detecting a fragment of specific cleavage derived from ^{m}C as a positive band on a sequencing gel. Previously, Cadet and co-workers reported one-electron photooxidation of ^{m}C under sensitization by 1,4-naphthoquinone (NQ)[8] to produce an alkali labile oxidized form of 5-formylcytosine via formation of ^{m}C radical cation followed by its deprotonation.[8a] This mode of photosensitized one-electron oxidation may be applied to DNA cleavage at the methylation site. In the preliminary study,[9] we identified a large difference in reactivity between the modified ^{m}C and normal C residues

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in DNA toward photooxidation sensitized by NQ that is tethered to oligodeoxynucleotides (ODN) with a well specified complementary base sequence so as to form a duplex with the cytosine-methylated DNA specimen. The photosensitization of an NQ-tethered duplex showed that ^{m}C was efficiently one-electron oxidized into 5-formylcytosine, while the corresponding normal C did not undergo such an oxidation. Treatment with hot piperidine led to strand cleavage at the resulting 5-formylcytosine residue, thus a positive band being observed at the position of the original ^{m}C on a sequencing gel. However, our previous system for identification of the ^{m}C site[9] has a drawback for further improvement in that oxidative strand cleavage at a G doublet competitively occurs as a consequence of charge transport through DNA duplex.[10] In view of evidence that ^{m}C is usually generated in G-C-rich regions of DNA,[1,2] the presence of such a competitive strand cleavage at a G doublet should prohibit distinct detection of a ^{m}C site on a sequencing gel. In this regard, an ambiguity comes from our previous experiments[9] employing an expedient DNA base sequence, in which ^{m}C was located apart from G by an intervening A-T bridge.

The context of previous research prompted us to further identify the NQ-sensitized photooxidation of ^{m}C into strand cleavage in a naturally occurring DNA base sequence, in which ^{m}C exists adjacent to G. Thus, we herein characterized the oxidation reactivity of DNA with a partial sequence of the p53[2a] gene under photosensitization by NQ-bearing ODNs, evaluating the regional effect of NQ on the oxidative strand cleavage at ^{m}C and the positive charge (hole) transfer to a G doublet. We synthesized ODNs with several base sequences, in which the NQ chromophore was placed at the interior of the strand so as to be immobilized at a specific position upon duplex formation. In accord with our previous reports,[9] photoirradiation of a duplex of ^{m}C-containing ODN and its complementary NQ-bearing ODN resulted in strand cleavage at the ^{m}C site with high accuracy upon treatment with hot piperidine, while the corresponding normal C sites in the duplex did not undergo such a strand cleavage. Evidence was also obtained that competitive strand cleavage at the G site was significantly suppressed, notwithstanding the possible occurrence of the positive charge transfer. The suppression of superfluous strand cleavage at G is presumably due to a rapid charge recombination process between the G radical cation and the neighboring NQ radical anion. Thus, optimization of the regional position of the photosensitizing NQ chromophore relative to the target ^{m}C in the naturally occurring DNA base could produce exclusive strand cleavage at ^{m}C without unfavorable cleavage at G, thereby allowing distinct detection of the target ^{m}C on a sequencing gel.

Results and Discussion

As shown in Scheme 1, we have synthesized ODNs possessing an NQ chromophore at the interior of a strand. Carboxylic acid **1** synthesized from 2-methyl-1,4-naphthoquinone and succinic acid via oxidative decarboxylation[11] was coupled with hydrazine to give **2**. The photosensitizing NQ-tethered ODNs were prepared by coupling of **2** with ODNs possessing a formyl group, as synthesized from an ODN 1(D) with a triol using $NaIO_4$ treatment.[12] The crude NQ-tethered ODNs were purified by reversed phase HPLC, and incorporation of the NQ chromophore into ODN strand was confirmed by enzymatic digestion and MALDI-TOF mass spectrometry. ODN 7 tethering NQ to the strand end was synthesized as described previously.[9] The ODNs used in this study are summarized in Figure 1.

ODN 1(X) 5'-CTC TCT GCG CC**X** GTC TCT-3'
X = G, D, NQ_1
ODN 2(Y) 5'-AGA GAC **Y**GG CGC ACA GAG-3'
Y = ^{m}C, C, T, A, G
ODN 3 5'-AGA ^{z}**G**AC ^{m}**C**GG CGC ACA GAG-3'
ODN 4 5'-AGA GAC ^{m}**C**GG C^{z}**G**C ACA GAG-3'
ODN 5 5'-AGA GAC ^{m}**C**GG ^{m}**C**GC ACA GAG-3'
ODN 6 5'-CTC GTG CGC C-3'
ODN 7 5'-CTC GTG CGC C**NQ_2**-3'

