

# Locating the uracil-5-yl radical formed upon photoirradiation of 5-bromouracil-substituted DNA

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## ABSTRACT

In a previous study, we found that 2-deoxyribonolactone is effectively generated in the specific 5-bromouracil (<sup>Br</sup>U)-substituted sequence 5'-(G/C)[A]<sub>n=1,2</sub><sup>Br</sup>U<sup>Br</sup>U-3' and proposed that a formed uracil-5-yl radical mainly abstracts the C1' hydrogen from the 5'-side of <sup>Br</sup>U<sup>Br</sup>U under 302-nm irradiation condition. In the present work, we performed photoirradiation of <sup>Br</sup>U-substituted DNA in the presence of a hydrogen donor, tetrahydrofuran, to quench the uracil-5-yl radical to uracil and then subjected the sample to uracil DNA glycosylase digestion. Slab gel sequence analysis indicated that uracil residues were formed at the hot-spot sequence of 5'-(G/C)[A]<sub>n=1,2</sub><sup>Br</sup>U<sup>Br</sup>U-3' in 302-nm irradiation of <sup>Br</sup>U-substituted DNA. Furthermore, we found that the uracil residue was also formed at the reverse sequence 5'-<sup>Br</sup>U<sup>Br</sup>U[A]<sub>n=1,2</sub>(G/C)-3', which suggests that both 5'-(G/C)[A]<sub>n=1,2</sub><sup>Br</sup>U<sup>Br</sup>U-3' and 5'-<sup>Br</sup>U<sup>Br</sup>U[A]<sub>n=1,2</sub>(G/C)-3' are hot-spot sequences for the formation of the uracil-5-yl radical.

## INTRODUCTION

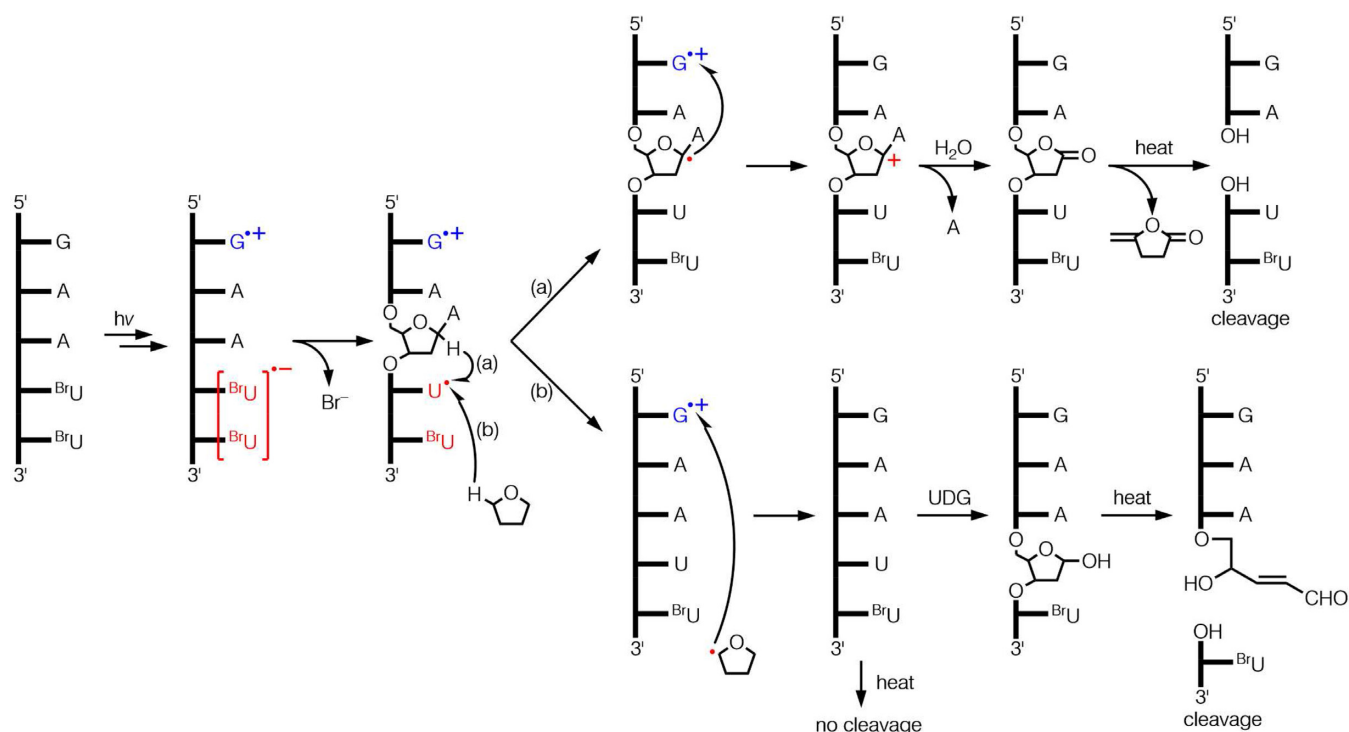
The migration of negative and positive charges (excess electrons and holes) in DNA has recently attracted considerable research interest in the fields of both biology and engineering. For example, hole transfer in DNA may promote oxidative DNA damage from a remote site, which may define mutation hot-spots in the genome (1–3). Electron transfer in DNA is used during the repair of UV-induced cyclobutane pyrimidine dimers in DNA, in which electron transfer from FADH<sup>-</sup> to the dimer lesion is crucial for DNA repair by photolyase (4,5). In engineering applications, charge transfer in DNA is expected to be used for the development of DNA-based electronic nanodevices (6–9).

