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EFFECTS OF A MODIFIED DYE-LABELED NUCLEOTIDE SPACER ARM ON INCORPORATION BY THERMOPHILIC DNA POLYMERASES

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□ *The ability of eight commercially available thermophilic DNA polymerases to sequentially incorporate fluorescently labeled nucleotides sequentially was analyzed by a gel based primer extension assay. Cy5-dUTP or a variant nucleotide in which the linker had been lengthened by 14 atoms between the dye and the nucleobase were compared. We found that the Cy5-dUTP with a longer linker resulted in longer primer extension lengths. Furthermore, some of the assayed polymerases are capable of extending the primer to the full or near full length of 30 nucleotides using dye-labeled nucleotides exclusively.*

Keywords DNA polymerase; Fluorescent nucleotide; Sequencing by synthesis

INTRODUCTION

The ability of DNA polymerase to sequentially incorporate dye-labeled nucleotides has profound implications in the field of DNA sequencing, especially in the single-molecule sequencing-by-synthesis paradigm.[1,2] Sequencing by synthesis generally entails the sequential addition of fluorophore-labeled deoxynucleoside triphosphates (dNTPs) by DNA polymerase. If all dNTPs are labeled with the same fluorophore, it is necessary to add each known dNTP separately to the template in order to

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obtain sequence information. If each dNTP is labeled with a unique fluorophore, it may be possible to add all four dye-labeled dNTPs at once and observe the polymerase extending the template sequence through time.

In each of these scenarios, the number of bases that the polymerase is able to extend a template sequence solely using dye labeled dNTPs is limited, likely because the bulk of the fluorophore side chain stemming off of the nucleotide sterically inhibits the polymerase from extending the template. In support of this theory, dNTPs containing cleavable linkers allow template extension in an unhindered manner.[3–5] Others have shown that by increasing the length of this side chain, DNA polymerase is able to incorporate more fluorophore-labeled dNTPs into the growing primer.[6] Scattered reports of conditions under which DNA polymerases is able to synthesize DNA exclusively from labeled nucleotides exist,[7–9] however, an established protocol capable of generating high product yield independent of template sequence has remained elusive. The efficiency of labeled nucleotide incorporation by DNA polymerase depends on many factors and is influenced both by the dye label as well as the nucleobase substrate.[10]

Although a correlation between fluorophore-dNTP linker length and the ability of DNA polymerase to incorporate a greater number of dye-labeled nucleotides into a given template DNA molecule has been qualitatively established, the extent of this linker length effect on subsequent nucleotide incorporations is unknown. We designed a gel-based assay to directly examine the extent of primer extension by DNA polymerase in the presence of either commercially available Cy5-dUTP (Cy5-10-dUTP) or a Cy5-dUTP variant in which the linker has been lengthened by 14 atoms (Cy5-24-dUTP) (see Figure 1). We assayed eight thermophilic DNA polymerases using either Cy5-10-dUTP or Cy5-24-dUTP as substrate. All of the assayed polymerases generated products of greater length when provided with Cy5-24-dUTP as substrate. The extent of increased product length varied for individual DNA polymerases.

MATERIALS AND METHODS

Nucleotides

dTTP was purchased from Roche (Indianapolis, IN). Cy5-10-dUTP was purchased from Amersham Biosciences (Piscataway, NJ). Cy5-24-dUTP was prepared in a manner similar to the protocol developed by Waggoner[6] as follows: Cy5-10-OSu was coupled to 6-(6-amino-hexanoylamino)-hexanoic acid (11) under standard conditions to produce Cy5-24-OH. After activation to the succinimidyl ester, Cy5-24-OSu was reacted with AP-dUTP to afford Cy5-24-dUTP. ESI-MS: [M-1] 1384.3, [M + 4Na] 1473.3.

