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Enzymatic Synthesis of Labeled DNA by PCR Using New Fluorescent Thymidine Nucleotide Analogue and Superthermophilic KOD Dash DNA Polymerase

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Abstract—Triphosphate of a new fluorescent labeled thymidine analogue was incorporated as a substrate for PCR using KOD Dash DNA polymerase forming the corresponding fluorescent labeled DNA which is useful for a DNA probe.

Fluorescence-labeled DNA probes are important tools for biological and biochemical studies and for diagnostic use. A variety of methods for the synthesis of the fluorescent DNA have been reported over the past 10 years.[1–3] Probes containing higher density of labels would increase detection sensitivity. Polymeric chain reaction is very useful for the simultaneous labeling and the amplification of DNA with high density of the fluorophore, if a fluorescent nucleotide can be accepted by DNA polymerase as a substrate. It is reported that Taq and Vent DNA polymerases can use 5′-triphosphates of 2′-deoxyuridine derivatives bearing a (*E*)-propenyl or propynyl linker at C5 position, but no other modified substrate analogue.[7–11] Thus, 2′-deoxyuridine derivatives bearing a fluorophore via 3-aminopropenyl or 3-aminopropynyl linker at C5 position have been developed and used as a fluorescent-labeled substrate for Taq DNA polymerase, although co-existence of the natural substrate TTP is required for the synthesis of a full-length DNA probe.[3–6] For example, Ried et al. reported synthesis and application of the DNA probes by PCR using Taq DNA polymerase and the fluoresence-labeled deoxyuridine derivative with a 3-aminopropenyl linker.[4] Cyanine dye-labeled deoxyuridine derivatives with a 3-aminopropenyl linker can be also accepted by Taq DNA polymerase yielding the corresponding fluorescent labeled DNA probe.[5,6] Although they are now commercially available, more efficient labeled nucleotides and enzymes are required to prepare the labeled DNA probes for use in the diagnosis and in the molecular biology.

Previously, we reported the synthesis of thymidine analogues bearing a methylene group at the C5 α-position with an amino-linker arm, and their introduction into oligodeoxyribonucleotides[12,13] using a DNA synthesizer. Very recently, we reported that triphosphates of the thymidine analogues could be accepted by KOD Dash DNA polymerase as a substrate in the PCR.[14] The triphosphate of the thymidine analogue with amino-linker was further reacted with fluorescein isothiocyanate, yielding a new fluorescence-labeled thymidine analogue. Here we report preparation of the fluorescent nucleotides and their application for the synthesis of fluorescence-labeled DNA by PCR using KOD Dash DNA polymerase. Post-labeling of the modified DNA prepared from the thymidine analogue by PCR was also carried out by reaction with fluorescein isothiocyanate.

5′-Triphosphate of 5 - *N* - (6 - aminohexyl)carbamoylmethyl-2′-deoxyuridine (**1**) was synthesized by the method described previously.[14,15] The terminal amino group of the nucleotide was reacted with 4.4 equivalent of fluorescein isothiocyanate in 0.25 M carbonate buffer (pH 9.2) containing DMF to furnish the fluorescence labeled-thymidine analogue (**2**) in 28% yield, after purification by HPLC. The nucleotide **1** was reacted with *N*-[ε-trifluoroacetylcaproyloxy]succinimide ester,

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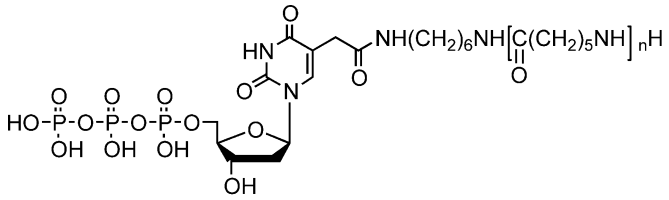

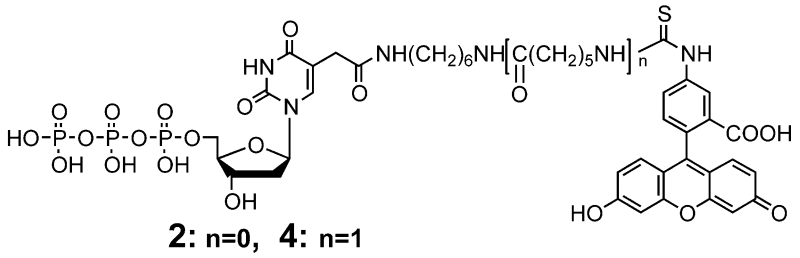

and subsequent removal of trifluoroacetyl group by treatment with ammonium hydroxide gave the nucleotide with a longer amino-linker **3**, which was further labeled with fluorescein isothiocyanate yielding **4**, to examine the effect of the length of the linker arm on the PCR. The nucleotides, **2**, **3** and **4**, were purified by preparative HPLC on ODS-silica gel and confirmed by ESI-mass spectrum.[16]

