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# Photoregulation of DNA polymerase I (Klenow) with caged fluorescent oligodeoxynucleotides

XinJing Tang, Julia L. Richards, Adam E. Peritz and Ivan J. Dmochowski*

*Department of Chemistry, University of Pennsylvania, 231 South 34th Street, Philadelphia, PA 19104-6323, USA*

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**Abstract**—The DNA polymerase reaction by Klenow fragment (KF) was efficiently regulated with UV light using a 25-mer caged fluorescent oligodeoxynucleotide (CFO) as the template. The CFO was functionalized with a fluorescein reporter (Fl) and photocleavable DABSYL quencher moiety (Dab). With Fl and Dab at adjacent cytidines in the middle at the template, KF was blocked from extending a complementary 12-mer primer. Upon UV photolysis of the DABSYL blocking group under aerobic conditions, fluorescein emission was restored and 50% of the primers were fully extended by KF.


The ability to trigger biomolecular events using photoactivatable 'caged' compounds has found wide usage in chemistry and biology.[1–7] Caged DNA or RNA, whose function is transiently blocked by a photolabile moiety, offers exciting possibilities to control protein–nucleic acid interactions for applications in biochemistry, biotechnology, and gene regulation. However, there are still few examples of caged nucleic acids, due to synthetic and experimental challenges. Bai et al. successfully incorporated a photolabile group at the 5′ or 3′ ends of a DNA oligonucleotide.[6,8] However, it has been more challenging to attach a suitably photoactive moiety to the middle of the oligodeoxynucleotide (ODN), where it can maximally perturb DNA function.[9,10] Synthetic methods developed in our lab[11] and elsewhere[12,13] allow the site-specific attachment of photoactive blocking groups to ODNs. The incorporation of a fluorescent reporter is unique to our system and allows real-time monitoring of the photoactivation process. This holds considerable advantages for many biological applications, but requires further development for in vivo studies. Herein, we demonstrate the ability of caged fluorescent oligodeoxynucleotides (CFOs) to regulate primer extension by the Klenow fragment (KF) of DNA polymerase I.

DNA polymerases are involved in all DNA syntheses occurring in Nature. These enzymes are the workhorses in numerous molecular biology core technologies, such as the polymerase chain reaction (PCR), cDNA cloning, genome sequencing, and detection of nucleotide variation within genes.[14,15] The Klenow fragment of *Escherichia coli* DNA polymerase I retains the $5' \rightarrow 3'$ polymerase and $3' \rightarrow 5'$ (proofreading) exonuclease activities. KF serves as a useful model in our lab for controlling protein–DNA interactions with light. One previous study on the photoregulation of DNA polymerase, used azobenzene attached to a short modulator oligonucleotide.[16] Unfortunately, by introducing this second primer, the overall efficiency of the polymerase was reduced. Moreover, the short timescale of the azobenzene *cis* → *trans* re-isomerization makes this photoswitchable moiety too unstable for most biological experiments. Therefore, we set out to develop CFOs to modulate KF activity using kinetic and thermodynamic data that had been collected with modified ODNs.[17] Recent studies showing that a TT dimer in the template can inhibit primer extension by DNA polymerase suggested that modifying adjacent bases of the template could block DNA synthesis.[18]

Templates **1**–**6** (Fig. 1) were synthesized using standard phosphoramidite chemistry in an automated DNA synthesizer. The cytosine phosphoramidites modified with fluorescein or pendant amine were synthesized and incorporated as described previously.[11] The 25-mer oligodeoxynucleotides were subsequently cleaved from the solid support and purified. Oligos **3** and **6** were synthesized through reaction with the photoactive quencher

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* Corresponding author. Tel./fax: +1 215 898 2037; e-mail: ivandmo@sas.upenn.edu

