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Replication of cyclobutane pyrimidine dimer analogue by Ex Taq DNA polymerase

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**ABSTRACT**

We previously reported an efficient and reversible template-directed photoligation using 5-carboxyvinyl-2'-deoxyuridine ($^{CV}$U)-containing ODN at the 5'-terminal. This method forms d(T- $^{CV}$U) as a cyclobutane pyrimidine dimer (CPD) analogue between the 3'-terminal thymidine and the 5'-terminal $^{CV}$U of two oligodeoxynucleotides (ODNs). In this study, we performed PCR using a DNA template containing d(T- $^{CV}$U). Then, we found that two adenines were incorporated opposite the d(T- $^{CV}$U).

**1. INTRODUCTION**

Nucleic acid templated syntheses prior to the current decade predominantly used DNA or RNA templates to mediate ligation reactions that generate oligomers of DNA, RNA or structural analogues of nucleic acids [1–6] .In addition to analogues of the phosphoribose backbone, products that mimic the structure of stacked nucleic acid aromatic bases have also been generated by DNA-templated synthesis [7–9]. We previously described a reversible template-directed photoligation mediated by [2 + 2] cycloaddition that was modeled on the structure of the cyclobutane pyrimidine dimer (CPD) between 3'-terminal thymidine and 5'-terminal 5-carboxy-2'-deoxyuridine (Fig. 1) [10–15]. We modified nucleosides containing various DNA functional groups that react after hybridization by photoirradiation have been extended to the SNPs detection system [14]. While non-enzymatic ligation methods may offer some advantages, one limitation of the photoligation strategy relative to enzymatic methods is the fact that the CPD analogue as the ligated structures differs from that of the natural DNA junction, which is likely to interfere with further manipulation (such as amplification). However, *in vitro* analyses have shown that some DNA polymerases can copy distorted DNA templates containing a UV-induced lesion [16–18]. Additionally, because a single bond is included in the structure, d(T- $^{CV}$U) has higher flexibility than usual CPD (Figure 2). Therefore, we expect that d(T- $^{CV}$U) as a CPD analogue is more

suitable for primer extension than CPD. In this study, we examined some DNA polymerases that can accept the DNA template containing d(T- $^{CV}$U) during the PCR, and we found that Ex Taq DNA polymerase can read a template DNA containing d(T- $^{CV}$U). Additionally, we determined that two adenines were incorporated opposite d(T- $^{CV}$U).

## 2. EXPERIMENTAL SECTION

### *2.1. General method and materials*

Dioxane, pyridine, DMTrCl, was purchased from Kanto Kagaku Reagent Division. 5-Iodo-2'-deoxyuridine was purchased from Tokyo Chemical Industry CO., LTD. Ethyl trifluoroacetate, palladium (II) acetate, $PPh_3$, methyl acrylate and 2-cyanoethyl *N,N,N',N'*-tetra-isopropyl-phosphorodiamidite were purchased from Aldrich. DMAP was purchased from ACROS ORGANICS. 1*H*-tetrazole was purchased from Glen Research. The reagents for the DNA synthesizer such as $I_2$ solution ($I_2$/ $H_2O$/ pyridine/tetrahydrofuran, 3: 2: 19: 76), A-, G-, C-, and T-β-cyanoethyl phosphoroamidites were purchased from Glen Research. Photoligation products were purified by Dynabeas M-280 streptavidin (Roche). Other regents were purchased at the highest commercial quality and used without further purification unless otherwise stated. Calf intestine alkaline phosphatase (AP) (1500 units) was purchased from Roche. Nuclease P1 (500 units) was purchased from Yamasa. Reactions were monitored on TLC plates precoated with Merck silica gel 60 $F_{254}$. Kanto Chemical Silica Gel 60 N was used for silica gel column chromatography. $^1$H-NMR spectrum was recorded on Varian Gemini-300 (300 MHz). Coupling constant (*J* value) are reported in hertz. The chemical shift is reported in δ(ppm) relative to residual chloroform (δ = 7.24) and DMSO (δ = 2.49) as internal standards. ODNs were synthesized on an Aplid Biosystems 3400 DNA Synthesizer. Reverse phase HPLC was performed on a Chemcobond 5C18 ODS column (4.6 x 150 mm) with a JASCO PU-2080, MX-2080-32, DG-2080-53 system equipped with a JASCO UV 2075 spectrometer at 260 nm. Mass spectra were recorded on a Voyager-DE PRO-SF, Applied Biosystems. Capillary gel electrophoresis was performed with P/ACE™ MDQ, BECKMAN COULTER. The denaturing gel (eCAP™ ssDNA 100-R Kit) purchased from BECKMAN COULTER containing Tris-Borate buffer and urea was prepared according to the instruction manual. BECKMAN COULTER DU 800 UV/Visible spectrophotometer was used for absorption spectra measurements.

