Published online 17 March 2009

Nucleic Acids Research, 2009, Vol. 37, No. 8 ***e58***
doi:10.1093/nar/gkp150

Restriction enzyme-free mutagenesis via the light regulation of DNA polymerization

Douglas D. Young, Hrvoje Lusic, Mark O. Lively and Alexander Deiters*

Department of Chemistry, North Carolina State University, Raleigh, NC 27607-8204, USA

Received November 24, 2008; Revised February 17, 2009; Accepted February 20, 2009

ABSTRACT

The effects of photocaged nucleosides on the DNA polymerization reaction was investigated, finding that most polymerases are unable to recognize and read through the presence of a single caging group on the DNA template. Based on this discovery, a new method of introducing mutations into plasmid DNA via a light-mediated mutagenesis protocol was developed. This methodology is advantageous over several common approaches in that it requires the use of only two polymerase chain reaction primers, and does not require any restriction sites or use of restriction enzymes. Additionally, this approach enables not only site-directed mutations, but also the insertion of DNA strands of any length into plasmids and the deletion of entire genes from plasmids.

INTRODUCTION

Site-directed mutagenesis represents an invaluable molecular biology tool for the modification of DNA sequences, which is necessary for the investigation of protein function, as well as protein and genetic engineering. Numerous mutagenesis methods have been developed, based on DNA polymerase catalyzed oligonucleotide synthesis (1,2). DNA polymerases are essential enzymes for DNA synthesis applications, but also for the propagation of genetic information *in vivo*. These enzymes are responsible for the polymerization and replication of DNA using a template strand of DNA and deoxynucleotide triphosphates as monomeric building blocks, effectively catalyzing the formation of phosphodiester bonds (3). However, DNA polymerases are not capable of the *de novo* synthesis of DNA and thus require a 'primer' possessing a free 3′ hydroxyl group to initiate the polymerization. Numerous homologs of DNA polymerases exist in several species (5 prokaryotic and over 15 eukaryotic DNA polymerases are known) and their functions have been extensively studied (3). We hypothesized that obtaining photochemical control over their enzymatic activity would enable us to develop a new and versatile DNA mutagenesis technology. This methodology will enable the mutation, insertion, and deletion of any number of bases (only limited by DNA synthesis) in any plasmid without the use of restriction sites and restriction enzymes. In order to achieve this we explored the response of DNA polymerases to the presence of a photo-labile protecting group (caging group) installed on either the DNA template being amplified or the primers utilized to amplify the DNA. In previous studies different modifications both in the DNA phosphodiester backbone of the template, as well as on the nucleobases themselves have been incorporated (4,5).

Photocaging is an established approach towards achieving spatial and temporal control over biological processes through irradiation with UV light (6–11). The term 'caging' refers to the installation of a photolabile group on a biologically active molecule which abolishes the function of the biomolecule until it is irradiated with light of the appropriate wavelength. This concept has previously been employed in the caging of DNA, RNA, proteins and biologically active small molecules (6–11). We have developed the NPOM (6-nitropiperonyloxymethyl) caging group and employed it in the preparation of caged thymidine and deoxyguanosine phosphoramidites (12–14). These caged nucleotides can be incorporated into oligonucleotides via conventional solid-phase DNA synthesis under standard conditions (Figure 1). Recently, by our group and others, a photo-caged base has been employed in polymerase chain reaction (PCR) primers (15–17). Depending on the specific polymerase employed, different effects were observed ranging from termination of polymerization to enzymatic proof-reading of the abnormal base to yield a correct, full-length product.

Previously, we discovered that NPOM caging groups installed every five to six bases in a DNA oligomer (e.g. a 19-mer) effectively inhibit hybridization to its DNA or RNA complement (13,15,29). However, efficient hybridization was still observed in the presence of a single NPOM caging group (15). Here, we are reporting the effects and applications of NPOM caging groups installed on a DNA template during polymerase catalyzed replication. Based on the steric bulk of the caging group, we hypothesized that it may be feasible to prevent polymerase

*To whom correspondence should be addressed. Tel: +1 919 513 2958; Fax: +1 919 515 5079; Email: alex_deiters@ncsu.edu