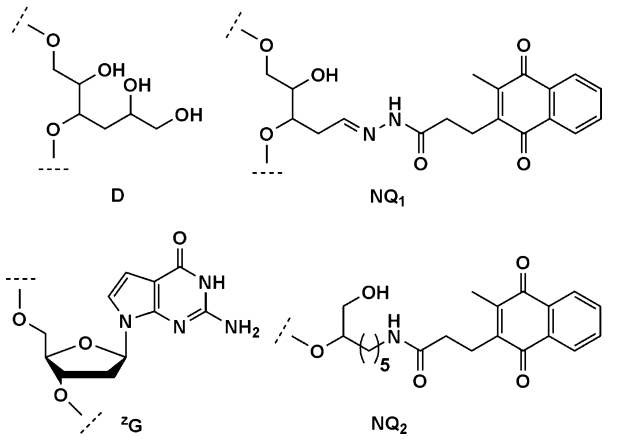
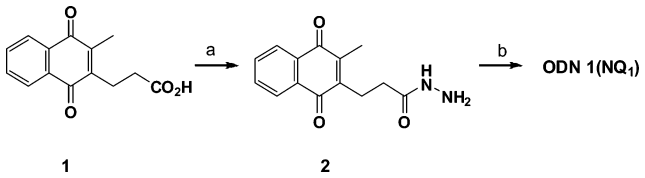


Figure 1. Structures and sequences of the oligodeoxynucleotides used in this study.

Scheme 1. Synthesis of Oligodeoxynucleotides Possessing a NQ Chromophore[a]



[a] Conditions: (a) Hydrazine, EDCI, HOBt, DMF, 60%; (b) ODN 1(D), 0.5 mM $NaIO_4$, 0.1 M NaOAc (pH 6.0).

The NQ-tethered ODN was hybridized with corresponding complementary DNA strand to form a duplex, the stability of which was determined by monitoring melting temperatures (T_m) in 2 mM sodium cacodylate buffer (pH 7.0) containing 20 mM NaCl. The ODN 1(D)/ODN 2(^{m}C) duplex containing a pair of abasic site and ^{m}C showed a lower T_m value by 14 °C than the reference duplex with a G-^{m}C base pair, probably due to partial absence of hydrogen bonding. The T_m value of ODN 1(NQ_1)/ODN 2(^{m}C) duplex was similar to that of ODN 1(D)/ODN 2(^{m}C) duplex, indicating that incorporation of the NQ chromophore could not hinder duplex formation. On the other hand, the circular dichroism spectrum showed a positive peak at 278 nm and a negative peak at 246 nm (Figure 2), indicating that the global structure of the ODN 1(NQ_1)/ODN 2(^{m}C) duplex was

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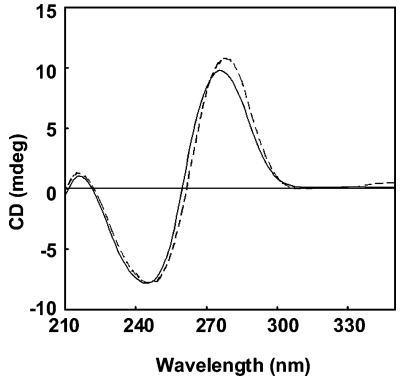

Figure 2. CD spectra of DNA duplexes (2.5 μM) consisting of ODN 1(G)/ODN 2(^{m}C) (solid line) or ODN (NQ_1)/ODN 2(^{m}C) (dashed line). All measurements were carried out in 2 mM sodium cacodylate buffer (pH 7.0) and 20 mM NaCl at 25 °C.

retained as a B-form as in the case of normal duplex ODN 1(G)/ODN 2(^{m}C).

We performed photosensitized one-electron oxidation of the modified ODN with the NQ chromophore at the interior of a strand for selective cleavage at the ^{m}C site in a partial sequence of human p53 gene corresponding to codons 280−285 of exon 8. In this study, we targeted the ^{m}C in codon 282 that has been marked as a methylation hot spot.[2a] Photoirradiation at λ_{ex} = 312 nm of the duplex consisting of ODN 1(NQ_1) with ^{32}P-5′-end-labeled ODN 2(C) or ODN 2(^{m}C), respectively, was carried out in sodium cacodylate buffer (pH 7.0) containing 20 mM NaCl at 0 °C in air, and the reaction was analyzed by polyacrylamide gel electrophoresis after treatment with hot piperidine. As shown in Figure 3, a specific strand cleavage occurred at ^{m}C in the ODN 1(NQ_1)/ODN 2(^{m}C) duplex after 2 h photoirradiation, whereas only a background level of strand cleavage at the corresponding normal C was observed for the ODN 1(NQ_1)/ODN 2(C) duplex. These results clearly indicate that photoexcitation of the NQ chromophore located at the interior of a strand can one-electron oxidize ^{m}C into its radical cation intermediate, which undergoes subsequent deprotonation and addition of oxygen[8a] to cause strand cleavage exclusively at ^{m}C. In a control photoreaction of a duplex consisting of ODN 1(D)/^{32}P-5′-end-labeled ODN 2(^{m}C) duplex consisting of an abasic site instead of the NQ chromophore, we could not observe a cleavage band at the target ^{m}C site, indicating that the photoirradiation of the NQ chromophore is responsible for the oxidative strand cleavage at ^{m}C.[13]