### *2.2. Synthesis of oligonucleotides*

The synthesis of the photoreactive nucleosides and the corresponding phosphoramidite building block of 5-carbamoylvinyl-2'-deoxyuridine ($^{CV}$U) as well as the synthesis of corresponding oligodeoxynucleotides followed standard routes in DNA-chemistry.$^{CV}$U was synthesized from 5-Iodo-2'-deoxyuridine; the scheme was reported previously [11]. Oligonucleotides were prepared by the β̃(cyanoethyl)phosphoramidite method on a controlled pore glass supports by using an Applied Biosystems Model 3400 synthesizer. The 0.1 M acetonitrile solution of $^{CV}$U was used in automated synthesis, the oligonucleotides were cleaved from the support by conc. aqueous ammonia for 1h. deprotected by heating the solutions at 55 °C for 8 h, and purified by reverse phase HPLC. The purity and concentration of all nucleotides were determined by digestion with AP, Nuclease P1 to 2'-deoxymononucleotide at 37 °C for 3 h. MALDI-TOF MS: calculated for ODN **1** [$(M+H)^+$] calcd 7714.01 for $[M + H]^+$, found 7714.29.

*2.3. Photoligation of DNA oligomer*

The reaction mixture (total volume 60 $\mu$L) containing ODN **1** and ODN **2** (each 10 $\mu$M strand concentration) in the presence of template ODN **3** (10 $\mu$M strand concentration) in sodium cacodylate buffer (50 mM, pH 7.0) and sodium chloride (100 mM) was irradiated with a 25 W transilluminator (366 nm) at 0 °C for 1 h (Step 1 in Scheme 1).

*2.4. Purification of ODN **4** for PCR*

The resulting ligated ODN **4** was subsequently immobilized on streptavidine-magnetic beads (Step 2 in Scheme 1). The ligated ODN **4** was purified from unreacted ODN **2** and template ODN **3** on streptavidin-linked magnetic beads by washing twice with 10 mM tris-HCl (pH 7.5), 1 mM EDTA and 1 M NaCl (2 min, 80 °C) and eluting under 10 mM EDTA and 95% formamide (5 min, 60 °C) (Step 3, 4 in Scheme 1).

*2.5. PCR amplification of ODN **4***

*PCR by Ex Taq polymerase*

Real-time PCR was set up using a 25 $\mu$L volume, with 10 nM ODN **4**, 10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, each dNTP 0.4 mM, 0.3 $\mu$M primer pair, 1 x SYBER Green and 2.5 unit ExTaq DNA

polymerase. The PCR consisted of denaturation at 94 °C for 10 min, and 50 cycles of 94 °C for 10 s, 60 °C for 20 s and 72 °C for 20 s, and was followed by a final extension of 72 °C for 10 min.

*PCR by rTaq polymerase*

Real-time PCR was set up using a 25 $\mu$L volume, with 10 nM ODN **4**, 10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, each dNTP 0.4 mM, 0.3 $\mu$M primer pair, 1 x SYBER Green and 2.5 unit rTaq DNA polymerase. The PCR consisted of denaturation at 94 °C for 10 min, and 50 cycles of 94 °C for 10 s, 60 °C for 20 s and 72 °C for 20 s, and was followed by a final extension of 72 °C for 10 min.

*PCR KOD dash polymerase*

Real-time PCR was set up using a 25 $\mu$L volume, with 10 nM ODN **4**, 10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, each dNTP 0.4 mM, 0.3 $\mu$M primer pair, 1 x SYBER Green and 2.5 unit KOD dash DNA polymerase. The PCR consisted of denaturation at 94 °C for 10 min, and 50 cycles of 94 °C for 10 s, 60 °C for 30 s and 74 °C for 60 s, and was followed by a final extension of 74 °C for 10 min.

*PCR by Vent exo(-) polymerase*

Real-time PCR was set up using a 25 $\mu$L volume, with 10 nM ODN **4**, 10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, each dNTP 0.4 mM, 0.3 $\mu$M primer pair, 1 x SYBER Green and 2.5 unit vent (exo-) DNA polymerase. The PCR consisted of denaturation at 94 °C for 10 min, and 50 cycles of 94 °C for 10 s, 60 °C for 20 s and 72 °C for 20 s, and was followed by a final extension of 72 °C for 10 min.

## RESULTS AND DISCUSSION

### *3.1. Synthesis of oligonucleotides*

$^{CV}$U was prepared according to the method reported previously [11]. ODN containing $^{CV}$U, 5’-d($^{CV}$UGGAGCTGCTTGGCGCGCTCCCCGT)-3’ (ODN **1**) was synthesized using phosphoramidite of $^{CV}$U according to conventional DNA synthesis. ODN **1** was characterized by the nucleoside composition and MALDI-TOF MS (calcd 7714.01 for $[M + H]^+$, found 7714.29). The ODNs used in this study are summarized in Table 1.

