Pathways of excess electron transfer in phenothiazine-tethered DNA containing single-base mismatches†

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The effects of local structural disorder on excess electron transfer (EET) in DNA were investigated by evaluating photoinduced electron transfer in phenothiazine (PTZ)-modified oligodeoxynucleotides bearing single-base mismatches. Unexpectedly, more efficient electron transfer was observed for the mismatched duplexes than for the complementary DNA, suggesting that distraction of hydrogen bond interaction at the mismatch site enables electron injection or hopping beyond the mismatch sites. It was also anticipated that water accessibility of the mismatched nucleobases could affect EET because protonation of the electron-captured pyrimidine intermediates became competitive to EET, especially at the mismatch sites.

Introduction

Efficient and high-throughput detection of single nucleotide polymorphisms (SNPs) in DNA has been explored intensively in the past decade, because SNPs are important markers in the diagnosis of particular genetic diseases. Among the new technologies for detecting SNPs, charge transfer-based methods for discriminating normal from mismatched base pairs in DNA have been developed recently.[1,2] Duplex DNA forms well π-stacked nucleic acid bases inside the helix, and the structural disorder induced by mismatched bases is believed to affect the long-range hole transfer through the π-way.[3] Based on the charge transfer property, several types of sensitive electrochemical methods have been reported for discriminating SNP-containing DNA so far.[2] For example, redox-active intercalators bound to DNA modified on gold surfaces have been developed for detecting a single-base mismatch in target DNA by monitoring electrochemical signals.[2a,b] Majima and coworkers recently reported a strategy for the optical detection of SNPs on a single-molecule basis, in which the bleaching of the DNA-bound fluorescent probe can be detected as a signal of hole transfer in target DNA.[2d]

It has been suggested that the electron transfer efficiency through the LUMOs (excess electron transfer, EET) of the DNA duplex could also be sensitive to structural perturbation.[4] So far, several types of photo-induced electron donors, including flavins,[5] stilbene derivatives,[6] diaminonaphthalenes,[7] phenothiazine (PTZ),[8] and pyrene derivatives[9] have been synthesized for the chemical detection of EET in DNA. The mechanism of EET through DNA base pairs is not understood fully, but it seems that the reaction proceeds predominantly by a thermally activated hopping mechanism between the base pairs, especially pyrimidine bases at ambient temperatures.[10] In this context, we previously synthesized PTZ-tethered DNA containing 5,6-dihydroxy-5,6-dihydrothymidine (thymidine glycol, Tg) as a major damaged DNA structure and demonstrated that excess electrons could be transported beyond the A/Tg site.[8b] The result could be explained by low electron affinity of Tg, relatively long lifetime of the corresponding radical anion, and flexibility of the duplex structures. Because of our interest in the potential application of this type of DNA-electron donor conjugates to a SNP probe and in understanding the effect of modified DNA bases on the EET efficiency, we have prepared internally and 5′-teminally PTZ-tethered DNA duplexes containing mismatched base pairs and have studied their EET properties. As described below, we found that this type of probe shows distinctive signals for single-base mismatches that are dependent on both the structural stability of DNA and the redox properties of the nucleobases at the mismatch site.

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Results and discussion

Internally PTZ-tethered oligodeoxynucleotides

To incorporate PTZ into duplex DNA, we prepared oligodeoxynucleotides (ODNs) containing the UniLink amino modifier in the middle of the sequences. Wagner and Wagenknecht have reported recently on the synthesis of similar PTZ conjugates based on phosphoramidite chemistry.[8a,b] Also, Majima and coworkers have developed DNA assemblies containing PTZ as a hole acceptor.[8d,e] In this study, we employed a postsynthetic strategy, in which the amino linker and succinimidyl ester of PTZ were coupled under mild conditions, to avoid unwanted oxidation of PTZ during the DNA synthesis. Succinimidyl ester of PTZ was synthesized as reported previously[8b] and incubated in acetonitrile solution in the presence of amino-modified ODNs (Chart 1). 5-Bromo-2′-deoxyuridine (^{Br}U) was inserted as an electron acceptor.