5-Bromouracil (<sup>Br</sup>U) is a photoreactive analogue of thymine that has been used as an electron acceptor in probes for electron transfer because of the generation of

reactive uracil-5-yl radicals. Thymine in DNA can be replaced with <sup>Br</sup>U easily and the replacement results in almost intact functional properties of thymine even in living cells. Therefore, <sup>Br</sup>U-substituted DNA has been used as a probe to reveal details of a number of protein–DNA interactions (10–18). We have examined the photoreactivity of <sup>Br</sup>U-substituted DNA and reported that uracil-5-yl radicals are effectively generated in 5'-(G/C)[A]<sub>n=1,2</sub><sup>Br</sup>U<sup>Br</sup>U-3' sequences in double-strand DNA, resulting in the selective formation of 2-deoxyribonolactone residues. These sequences are referred to as a 'hot-spot sequence of <sup>Br</sup>U-substituted DNA'. Charge transport is essential for photoreaction of <sup>Br</sup>U because G has the lowest oxidation potential among A, T, G and C bases. In our earlier study, we demonstrated that G was the electron donor in the hot-spot sequence (19).

The proposed photoreaction mechanism at the 5'-GAA<sup>Br</sup>U<sup>Br</sup>U-3' sequence in double-strand DNA is shown in Scheme 1 (a). Initially, electron transfer through the  $\pi$ -stack from G occurs upon 302-nm UV irradiation and the electron is trapped by <sup>Br</sup>U<sup>Br</sup>U to form an anion radical. Release of Br<sup>-</sup> generates the uracil-5-yl radical, which then abstracts mainly the C1' hydrogen from the adjacent 2'-deoxyadenosine residue at the 5'-side. The C1' radical is then oxidized to the C1' cation by the electron-donated radical cation residue G<sup>•+</sup>. It has been suggested that trapping of G<sup>•+</sup> by O<sub>2</sub> is very slow (in the order of milliseconds), which may explain why reduction of G<sup>•+</sup> through back electron transfer from the C1' radical is favored over oxidation of G after charge separation in the hot-spot sequence (19). After regeneration of G, reaction of the C1' cation with H<sub>2</sub>O generates 2-deoxyribonolactone with concomitant release of adenine (19). The sites of 2-deoxyribonolactone in the DNA sequence were detected as DNA cleavage bands after facile thermal degradation. The abstraction of hydrogen by the uracil-5-yl radical is atom-specific and is highly dependent on the conformation of the DNA, which can form structures such as A-form, B-form, Z-form, bent DNA and G-quadruplexes (20). In contrast, in the presence of a hydrogen donor, the forming uracil-5-yl radical is quenched to produce uracil as shown in Scheme 1 (b). The sites of

