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Synthesis of an artificial hole-transporting nucleoside triphosphate, $d^{MD}ATP$, and its enzymatic incorporation into DNA

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Abstract—We report on the enzymatic synthesis of DNA wires by successive incorporation of artificial nucleotides. We synthesized an efficient hole-transporting nucleoside triphosphate, $d^{MD}ATP$, and have examined the screening of polymerases and reverse transcriptases for enzymatic incorporation of $d^{MD}ATP$ into DNA. We found that KOD Dash is an effective enzyme for the enzymatic synthesis of $d^{MD}A$-containing DNA. Furthermore, the efficiency of enzymatic incorporation was remarkably improved by the addition of manganese chloride. By applying the PCR method to the synthesis of DNA containing a run of ^{MD}A, the ^{MD}A run was amplified up to 6×10^6 times. ^{MD}A runs prepared by the enzymatic method exhibited a high hole-transporting ability.

1. Introduction

In recent years, DNA has attracted much attention as a conductive biopolymer,[1–4] and a number of mechanistic and physical studies on DNA hole transport have been reported so far.[5–11] In regards to improving the hole transport efficiency to DNA, we have recently developed an artificial nucleobase, methoxybenzodeazaadenine (^{MD}A).[12] This artificial nucleobase is able to mediate hole transport effectively, and, at the same time, is not oxidatively decomposed. Recently, it has also been reported that ^{MD}A is available as a building block for a DNA logic gate system.[13]

The development of an efficient synthetic method for long DNA sequences containing multiple ^{MD}A bases is very important for further development of such DNA devices. We have so far prepared ^{MD}A runs via the conventional solid phase synthesis. On the other hand, DNA is easily elongated with DNA polymerases, and amplified up to several million times by means of the polymerase chain reaction (PCR). If the method for successive incorporation of ^{MD}A into DNA using DNA polymerases is developed, it would be an efficient technique for preparation and amplification of molecular wires. Much effort in enzymatically incorporating artificial nucleotides using DNA polymerases have been reported in the literature.[14–23] However, there is no precedent in which the artificial nucleobase containing an extended π-electron system, such as ^{MD}A, is efficiently and successively incorporated to DNA using polymerases. Therefore, we have synthesized the 5′-triphosphate salt of ^{MD}A ($d^{MD}ATP$), and have examined the screening of polymerases for successive incorporation of $d^{MD}ATP$ into DNA.

Here, we report on the enzymatic synthesis of DNA wires by successive incorporation of ^{MD}A. We synthesized $d^{MD}ATP$, and examined the screening of polymerases and reverse transcriptases for enzymatic incorporation of $d^{MD}ATP$ into DNA. Furthermore, the efficiency of enzymatic incorporation was remarkably improved by the addition of manganese chloride. ^{MD}A runs were efficiently amplified by applying the PCR method. For resulting ^{MD}A runs, a highly efficient long-range hole transport was observed.

2. Results and discussion

2.1. Preparation of $d^{MD}ATP$

The preparation of the hole-transporting nucleoside, $d^{MD}A$, was readily accomplished in two steps from

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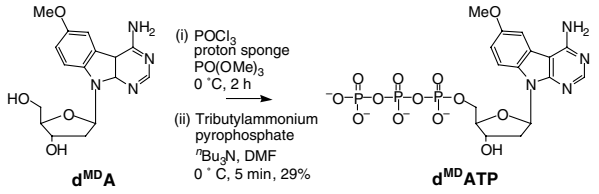

Scheme 1.

4-chloro-6-methoxy-1*H*-pyrimido[4,5-*b*]indole using a protocol reported in an earlier publication.[2,24,25] Phosphorylation of $d^{MD}A$ was carried out using a one-pot method (Scheme 1).[26] The nucleoside $d^{MD}A$ was phosphorylated directly with phosphorus oxychloride and tributylammonium pyrophosphate in the presence of dry proton sponge at 0 °C to give $d^{MD}ATP$ in 29% yield.