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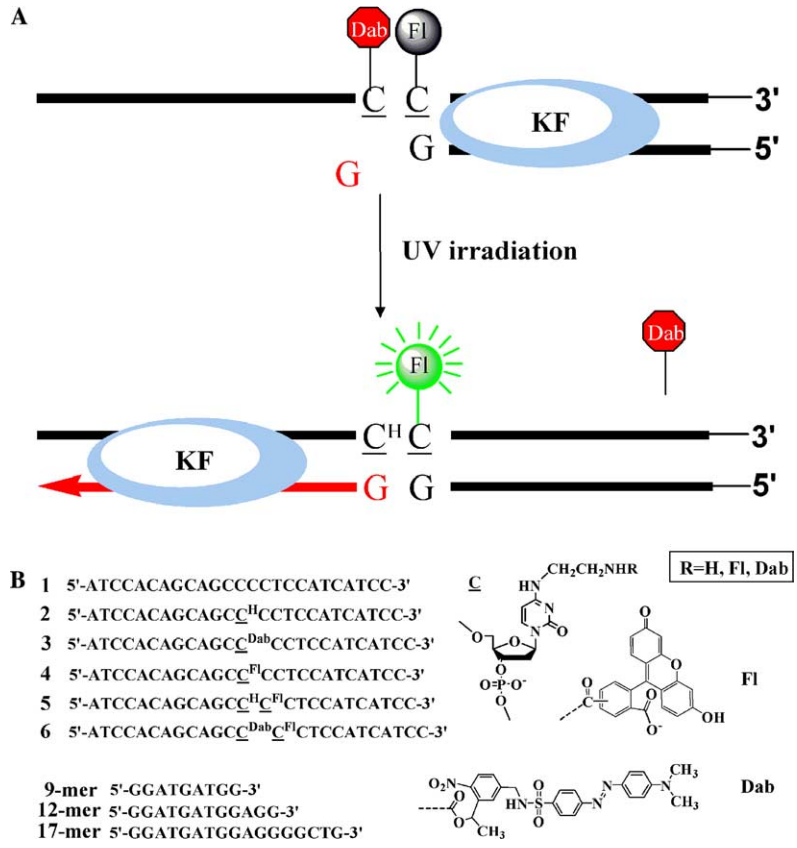


**Figure 1.** (A) Strategy for photoregulating primer extension by the Klenow fragment (KF). (B) Sequences of DNA templates and primers.

(Dab) and purified in 70% overall yield. CFO **6** contained a fluorescein reporter and photocleavable dimethylamino-azobenzenesulfonyl (DABSYL) quencher, attached at adjacent central cytidines (Fig. 1). The DABSYL moiety was attached to the oligonucleotide via a heterobifunctional 1-[5-(aminomethyl)-2-nitrophenyl]ethanol linker. This photocleavable spacer provides a convenient method for attaching diverse functionality to the oligonucleotide after solid-phase synthesis.

The 3′ end of the 12-mer primer was designed to terminate at the complementary $C^{Fl}$ of template **6**. Thus, primer extension using **6** required KF to incorporate dG opposite the sterically bulky $C^{Dab}$.[19] After UV irradiation of the reaction mixture (337 nm laser, 60 Hz, ~12.7 mW/cm$^2$), the yield of fully extended primer was 50% at 60 min, versus only 2.5% without irradiation (Fig. 2).

The efficiency of 12-mer primer extension depended greatly on the type of blocking group(s) and the number of modified bases on the template. With either the photocleavable DABSYL group (template **3**) or fluorescein (template **4**) as the only cytidine modification, the primer was fully extended within 20 min (Fig. 3). By comparison, with unmodified **1** or amino-linked cytidine **2**, the primer was fully extended within 5 min (Fig. 3). Thus, individually, the DABSYL and fluorescein moieties had very little effect on KF. The ethylamino-linked cytidine, $C^{H}$, had no apparent effect on primer extension at the time resolution of these experiments.

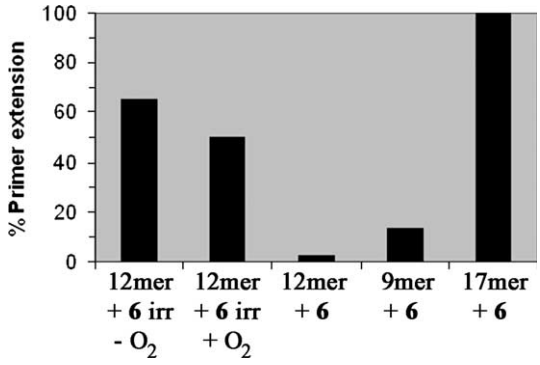


**Figure 2.** The percentage of primers (12-mer, 9-mer, and 17-mer) fully extended through the 25-mer template, observed 60 min after the addition of KF, incubation at 37 °C. Percentage of primer extension was determined with the phosphorimager by integrating the intensity of the 25-mer peak, dividing by the total intensity of the bands corresponding to the 12-mer and 25-mer, and multiplying by 100. The first two bars show the result of photoactivating the 25-mer template **6**, in the absence (bar 1) and presence (bar 2) of dioxygen. The deoxygenated sample was irradiated for 5 min with a Xe lamp (355 nm, 36 mW/cm$^2$). The oxygenated sample was irradiated for 40 min at 0 °C with a 337 nm laser (60 Hz, ~12.7 mW/cm$^2$). Only 2.5% of the 12-mer primers were extended by KF without irradiation (bar 3). 13.6% and 100% of the 9-mer and 17-mer primers were extended by KF without irradiation, bars 4 and 5, respectively.