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Scheme 1.

uracil formation could be detected as DNA cleavage bands after reaction with uracil DNA glycosylase (UDG) and subsequent heat treatment.

In the present work, we analyzed 302-nm irradiated  $^{Br}U$ -substituted DNA by polyacrylamide gel electrophoresis (PAGE) and developed a method for detecting the region in which a uracil-5-yl radical formed. The method was also effective in detecting quenched radicals with an excess of hydrogen donor, such as tetrahydrofuran (THF), treating the sample with UDG. The present method provides an alternative way to locate the uracil-5-yl radical in 302-nm irradiated  $^{Br}U$ -substituted DNA and provides further insight into the hot-spot sequence.

## MATERIALS AND METHODS

### Preparation of primers and DNA templates

In this experiment, two DNA fragments containing 298 and 383 bp were constructed using pUC18 and pGEM-3z/601 plasmids vectors respectively to perform photoreaction. Primers were synthesized for pUC18 sequence; forward primer: 5'-GCA GGT CGA CTC TAG AGG AT, reverse primer: 5'-GAG TCA GTG AGC GAG GAA G and for 601 sequence; *forward primer*: 5'-TAA TAC GAC TCA CTA TAG GG, reverse primer: 5'-ATT TAG GTG ACA CTA TAG. In addition, Texas-Red-labeled primers which have the same sequences were also used. All of the primers were purchased from Sigma-Aldrich.