We examined the incorporation of the triphosphates of fluorescence labeled thymidines in place of TTP during the PCR using KOD Dash DNA polymerase. pUC18 plasmid DNA, and DNA **A**, 5′-GGAAACAGCT ATGACCATGATTAC-3′ and DNA **B**, 5′-CGACGTT GTAAAACGACGGCCAGT-3′ were used as a template and primers, respectively, for the PCR. The PCR were carried out at 94 °C/1 min, 30 cycles of 94 °C/30 s–52 °C/30 s–74 °C/1 min, and 74 °C/5 min in the reaction mixture (20 μL) containing 0.5 ng/μL DNA template (pUC18 2686 bp), 0.4 μM of each primer, natural dNTPs or modified dNTP mix (0.2 mM of each nucleotide), 0.5 unit/10 μL of the enzyme (0.05 unit/10 μL of the enzyme for natural substrate TTP) in the buffer supplied by the maker for the DNA polymerase reaction. The reaction mixture was quenched by addition of formamide–dye solution and PCR products were analyzed by 2% agarose gel electrophoresis and visualized by staining with ethidium bromide. The formation of the fluorescence-labeled DNA can be detected without ethidium bromide staining. The PCR with the natural substrate, TTP and the modified substrate **1** demonstrated formation of the 108 base pair DNA product as expected from the sequence of primers and template (Fig. 1). The modified substrate bearing a long amino-linker arm, **3** also worked as a substrate in the PCR forming the 108mer DNA. The modified DNA products showed slower mobility shift on electrophoresis depending on the mass increase and the charge of the substituent group. The PCR with the fluorescence-labeled TTP analogues, **2** and **4**, could not form the corresponding full-length 108mer DNA. Successful PCR with this template and primers requires the incorporation of 49 modified substrates with a single stretch of four successive thymidine residues. A bulky fluorescence group in **2** or **4** likely suppresses the successive incorporation of the modified substrate.

We have further examined combination use of the fluorescent labeled-TTP, **2** and TTP or **1** for the synthesis of the full-length fluorescence-labeled DNA, as multiple molecules of **2** may be incorporated at isolated positions, but successive incorporations of **2** can be avoided by incorporation of TTP or **1**. Figure 2 shows the formation of the PCR products from the combination of **2** and **1** (I: lanes 3–8) and **2** and TTP (II: lanes 10 and 11), before (A) and after ethidium staining (B). Fluorescence without ethidium staining showed the

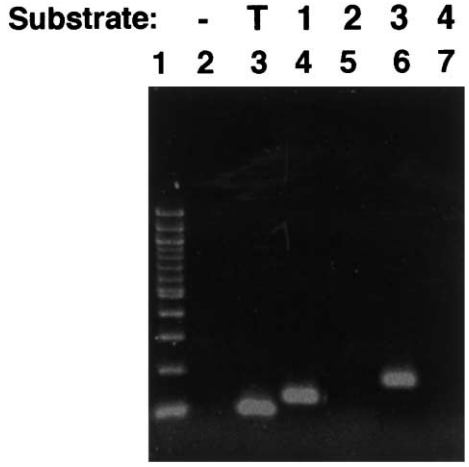

Figure 1. PCR assays of TTP analogues using KOD Dash DNA polymerase. The mixture (20 μL) for PCR contained 0.5 ng/μL DNA template (pUC18 2686 bp), 0.4 μM of each primer, natural dNTPs or modified dNTP mix (0.2 mM of each nucleotide), 0.5 unit/10 μL or 0.05 unit/10 μL of DNA polymerase in the buffer supplied by the maker for the DNA polymerase reaction. PCR assays were carried out at 94 °C /1 min, 30 cycles of 94 °C/30 s–52 °C/30 s–74 °C/1 min, and 74 °C/5 min. The reaction mixture was quenched by addition of formamide–dye solution and PCR products were separated by 2% agarose gel electrophoresis. The gel was visualized after staining with ethidium bromide. Lane 1: marker DNA (100–1200 bp); lane 2: negative control without TTP; lane 3: positive control: TTP + dNTP; lane 4: **1** + dNTP, lane 5: **2** + dNTP; lane 6: **3** + dNTP; lane 7: **4** + dNTP (dNTP: dATP + dCTP + dGTP).