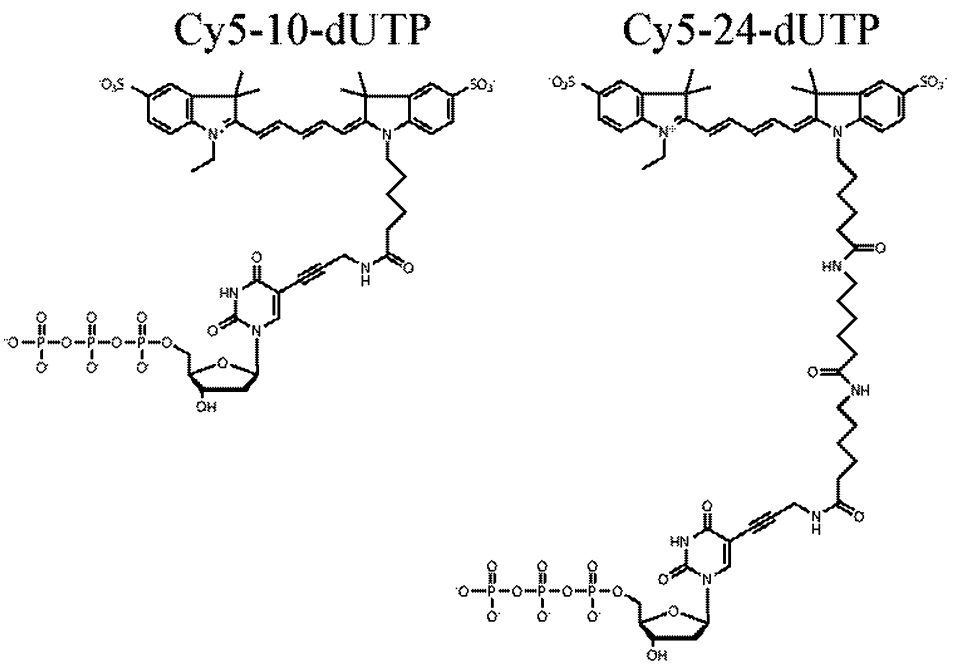

FIGURE 1 Commercially available Cy5-dUTP (Cy5-10-dUTP) and longer linker derivative Cy5-24-dUTP. Cy5-24-dUTP contains two additional aminohexanoic acid fragments relative to Cy5-10-dUTP. These extra groups increase the distance between Cy5 and the deoxynucleoside triphosphate by 14 atoms.

Template Preparation

Synthetic oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Annealing of primer with template was performed by mixing 3 nmol of Cy3-labeled primer (5′-Cy3-GTCTGGGCTTTTGGTTTGTGGG-3′) with 3 nmol of template (5′-$[A]_{30}$CCCACAAACCAAAAGCCCAGAC-3′) in 50 μL annealing buffer (150 mM NaCl, Tris-HCl, pH 7.2), heating the mixture for 5 min at 100°C, and cooling to room temperature over 1 h. In order to remove any unannealed DNA, the mixture was then treated with 50 units of Exonuclease I (New England Biolabs, Beverly, MA) and allowed to incubate at 37°C for 2 h. Annealed duplex DNA was purified from the reaction using the QiaQuick Nucleotide Removal Kit (Qiagen, Valencia, CA).

Primer Extension

Primer extension reactions were performed using 15 pmol of annealed duplex DNA in 50 μL reaction buffer (Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 12.5 mM dithiothreitol) containing 25 μM of the appropriate nucleotide and 1 unit of the appropriate DNA polymerase (LA Taq (Takara Mirus Bio, Madison, WI), Q-BioTaq (Qbiogene, Irvine, CA), Vent Exo$^-$ (New England

Biolabs, Bevarly, MA), Sequitherm (Epicentre, Madison, WI), Deep Vent Exo$^-$ (New England Biolabs), ThermalAce (Invitrogen, Carlsbad, CA), Thermus (Chimerx, Milwaukee, WI), and Taq (Qiagen, Valencia, CA). Reactions were allowed to incubate for 3 h at 60°C after which they were stopped by the addition of 2 μL 100 mM EDTA. Excess nucleotides and DNA polymerase were removed from each reaction using the QiaQuick Nucleotide Removal Kit. Extension reaction product was eluted from the kit's column using 50 μL 90% formamide.