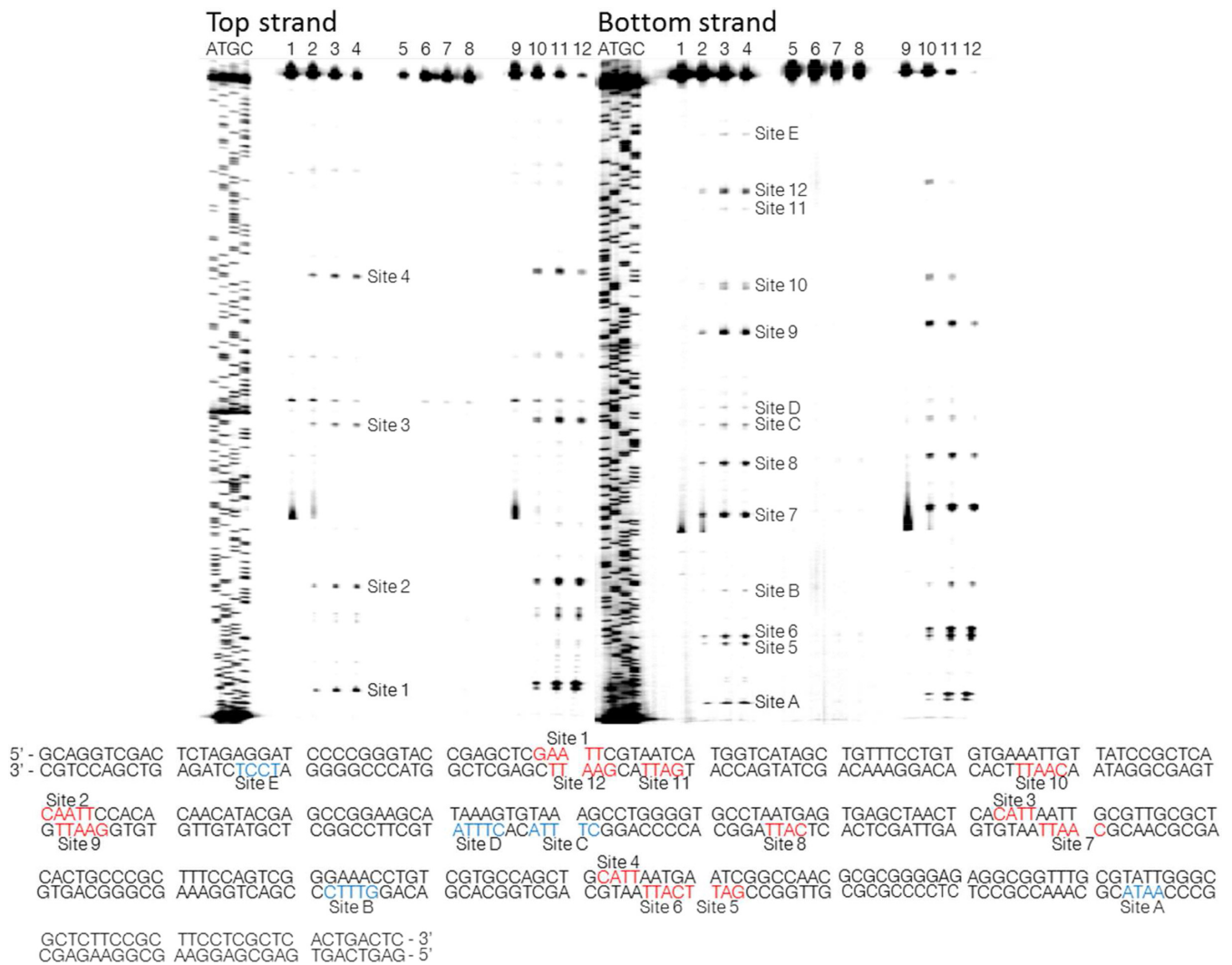
### Preparation of $^{Br}U$ substituted DNA fragment by PCR

Master mix recipe per polymerase chain reaction (PCR) consisted of 20  $\mu$ l of 10x buffer (500-mM KCl, 100-mM

Tris-HCl, 25-mM  $MgCl_2$ , pH 8.3), 20  $\mu$ l each of 2-mM dATP, dGTP, dCTP and d $^{Br}UTP$ , 6  $\mu$ l each of 10  $\mu$ M forward and reverse primers, 2  $\mu$ l of 10-U *Taq* DNA Polymerase, 50 ng of DNA template and Milli-Q water (total reaction volume was 200  $\mu$ l). We used Texas-Red-labeled forward primer and non labeled reverse primer for preparing top strand Texas-Red-labeled DNA and in the case of bottom strand labeled DNA reverse is the case. Amplification conditions were as follows: 95°C at 2 min (denaturing and heat activation of *Taq* DNA Polymerase); 30 cycles of (i) 95°C 20 s, (ii) 50°C 30 s and (iii) 68°C 30 s (annealing and extension); 68°C 5 min (final extension); cool at 12°C (end of amplification). Products were purified by GenElute™ PCR Clean-Up Kit (Sigma-Aldrich), and confirmed by 2% agarose gel. The purified samples were quantified by Nano Drop 1000 (Thermo Fisher Scientific).