2.2. ^{MD}A incorporation method

As a first step, we examined a primer extension assay using a variety of DNA polymerases employing a duplex consisting of a ^{32}P-labeled 17-mer primer (**ODN1-P1**) and a 43-mer template (**ODN1-T1**), which contained only one $d^{MD}ATP$ incorporation site (Fig. 1a). Five representative DNA polymerases and reverse transcriptases were examined for ^{MD}A incorporation during DNA polymerization, such as KOD Dash DNA polymerase (KOD Dash), *Bst* DNA polymerase large fragment (Bst), M-MuLV reverse transcriptase (M-MuLV), SuperScript II RNase H^- reverse transcriptase (SS II), and Deep Vent (*exo*$^-$) DNA polymerase (Deep Vent). The polymerization reactions were carried out either at 37 °C for 1 h for M-MuLV and SS II, or for 30 cycles between 37 °C for 0.5 min and 72 °C for 0.5 min for the thermophilic polymerases, such as KOD Dash, Bst, and Deep Vent. These reactions were carried out in a reaction mixture (30 μL) containing 3 μM **ODN1-T1**, ^{32}P-labeled **ODN1-P1** (ca. 0.4 μM, 24 kcpm), TTP, dCTP, dGTP, and $d^{MD}ATP$ mix (0.8 mM of each nucleotide), and polymerase (at the concentration recommended by the supplier) in the buffer supplied for each polymerase. After a primer extension assay, the reaction mixture was quenched by adding a formamide-dye solution, and then the reaction products were analyzed using polyacrylamide gel electrophoresis (PAGE). The results are shown in Figure 1b (lanes 5, 7, 9, 11, and 13). The 43-mer full-length DNA was produced in the polymerization reaction with KOD Dash, Bst, and SSII. In particular, KOD Dash DNA polymerase exhibited the highest activity (lane 5). However, the polymerase activities were, overall, relatively low. M-MuLV and Deep Vent gave no full-length primer extension product (lanes 9 and 13, respectively). The 22-mer DNA was mainly produced during DNA polymerization with M-MuLV, SS II, and Deep Vent, suggesting that the incorporation of the ensuring base of ^{MD}A was problematic.

The addition of manganese ions (Mn^{2+}) is known to relax the substrate specificity of many DNA polymerases.[27–29] Thus, to improve the efficiency of our DNA synthesis, we added Mn^{2+} to the reaction mixture.

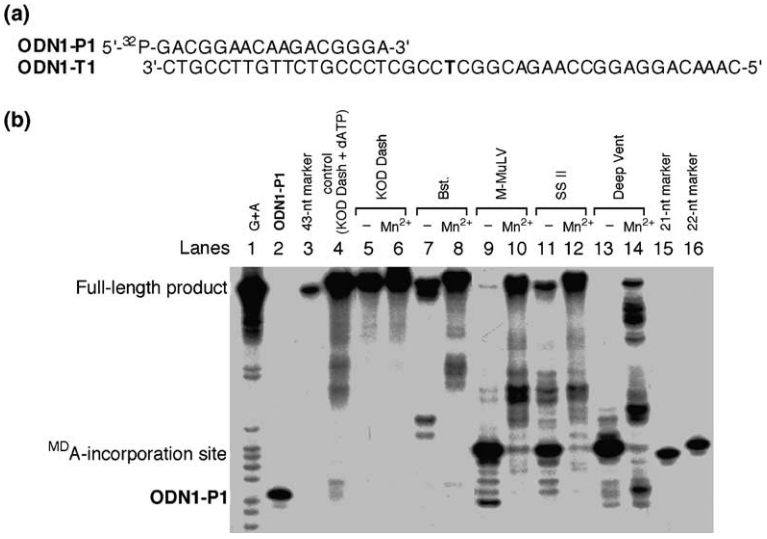

Figure 1. (a) Sequences of a primer, **ODN1-P1**, and a template, **ODN1-T1**, used for a primer extension assay for one ^{MD}A incorporation. (b) PAGE analysis of the primer extension assay. The polymerization reactions were carried out at 37 °C/1 h for M-MuLV reverse transcriptase (M-MuLV) (200 U) and SuperScript II reverse transcriptase, RNase H^- (SS II) (200 U) or 30 cycles of 37 °C/0.5 min–72 °C/0.5 min for KOD Dash DNA polymerase (KOD Dash) (2.5 U), *Bst* DNA polymerase large fragment (Bst) (8 U) and Deep Vent (*exo*$^-$) DNA polymerase (Deep Vent) (2 U) in the reaction mixture (30 μL) containing 3 μM DNA template, primer (ca. 0.4 μM, 24 kcpm), TTP, dCTP, dGTP, and $d^{MD}ATP$ mix (0.8 mM of each nucleotide), and polymerase in the buffer supplied for each polymerase. The reaction mixture was quenched by the addition of a formamide-dye solution, and then the reaction products were analyzed by PAGE. Lane 1, Maxam-Gilbert G + A sequencing; lane 2, **ODN1-P1**, lane 3, 43-mer DNA marker; lane 4, with KOD Dash in the presence of four natural dNTPs; lane 5, with KOD Dash in the absence of $MnCl_2$; lane 6, with KOD Dash in the presence of 1.5 mM $MnCl_2$; lane 7, with Bst in the absence of $MnCl_2$; lane 8, with Bst in the presence of 1.5 mM $MnCl_2$; lane 9, with M-MuLV in the absence of $MnCl_2$; lane 10, with M-MuLV in the presence of 1.5 mM $MnCl_2$; lane 11, with SS II in the absence of $MnCl_2$; lane 12, with SS II in the presence of 1.5 mM $MnCl_2$; lane 13, with Deep Vent in the absence of $MnCl_2$; lane 14, with Deep Vent in the presence of 1.5 mM $MnCl_2$; lane 15, 21-mer DNA marker; lane 16, 22-mer DNA marker.