### *3.2. Photoligation of DNA oligomer*

We determined the feasibility of the template-directed photoligation via ODN **1** (Step 1 in Scheme 1). When ODN **1** and biotinylated ODN **2** (55 mer) were irradiated at 366 nm for 1 h in the presence of

template ODN **3**, we observed the appearance of the peak of ligated ODN **4** (80 mer) as determined by capillary gel electrophoresis (CGE) with the disappearance of ODN **1** and ODN **2** (Figure 3b).

### *3.3. Purification of ODN 4 for PCR*

The resulting ligated ODN **4** was subsequently immobilized on streptavidine-magnetic beads. The biotinylated ODN was then recovered by elution with 95% formamide and used as a template in PCR with dNTPs. We observed the residue of the peak of ligated product ODN **4** in 66% yield as determined by CGE analysis (Figure 3c).

### *3.4. PCR amplification of ODN 4*

We examined the ODN **4** containing d(T-$^{CV}$U) during the PCR using four thermostable DNA polymerase, KOD dash, Vent (exo-), rTaq, and ExTaq DNA polymerase. ODN **4**, and primer F, 5'-CACCTGCGCAAGCTGCGTAA-3' and primer R, 5'-ACGGGGAGCGCGCCAAGCAG-3' were used as a template and primers, respectively, for the PCR assay. The PCR did not demonstrate formation of the 80 base pair DNA product when using KOD dash, Vent (exo-), and rTaq DNA polymerase (data not shown). Only Ex Taq DNA polymerase could accept ODN **4** and form 80 bp PCR products. Real time PCR system Ct values were correlated with the four levels of concentration of template ODN **4** that were set to 10 nM, 1 nM, 0.1 nM, and 0.01 nM. This result suggests that ODN **4** was used as a template for PCR. Additionally, we used 80 mer DNA as reference molecules that were set to the same concentration. We obtained Ct values ranging from 10.8 to 36.2 cycles for 80 mer DNA and from 29.4 to 36.3 cycles for ODN **4** (Figure 4). These results show that the amplification of using ODN **4** is slower than 80 mer DNA, and it is thought that a CPD part included in ODN **4** delays the progress of polymerase. Figure 5 shows the results of the PCR with various concentrations of the substrate ODN **4** as analyzed by microchip gel electrophoresis. The results indicate that the enzyme read through the CPD analogue in the DNA template, accepted the complementary substrate, and continued the chain elongation until the end of the template ODN **4**. And then, we investigated the sequence of the elongation products. The sequencing results

showed that the PCR products contain two adenines opposite d(T-$^{CV}$U), in contrast to the cDNA of two thymidines at that position (data not shown).

## 4. CONCLUSION

This study demonstrates that d(T-$^{CV}$U)-containing DNA were substrates for Ex Taq DNA polymerase yielding the corresponding PCR products. Additionally, two adenines were preferentially inserted opposite d(T-$^{CV}$U). Employing these methods, we are currently investigating a new CPD analogue that was synthesized from other photosensitive nucleosides.

## 5. ACKOWLEDGMENT

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## Figure and Captions

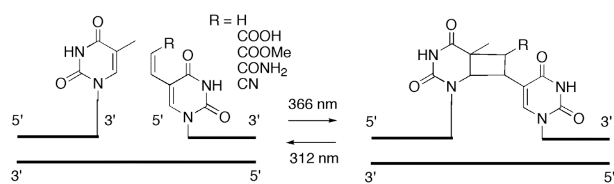



**Figure 1.** The template-directed reversible photoligation of DNA oligomer via 5-vinyldeoxyuridine derivative at the 5´ end.

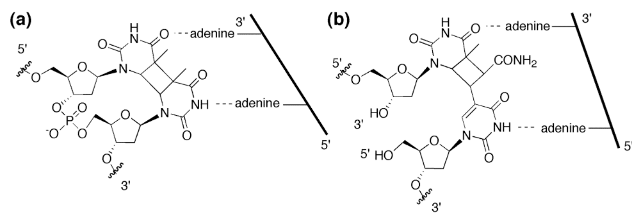
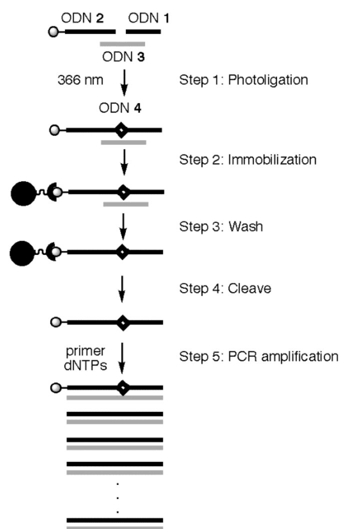