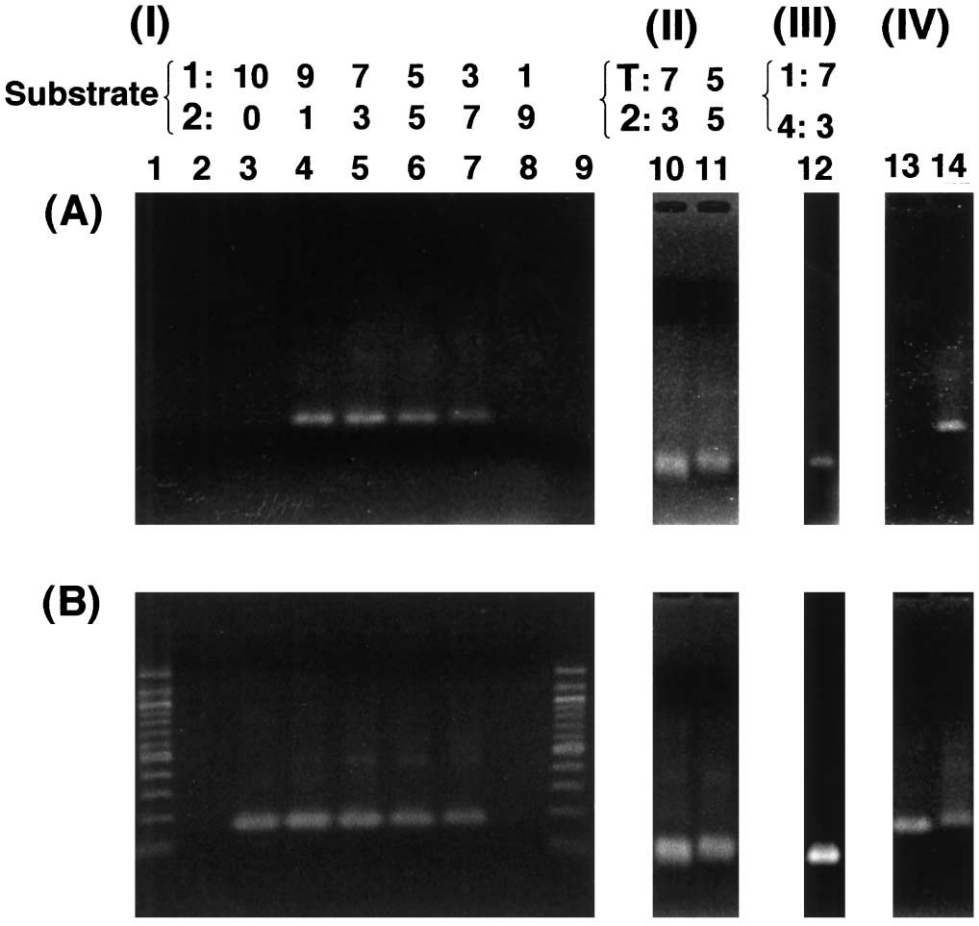

Figure 2. Synthesis of fluorescent-labeled DNA by PCR using KOD Dash DNA polymerase. PCR assays were carried out in the same way as those in Figure 1, except the reaction time for the fluorescent labeled thymidine analogues (I, II and III), 30 cycles of 94 °C/30 s–52 °C/1 min–74 °C/2 min. The gel was analyzed directly (A) and after staining with ethidium bromide (B). Lanes 1 and 9: marker DNA (100–1200 bp); lane 2: negative control without TTP; lane 3: positive control **1**; lane 4: **1** + **2** (9:1); lane 5: **1** + **2** (7:3); lane 6: **1** + **2** (1:1); lane 7: **1** + **2** (3:7); lane 8: **1** + **2** (1:9); lane 10: TTP + **2** (7:3); lane 11: TTP + **2** (1:1); lane 12: **1** + **4** (7:3); lane 13: **1**; lane 14: same as lane 13 but after most labeling with fluorescein isothiocyanate. All reaction mixtures contained dNTP (dATP + dCTP + dGTP) in addition to TTP or TTP analogues.

formation of fluorescent DNA. Small ratio of **2** against TTP or **1** gave the PCR product in large quantity but with low or no fluorescent labeling. On the other hand, large ratio of **2** to TTP or **1** suppressed the polymerization yielding low or no DNA product although with high labeling concentration. Thus, use of **2** and TTP or **1** in 5:5 to 3:7 ratio gave the best result for synthesis of fluorescence labeled DNA in reasonable yields. Similarly combined use of **4** and **1** in 3:7 ratio gave the full length 108mer fluorescent DNA by PCR (III: lane 12).

We further examined the post-labeling of the modified DNA obtained from **1** by PCR. The PCR product from **1** was isolated by ethanol precipitation and reacted with fluorescein isothiocyanate to furnish the fluorescence labeled DNA as shown in Figure 2 (IV: lane 14). A DNA probe with other fluorophore such as cyanine dye could be obtained similarly by introduction of an amine-reactive fluorophore derivative into the modified DNA from **1**.

In conclusion, PCR using a new fluorescent nucleotide with KOD Dash DNA polymerase provides a new entry for the synthesis of a DNA probe with a high density of fluorescence, and complements the previous method of the DNA probes synthesis in which fluorescence labeled deoxyuridine derivatives with a 3-aminopropenyl linker and Taq DNA polymerase are used.[4–6] The corresponding fluorescent DNA can be also obtained by post-labeling of the modified DNA from the TTP with an amino-linker arm.

References and Notes

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