As a result of the addition of 1.5 mM of manganese chloride to the standard reaction mixtures, a drastic improvement in polymerase activity was observed. In the case of polymerization with SS II, the fraction of full-length product increased from 20% in the absence of Mn^{2+} to 71% in the presence of Mn^{2+} (lane 12). Similarly, the increase in the yield of the full-length primer extension product by the addition of Mn^{2+} was observed also for Bst, M-MuLV, and Deep Vent.

2.3. Synthesis of an ^{MD}A run using a primer extension assay

The successful ^{MD}A incorporation using DNA polymerases prompted us to apply this primer extension system to the synthesis of an ^{MD}A run using a template sequence that included 10 successive T nucleotides. We examined the extension of **ODN1-P1** on a T10 template (**ODN1-T10**) with KOD Dash, which exhibited the greatest activity in the assay described above (Fig. 2a). The assay was carried out under the same reaction conditions in the presence or absence of Mn^{2+}, as noted in Figure 1b. The production of full-length DNA was negligible in the absence of Mn^{2+} under the conditions, but on the addition of Mn^{2+} to the reaction mixture, the full-length DNA was produced in 8% yield. The synthesis of DNA containing ^{MD}A runs was accomplished using the polymerization system with KOD Dash and Mn^{2+}.

2.4. Amplification of an ^{MD}A run using the PCR method

In an effort to further improve the synthetic yield of ^{MD}A runs, we applied a PCR amplification to our system. Since d^{MD}ATP is acceptable to thermophilic DNA polymerase as a substrate under the reaction conditions described above, PCR would be a very useful method for the preparation and amplification of ^{MD}A-containing DNA with a high density of the hole-transporting units. We examined the incorporation of d^{MD}ATP during a PCR using KOD Dash. The cyclic PCR experiments were carried out over 28 cycles at 96 °C for 10 s, to 37 °C for 0.5 min, to 72 °C for 0.5 min. The reaction was carried in a reaction mixture (25 μL) containing 1 pM of DNA template (**ODN1-T10**), and 50 μM each of the primers (**ODN1-P1** and **ODN1-P2**) in the presence of 1.5 mM manganese chloride using KOD Dash (4 units) (Fig. 2a). The resulting duplex was detected by monitoring the enhancement of fluorescence of SYBR Green I using the GeneAmp® 5700 Sequence Detection System (Applied Biosystems) (Fig. 2b). Under the reaction conditions, a large enhancement of the fluorescence of SYBR Green I at 555 nm was observed, suggesting that the primers were efficiently elongated. From the increment of the fluorescence intensity of SYBR Green I, the efficiency of the primer extension was estimated at 12%, corresponding to a 6×10^6-fold amplification of the duplex. The PAGE analysis of the PCR products revealed the generation