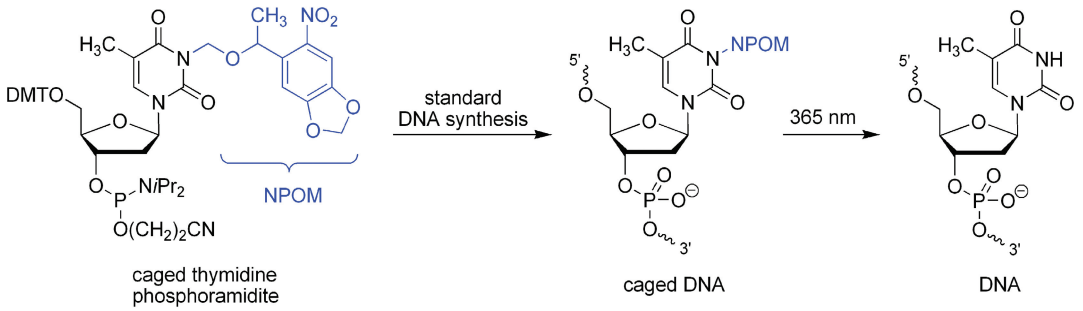

Figure 1. NPOM caged, 5′ dimethyltrityl (DMT) protected thymidine phosphoramidite and its incorporation into synthetic DNA. The caged DNA can be effectively decaged through a brief irradiation with UV light of 365 nm.

extension. This approach would then afford the development of a facile methodology for the light-mediated site-directed mutagenesis, as well as the addition and removal of DNA to and from plasmids.

MATERIALS AND METHODS

Materials

All DNA polymerases were obtained from New England Biolabs and used with the supplied buffers. Non-caged DNA controls were obtained from Integrated DNA Technologies (IDT), and pGFPuv was obtained from Clontech. The caged thymidine monomer was readily prepared according to the previously reported route (13); however, it is currently commercially available from Berry & Associates Inc. (Dexter, MI). Control mutagenesis reactions were perfomed using a Stratagene QuikChange Site-Directed Mutagenesis Kit, following standard protocols. Oligonucleotides were end labelled using γ^{32}P-ATP (MP Biomedicals) and T4 Kinase (New England Biolabs) at 37°C for 1 h, and then purified using TE Midi Select-D, G25 microcentrifuge spin columns (Shelton Scientific). Buffers employed in the PCR and extension reactions were provided by the vendor of the corresponding DNA polymerase.

DNA synthesis

DNA synthesis was performed using an Applied Biosystems (Foster City, CA) Model 394 automated DNA/RNA Synthesizer using standard β-cyanoethyl phosphoramidite chemistry. All caged oligonucleotides were synthesized using 40 nmol scale, low volume solid phase supports obtained from Glen Research (Sterling, VA). Reagents for automated DNA synthesis were also obtained from Glen Research. Standard synthesis cycles provided by Applied Biosystems were used for all normal bases using 2 min coupling times. The coupling time was increased to 10 min for the positions at which the caged thymidine modified phosphoramidites were incorporated. Each synthesis cycle was monitored by following the release of dimethoxytrityl (DMT) cations after each deprotection step. No significant loss of DMT was noted following the addition of the caged-T for any of the oligonucleotides, so 10 min was sufficient to allow maximal coupling of the caged-T. Yields of caged oligonucleotides were close to theoretical values routinely obtained.

Primer extension reactions

Three templates **D1** (5′ CGCACCCAGGCTAGCTACA ACGACTCTCTCCG 3′), **D2** (5′ CGCACCCAGGCTAG CTACAACGACTCTCTCCG 3′), and **WT** (5′ CGCACC CAGGCTAGCTACAACGACTCTCTCCG 3′) were resuspended in sterile water to a final concentration of 10 μM. The template (2 μl of a 10 μM solution) was then incubated with end-labelled ^{32}P primer 5′ AATGGGCGG AGAGAG 3′ (2 μl of a 10 μM solution), dNTPs (2 μl each of a 1 mM solution), and with either DNA Polymerase I, T7 DNA Polymerase, or T4 DNA Polymerase (1 μl, five units) in the appropriate buffer (5 μl of a 10× solution provided by New England Biolabs with each enzyme), and the reaction was brought to a final volume of 50 μl with dH_2O (38 μl). Extension reactions were allowed to progress for 1 h at 37°C. The DNA polymerase was then deactivated at 70°C for 10 min, and the reaction was separated on a 25% polyacrylamide gel (400 V, 40 min) and imaged on a Storm Phosphorimager.