### DNA 302-nm photoreaction

Each of the following reaction mixture contained 10-nM  $^{Br}U$  DNA fragment and 10-mM sodium cacodylate buffer (pH 7.0). THF(-)UDG(-) reaction mixture did not contain THF, THF(+)/UDG(-/+ ) reaction mixture contained 500-mM THF. The volume of each sample was 13  $\mu$ l. Irradiation was performed using a 3UV™ Transilluminator (UVP) at 0°C for 0, 1, 3 and 5 min, with 302 nm under aerobic condition. The distance between UV lamp and photoreaction mixture was 10 cm. After 302-nm irradiation, 1.5  $\mu$ l of 10x UDG buffer (200 mM of Tris-HCl, 10-mM ethylenediaminetetraacetic acid (EDTA), 10-mM dithiothreitol (DTT), pH 8.0) and 1.25 U of UDG were added to the THF(+)/UDG(+) reaction sample and incubated at 37°C for 1 h. Finally all reaction mixtures were



**Figure 1.** Results of slab gel sequence analysis of DNA fragment (298 bp) after photoirradiation and heat treatment (95°C, 10 min). Lanes 1–4: THF(–)UDG(–) samples were irradiated for 0, 1, 3 and 5 min. Lanes 5–8: THF(+)UDG(–) samples were irradiated for 0, 1, 3 and 5 min. Lanes 9–12: THF(+)UDG(+) samples were irradiated for 0, 1, 3 and 5 min.

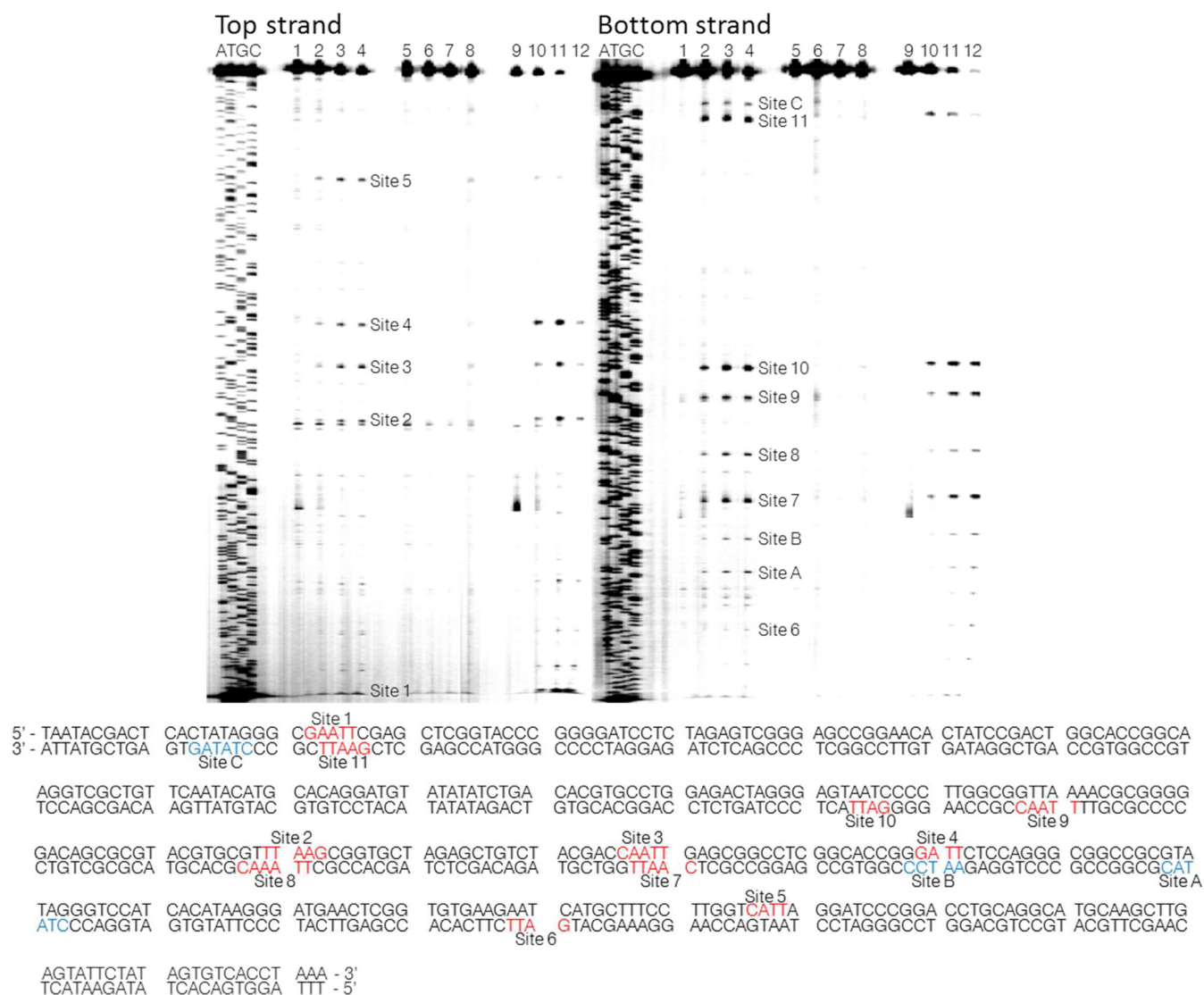
dried using a vacuum and 8 μl of Loading Dye (loading dye was prepared using 300 μl of 0.5 M EDTA, 200 μl of Milli-Q water, 10 ml of formamide and 2.5 mg of New fuchsin) were added to each sample and heated at 95°C for 10 min.