Because PTZ has an absorption band in the wavelength range longer than 330 nm, selective excitation of PTZ without excitation of ^{Br}U (absorption band: $\lambda < 320$ nm) is possible. Upon photoexposure of the duplexes to UV light (365 nm) at 4 °C, electrons are injected from PTZ and trapped irreversively by ^{Br}U, generating a uracil-5-yl radical as a consequence of bromide

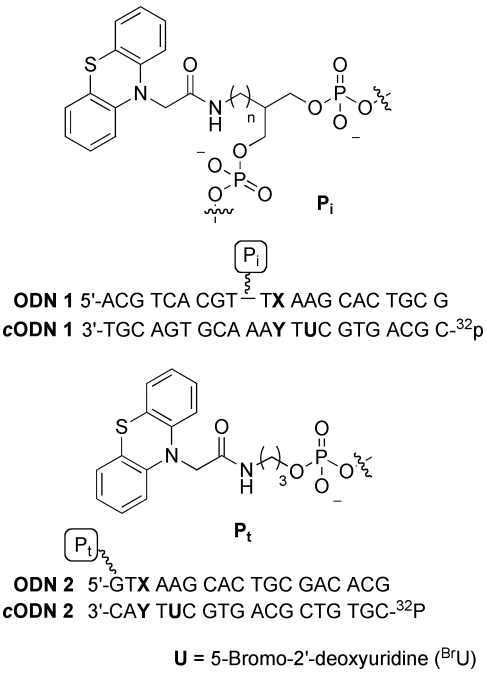


Chart 1 Structures of the oligodeoxynucleotides synthesized in this study.

anion release. The uracilyl radical abstracts hydrogen from the 5′-adjacent deoxyribose to form alkaline-labile structures; thus, electron trap by ^{Br}U could be detected as the DNA fragmentation after incubation in the presence of hot piperidine (Fig. 1).[11]

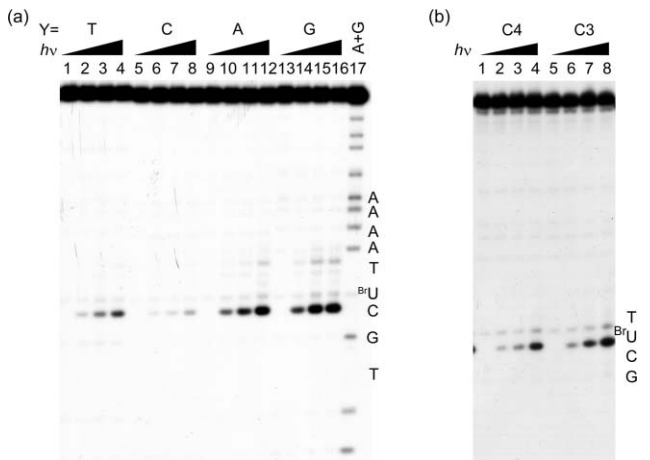


Fig. 1 (a) Gel electrophoresis images of photo-irradiated ODN 1/*c*ODN 1 [X = A; Y = T (lanes 1–4), C (lanes 5–8), A (lanes 9–12), and G (lanes 13–16)] after piperidine treatment. Duplex DNA (1 μM) in phosphate buffer (10 mM phosphate, 90 mM NaCl, pH 7.0) was photo-irradiated (365 nm, 0–6 h) under a N_2 atmosphere at 4 °C. (b) Effect of linker length of the electron donor [C4 linker (lanes 1–4) and C3 linker (lanes 5–8)] on EET in ODN 1/*c*ODN 1 (X = A, Y = T).

The thermal melting behaviours of the duplexes ODN 1/*c*ODN 1 were investigated by increasing temperature from 5 °C to 80 °C (Table 1). Introduction of the amino linker reduced the thermal stability of the duplex, but slight stabilization was observed after the PTZ modification, which implies that PTZ can intercalate between the base pairs because of the aromaticity of the PTZ ring.

Table 1 Electron transfer yields as evaluated by the strand cleavage at the 5′-adjacent base of ^{Br}U and melting points (T_m) of the duplexes ODN 1/*c*ODN 1[a]

X/Y	Strand Cleavage[b] (% h^{-1})	T_m/°C
A/G	10.2	60.1
A/A	6.6	59.3
A/T	1.8	63.7
A/C	1.0	59.5

[a] 1 μM DNA, 10 mM phosphate buffer (pH 7.0), 90 mM NaCl. [b] Each analysis was repeated at least three times. Initial strand cleavage rates were obtained by fitting cleavage curves (% scission product plotted against total material) to first-order exponential curves.