Caged primers in the PCR

Standard PCRs were conducted using either **WT**, **P1**, **P2** or **P3** forward primers (5 μl of a 10 μM solution, Table 1), the end-labelled ^{32}P reverse primer 5′ AGCGATCGCTAT TTTCCATG 3′ (5 μl of a 10 μM solution), plasmid template (1 μl of a 1 ng/μl solution), dNTPs (2 μl each of a 1 mM solution), and the appropriate buffer (5 μl of a 10× solution provided by New England Biolabs with each enzyme). The reaction mixture was brought to a final volume of 49 μl with dH_2O (31 μl), followed by the addition of either *Taq* Polymerase (1 μl, five units) or Phusion Polymerase (1 μl, two units). The following PCR program was used: 95°C (2 min), followed by 30 cycles of 95°C (30 s), 50°C (30 s) and 72°C (1 min). The reactions were then separated on a 20% polyacrylamide gel (400 V, 30 min) and imaged on a Storm Phosphorimager.

Optimized mutagenesis protocol

Primers to accomplish point mutations (**P4**–**P10**) and deletions (**P11**–**P14**) with varying numbers of caging groups and base pair overlaps were designed and synthesized

(Table 2). Primers were designed in an analogous fashion to standard PCR primers (i.e. appropriate T_m/GC ratio), with the caged nucleoside at a position immediately prior to the sequence that is to be introduced into the plasmid. The new sequence should be exactly complementary in both primers and should optimally be at least 10 bases in length to facilitate adequate nick repair. It is important to note that the selection of primers is key, and for some sequences, longer hybridization arms are required (see GFP deletion in Table 3), which in turn requires additional caging groups to prevent undesirable hybridization during the PCR (see 'Results and Discussion' section). The forward and reverse primers (5 µl each of a 10 µM solution), the pGFPuv template (1 µl of a 0.1 ng/µl solution), dNTPs (2 µl each of a 1 mM solution), Phusion GC Buffer (5 µl of a 10× stock, New England Biolabs), Phusion DNA Polymerase (1 µl, two units) and dH_2O (31 µl) were mixed and subjected to the following PCR program: 95°C (2 min), followed by 40 cycles of 95°C (30 s), 40°C (60 s) and 72°C (3.3 min), with a final extension at 72°C (2 min). An identical PCR reaction was then repeated, using 5 µl of the previous reaction as the template, followed by purification with a PCR cleanup kit (Promega). The PCR tube containing the purified product (50 µl) was then placed on a transilluminator and irradiated for 8 min at 365 nm (25 W), followed by subjection to a hybridization protocol (95°C for 5 min, three cycles of 65°C for 5 min and 30°C for 15 min). The hybridized DNA (2 ng) was then transformed (30 min at 4°C, 45 s at 42°C and 2 min at 4°C) into chemically competent Top 10 cells (20 µl, 4×10^7 cfu) followed by a 1 h recovery at 37°C in 100 µl of SOC media (Q-Biogene), and all 120 µl were plated on ampicillin (50 µg/ml) LB (Luria-Bertani) plates. Colonies were assessed for mutations via visual observation of GFP fluorescence, by colony-PCR screens, and through DNA sequencing.

RESULTS AND DISCUSSION

Primer extension reactions

Our experiments commenced with investigations into the effects of caged nucleotides on DNA polymerase catalyzed primer extensions (Figure 2). In order to achieve this, we incorporated a single-caged thymidine residue into a short oligonucleotide (32 bases) at two different positions (templates **D1** and **D2**), and examined the reaction of various polymerases to the presence of the caging group. Depending on the position of the caging group, different length products of polymerase extensions should be obtained if the polymerase is incapable of reading beyond the caging group. Previous research by Dmochowski *et al.* using the Klenow fragment of DNA Polymerase I suggests that the polymerase is capable of reading through a caging group depending on the distance of the 3′ primer terminus from the caging group. However, they found that when the 3′ end of the primer is aligned directly with the caged nucleotide in the template, prohibiting the enzyme from generating any velocity, polymerization does not occur until the caging group is removed through light irradiation (17).

Table 1. Primer set employed in the analysis of PCR read-through

Primer	Sequence	Function
WT	5′ CTGATTTCGACCAGGTT 3′	Forward PCR primer
P1	5′ CTGATT**T**CGACCAGGTT 3′	Forward PCR primer
P2	5′ CTGA**TT**$\overline{\mathbf{T}}$CGACCAGGTT 3′	Forward PCR primer
P3	5′ CGAC$\overline{\mathbf{CA}}$GGTT 3′	Truncated PCR primer

The caged thymidine is indicated by a $\overline{\mathbf{T}}$.