Most interestingly, when both fluorescein and the photocleavable DABSYL were attached (template **6**), the blocking effect was substantially enhanced, as had been hypothesized. With the 12-mer primer (Fig. 3), it

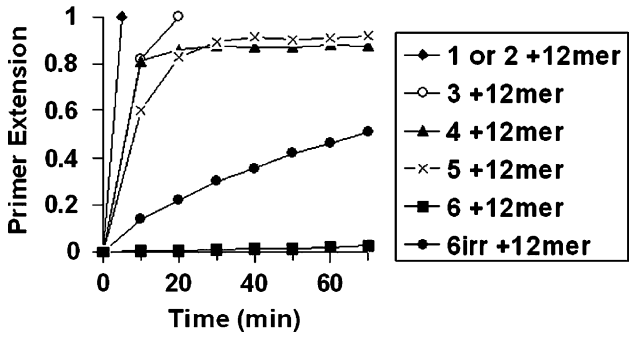


**Figure 3.** Kinetics of 12-mer primer extension by KF at 37 °C using templates **1**–**6**. The rates for extension of primers **1** and **2** (♦) were indistinguishable by this method. KF primer extension with templates **3** (○), **4** (▲), and **5** (×) exhibited similar kinetic behavior. KF primer extension with template **6** (■) was almost completely blocked even after 70 min, but upon UV irradiation to generate **6 irr** (●), the 12-mer was extended, although at a slower rate. Primer extension was quantified by PAGE analysis, where the normalized values on the *y*-axis equal the intensity of the 25-mer (fully extended) band divided by the total intensity of the bands corresponding to the 12-mer and 25-mer.

appeared that equilibrium was rapidly established between polymerase and exonuclease activities. Shorter (9-mer) and longer (17-mer) primers were tested under the same conditions, to determine the effective range of the DABSYL. The 17-mer primer was rapidly extended to 25 nt (Fig. 2). This result agreed with previous studies showing that KF has a footprint of ±4 nucleotides.[20] Some of the 17-mer was also cut back to 12 nt. With the shorter 9-mer, the DABSYL group had relatively little effect on KF reactivity until the primer was extended to 12 nt (Supplementary data). At this position, KF had to incorporate dG across from the DABSYL, and the enzyme appeared to stall. Although the reaction was sluggish with the 9-mer, 13.6% extension was observed at 60 min (Fig. 2). Thus, with a 'running start' KF extended more efficiently through the photocleavable DABSYL. This agrees with studies showing that shorter primers allow DNA polymerases to bypass a TT dimer in the middle of the template.[18]

The KF binding affinities for DNA duplexes of templates with the 12-mer primer were determined by gel-shift assays.[21] The $K_{D\cdot DNA}$ values were 140 nM for template **5** (containing the fluorescein-modified cytosine but no photocleavable DABSYL) and 150 nM for template **6**, compared to 100 nM for the template **1**-primer duplex.[19] Thus, $K_{D\cdot DNA}$ values cannot account for the large difference in primer extension rates between templates **5** and **6**. Furthermore, the melting temperatures ($T_m$) of duplexes of templates **1**–**6** and the 12-mer primer were virtually identical, 51 ± 1 °C. Thus, DABSYL and fluorescein worked cooperatively to stall KF primer extension without greatly affecting DNA duplex structure or initial KF binding.