**Figure 2.** Structure of thymine dimer (a), and d(T-$^{CV}$U) photoadduct (b).

**Table 1.** List of Sequences Used in This Study

| | Oligonucleotide sequences (5' → 3') |
|---|---|
| ODN **1** | $^{CV}$UGGAGCTGCTTGGCGCGCTCCCCGT |
| ODN **2** | Biotin-CACCTGCGCAAGCTGCGTAAGCGGCTC CTCCGCGATGCCGATGACCTGCAGAAGT |
| ODN **3** | GCCAAGCAGCTCCAACTTCTGCAGGTCA |
| Primer F | CACCTGCGCAAGCTGCGTAA |
| Primer R | ACGGGGAGCGCGCCAAGCAG |
| 80 mer DNA | CACCTGCGCAAGCTGCGTAAGCGGCTCCTCC GCGATGCCGATGACCTGCAGAAGTTGGAGCT GCTTGGCGCGCTCCCCGT |




**Scheme 1** Strategy for the synthesis of template ODN for PCR.

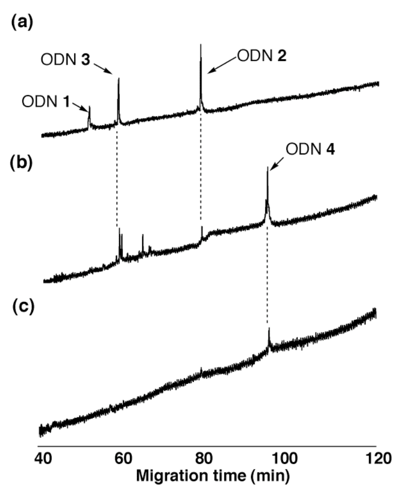
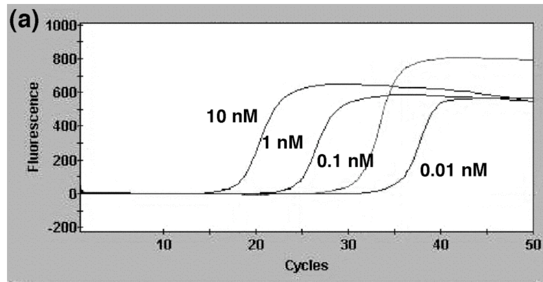



**Figure 3.** Capillary gel electrophoresis of photoreaction of ODN **1** (10 $\mu$M) and ODN **2** (10 $\mu$M) in the presence of template ODN **3** (10 $\mu$M): (a) before photoirradiation, (b) after irradiation at 366 nm for 5 min at 0 °C, 42% yield, (c) after purification from unreacted ODN **2** and template ODN **3** by streptavidin-linked magnetic beads.



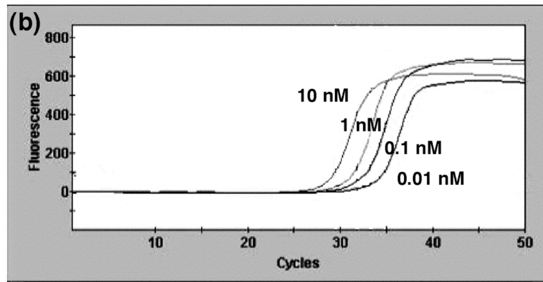




**Figure 4.** PCR product detection in real time: (a) amplification plot generated by 80 mer DNA; (b) amplification plot generated by DNA **4**.

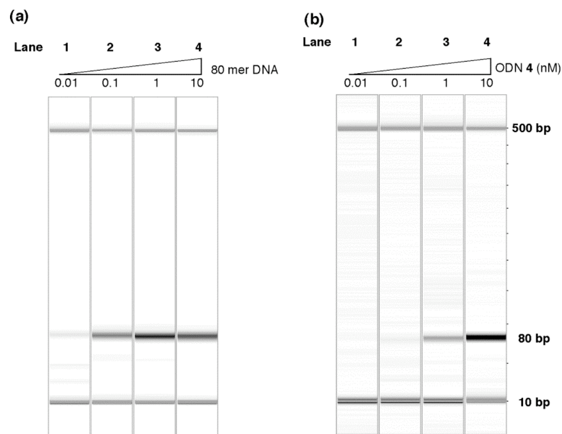



**Figure 5.** Microchip electrophoresis of the PCR of 80 mer DNA and ODN **4** with Ex Taq DNA polymerase. Concentrations of the 80 mer DNA in lanes 1 to 4: 0.01 nM (a), 0.1 nM, 1 nM and 10 nM. Concentrations of the ODN **4** in lanes 1 to 4: 0.01 nM, 0.1 nM, 1 nM and 10 nM (b).