In contrast to terminal NQ-tethered ODNs reported previously,[9] it is remarkable that possible oxidative strand cleavage at G doublets, which may be induced by a hole transfer process, was suppressed to a substantial extent in the ODN 1(NQ_1)/ODN 2(^{m}C) duplex. Recently, Majima and co-workers have shown that the charge recombination rate between phenothiazine radical cation and naphthalimide radical anion on ODNs, which are separated by several A-T base pairs, decreased as the mutual distance increased.[14] Barton and co-workers also reported that no DNA damage occurred in spite of G oxidation by excited

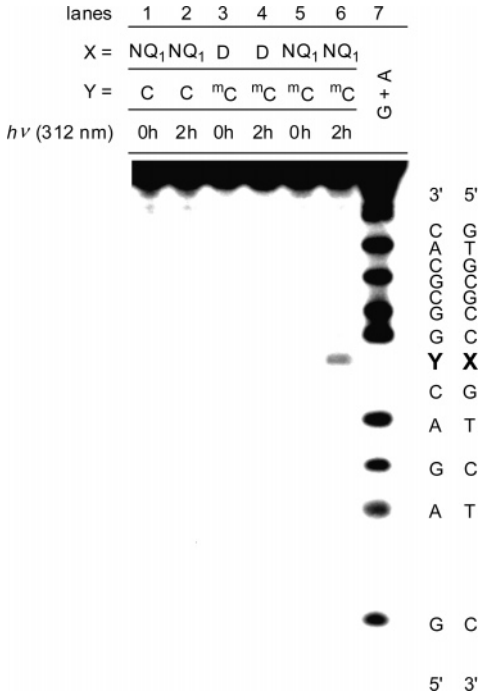

Figure 3. Representative autoradiogram of denaturing gel electrophoresis for ODN 1(X) and ^{32}P-5′-end-labeled ODN 2(Y) upon 312 nm photoirradiation for 0 h (lanes 1, 3, and 6) and 2 h (lanes 2, 4, and 6) in the presence of 20 mM NaCl in 2 mM sodium cacodylate buffer (pH 7.0) at 0 °C. After hot piperidine treatment (90 °C, 20 min), the samples were electrophoresed through a denaturing 15% polyacrylamide/7 M urea: lane 1 and 2, ODN 1(NQ_1)/ODN 2(C) duplex; lanes 3 and 4, ODN 1(D)/ODN 2(^{m}C) duplex; lane 5 and 6, ODN 1(NQ_1)/ODN 2(^{m}C) duplex; lanes 7, Maxam−Gilbert G+A sequencing lanes.

thionine linked with the DNA strand, because of rapid charge recombination that permits no net reaction.[15] In these contexts, it is not surprising that in the present experiment exclusive strand cleavage at ^{m}C occurred without competitive oxidative cleavage at G doublets upon NQ-sensitized photooxidation. As shown in Figure 4, the positive charge of the ^{m}C radical cation generated by NQ-sensitized one-electron photooxidation may transfer to the neighboring G bases to produce G radical cations. The resulting G radical cation is most likely to undergo charge recombination with the adjacent NQ radical anion, which may occur more rapidly than its irreversible reaction with oxygen or water leading to degradation and cleavage of the ODN strand. Thus, the presence of a rapid charge recombination process could suppress strand cleavage at G sites.[16] Otherwise a long-lived charge separation state of NQ-radical anion and G radical cation is created by an A-T bridge,[14] thereby leading to strand cleavage not only at ^{m}C but also at G, as reported in the previous paper.[9]

In competition with the positive charge-transfer process, the ^{m}C radical cation as generated by direct NQ-sensitized photooxidation of ^{m}C would be deprotonated and react irreversibly with molecular oxygen, thus forming an alkali labile oxidative product.[8c] Although the rate of deprotonation of the ^{m}C radical

(13) We also carried out the photoreaction of an equimolar mixture of ODN 1(NQ_1) and non-complementary ODN bearing the ^{m}C site (5′-AAAAGT^{m}-CGGTATATAGAG-3′). Although we carried out the photosensitized reaction for 2 h, the strand cleavage at ^{m}C was not observed (see Figure S1).

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(16) Although we have not yet confirmed any potential damage on the ODN bearing NQ chromophore, the amount of such ODN damage may be negligibly small in view of the fact that photoinduced primary intermediates of NQ radical anion and G radical cation undergo rapid charge recombination. It is however presumable that prolonged photoirradiation or location of hole trapping site such as 7-deazaguanine on the ODN strand may result in a strand damage.