Table 3. Light-mediated mutagenesis results

Mutation	Primer set	Positive colonies	Negative colonies	Total colonies	Mutation efficiency (%)
TAA stop introduction	**P4** & **P5**	26	0	26	100
AAA stop removal	**P9** & **P10**	68	6	74	92
GFP deletion	**P11** & **P12**	9	1	10	90

Table 2. Primers for light controlled mutagenesis

Primer	Sequence	Function
P4	5′ AATAAA**T**GAGTAAAGGAGAAGAAC 3′	GFP stop codon forward
P5	5′ ATTTAT$\overline{\mathbf{T}}$CTACCGGTACCCGG 3′	GFP stop codon reverse
P6	5′ AATAAA$\overline{\mathbf{T}}$GAG**T**AAAGGAGAAGAAC 3′	GFP stop codon forward
P7	5′ ACTCA**T**$\overline{\mathbf{T}}$AT**T**C$\overline{\mathbf{T}}$ACCGGTACCCGGG 3′	GFP stop codon reverse
P5	5′ ACCGG$\overline{\mathbf{T}}$AGA$\overline{\mathbf{A}}$ATAAA**T**GAG**T**AAAGGAGAAGAACAA 3′	GFP stop codon forward
P6	5′ ACTCA$\overline{\mathbf{T}}$$\overline{\mathbf{T}}$TAT**T**CTAC$\overline{\mathbf{C}}GG\overline{\mathbf{T}}$$\overline{\mathbf{A}}$CCCGGGGATCCTCT 3′	GFP stop codon reverse
P7	5′ ACCGG$\overline{\mathbf{T}}$AGAA$\overline{\mathbf{T}}$AAATGA$\overline{\mathbf{G}}$$\overline{\mathbf{T}}$AAAGGAGAAGAACAA 3′	GFP stop codon forward
P8	5′ ACTCATTTATTCTACCGG$\overline{\mathbf{T}}$$\overline{\mathbf{A}}$CCCGGGGATCCTCT 3′	GFP stop codon reverse
P9	5′ AAAAAA**T**GAGTAAAGGA$\overline{\mathbf{G}}$AAGAAC 3′	GFP start codon forward
P10	5′ ATTTTTT$\overline{\mathbf{C}}$TACCGGTACCCGGG 3′	GFP start codon reverse
P11	5′ ACCGG$\overline{\mathbf{T}}$ACCCGGGGA**T**CCTCTAGAGTCGACC 3′	GFP deletion forward
P12	5′ A**T**CCCC$\overline{\mathbf{C}}$GGG**T**ACCGG$\overline{\mathbf{T}}$$\overline{\mathbf{T}}$GAATTCCAACTGAG 3′	GFP deletion reverse
P13	5′ A$\overline{\mathbf{C}}$CGGTACC$\overline{\mathbf{C}}$GGGGA$\overline{\mathbf{T}}$CCTCTAGAGTCGACC 3′	GFP deletion forward
P14	5′ ATCCCCGGGTACCGG$\overline{\mathbf{T}}$$\overline{\mathbf{T}}$GAATTCCAACTGAG 3′	GFP deletion reverse

The caged thymidine is indicated by a $\overline{\mathbf{T}}$.

Figure 2. DNA polymerization through extension of a primer using a 32 nt template with a caged thymidine (blue square) 17 or 21 nt from the 3′ end of the template. A single caging group blocked polymerization by T4 and T7 DNA polymerase.

Three different polymerases were examined: DNA Polymerase I (polymerase family A), T7 DNA Polymerase (polymerase family A), and T4 DNA Polymerase (polymerase family B) were selected due to their different fidelities (ability to exactly copy templates) and different exonuclease activities. Family A polymerases share similar sequence homologies and are known for their replicative and repair capabilities, whereas family B polymerases are known mostly for their replicative properties. T4 DNA polymerase has the highest degree of 3′ → 5′ proof-reading capacity, while T7 DNA polymerase has a high fidelity and rapid extension rate (18,19). DNA Polymerase I, on the other hand, possesses low proof-reading ability and has a 5′ → 3′ exonuclease function.

After radioactively labelling a DNA primer for the reaction, each polymerase was examined in the presence of a non-caged DNA template (**WT**), and the two caged templates (**D1** and **D2**). Extension reactions employed the labeled primer (1 pmol), template (1 pmol) and one of the polymerases (New England Biolabs, three units) and were allowed to proceed for 30 min at 37°C. Extension products were then analyzed via polyacrylamide gel electrophoresis and imaged on a STORM phosphorimager (Figure 3).