Photolysis of the DABSYL was concomitant with an increase in fluorescein emission ($\lambda_{max}$ = 520 nm), which allowed the reaction to be monitored with a fluorometer. We showed previously that the photocleavable DABSYL can be 99% removed with a Xe lamp (355 nm, 36 mM/cm$^2$) in SCC buffer in 3 min.[11] Under the conditions of KF extension, photolysis was 95% complete in 5 min, as determined by fluorescence spectroscopy and HPLC. With the Xe lamp, the yield of primer extension was 30–40% and was increased to 50% using a pulsed $N_2$ laser. Adding KF and/or the primer after template irradiation made no improvement. Irradiating template **3**, containing only the photocleavable DABSYL and no fluorescein, in the presence of primer led to 99% extension after 10 min, which was virtually identical to the non-irradiated sample (Fig. 3). These studies indicated that the enzyme and primer were not damaged by low-level UV exposure. Furthermore, the photochemical bond cleavage of DABSYL in **3** was not inherently destructive to the DNA or enzyme. Indeed, the high yields of DNA uncaging with template **3** in aerobic solution compared favorably with previous studies using different photocleavable moieties.[3,13]

In order to understand the sub-optimal KF primer extension with photoactivated template **6**, we turned our attention to fluorescein. This fluorophore is a well-known photosensitizer for oxygen. This supports the finding that deaerating the solution before irradiating with the Xe lamp roughly doubled the efficiency of primer extension to 65% (Fig. 2). To resolve the remaining discrepancy in KF primer extension yields between templates **3** and **6**, we studied the products formed by irradiating **6** (known as **6 irr**). Perplexingly, **6 irr** looked virtually identical to **5** by HPLC, giving a single peak with the same retention time. However, purified **6 irr**, unlike **5**, was a poor template for the KF reaction. This agreed with the kinetic data for **6 irr** generated in situ (Fig. 3). Non-specific oxidative damage to **6** would be expected, in most cases, to have little effect on KF activity, and a much greater effect on the HPLC retention time. Further investigations showed that photoirradiating template **4**, which contained fluorescein but no adjacent amino linker, had no effect on KF primer extension. Thus, the ethylamino group played a role in blocking KF, but only in the presence of the nearby photoexcited fluorescein. No changes in the fluorescein absorption or emission spectral profiles were observed upon photoactivating templates **4**–**6**. These data taken together suggest a subtle perturbation of the DNA structure that is consistent with an intramolecular cross-link between the amino linker and adjacent fluorescein. The slower rate of primer extension with **6 irr** relative to **5** (Fig. 3) indicated that most of template **6** was modified during UV irradiation. This lowered the concentration of competent template, which was particularly significant since the template concentration was initially equimolar with the primer.

Despite the apparent crosslinking of **6**, it was possible to photoregulate KF primer extension 20-fold under aerobic conditions. Incorporating a less electrophilic fluorophore, or placing the fluorophore farther from the amino linker, should result in a more robust template and give even higher yields of DNA synthesis. These experiments are underway. We conclude that this photochemical strategy is sufficiently flexible to allow many other biological investigations of protein–DNA interactions.

Previous efforts to control gene expression with light have relied mostly on modifying plasmids or mRNA strands non-discriminately with multiple benzylated blocking groups.[3,5,7] Matsunaga et al. recently demonstrated the photoregulation of RNase H using a 20-mer DNA sense strand decorated with five azobenzenes.[22] The use of multiple photoactive groups in these examples delivered modest biological effects and necessitated high UV exposure, due to low quantum efficiencies and absorption from endogenous chromophores. Our CFOs and Heckel's caged ODNs[12,13] benefit from the site-specific incorporation of a single photoactive moiety. CFO **6** was also designed to limit near-UV absorption from chromophores (i.e., fluorescein) other than the photoactive linker, in order to maximize the quantum uncaging efficiency.

CFOs address the most critical synthetic and experimental obstacles to using caged oligonucleotides for biological investigations. We showed previously that CFOs can be photoactivated inside living zebrafish embryos without developmental consequences.[11] Importantly, the fluorescent reporter identified regions in the embryo where the CFO had been activated. Herein, we demonstrated the efficient regulation of KF activity using a single photocleavable group working in tandem with a fluorescent reporter. In these KF assays, the fluorescent reporter allowed the rapid optimization of the photocleavage conditions, without requiring extensive HPLC or mass spectrometric analysis. The results from these studies are guiding the development of other caged fluorescent compounds in the lab. By modulating specific protein–nucleic acid interactions, CFOs will provide new avenues for controlling gene expression in vivo.

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## Supplementary data

Details about thermal denaturation methods, KF primer extension studies, binding constant measurements, and kinetics experiments can be found, in the online version, at doi:10.1016/j.bmcl.2005.08.058.

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