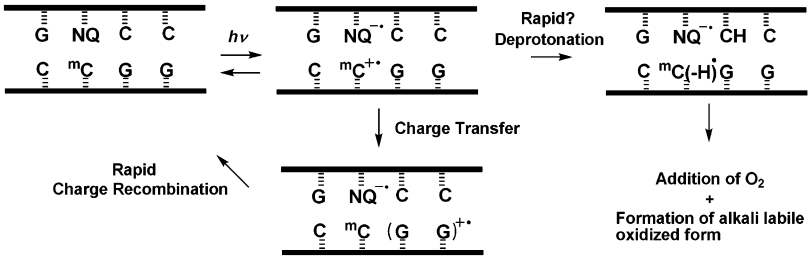

Figure 4. Plausible photochemical reaction mechanism for exclusive DNA strand cleavage at 5-^{m}C and charge-transfer process.

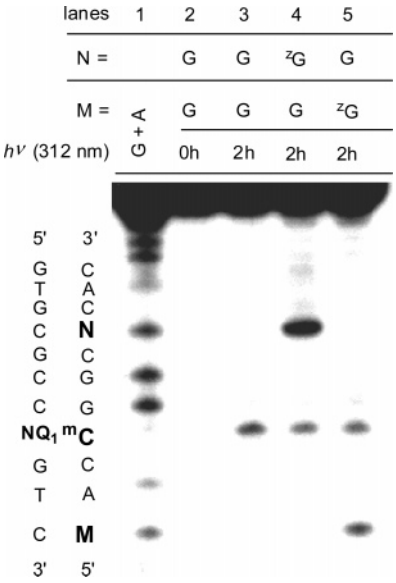

Figure 5. Representative autoradiogram of denaturing gel electrophoresis for ^{32}P-5′-end-labeled ODNs possessing ^{z}G unit and ODN 1(NQ$_1$) upon 312 nm photoirradiation for 0 h (lanes 2) and 2 h (lanes 3, 4, and 5) in the presence of 20 mM NaCl in 2 mM sodium cacodylate buffer (pH 7.0) at 0 °C. After hot piperidine treatment (90 °C, 20 min), the samples were electrophoresed through a denaturing 15% polyacrylamide/7 M urea: lane 1, Maxam−Gilbert G+A sequencing lanes; lanes 2 and 3, ODN 1(NQ$_1$)/ODN 2(^{m}C) duplex; lane 4, ODN 1(NQ$_1$)/ODN 4 duplex; lane 5, ODN 1(NQ$_1$)/ODN 3 duplex.

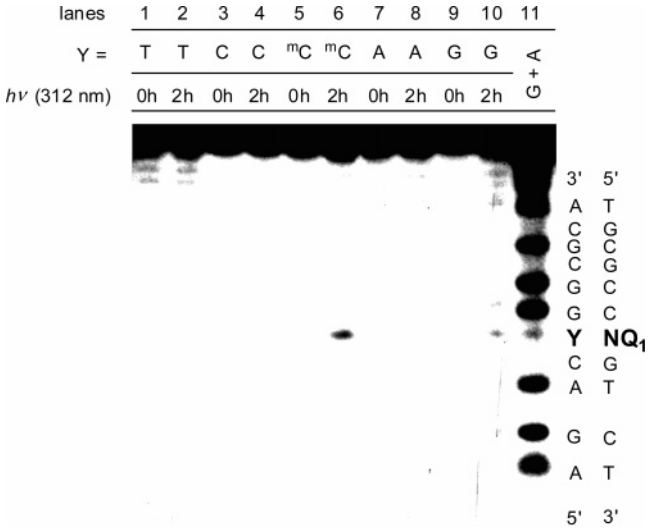

Figure 6. Representative autoradiogram of denaturing gel electrophoresis for ODN 1(NQ$_1$) and ^{32}P-5′-end-labeled ODN 2(Y) upon 312 nm photoirradiation for 0 h (lanes 1, 3, 5, 7, and 9) and 2 h (lanes 2, 4, 6, 8, and 10) in the presence of 20 mM NaCl in 2 mM sodium cacodylate buffer (pH 7.0) at 0 °C. After hot piperidine treatment (90 °C, 20 min), the samples were electrophoresed through a denaturing 15% polyacrylamide/7 M urea: lane 1 and 2, ODN 1(NQ$_1$)/ODN 2(T) duplex; lanes 3 and 4, ODN 1(NQ$_1$)/ODN 2(C) duplex; lane 5 and 6, ODN 1(NQ$_1$)/ODN 2(^{m}C) duplex; lanes 7 and 8, ODN 1(NQ$_1$)/ODN 2(A) duplex; lane 9 and 10, ODN 1(NQ$_1$)/ODN 2(G) duplex; lane 11, Maxam−Gilbert G+A sequencing lane.

cation into a methyl-centered radical is unknown, it is at least presumable that irreversible product formation from the ^{m}C radical cation may occur on a time scale region comparable to the charge-transfer process.