Product Analysis

Electrophoretic separation of primer extension reaction products was performed using denaturing 15% (w/v) polyacrylamide TBE-urea gels (Invitrogen, Carlsbad, CA). Two microliters of denatured GeneScan LIZ120 (Applied Biosystems, Foster City, CA) was used as a size standard. Ten microliters of extension reaction product (approximately 2 pmol) was mixed with 10 μL of 2X TBE-urea preparative buffer (Invitrogen) containing no dyes. This mixture was heated to 100°C for 5 min to denature primer product from template and immediately transferred to an ice slurry. Samples were loaded onto the gel and ran in 1X TBE at constant 180 V for 38 min at 55°C. Gels were transferred to 500 mL of fixing solution (10% acetic acid, 10% methanol) and gently shaken for 1 h, after which they were washed 3 times (15 min each wash) with 500 mL of ultra pure water. Gels were imaged on a Typhoon 8600 variable mode imager (Amersham Biosciences, Piscataway, NJ) at high sensitivity and 100 μm resolution. Cy3 fluorescence was visualized using the green (532 nm) laser as excitation source (PMT setting at 700 V) and a 555 nm BP 20 emission filter. Cy5 and LIZ were detected using the red (633 nm) laser (PMT setting at 800 V) as excitation source and a 670 nm BP 30 emission filter. Images were processed and channel cross-contamination was removed using the Typhoon's IQ Solutions software package.

RESULTS AND DISCUSSION

We designed a primer extension assay to screen several commercially available thermophilic DNA polymerases for their ability to incorporate a Cy5-labeled dNTP. Additionally, we sought to compare the extent to which these polymerases were able to extend the primer using a Cy5-dUTP variant that contained a longer spacer arm between the dye and the dNTP.

To validate that the buffer conditions were appropriate for the given polymerases, we first performed the primer extension assay using unlabeled dTTP as polymerase substrate. As shown in Figure 2 (lanes 3–10), under these conditions, each of the tested polymerases extended all detectable primer to full or near full length (52 bases). Slight differences in product size may

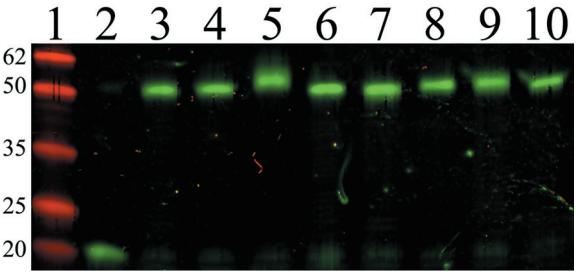

FIGURE 2 Size separation of primer extension reactions on a 15% (w/v) denaturing polyacrylamide gel imaged on a Typhoon 8600 scanning imager. Lane 1 shows the GeneScan LIZ120 size standard (red). Numbers on the left indicate size in bases. Lanes 2–10 are primer extension reactions using dTTP as polymerase substrate in which green represents fluorescence from the Cy3-labeled primer. Lane 2 shows the negative control in which no polymerase was added to the reaction. Lanes 3–10 are positive controls and represent primer extension reactions in which the following thermophilic DNA polymerases were used: LA Taq (lane 3), Q-BioTaq (lane 4), Vent Exo$^-$ (lane 5), Sequitherm (lane 6), Deep Vent Exo$^-$ (lane 7), ThermalAce (lane 8), Thermus (lane 9), and Taq (lane 10).

be due to template independent extension of the fully extended strand by one or a few bases at its 3′ end, a known property of some thermophilic DNA polymerases exploited in molecular subcloning of PCR products into plasmid vectors.[12] Figure 2 (lane 2) shows the unextended primer of 22 bases under identical conditions in the absence of polymerase.

The assay was repeated with both commercially available Cy5-dUTP (Cy5-10-dUTP) and a longer-linkered variant (Cy5-24-dUTP) as substrate (Figure 3). When provided with Cy5-10-dUTP as substrate, most polymerases