### Analysis using denaturing polyacrylamide gel electrophoresis

DNA sequence ladder was prepared by Thermo Sequenase Dye Primer Manual Cycle Sequencing Kit. DNA photoreaction samples were analyzed with sequence ladder by the slab gel sequencer, SQ5500E (HITACHI), filled with denaturing polyacrylamide gel. A total of 1.4 μl of loading samples were applied for the sequencing. Capillary sequencer, 3130xL Genetic Analyzer (Applied Biosystems), also can be used for the analysis instead of slab gel sequencer (Supporting Information S8).

### RESULT AND DISCUSSION

Two different <sup>Br</sup>U-substituted DNA fragments (298 and 383 bp) were used for photoreaction. These were prepared by PCR under usual PCR conditions except that d<sup>Br</sup>UTP was used instead of 2-deoxythymidine triphosphate (dTTP). The results after photoirradiation of the top and bottom strand of the pUC18 fragment (298 bp) sequence are shown in Figure 1. Consistent with previous results, bands arising from selective cleavages were observed after 302-nm UV irradiation and subsequent heat treatment (95°C for 10 min). Slab gel sequence analysis (lanes 1–4) revealed DNA fragments that corresponded to cleavage at four hot-spot sequences (sites 1–4) in the top strand and eight hot-spot sequences (sites 5–12) in the bottom strand (lane 1–4). In the presence of 500-mM THF almost all the cleaved fragment bands disappeared, suggesting that most of uracil-5-yl radicals were quenched by THF under these conditions (lanes 5–8). However, after treatment with UDG and subsequent heating at 95°C for 10 min, bands corresponding



**Figure 2.** Results of slab gel sequence analysis of DNA fragment (383 bp) containing 601 sequence after photoirradiation and heat treatment (95°C, 10 min). Lanes 1–4: THF(–)UDG(–) samples were irradiated for 0, 1, 3 and 5 min. Lanes 5–8: THF(+ )UDG(–) samples were irradiated for 0, 1, 3 and 5 min. Lanes 9–12: THF(+ )UDG(+ ) samples were irradiated for 0, 1, 3 and 5 min.

to cleavage at the hot-spot sequence appeared (lanes 9–12). Because abasic sites are known to be cleaved upon heating (21), these results indicate that such sites are formed within the hot-spot sequence by the action of UDG. We also found some additional cleavages at sites (sites A–E) which are not from the exact hot spot sequence that we proposed. The bands appeared after the cleavage at lanes 9–12 was found to be more intense than that at lane 1–4. The reason for the different intensity bands in both cases could be explained by the selectivity of the uracil-5-yl radical. For instance, uracil-5-yl radical is also known to competitively abstract C2'- $\alpha$  hydrogen to form erythrose-containing sites, which only undergo DNA cleavage upon heating under alkaline conditions (22,23). The amount of thermal cleavage of 2-deoxyribonolactone is less than the amount of uracil-5-yl radical form in total. Thus, the present method of quenching with THF prevents the uracil-5-yl radical to abstract hydrogen from either C1' or C2'- $\alpha$  and subsequent UDG

treatment give only heat labile abasic site resulting higher amount of cleavage.