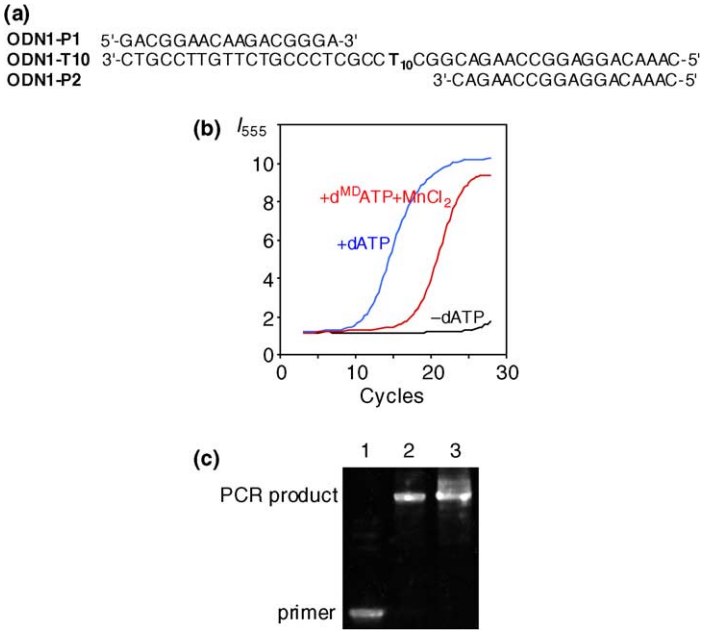

Figure 2. Enzymatic synthesis of $^{MD}A_{10}$-containing DNA. (a) Primers and a template used in these studies. **ODN1-P1** and **ODN1-T10** were used in primer extension assay. In PCR amplification, **ODN1-P2** was also added. (b) SYBR Green fluorescence profile of the real time PCR. The PCR was carried out at 28 cycles of 96 °C/10 s–37 °C/0.5 min–72 °C/0.5 min in the reaction mixture (25 μL) containing 1 pM **ODN1-T10**, 2.5 μM each of **ODN1-P1** and **ODN1-P2**, TTP, dCTP, dGTP, and d^{MD}ATP mix (0.8 mM of each nucleotide), KOD Dash (4 U) in the buffer supplied for each polymerase in the presence of 1.5 mM $MnCl_2$. The reaction was simultaneously monitored by the fluorescence emission of SYBR Green I at 555 nm with the excitation at 485 nm. The result is drawn by a red line. A blue line shows the result for the mixture containing TTP, dCTP, dGTP and dATP mix. A black line shows the result for the mixture containing TTP, dCTP, and dGTP mix without dATP. (c) PAGE analysis of PCR products. After PCR reaction as described above, the reaction mixtures were analyzed with 15% polyacrylamide/7 M urea. The resulting gel was stained with SYBR II, and then illuminated with a 290–365 nm transilluminator. The image was taken through a 380 nm long pass emission filter. Lane 1, 20 μM primer solution; lane 2, the result for the PCR mixture containing d^{MD}ATP in the presence of 1.5 mM $MnCl_2$; lane 3, the result for the PCR mixture containing dATP.

of full length DNA (Fig. 2c). ESI mass spectroscopic analysis also supported the production of full-length DNA containing ^{MD}A 10-mer ([M−11H]$^{11-}$, calcd 1539.2, found 1539.5; [M−20H]$^{20-}$, calcd 844.5, found 844.5).

2.5. Hole transport through an enzymatically synthesized ^{MD}A run

Having established the enzymatic synthesis of DNA containing a successive ^{MD}A sequence, we examined the hole transport reactions through an extended DNA sample containing an ^{MD}A run. For the execution of the hole transport reaction, a DNA strand containing ^{MD}A runs was synthesized using a T10 template, **ODN2-T10**, and KOD Dash in the presence of Mn^{2+} from a primer **ODN2-P1** (Fig. 3a and b). After the extended product, **ODN2-P1(ex)**, was isolated from the gel, we examined the photo-induced hole transport reactions. The extended DNA was hybridized with the strand, **ODN2-T10(U*)**, which contains a photosensitizer for hole injection to DNA, cyanobenzophenone-substituted uridine (**U***).[30] GGG steps, which are known as effective