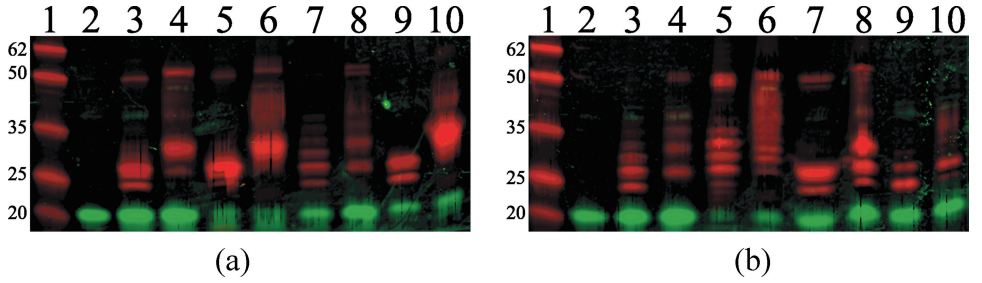

FIGURE 3 Size separation of primer extension reactions using Cy5-10-dUTP and Cy5-24-dUTP as polymerase substrates. Extension products were separated on 15% (w/v) denaturing polyacrylamide gels and imaged using a Typhoon 8600 imaging system. For both A and B, lane 1 shows the GeneScan LIZ120 size standard (red). Numbers on the left indicate size in bases. A) Lane 2 shows the negative control in which no polymerase was added to the reaction containing Cy5-10-dUTP. Lanes 3, 5, 7, and 9 used Cy5-10-dUTP as polymerase substrate, while lanes 4, 6, 8, and 10 used Cy5-24-dUTP as polymerase substrate. Red represents fluorescence from incorporated Cy5-labeled nucleotides and green represents fluorescence from the Cy3-labeled primer. The following DNA polymerases were used: LA Taq (lanes 3 and 4), Q-BioTaq (lanes 5 and 6), Vent Exo$^-$ (lanes 7 and 8), and Sequitherm (lanes 9 and 10). B) Lane 2 shows the negative control in which no polymerase was added to the reaction containing Cy5-24-dUTP. Lanes 3, 5, 7, and 9 used Cy5-10-dUTP as polymerase substrate, while lanes 4, 6, 8, and 10 used Cy5-24-dUTP as polymerase substrate. The following DNA polymerases were used: Deep Vent Exo$^-$ (lanes 3 and 4), ThermalAce (lanes 5 and 6), Thermus (lanes 7 and 8), and Taq (lanes 9 and 10).

were able to extend the primer to varying degrees by approximately 5–10 bases. Notably, several polymerases appear to convert at least a portion of primer to full or near full length product (LA Taq, Figure 3A (lane 3), ThermalAce Figure 3B (lane 5), and Thermus, Figure 3B (lane 7)).

When Cy5-24-dUTP is instead provided as substrate, the length of the longest extended primer for each polymerase was greater. LA Taq (Figure 3A, lane 4), Q-Bio Taq (Figure 3A, lane 6), Deep Vent Exo$^-$ (Figure 3B, lane 4), Thermal Ace (Figure 3B, lane 6), and Thermus (Figure 3B, lane 8) appear to extend a portion of the primer to full or near full length. Strikingly, ThermalAce produces product ranges between approximately 30 and 52 bases (primer extended by 8–30 nucleotides, respectively).

We were surprised to observe that a large fraction of primer remained unextended in the assay when labeled-dUTP was provided as substrate. With the exception of Q-Bio Taq and Thermal Ace, the majority of primer appears to run the same length as negative controls containing no polymerase (Figures 3A and 3B, lane 2). Possibly, although sufficient time was given to fully extend the template with dTTP, more time may be needed with labeled dNTPs. Alternatively, labeled dNTPs may interfere with polymerase docking or the initiation of synthesis for our chosen template.

We originally sought to quantitate extension product by labeling our primer with Cy3. However, as seen in Figure 3, although incorporated Cy5 nucleotides are readily detected, we were unable to detect any significant Cy3 fluorescence from the extended products. One possibility is that multiple incorporation events enhance Cy5 fluorescence levels stoichiometrically. Additionally, Cy5 is an acceptor for Cy3 fluorescence energy. Although 22 bases (the distance between the Cy3 tag and the first incorporation site) exceeds the Forster radius for the Cy3/Cy5 fluorescent resonant energy transfer (FRET) pair, the sheer number of Cy5 molecules on the extended product and within the three dimensional space of the gel may absorb this energy and mask Cy3 fluorescence.

In conclusion, we compared the ability of eight commercially available thermophilic DNA polymerases to extend a primer utilizing only Cy5-10-dUTP or Cy5-24-dUTP as substrate. Our findings show that using the longer linkered Cy5-24-dUTP results in greater extension lengths as well as an increase in the amount of product extended to full or near full length.

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