The first lane of each gel contains the polymerase product of the non-caged DNA template (**WT**), yielding a full-length product of 38 bases. The extension reaction was then conducted in the presence of a caged template with photolabile thymidines at different positions. The template **D1** has 11 base pairs prior to the caging group, while the template **D2** only provides seven bases prior to the caging group. Ideally, this will afford insight into the behavior of the polymerase before encountering the caged thymidine residue. The reaction utilizing **D1** with both T4 and T7 Polymerases yields a 12 base pair shorter product than the full-length template (Figure 3A and B; lane 2). Additionally, reaction with **D2** and both T4 and T7 affords a 16 base pair shorter product than the full-length product (Figure 3A and B; lane 3). These data suggest that T4 and T7 DNA polymerase are unable to continue DNA polymerization past an NPOM caged thymidine nucleotide. In the absence of a caging group the ^{32}P-labelled primer is completely converted into full-length product; however, in the presence of a caging group polymerase efficiency is decreased leading to remaining unextended primer (15 nt band in Figure 3). Conversely, DNA Polymerase I appears to read through the caged nucleotide, producing full-length product in all three cases (Figure 3C; lanes 1, 2 and 3). This corroborates the findings of Dmochowski *et al.* (17), as the Klenow fragment used by them is simply DNA Polymerase I without the 5′ → 3′ exonuclease domain. This enzyme's proof-reading capabilities thus are comparable to

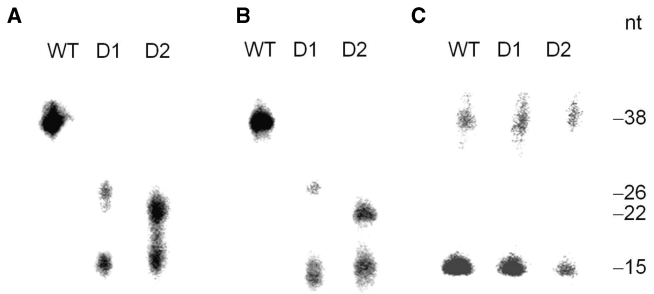

Figure 3. Effects of a caged thymidine nucleobase on the DNA polymerization by mesophilic DNA polymerases. (**A**) Products resulting from T4 DNA Polymerase extension. When using the non-caged template full-length product (38 nt) is obtained; however, using either **D1** or **D2** caged templates, polymerization is halted, leading to truncated product (26 or 22 nt, respectively). (**B**) Products resulting from T7 DNA Polymerase extension. Similar truncations are observed as with T4 DNA Polymerase. (**C**) Products resulting from DNA Polymerase I extension, demonstrating a polymerase read-through to yield full-length product (38 nt) in all cases.

DNA Polymerase I, and the polymerase is able to read through the caging group. In all cases the DNA Polymerase I extension efficiency is lower than that observed in the case of the other two polymerases, as large quantities of unreacted primer remain after a 1 h extension reaction, even in the case of the non-caged DNA template.

PCR

Based on the results of the primer extension reaction, we next investigated the effects of caged nucleotides on the polymerization of DNA with hyperthermophilic DNA polymerases. Thus, we employed caged primers with either a single or three consecutive caged thymidine nucleosides. In order to visualize truncated products we amplified a 45-mer with either *Taq* DNA polymerase or Phusion DNA polymerase (Figure 4).

Each PCR was conducted with one of the forward primers (**WT**, **P1**, **P2** or **P3**), a radioactively labelled reverse primer (Integrated DNA Technologies; 50 pmol), DNA template (1 ng), dNTPs and either *Taq* DNA polymerase (New England Biolabs, five units) or Phusion DNA Polymerase (New England Biolabs, two units) for 30 cycles. The PCR was then run on a polyacrylamide gel to identify PCR termination by the caged nucleotide. The gel was imaged on a STORM phosphorimager via detection of the ^{32}P-labelled PCR product. Primer **P3** was designed to generate a PCR product of the same length as a product resulting from polymerase termination (Figure 5).

The non-caged primer afforded full-length PCR product for both enzymes (Figure 5A and B; lane 1), while, as expected, reactions conducted with primer **P3** yielded products which were seven bases shorter (Figure 5A and B; lane 2). PCRs with both the singly caged primer **P1** and the triply caged primer **P2** afforded truncated products the same size as the **P3** product (Figure 5A and B; lanes 3 and 4). These results demonstrate that both *Taq* DNA polymerase and Phusion DNA polymerase are stopped

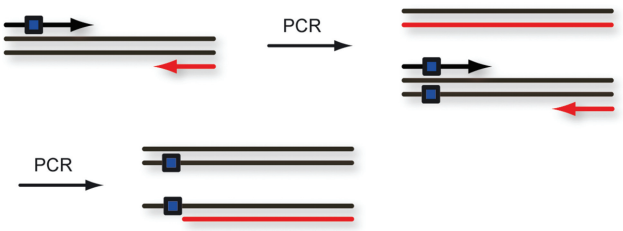

Figure 4. DNA polymerization by PCR using a caged primer (17 nt) containing the first caged thymidine (blue square) 10 nt from the 5′ end. PCR generates a caged template which results in a stop of *Taq* and Pfu polymerase due to the presence of a single-caged thymidine.