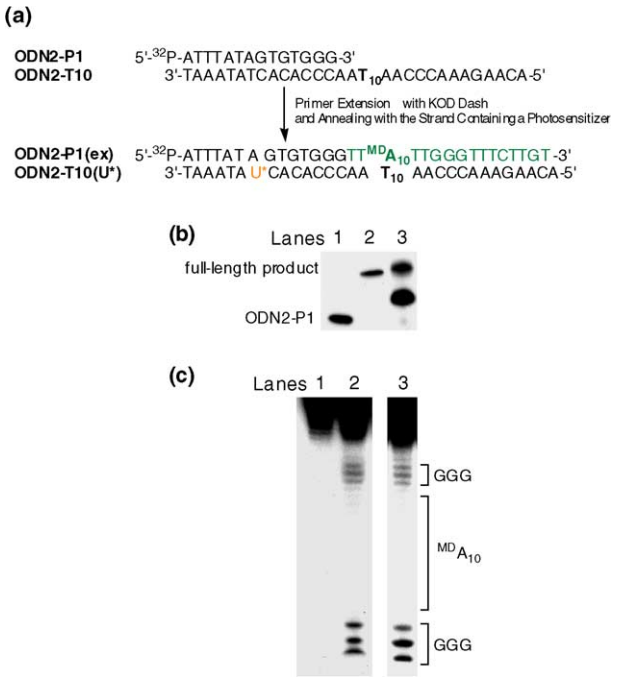

Figure 3. Synthesis and evaluation of DNA wire. (a) Primers and a template used for the enzymatic preparation of DNA wire. (b) PAGE analysis of the primer extension assay. The PCR were carried out at 30 cycles of 37 °C/0.5 min–72 °C/0.5 min in the reaction mixture (30 μL) containing 3 μM **ODN2-T10**, **ODN2-P1** (ca. 0.4 μM, 24 kcpm), TTP, dCTP, dGTP and d^{MD}ATP mix (0.8 mM of each nucleotide), KOD Dash (4 U) in the buffer supplied for each polymerase. The reaction mixture was quenched by the addition of a formamide-dye solution, and then the reaction products were analyzed by PAGE. Lane 1, **ODN2-P1**, lane 2, 39-mer DNA marker; lane 3, with KOD Dash in the presence of 1.5 mM $MnCl_2$. (c) PAGE analysis of the hole transport reaction of duplex **ODN2-P1(ex)/ODN2-T10(U*)**. The duplexes in 10 mM sodium cacodylate (pH 7.0) were irradiated (λ = 312 nm) at 0 °C for 45 min followed by a hot piperidine treatment (90 °C, 20 min). Lane 1, control lane (no photoirradiation); lane 2, Maxam-Gilbert G + A sequencing lane; lane 3, photoirradiated duplex.

hole traps,[31,32] were incorporated into both sides of the ^{MD}A run. The hole transport reaction was triggered by photoirradiation using a transilluminator (312 nm) at 0 °C. The result of the hole transport reaction was visualized by PAGE analysis following the hot piperidine treatment of the reaction products. From Figure 3c, the ratio of oxidative strand cleavage at the distal GGG (G_b) versus the proximal GGG (G_a) steps was estimated to be 0.60. The extended duplex has a high hole-transporting efficiency, as high as that reported for ^{MD}A runs in an earlier paper.[12] Thus, it was established that the facile synthesis of DNA wires possessing a good hole-transporting ability has been accomplished by successive incorporation of ^{MD}A using an enzymatic method.

3. Conclusions

We have demonstrated that ^{MD}A-containing DNA wires can be synthesized using polymerases. The extension of DNA wires was achieved by successive incorporation of d^{MD}ATP using DNA polymerases, especially KOD Dash, in the presence of manganese ions. By using the PCR method, DNA wires, which exhibit a high enough hole transport efficiency, were greatly amplified. The development of this method for the successive incorporation of artificial nucleotides, such as ^{MD}A, will contribute to a significant advancement in artificial DNA technology, for use in DNA wires and DNA logic gates.

4. Experimental

4.1. General

Chemical shift values (δ) are reported relative to H_3PO_4 (85%) for ^{31}P NMR (external standard). JASCO V-500 UV–vis spectrometer was used for UV measurement. [γ-^{32}P] ATP (370 Mbq/L) was obtained from Amersham. A GIBCO BRL Model S2 Sequencing Gel Electrophoresis Apparatus was used for polyacrylamide gel electrophoresis (PAGE). T4 DNA ligase was purchased from TaKaRa. dNTP mix (PCR grade) was purchased from Roche Diagnostic. KOD Dash DNA polymerase was purchased from TOYOBO. M-MuLV reverse transferase, Deep Vent (*exo*$^-$) DNA polymerase and *Bst* DNA polymerase large fragment were purchased from New England BioLabs Inc. SuperScript II RNase H$^-$ reverse transcriptase was purchased from Invitrogen.