To confirm whether the positive charge-transfer process may be involved in the present base sequence, an attempt was also made to trap the migrated positive charge of radical cation by monitoring the degradation of 7-deazaguanine (^{z}G) as an efficient trap agent for the radical cation.[17] We prepared a duplex of ODN 3 and ODN 4 bearing ^{z}G instead of a G located far from the ^{m}C and NQ sites, which was photoirradiated at λ_{ex} = 312 nm in a similar manner. As shown in Figure 5, we observed intense spots corresponding to strand cleavages at ^{z}G as well as ^{m}C upon photoirradiation, indicating that the radical cation injected by NQ-sensitized photooxidation could migrate to ^{z}G and was therein trapped. In this light, the present photoreaction system potentially involves a positive charge-transfer process, while a rapid charge-recombination process would occur to suppress possible oxidative strand cleavage at the G doublet.

In separate experiments, we also pursued the NQ-sensitized photooxidation of duplexes with ODN 2(T), ODN 2(G), and ODN 2(A), in which T, G, and A bases are arranged, respectively, at the target site identical to ^{m}C in ODN 2(^{m}C) or C in ODN 2(C) (Figure 6). In contrast to the strong strand cleavage seen at ^{m}C, the replacement C, T, or A for the target ^{m}C did not undergo photooxidation to cause strand cleavage, whereas a slight amount of strand cleavage occurred at the consecutive Gs that are known to be more easily oxidized. Cadet and co-workers reported that dG is the most efficiently degradable nucleoside among the DNA constituents due to charge transfer in the NQ-sensitized photooxidation.[8b] The present observation implicates that DNA base radical cations were generated as the possible primary intermediates and their positive charge may migrate to the G doublet to produce the corresponding G radical cation, most of which undergoes charge recombination with the neighboring NQ radical anion to suppress overall strand cleavage. In addition, the suppressed strand cleavages at C and T may be explained in terms of the free energy change of charge separation (ΔG_{CS}). The ΔG_{CS} for photooxidation of C and T by NQ in the excited triplet state are evaluated as 0.04 and 0.18 eV, respectively, which are considerably smaller than the ΔG_{CS} value of 0.58 eV for ^{m}C,

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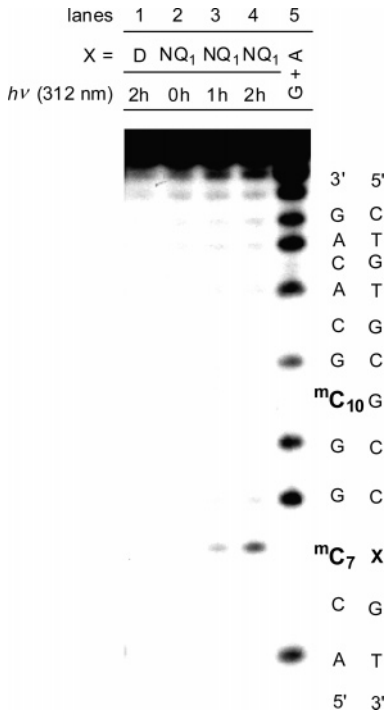

Figure 7. Representative autoradiogram of denaturing gel electrophoresis for ^{32}P-5′-end-labeled NQ-tethered duplex possessing dual 5-^{m}C site. Photoreactions (312 nm) of ODN 1(X)/ODN 5 duplex was carried out in the presence of 20 mM NaCl in 2 mM sodium cacodylate buffer (pH 7.0) at 0 °C. After hot piperidine treatment (90 °C, 20 min), the samples were electrophoresed through a denaturing 15% polyacrylamide/7 M urea: lanes 1, Maxam−Gilbert G+A sequencing lanes; lane 2, photoreaction of ODN 1(D)/ODN 5 duplex; lanes 3−5, photoreactions ODN 1(NQ_1)/ODN 5 duplex. ODNs in lanes 1−3 were photoirradiated for 0, 1, and 2 h, respectively.

i.e., the NQ-sensitized photogeneration of radical cations of C and T is more difficult than that of ^{m}C.[18]

An attempt was also made to demonstrate a sequence-selective cleavage at the ^{m}C site in DNA using ODN 5 with an ^{m}C residue at the dual site on the same partial sequence of p53 gene. We evaluated the photoreaction of ODN 1(NQ_1) in the presence of ODN 5. Figure 7 shows representative gel pictures of specific strand cleavage at the ^{m}C site, which were observed after 312 nm photoirradiation for 2 h at 0 °C. Reflecting the location of the NQ chromophore in the ODN 1(NQ_1)/ODN 5 duplex, exclusive strand cleavage occurred at $^{m}C_7$ that was in a position ahead of the NQ chromophore, while substantially no strand cleavage was observed at $^{m}C_{10}$ because of its position far from the NQ chromophore. These results assume crucial importance of a close interaction between the NQ chromophore and ^{m}C to induce site-selective strand cleavage of DNA and indicate that the NQ-sensitized photooxidation proposed herein can cleave DNA strand at a given target ^{m}C by matching base pairs in a sequence-selective manner.