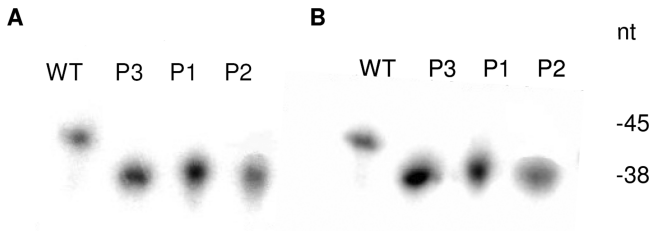

Figure 5. Effects of a caged thymidine nucleobase on the PCR. (**A**) PCR product using Phusion DNA Polymerase. Polymerization is halted in the presence of a caging group, which is confirmed using the truncated primer **P3** that affords the same length product. (**B**) PCR product using *Taq* DNA polymerase. Polymerization is halted in the presence of a caging group, which is confirmed using the truncated primer **P3** that affords the same length product.

by a single-caged thymidine. Interestingly, despite the similarities in both structure and fidelity of the *Taq* polymerase and DNA polymerase I (20), the *Taq* enzyme halted in response to the caging group. However, previous literature has suggested that polymerases within the same family can behave differently in response towards a perturbation in the DNA template (21). Although, we do not understand this behavior yet and future work is required to examine this phenomenon, this property of the *Taq* polymerase enabled a light-mediated mutagenesis and cloning methodology.

Light-mediated mutagenesis and cloning

The ability of caging groups to stop DNA polymerization by polymerases enables the enzymatic synthesis of double-stranded DNA with a 5′ single-stranded DNA overhang of virtually any size (red DNA strand in Figure 6). When applied to the construction of plasmid DNA and molecular cloning, it provides the opportunity to easily and rapidly introduce single or multiple base mutations and to insert synthetic DNA of any length into plasmids. Moreover, it enables the deletion of any sequence of DNA from any plasmid. Importantly, all these processes can be conducted without restriction sites, endonucleases and ligases. Site-directed mutagenesis and related modifications of plasmid DNA are important tools for the introduction of stop codons, gene knock-outs and alterations in the codon set to exchange amino acids (22–24).

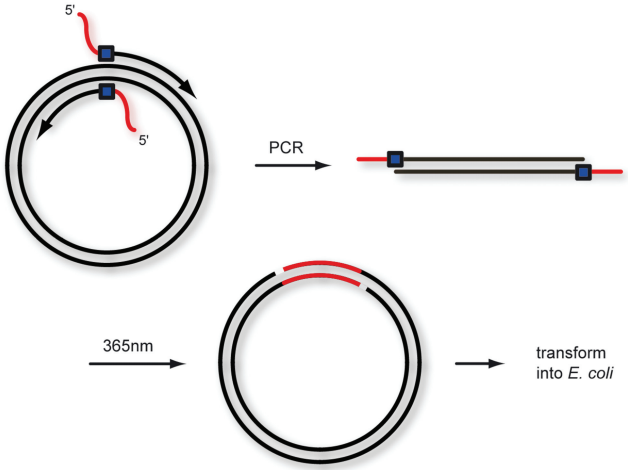

Figure 6. The blocking of a polymerase using a caged thymidine (blue square) can be used for the insertion of new DNA (red) into a plasmid via PCR. This strategy also allows for the site-directed mutagenesis or deletion of DNA. The installation of a caged thymidine enables the generation of double stranded DNA with long single stranded overhangs (red). Removal of the caging group through a brief UV irradiation at 365 nm generates a nicked plasmid which can be transformed into *E. coli* where it is propagated after nick repair.

The most commonly used, commercially available QuikChange™ mutagenesis kit from Stratagene provides a very fast, 1-day mutagenesis protocol (25). A requirement of this protocol are (partially) overlapping primers to replicate the plasmid DNA, which, due to their complementarity, only enable linear DNA amplification, resulting in the need to digest the parental plasmid. Moreover, this method is restricted to primer pairs of 25–45 bases with a T_m >78°C to prevent primer-dimer formation and to favor primer-template annealing; particularly for primer pairs with multiple mismatches, limiting the number of mutated, deleted or inserted bases to a maximum of 12. Other commercially available approaches exist to modify plasmids; however they possess several disadvantages. Most notably, they require the use of a ligation reaction or other enzymatic processing, which decreases the overall efficiency of the process (examples include Clontech's Transformer and Promega's Altered Sites). Restriction enzyme free cloning protocols have been developed, but rely upon the use several primers (up to eight) and thus require complex experimental design (26–28).