^{32}P-Radiolabeled *c*ODN 1 was hybridized with the PTZ conjugate ODN 1, and photoexposed at 365 nm under anoxic conditions, and the alkali-treated samples (90 °C, 20 min) were subjected to polyacrylamide gel electrophoresis (PAGE) analysis. A major strand cleavage in *c*ODN 1 was observed at the 5′-adjacent C to ^{Br}U. The intensity of the corresponding fragment bands should correlate with the amount of electrons captured by ^{Br}U. To investigate the effects of mismatched bases inserted between PTZ and the acceptor ^{Br}U, the photoreactivity of ODN 1/*c*ODN 1 was quantified by varying the base Y at the counter position of X (= A) (Fig. 1). Table 1 shows the strand cleavage efficiencies for duplex ODN 1/*c*ODN 1. The purine-purine mismatch base pair at X/Y seemed to have greater electron transfer efficiency compared with the complementary (X/Y = A/T) and pyrimidine-purine mismatch (X/Y = A/C) base pairs. Our previous studies showed that EET through the damaged base structure Tg occurs in a good yield [5.0% h^{-1} for duplex ODN 1/*c*ODN 1 (X/Y = A/Tg)], which may be ascribed to the low electron affinity of Tg and structural fluctuation induced by the mismatch.[8b] Such local structural changes might increase the direct electron transfer from PTZ to ^{Br}U. To investigate the effects of the linker length, we synthesized an alternative duplex ODN 1/*c*ODN 1 (X/Y = A/T) with a shorter linker (Chart 1, n = 3), which might reduce the chance of direct electron transfer. As shown in Fig. 1b (C3 linker *vs.* C4 linker), however, the duplex with the shorter linker had almost the same electron transfer efficiency.[12] This suggests that direct electron transfer between photoexcited PTZ and ^{Br}U is not the major pathway for the EET in ODN 1/*c*ODN 1.

5′-End PTZ-tethered oligodeoxynucleotides

To understand better the structural effects on the EET reaction in DNA, PTZ was introduced at the 5′ end of the strands containing mismatch bases (ODN 2), because the terminal modification could provide further stabilization compared with ODN 1/*c*ODN 1 containing an abasic linker site in the middle of the sequences. The duplex dissociation temperatures of the corresponding duplexes (ODN 2/*c*ODN 2) were increased by the conjugation to PTZ.

Upon photoirradiation with UV light, strand cleavage products were generated from the duplexes ODN 2/*c*ODN 2 in a similar manner to that of the internal-PTZ conjugates ODN 1/*c*ODN 1 (Fig. 2). Again, purine-purine mismatch ODN 2/*c*ODN 2 (X/Y = A/A or A/G) showed relatively high strand cleavage at the 5′-adjacent C of ^{Br}U in yields of 3.3–5.8% h^{-1}. In contrast, the

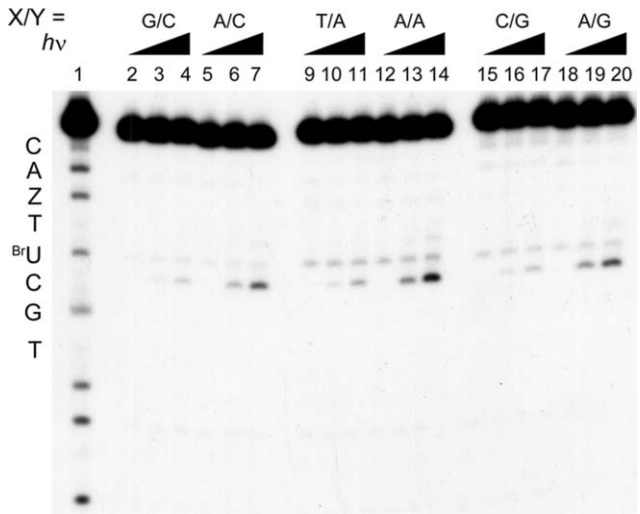


Fig. 2 A gel electrophoresis image of photo-irradiated ODN 2/*c*ODN 2 after piperidine treatment. Duplex DNA (1 μM) with a variable site (X/Y) in phosphate buffer (10 mM phosphate, 90 mM NaCl, pH 7.0) was photo-irradiated (365 nm, 0–2 h) under an N_2 atmosphere at 4 °C.

complementary duplexes containing G/C or A/T base pairs as intervening sequences produced lower yields of electron transfer (0.6–0.9% h^{-1}). It is noteworthy that switching the complementary base pairs from A/T (yield: 0.9% h^{-1}) to T/A (0.9% h^{-1}) or from G/C (0.6% h^{-1}) to C/G (0.6% h^{-1}) had no effect on the electron transfer reaction. This result indicates that orbital coupling between adjacent bases might be less important in our PTZ-ODN conjugates.