4.2. Preparation of ^{MD}A triphosphate

d^{MD}A (70 mg, 0.21 mmol) and *N*,*N*,*N*′,*N*′-tetramethyl-1,8-naphthalenediamine (dry proton sponge) (0.23 g, 1.1 mmol, 5 equiv) were dissolved in anhydrous acetonitrile, and co-evaporated three times. The residue was dissolved in trimethylphosphate (3.0 mL). The mixture was stirred at room temperature under Ar, and then cooled to 0 °C. After 15-min stirring at 0 °C, phosphorus oxychloride (25 μL, 0.27 mmol, 1.3 equiv) was added. After 2 h of stirring at 0 °C, tributylamine (0.20 mL,

0.84 mmol, 4.0 equiv) was added to the reaction mixture. Subsequently, tributylammonium pyrophosphate (1.0 g, 2.1 mmol, 10 equiv) in 3 mL of anhydrous DMF was also added to the mixture. After 5 min, the reaction mixture was poured into 40 mL of diethyl ether–acetone–$NaClO_4$-saturated acetone (10:9:1) with stirring. The precipitate was washed with ether twice and collected. The white solid was dried in vacuo, and redissolved in a small volume of 1 M triethylammonium bicarbonate buffer (pH 7.5). The solution purified via reverse phase HPLC (0–60% acetonitrile over 20 min in 0.1 M triethylammonium acetate buffer, pH 7.0) afford triphosphates as a white solid (29% yield). ^{31}P NMR (D_2O) δ −5.11 (d, J = 18.3 Hz), −6.36 (d, J = 18.3 Hz), −18.0 (t, J = 18.3 Hz).

4.3. Preparation of ^{32}P-5′-end-labeled DNA

The DNAs (4000-pmol strand) were 5′-end-labeled by phosphorylation with 4 μL of [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase using a standard procedure. The 5′-end-labeled DNA was purified by 15% denaturing polyacrylamide gel electrophoresis (PAGE), and isolated by the crush and soak method.

4.4. Primer extension reactions with ^{MD}A triphosphate

The reactions were carried out at 37 °C/1 h for M-MuLV reverse transcriptase (M-MuLV) (200 U) and SuperScript II reverse transcriptase, RNase H$^-$ (SS II) (200 U) or 30 cycles of 37 °C/0.5 min–72 °C/0.5 min for KOD Dash DNA polymerase (KOD Dash) (2.5 U), *Bst* DNA polymerase large fragment (Bst) (8 U) and Deep Vent (*exo*$^-$) DNA polymerase (Deep Vent) (2 U) in the reaction mixture (30 μL) containing 3 μM DNA template, primer (ca. 0.4 μM, 24 kcpm), TTP, dCTP, dGTP, and d^{MD}ATP mix (0.8 mM of each nucleotide), and polymerase in the buffer supplied for each polymerase. If necessary, 1.5 mM manganese chloride as an additive was added into the reaction mixture. The reaction was terminated by addition of 80% formamide loading buffer (a solution of 80% v/v formamide, 1 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue), and electrophoresed in 15% denatured polyacrylamide gel containing 7 M urea. The polyacrylamide gel was transferred to a cassette, and stored at −80 °C with Fuji X-ray film.

4.5. Hole transport experiment with PAGE analysis

^{32}P-5′-end-labeled DNA was hybridized to the complementary strand containing cyanobenzophenone-substituted deoxyuridine in 10 mM sodium cacodylate buffer (pH 7.0). Hybridization was achieved by heating the sample at 90 °C for 3 min and slowly cooling to room temperature. Photoirradiation was carried out in a 100 μL total volume containing 30 kcpm ^{32}P-5′-end-labeled DNA and their complementary strands (2 μM strand concn) in 10 mM sodium cacodylate buffer at pH 7.0. The reaction mixtures were irradiated with a transilluminator (312 nm) at a distance of 3 cm at 0 °C for 45 min. After irradiation, all reaction mixtures were precipitated with the addition of 10 μL of 3 M sodium acetate, 20 μL of herring sperm DNA (50 μM base pair concn) and 800 μL of ethanol. The precipitated DNA was washed with 100 μL of 80% cold ethanol and dried in vacuo. The precipitated DNA was resolved in 50 μL of 10% piperidine (v/v), heated at 90 °C for 20 min, evaporated by vacuum rotary evaporation to dryness, and resuspended in 5–20 μL of 80% formamide loading buffer (a solution of 80% v/v formamide, 1 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue). All reactions, along with Maxam-Gilbert G + A sequencing reactions, were conducted with heating at 90 °C for 1 min, and quickly chilled on ice. The samples (1 μL, 3–10 kcpm) were loaded onto 15% denaturing 19:1 acrylamide–bisacrylamide gel containing 7 M urea, and electrophoresed at 1900 V for approximately 1.5 h, and transferred to a cassette and stored at −80 °C with Fuji X-ray film.

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