Our general mutagenesis approach is shown in Figure 6, where a PCR with a plasmid template and a set of caged primers yields a linearized plasmid with sticky ends (red sequence). Upon light irradiation, the caging groups are removed, enabling circularization of the plasmid via hybridization, and affording transformation into *Escherichia coli*. Intracellular repair of the remaining nicks provides a mutagenized plasmid, with any length and sequence of new DNA (red) added. Due to the non-complementary nature of the primer sequence annealing to the plasmid, exponential amplification is achieved in the PCR, affording an excess of mutagenized DNA over

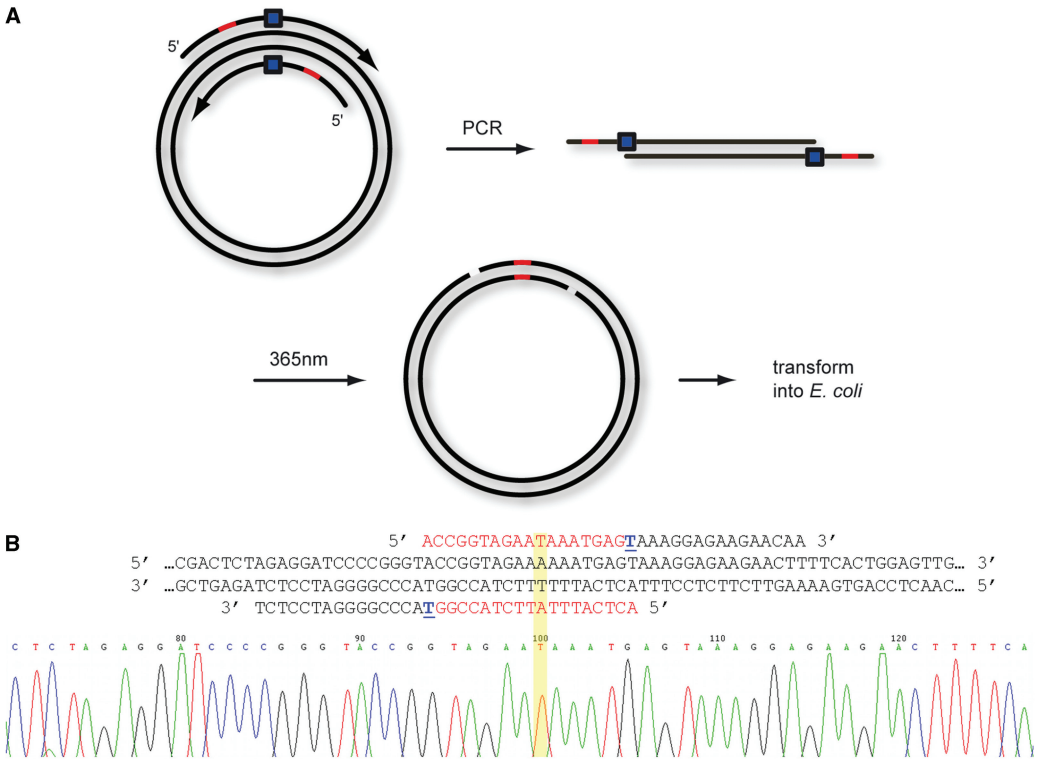

Figure 7. Site-directed mutagenesis of pGFPuv to introduce a stop codon. (**A**) General scheme leading to the introduction of a mutation (red) into a plasmid. The caged thymidines (blue squares) inhibit polymerase read-through generating 'sticky-ends' which can be employed to circularize the plasmid after decaging with a brief UV irradiation. (**B**) Primers (**P7** and **P8**) aligned with the pGFPuv DNA sequence demonstrating the mutation, and the sequencing results confirming the mutation of the DNA, introducing a thymidine residue in place of the adenosine residue effectively introducing a stop codon. **T** = NPOM caged thymidine.

template DNA. The length of the mutagenized or inserted DNA (red sequence) is only limited by DNA synthesis. Moreover, no restriction enzymes or restriction sites are necessary for this mutagenesis approach.