To further evaluate the regional effect of the NQ chromophore on the sensitized photooxidative strand cleavage at ^{m}C, we also performed photoirradiation of a duplex of ODN 2(^{m}C) and ODN 7 with an NQ chromophore tethered to a flexible methylene linker at the strand end. As shown in Figure 8, along with strong strand cleavage at ^{m}C, a small amount of strand cleavage could be detected at the G doublet. It is likely that the charge-recombination process between the G radical cation and the NQ radical anion may become partially difficult to allow fixed charge separation to some extent, presumably due to the presence of a more flexible and longer methylene linker that increases in distance between the NQ chromophore and G in ODN 7. Thus, upon incorporation of the NQ photosensitizer into ODN, a more rigid linker structure should be employed so as to produce better contact of NQ with the target ^{m}C for exclusive strand cleavage at ^{m}C.

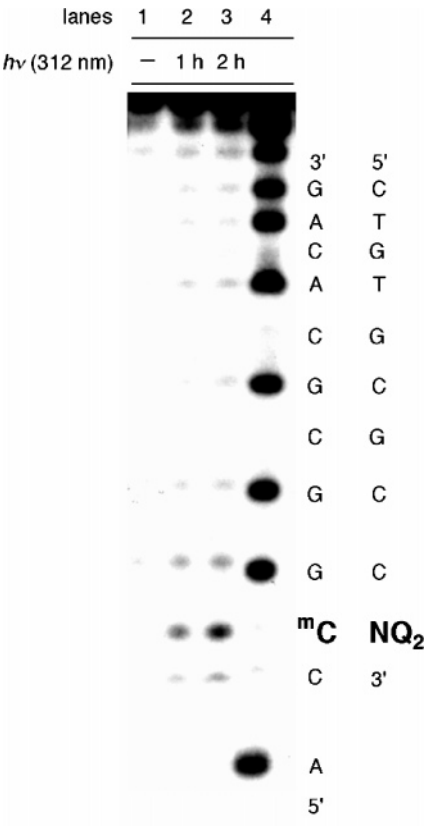

Figure 8. Autoradiogram of denaturing gel electrophoresis for ^{32}P-5′-end-labeled ODN 2(^{m}C) and ODN 7 upon 312 nm photoirradiation in the presence of 20 mM NaCl in 2 mM sodium cacodylate buffer (pH 7.0) at 0 °C. After hot piperidine treatment (90 °C, 20 min), the samples were electrophoresed through a denaturing 15% polyacrylamide/7 M urea. ODNs in lanes 1−3 were photoirradiated for 0, 1, and 2 h, respectively. Lane 4 shows Maxam−Gilbert G+A sequencing lane.

Table 1. Melting Temperature (T_m) of Duplexes with or without NQ Chromophore[a]

	ODN 1(G)/ ODN 2 (^{m}C)	ODN 1(D)/ ODN 2 (^{m}C)	ODN 1(NQ_1)/ ODN 2 (^{m}C)	ODN 6/ ODN 2 (^{m}C)	ODN 7/ ODN 2 (mC)
T_m (°C)	59.6	45.4	46.5	42.8	46.3

[a] All measurements of T_m were done in a buffer containing 20 mM NaCl, 2 mM sodium cacodylate at pH 7.0.

Conclusion

In summary, we have demonstrated a photochemical method for discrimination of a target ^{m}C in DNA, using a sensitizing NQ-tethered complementary ODN. We successfully established

(18) The free energy changes of charge separation (ΔG_{CS}) was estimated using Weller's equation (1): $\Delta G_{CS} = -(E_T + E_{rdn}) + E_{ox}$ (1) where E_T is the NQ triplet energy[18a] (2.52 eV), E_{rdn} is its reduction potential[18b] (−0.45 V vs SCE), and E_{ox} is the nucleobase oxidation potentials (^{m}C: 1.49 V, T: 2.11 V, C: 1.89 V vs SCE) which were calculated from ionizing potentials[18c] (^{m}C:[18d] 8.39 eV, T:[10c] 9.14, C:[10c] 8.87 eV). See (a) Melvin, T.; Bothe, E.; Schulte-Frohlinde, D. *Photochem. Photobiol.* **1996**, *64*, 769−776. (b)Wardman, P. *J. Phys. Chem. Ref. Data* **1989**, *18*, 1637−1755. (c) Burrows, C. J.; Muller, J. G. *Chem. Rev.* **1998**, *98*, 1109−1151. (d) Close, D. M. *J. Phys. Chem. B* **2003**, *107*, 864−867.

a discriminating method by which oxidative strand cleavage can be induced exclusively at a target ^{m}C in a partial sequence of the naturally occurring p53 gene by photosensitization of an NQ-tethered ODN in a sequence-selective manner. In practice, photoirradiation of a duplex formed between target ^{m}C-containing ODN and NQ-tethered complementary ODN gave rise to exclusive display of ^{m}C as a positive band on a sequencing gel. In the photochemical primary step, the target ^{m}C is most likely to be one-electron oxidized into the radical cation intermediate by the sensitization of NQ. The resulting ^{m}C radical cation may undergo rapid deprotonation and subsequent addition of molecular oxygen, leading to its degradation followed by strand cleavage at the target ^{m}C site. In contrast to ^{m}C-containing ODN, the ODN analog with replacement of C, T, A, or G at the ^{m}C site underwent less oxidative strand cleavage at the target base site, presumably because of charge-transfer and charge-recombination processes between the base radical cation and the NQ radical anion.