Effects of local structural disorder and proton transfer on EET in DNA

Considering the thermal stabilities of the duplexes, electron transfer efficiency might be related to some extent to structural fluctuation at the mismatch sites (Tables 1 and 2). According to theoretical investigations into the mechanisms of DNA-mediated EET and the electrochemical analysis of EET through mismatched bases, local structural disorder could lower the efficiency. Hole transfer through specific mismatch base pairs such as A/A could enhance the efficiency, possibly because the flexible structures enable the formation of active conformations for efficient hole transfer.[13] To access the possible formation of similar active conformations in our probes, we simply evaluated EET in ODN 1/*c*ODN 1 containing an abasic analog (X = A, Y = Ab). As shown in Fig. 3, efficient strand cleavage at the 5′-adjacent base of ^{Br}U was clearly observed.

Table 2 Time-dependent strand cleavage induced by electron transfer from photo-excited PTZ to ^{Br}U in duplex ODN 2/*c*ODN 2[a]

X/Y	Strand Cleavage[b] (% h^{-1})	T_m/°C
A/A	5.7	62.8
A/G	3.3	61.3
A/C	2.9	62.1
A/T	0.9	64.6
T/A	0.9	63.1
G/C	0.6	67.0
C/G	0.6	67.3

[a] 1 μM DNA, 10 mM phosphate buffer (pH 7.0), 90 mM NaCl. [b] Each analysis was repeated at least three times. Strand cleavage rates were obtained by fitting data (% scission product plotted against total material) to straight lines.

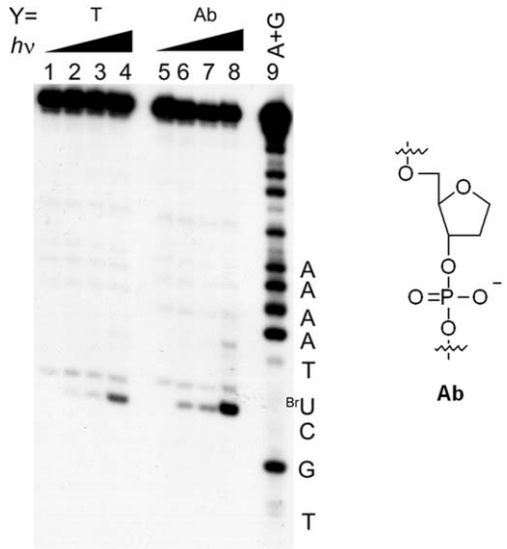
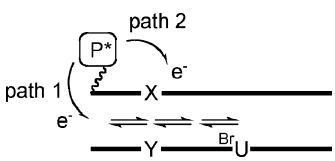


Fig. 3 A polyacrylamide gel image of photo-irradiated ODN 1/*c*ODN 1 containing either X/Y = A/T (lanes 1–4 and 9) or A/Abasic analog (Ab) (lanes 5–8). Duplex DNA (1 μM) in phosphate buffer (10 mM phosphate, 90 mM NaCl, pH 7.0) was photoirradiated (365 nm, 0–6 h) under an N_2 atmosphere at 4 °C, and then treated with piperidine.

In the PTZ-DNA conjugates, photo-excited PTZ transfers an electron onto the DNA duplex (Scheme 1, path 1). Considering the observations above, it is likely that single-base mismatch induces destabilization of the duplex especially around the electron donor site, which may lead to electron injection into sites beyond the mismatch (path 2). In addition, excess electrons might escape into the aqueous phase and become hydrated electrons, and some of them might revisit the DNA[14] because such unpaired nucleobases are more water accessible than the paired bases. Under such circumstances, injected electrons migrate forward to ^{Br}U, because back electron transfer to the PTZ radical cation ($PTZ^{+\bullet}$) through the mismatch site is expected to be less efficient.



Scheme 1 Pathways of electron injection from photo-excited PTZ (P*) to DNA duplex containing a single-base mismatch (X/Y).

