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 (CVU)-containing ODN at the 5'-terminal. This method forms d(T-CVU) as a cyclobutane pyrimidine dimer (CPD) analogue between the 3'-terminal thymidine and the 5'-terminal CVU of two oligodeoxynucleotides (ODNs). In this study, we performed PCR using a DNA template containing d(T-CVU). Then, we found that two adenines were incorporated opposite the d(T-CVU).

Keywords: Bioorganic Chemistry; Biopolymer; DNA technology

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 modelled on the structure of the cyclobutane pyrimidine dimer (CPD) between 3'-terminal thymidine and 5'-terminal 5-carboxy-2'-deoxyuridine (CVU) (Fig. 1) [10–15]. We modified nucleosides containing various DNA functional groups that react after hybridization by photolysis 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, \textit{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-CVU) has higher flexibility than usual CPD (Fig. 2). Therefore, we expect that d(T-CVU) 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-CVU) during the PCR, and we found that Ex Taq DNA polymerase can read a template DNA containing d(T-CVU). Additionally, we determined that two adenines were incorporated opposite d(T-CVU).

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\textsubscript{3}, methyl acrylate and 2-cyanoethyl N,N,N',N'-tetra-isopropyl-phosphorodiadime were purchased from Aldrich. DMAP was
purchased from ACROS ORGANICS. 1H-tetrazole was purchased from Glen Research. The reagents for the DNA synthesizer such as I2 solution (I2/H2O/pyridine/tetrahydrofuran, 3:2:19:76), A-, G-, C-, and T-β-cyanoethyl phosphoramidites were purchased from Glen Research. Photoligation products were purified by Dynabeads M-280 streptavidin (Roche). Other reagents 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 (1500 units) was purchased from Yamasa. Reactions were monitored on TLC plates precoated with Merck silica gel 60 F254. Kanto Chemical Silica Gel 60 N was used for silica gel column chromatography. 1H-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 a controlled pore glass supports by using an Applied Biosystems Model 3400 synthesizer. The 0.1 M acetonitrile solution of CVU was used in automated synthesis, the oligonucleotides were cleaved from the support by conc. aqueous ammonia for 1 h 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)+] calculated 7714.01 for [M+H]+, found 7714.29.

2.2. Synthesis of oligonucleotides

The synthesis of the photoreactive nucleosides and the corresponding phosphoramidite building block of CVU as well as the synthesis of corresponding oligodeoxynucleotides followed standard routes in DNA chemistry. CVU was synthesized from 5-iodo-2′-deoxouridine; the scheme was reported previously [11]. Oligonucleotides were prepared by the β[(cyanoethyl)phosphoramidite method on a controlled pore glass supports by using an Applied Biosystems Model 3400 synthesizer. The 0.1 M acetonitrile solution of CVU was used in automated synthesis, the oligonucleotides were cleaved from the support by conc. aqueous ammonia for 1 h 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′-deoxynucleonucleotide at 37 °C for 3 h. MALDI-TOF MS; calculated for ODN 1 [(M+H)+] calculated 7714.01 for [M+H]+, found 7714.29.

2.3. Photoligation of DNA oligomer

The reaction mixture (total volume 60 µL) containing ODN 1 and ODN 2 (each 10 µM strand concentration) in the presence of template ODN 3 (10 µM 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) (Steps 3, 4 in Scheme 1).
2.5. PCR amplification of ODN 4

2.5.1. PCR by Ex Taq polymerase

Real-time PCR was set up using a 25 μL volume, with 10 nM ODN 4, 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl₂, each dNTP 0.4 mM, 0.3 μM primer pair, 1× SYBER Green and 2.5 unit Ex Taq 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.

2.5.2. PCR by rTaq polymerase

Real-time PCR was set up using a 25 μL volume, with 10 nM ODN 4, 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl₂, each dNTP 0.4 mM, 0.3 μM primer pair, 1× 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 30 s and 74 °C for 60 s, and was followed by a final extension of 74 °C for 10 min.

2.5.3. PCR KOD dash polymerase

Real-time PCR was set up using a 25 μL volume, with 10 nM ODN 4, 10 mM Tris–HCl, 50 mM KCl, 2 mM MgCl₂, each dNTP 0.4 mM, 0.3 μM primer pair, 1× 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.

2.5.4. PCR by Vent exo(-) polymerase

Real-time PCR was set up using a 25 μL volume, with 10 nM ODN 4, 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl₂, each dNTP 0.4 mM, 0.3 μM primer pair, 1× 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.

3. Results and discussion

3.1. Synthesis of oligonucleotides

CVU was prepared according to the method reported previously [11]. ODN containing CVU, 5′-d(CVUGAGCTGCTTGGCGCGCTCCCCGT)-3′ (ODN 1) was synthesized using phosphoramidite of CVU 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 (Fig. 3b).

Table 1

| Oligonucleotide sequences (5′–3′) |
|----------------------------------|
| ODN 1   | CVUGAGCTGCTTGGCGCGCTCCCCGT       |
| ODN 2   | Biotin-CACCTGCGCAAGCTGCTAAGCGCGCTCTCCTCGCGATGCCGATGACCTGCAAGT |
| ODN 3   | GCCAAGCGCACTCCAACTCTGGCAGCTA   |
| Primer F| CACCTGCGCAAGCTGCTAAGCGCGCTCTCCCTCGCGATGCCGATGACCTGCAAGT |
| Primer R| AGCGCGCAAGCTGCTAAGCGCGCTCTCCCTCGCGATGCCGATGACCTGCAAGT |
| 80 mer DNA | CACCTGCGCAAGCTGCTAAGCGCGCTCTCCCTCGCGATGCCGATGACCTGCAAGT |

Scheme 1. Strategy for the synthesis of template ODN for PCR.
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 (Fig. 3c).

3.4. PCR amplification of ODN 4

We examined the ODN 4 containing d(T-CVU) during the PCR using four thermostable DNA polymerase, KOD dash, Vent (exo-), rTaq, and ExTaq DNA polymerase. ODN 4, and primer F, 5'-CACCTGCGCAAGCTGCCGCTAA-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, 1, 0.1, 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 (Fig. 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. Fig. 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-CVU), in contrast to the cDNA of two thymidines at that position (data not shown).

4. Conclusion

This study demonstrates that d(T-CVU)-containing DNA were substrates for Ex Taq DNA polymerase yielding the corresponding PCR products. Additionally, two adenines were preferentially inserted opposite d(T-CVU). Employing these methods, we are currently investigating a new CPD analogue that was synthesized from other photosensitive nucleosides.
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Fig. 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 1–4: 0.01 nM (a), 0.1, 1 and 10 nM. Concentrations of the ODN 4 in lanes 1–4: 0.01, 0.1, 1 and 10 nM (b).