Although the present photochemical system would be a promising candidate for a convenient method of discriminating ^{m}C in DNA, the low sensitivity of the detection due to the low yield (ca. 5%) of ^{m}C selective strand cleavage still remains to be improved for establishment of a more general method with higher sensitivity. Our current study focuses on construction of a highly sensitive protocol, by which one can detect ^{m}C from quite a small amount of genomic DNA, using improved photofunctionalized ODNs.

Experimental Section

General Methods. Melting points were determined by a Yanagimoto micro melting point apparatus and are uncorrected. ^{1}H NMR spectra were measured with JEOL JMN-AL-300 (300 MHz) or JEOL JMN-AL-400 (400 MHz) spectrophotometers. Coupling constants (*J* values) are reported in hertz. The chemical shifts are expressed in ppm downfield from tetramethylsilane ($\delta = 0$) or residual chloroform ($\delta = 7.24$) as an internal standard. ^{13}C NMR spectra were measured with JEOL JMN-AL-300 (75 MHz) or JEOL JMN-AL-400 (100 MHz) spectrophotometers. Mass spectra were recorded on a JEOL JMS-SX102A spectrometer, using a nitrobenzyl alcohol matrix. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry of oligonucleotides was performed on a JEOL LMS-ELITE MALDI-TOF MASS spectrometer with 2′,3′,4′-trihydroxyacetophenone as the matrix. Wakogel C-300 was used for silica gel column chromatography. Precoated TLC plates (Merck silica gel 60 F_{254}) were used for monitoring reactions. The oligonucleotides were purchased from Invitrogen. The reagents for DNA synthesizer such as A, T, G, C, and abasic phosphoramidite were purchased from Glen Research. Calf intestinal alkaline phosphatase (AP), nuclease P1 (P1) and phosphodiesterase I were purchased from Promega, Yamasa, and ICN, respectively. High-performance liquid chromatography (HPLC) was performed with Shimadzu 6A or HITACHI D-7000 HPLC systems. Sample solutions were injected onto a reversed phase column (Inertsil ODS-3, GL Sciences Inc., ϕ 4.6 mm × 150 mm or 10 mm × 150 mm). The solvent mixture of 0.1 M triethylamine acetate (TEAA), pH 7.0, and 100% acetonitrile was delivered as the mobile phase at a flow rate of 0.6 or 3.0 mL/min at 25 °C. The column eluents were monitored by UV absorbance at 254 nm or 260 nm. Photoirradiation at $\lambda_{ex} = 312$ nm was carried out using a Lourmat TFX-20M transilluminator. Gel electrophoresis was carried out on a Gibco BRL Model S2 apparatus. [γ-^{32}P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase (10 units/μL) were obtained from Amersham Bioscience and Nippon Gene, respectively. ODN 7 possessing NQ at the strand end was synthesized as described previously.[9] All aqueous solutions were prepared using purified water (Yamato, WR600A).

3-(Hydrazinocarbonylethyl)-2-methyl-1,4-naphthoquinone 2. To a solution of 3-(3-methyl-1,4-naphthoquinon-2-yl)-propionic acid **1** in DMF (1 mL) were added 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide monohydrochloride (157 mg, 0.82 mmol) and 1-hydroxybenztriazole (110 mg, 0.81 mmol) at 0 °C, and the mixture was stirred for 30 min. To the reaction mixture was added hydrazine monohydrate (64 mg, 2 mmol in 0.2 mL of DMF), and the mixture was stirred for 2 h at ambient temperature. After dilution with water, the reaction mixture was extracted with ethyl acetate. The organic layer was washed by brine, dried over anhydrous $MgSO_4$, filtered, and then concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, 50% ethyl acetate/hexane) to give **2** (64 mg, 60%) as pale yellow solid: mp 182−183 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.00 (br, 1H), 8.06−7.96 8 m, 2H), 7.89−7.81 (m, 2H), 4.15 (br, 2H), 2.79 (t, $J = 7.5$ Hz, 2H), 2.19 (t, $J = 7.5$ Hz, 2H), 2.11 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 183.8, 170.3, 143.5, 135.4, 131.6, 126.1, 32.1, 26.8, 12.4; FABMS (NBA) *m*/*z* 259 [(M + H)$^{+}$]; HRMS calcd for $C_{14}H_{15}N_2O_3$ [(M + H)$^{+}$], 259.1083, found 259.1082.