As discussed previously, EET through the G/C base pair was less efficient than the A/T base pair because the proton transfer between one-electron reduced C ($C^{-\bullet}$) and the counter guanine base G should be competitive with the electron hopping.[15]

$$C^{-\bullet} + G \rightarrow CH^{\bullet} + G(-H^{+})^{-}$$

More complicated results indicated that the yields of electron transfer were better in ODN 1/*c*ODN 1 (X/Y = A/C) than in ODN 1/*c*ODN 1 (A/T), whereas the opposite result was

obtained for ODN 2/*c*ODN 2 (A/C) and ODN 2/*c*ODN 2 (A/T). Considering the structural characteristics of the two probes, mismatched duplex ODN 1/*c*ODN 1 (A/C) might be more flexible and more water accessible than ODN 2/*c*ODN 2 (A/C), and thus the radical anion $C^{-\bullet}$ could be protonated by water to trap the excess electron irreversibly. However, $C^{-\bullet}$ in ODN 2/*c*ODN 2 (A/C) has less chance to gain a proton from water or from the counter base of A.

The observation that purine-purine mismatched ODNs always showed higher electron transfer efficiencies than pyrimidine base pairs might be because of large stacking surfaces of the purine-purine pairs. It has been shown that A/G forms a wobble base pair that can stack in the duplex.[16] In addition, the observation could be explained partly by the lower electron affinities of purine bases [EA(3′,5′-dGDP) = 0.95 eV; EA(3′,5′-dADP) = 1.59 eV)] than pyrimidines [EA(3′,5′-dCDP) = 1.99 eV; EA(3′,5′-dTDP) = 1.98 eV].[17] Pyrimidine bases are considered to be sink sites for EET in DNA, and the corresponding radical anions such as thymine radical anion are protonated into the stable neutral radical.[18]

$$T^{-\bullet} + H_2O \rightarrow T(6)H^{\bullet} + OH^-$$

In this context, the photo-excited state of PTZ could be quenched partly by mismatched T or C to generate reduced forms of pyrimidine bases. A recent pulse radiolysis study by Tagawa and coworkers has suggested that $T^{-\bullet}$ in single-stranded DNA might be protonated more spontaneously by the surrounding waters than that in double-stranded DNA,[19] which supports our observations described above.

Structure-dependent EET was discussed in this study based on the results of product analysis by gel electrophoresis. For further understanding the dynamics of EET in DNA, kinetic analyses of the forward and back electron transfers would be essential, because electron trap by ^{Br}U might be competitive with the back electron transfer to $PTZ^{+\bullet}$. Kinetic investigation on EET in our PTZ-DNA probes by the method of time-resolved spectroscopy is currently underway in our group.

Conclusions

Two types of PTZ-tethered ODNs were used to investigate photoinduced EET in mismatched DNA. Single-base mismatches including an abasic structure in the intervening sequence between PTZ and ^{Br}U enhanced the apparent yields of EET, possibly because the flexible structures at the mismatch site increase the chances of electron injection and hopping beyond the site. In addition, increasing water accessibility by inducing structural changes could affect electron migration efficiency because of protonation of the pyrimidine radical anions, which becomes more competitive than in the case of EET in the complementary DNA.

Experimental section

General methods

Reagents for DNA synthesis were purchased from Glen Research (Virginia, USA). ODNs were either purchased from Japan Bio-service (Sendai, Japan) or synthesized on an Applied Biosystems 3400 DNA Synthesizer. Mass spectrometry of ODNs were performed with a MALDI-TOF MS (PerSeptive Voager Elite, acceleration voltage 21 kV, negative mode) with 2′,3′,4′-trihydroxyacetophenone as a matrix, using T_{17} ([M–H]$^-$ 5108.37) and T_{27} ([M–H]$^-$ 8150.33) as the internal standards. Reversed phase HPLC was performed with a Hitachi D-7000 HPLC system. Sample solutions were injected on a reversed phase column (Inertsil ODS-3, GL Sciences, ϕ10 mm × 250 mm). Hybridization of DNA was achieved by heating the samples in a buffer (90 mM NaCl, 10 mM phosphate, pH 7.0) at 90 °C for 5 min and slowly cooling to room temperature. Photo-irradiation at 365 nm was carried out using a FTI-20L transilluminator (Funakoshi, Japan).

Synthesis of ODNs bearing amino-linkers

For preparation of ODN 2, the corresponding sequence containing an amino linker in the middle of it (NH_2-ODN 2, $n = 3$ or 4) were synthesized at a 1 μmol scale (500 Å CPG column) using β-cyanoethylphosphoramidite chemistry. For NH_2-ODN 2 ($n = 4$), UniLink aminomodifier (BD Biosciences) was used as the amino C4 phosphoramidite. NH_2-ODN 2 ($n = 3$) was synthesized from the corresponding amino-C3 phosphoramidite.[20] As a precursor of ODN 1, ODN with a 5′-amino C3 linker (NH_2-ODN 1) was purchased from Japan Bio-Service. ODNs were isolated by HPLC, and then further purified by polyacrylamide gel electrophoresis (PAGE). MALDI-TOF MS: NH_2-ODN 2 ($n = 3$), calcd for $C_{210}H_{271}N_{82}O_{126}P_{21}$ 6610.32, found 6609.97; NH_2-ODN ($n = 4$), calcd for $C_{211}H_{273}N_{82}O_{126}P_{21}$ 6623.35, found 6624.02.