To investigate the effect of THF and oxygen in the hot spot sequences under the photoirradiation condition, we irradiated a self-complementary DNA, 5'-CGAA<sup>Br</sup>U<sup>Br</sup>UCCG-3', in the presence or absence of THF, under air or argon and analyzed the irradiated samples by high performance liquid chromatography (HPLC). It was found that the uracil radicals generated upon photoirradiation were completely quenched via abstraction of H atom from THF present in excess amount rather abstracting H atom from its nearest nucleotide. As a result dehalogenated products, uracil, were generated instead of 2-deoxyribonolactone (Supplementary Data S1–3). While investigating the other parameters such as the effect of air or argon we obtained almost same HPLC profiles from photoirradiation under air and argon (Supplementary Data S4). These data suggests that oxygen in air does not have

much effect in this reaction. We also digested photoirradiated DNA samples to nucleosides by using P1 nuclease and Antarctic Phosphatase and traced the amount of oxidized guanine (Supplementary Data S4–7). Interestingly, 8-oxo-G was not detected in the presence of THF while a little amount of guanine radical cation became 8-oxo-G in the absence of THF in the photoirradiated DNA sample. These results also suggest that THF can quench guanine radical cation and reduce the generation of oxidized guanine.

We also analyzed the photoreaction of the DNA fragment (383 bp) containing 601 sequence as shown in Figure 2. This DNA fragment contains eight hot-spot sequences. Interestingly, in addition to these predicted sites, six additional sites were also found (Site 2, 8, 9 and Site A–C). The presence of Site 2, 8 and 9 confirmed that the 5'-BrU<sup>Br</sup>U[A]<sub>n=1,2,3</sub>(G/C)-3' motif can also work as a hot-spot sequence, referred as reverse hot spot sequences. However, the sites A–C are not from the exact hot spot sequences. In order to investigate the property of the motif under photoirradiation condition, we synthesized self-complementary DNA, 5'-GC<sup>Br</sup>U<sup>Br</sup>UAAGC-3' and analyzed through HPLC after photoirradiation with 302 nm. It was found that the irradiation mainly produced 5'-GCLUAAGC-3' (L denotes 2-deoxyribonolactone) from 5'-GC<sup>Br</sup>U<sup>Br</sup>UAAGC-3' (Supplementary Data S9–12). This result revealed that the reverse sequence can also produce 2-deoxyribonolactone by UV-irradiation and can be cleaved by heating after the irradiation. These results show that electron transfer through the hot-spot sequence upon photoirradiation of BrU-substituted DNA does not have 5'–3' or 3'–5' directionality.

## CONCLUSION

We analyzed two DNA fragments (298 and 383 bp) to locate uracil-5-yl radical upon 302-nm irradiation by using slab gel sequencer and capillary sequencer. In both cases, all thymine residues were replaced with BrU. By using a hydrogen donor, THF and subsequent UDG treatment, we found that, in addition to the known hot-spot sequence 5'-(G/C)[A]<sub>n=1,2</sub>BrU<sup>Br</sup>U-3', the reverse sequence 5'-BrU<sup>Br</sup>U[A]<sub>n=1,2</sub>(G/C)-3' also behaved in a similar way upon irradiation. Because an enormous range of proteins interact with DNA *in vivo* and there are some specific sites of particular interest, the present results may provide important information on the interface between nucleic acids and protein–DNA interactions.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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