Synthesis of Oligodoxynucleotides Possessing a Triol Group (ODN 1(D)). Synthesis of oligodeoxynucleotides was performed on an Applied Biosystems Model 392 DNA/RNA synthesizer by using standard phosphoroamidite chemistries. We used abasic phosphoramidite purchased from Glen Research for DNA synthesis to introduce a triol group into oligomers. After automated DNA synthesis, the oligomers were purified by reversed phase HPLC. Dried oligomers were resuspended in 200 μL of 80% acetic acid and incubated for 30 min at room temperature. After 30 min, to the reaction mixture was added an equal volume of deionized water, and the mixture was incubated for additional 4 h at room temperature to remove TBDMS protection. The fully deprotected oligomers were repurified by reversed phase HPLC. The purity and concentration of the oligomers were determined by complete digestion with AP, P1, and phosphodiesterase I at 37 °C for 4 h. Identities of synthesized oligomers were confirmed by MALDI-TOF mass spectrometry (ODN 1(D): calcd 5331.43, found 5331.40).

Incorporation of Naphthoquinone Chromophore into Oligodeoxynucleotides (ODN 1(NQ$_1$)). A mixture of 100 μM ODN 1(D) and 0.5 mM sodium periodate in 0.1 M NaOAc (pH 6.0, 80 μL) was incubated for 1 h at 4 °C in the dark. The reaction mixture was added to a solution of **2** in acetonitrile (5 mM, 80 μL) and then incubated for 14 h at ambient temperature. The reaction mixture was purified by reversed phase HPLC to give ODN 1(NQ$_1$). The purity and concentration of ODN were determined by complete digestion by AP, P1, and phosphodiesterase I at 37 °C for 12 h. Identities of synthesized ODNs were confirmed by MALDI-TOF mass spectrometry (ODN 1(NQ$_1$): calcd 5539.64, found 5539.25).

Melting Temperature (T_m) of Hybridized ODNs. A 1.5 μM solution of the appropriate ODN was dissolved in 2 mM sodium cacodylate buffer (pH 7.0) containing 20 mM NaCl. Melting curves were obtained by monitoring the UV absorbance at 260 nm with elevating temperature from 2 °C to 90 °C at a rate of 1 °C/min.

CD Spectrum. A 2.5 μM solution of duplex was dissolved in 2 mM sodium cacodylate buffer (pH 7.0) containing 20 mM NaCl. CD spectra of the solution were recorded at 25 °C on a JASCO J-700 spectrophotometer equipped with a Peltier temperature controller, using a UV cell with a 1 cm path length.

Preparation of 5′-^{32}P-End-Labeled ODNs. ODNs (400 pmol strand concentration) were labeled by phosphorylation with 4 μL of [γ-^{32}P]-ATP and 4 μL of T4 polynucleotide kinase using standard procedures.[19,20] The 5′-end-labeled ODNs were recovered by ethanol precipitation and further purified by 15% nondenaturing gel electrophoresis and isolated by the crush and soak method.[21]

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(21) Sambrook J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Plainview, NY, 1989.

Photooxidative Cleavage of ODNs. ^{32}P-5′-end-labeled ODNs (<400 nM strand concentration) were hybridized by their complementary ODNs possessing the NQ chromophore (500 nM) in 2 mM sodium cacodylate buffer (pH 7.0) containing 20 mM NaCl. Hybridization was achieved by heating the sample at 90 °C for 5 min and slowly cooling to room temperature. The ^{32}P-5′-end-labeled duplex was irradiated at 312 nm with a transilluminator at 0 °C. After irradiation, all reaction mixtures were precipitated with addition of 10 μL of herring sperm DNA or salmon sperm DNA (1 mg/mL), 10 μL of 3 M sodium acetate, and 800 μL of ethanol. The precipitated DNA was washed with 100 μL of 80% cold ethanol and then dried *in vacuo*. The precipitated DNA was resolved in 50 μL of 10% piperidine (v/v), heated at 90 °C for 20 min, and concentrated. The radioactivity of the samples was assayed using an Aloka 1000 liquid scintillation counter, and the dried DNA pellets were resuspended in 80% formamide loading buffer (a solution of 80% formamide (v/v), 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). All reactions, along with Maxam−Gilbert G + A sequencing reactions, were heat-denatured at 90 °C for 3 min and quickly chilled on ice. The samples ($3-5 \times 10^3$ cpm) were loaded onto 15% of polyacrylaamide/7 M urea sequencing gels and electrophoresed at 1900 V for 60−90 min, transferred to a cassette, and stored at −80 °C with Fuji X-ray film (RX-U).

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Supporting Information Available: Autoradiograms of a denaturing gel showing the photoreactions of ODN 1(NQ_1) and its noncomplementary ODN possessing ^{m}C site. This material is available free of charge via the Internet at http://pubs.acs.org.

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