Synthesis of PTZ-tethered ODNs

Succinimidyl ester of phenothiazine was prepared as reported previously.[8b] To phenothazine succinimidyl ester (3.0 mg, 8.4 μmol) in acetonitrile (200 μL) were added NH_2-ODN 1 or 2 (200 μM, 200 μL) and 100 μL of saturated $NaHCO_3$, and the reaction mixture was incubated at 37 °C for 12 h. Purification of the ODN was carried out by the use of HPLC. Elution using a linear gradient of MeCN/100 mM TEAA buffer (pH 7.0) (0–30%, over 60 min) at a flow rate of 3.0 mL/min, gave the desired products. MALDI-TOF MS: ODN 1 ($n = 4$, X = A), calcd for $C_{225}H_{282}N_{83}O_{127}P_{21}S$ 6863.63, found 6862.80; ODN 1 ($n = 3$, X = A), calcd for $C_{224}H_{280}N_{83}O_{127}P_{21}S$ 6849.19, found 6848.61; ODN 2 (X = A), calcd for $C_{192}H_{236}N_{76}O_{106}P_{18}S$ 5892.97, found 5893.55; ODN 2 (X = G), calcd for $C_{192}H_{236}N_{76}O_{107}P_{18}S$ 5908.96, found 5909.69; ODN 2 (X = C), calcd for $C_{191}H_{236}N_{74}O_{107}P_{18}S$ 5868.94, found 5869.85; ODN 2 (X = T), calcd for $C_{192}H_{237}N_{73}O_{108}P_{18}S$ 5883.95, found 5884.24. Their complementary ODNs containing 5-bromo-2′-deoxyuridine were purchased from Japan Bio-Services and further purified by PAGE.

Melting temperature measurements

Melting temperature (T_m) measurements were performed on a JASCO UV-vis Spectrophotometer V-530. Duplexes were formed by mixing 1.0 μM/each of the complementary DNA strands in phosphate buffer solution (10 mM, pH 7.0) containing NaCl (90 mM). The solutions were heated at 85 °C for 5 min and afterwards cooled to room temperature. The absorbance at 260 nm was measured by varing temperature of the solution from 5 °C to 80 °C with a heating rate of 0.5 °C min^{-1}. T_m were determined

as the maximum of the first derivative plots of the melting curves.

Photolysis and PAGE analysis

ODNs were radiolabeled at their 5′-termini by use of [γ-^{32}P]ATP (Amersham Biosciences, 10 mCi/mL) and T4 polynucleotide kinase (Nippon Gene, Tokyo, Japan). The labeling mixtures were subsequently centrifuged through a MicroBio-Spin 6 column (Bio-Rad) to remove excess unincorporated nucleotides. Complementary ODNs (1 μM, respectively) were annealed in N_2-saturated buffer (10 mM sodium phosphate pH 7.0, 90 mM NaCl) by heating at 85 °C followed by slow cooling to room temperature. Samples (20 μL) were photo-irradiated in microcentrifuge tubes under an N_2 atmosphere at 4 °C. The DNA samples were precipitated by adding 4 μL of 3 M sodium acetate (pH 5.5), 12 μL of herring sperm DNA (1 mg/mL) and 400 μL of ethanol and chilling the mixture to –20 °C, and then drying in a Speed Vac. As required, samples were also resuspended in 10 vol% piperidine (50 μL), heated at 90 °C for 20 min and then evaporated in a Speed Vac. The radioactivity of the samples was assayed using an Aloka 1000 liquid scintillation counter (Aloka, Tokyo, Japan), then resuspended in denaturing loading buffer (8 M urea, 40% sucrose, 0.025% xylene cyanol, 0.025% bromophenol blue), and loaded on a 15% acrylamide/bis-acrylamide (19 : 1) gel containing 7 M urea. After electrophoresis at 1700 V for approximately 60 min, the gel was transferred to a cassette, and stored at –80 °C with Fuji X-ray films (RX-U). Cleavage of the labeled strands was quantified by autoradiography using ATTO Densitograph software (version 3.0).

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