To demonstrate this approach, we synthesized primer sets that either stop GFP expression from a pGFPuv plasmid (by mutation of an AAA codon to a TAA stop codon upstream of the GFP gene) or that facilitate GFP expression by removing the TAA stop codon generated from the prior mutation (Figure 7, Table 2). Preliminary assessment of the successful mutagenesis was visually observed by the lack of GFP expression in bacterial cells transformed with pGFPuv harboring the AAA → TAA mutation.

The light-mediated mutagenesis protocol involved a typical PCR using caged primers (50 pmol), pGFPuv template (0.1 ng), deoxynucleotide triphosphates (50 pmol) and Phusion DNA Polymerase (New England Biolabs, two units). An identical PCR was then repeated, using 5 µl of the previous reaction as the template, followed by purification with a PCR cleanup kit (Wizard SV, Promega) to remove excess primers. Two sequential PCRs were conducted in order to dilute the template relative to the exponentially amplified mutated PCR product, and to compensate for the much higher transformation efficiency of the circular template plasmid compared to the nicked mutant vector (a second, alternative protocol is provided in the Supplementary Data). Purified product was then briefly irradiated (365 nm, 25 W, 8 min, transilluminator) and subjected to a hybridization protocol for efficient circularization. A reduced irradiation time of 2 min was sufficient as well, but led to a ~50% lower transformation efficiency. The nicked plasmid was then transformed into Top 10 cells and plated on ampicillin containing LB plates. Colonies were first assessed visually for a GFP phenotype, and several clones were sequenced to verify that the site-directed mutation and no other mutation occurred, despite the 8 min UV irradiation (Figure 7). This developed protocol does not require any restriction sites, restriction endonucleases or additional enzymes, lengthy incubations, or highly competent or specialized cells. It is important to note that despite the apparent introduction of a single point mutation, 17 new nucleotides have been introduced into the plasmid. This displays the feasibility of inserting any new sequence information into the plasmid DNA in a very straightforward 1-day experiment.

Overall, with relatively short single-stranded overhangs (8 nt) and in case of the introduction of mutations, efficient mutagenesis can be achieved using a single caging group to stop polymerization reactions. In case of longer overhangs, PCR efficiency can be increased by employing multiple caging groups to prevent undesired hybridization of the primer to another primer or the template. We already demonstrated that multiple caging groups

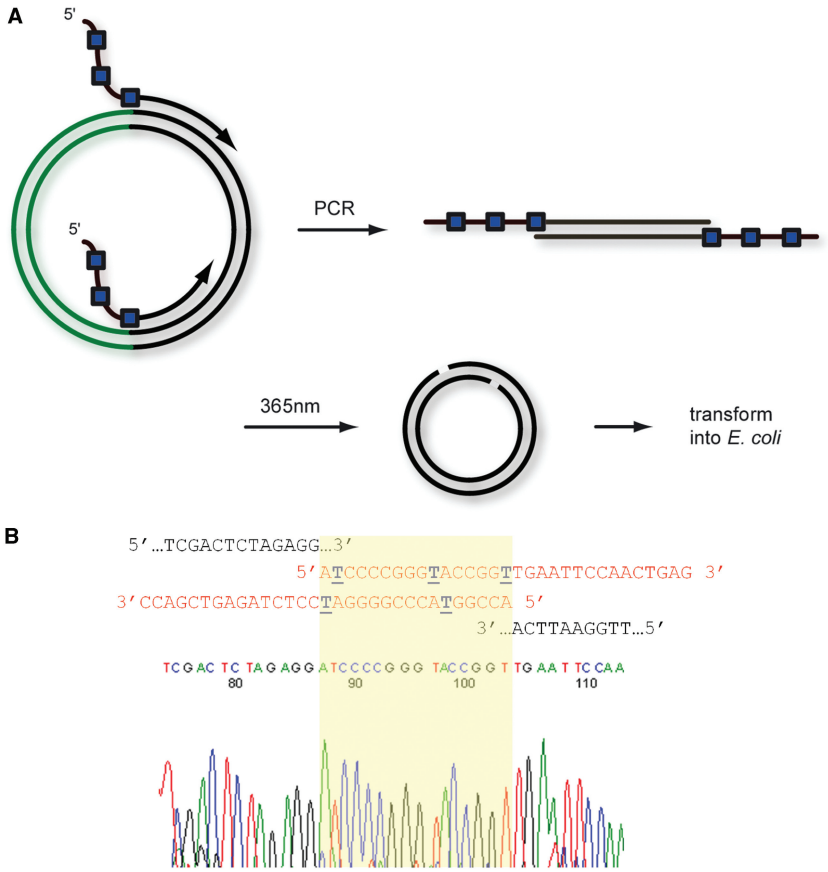

Figure 8. Strategy to delete entire sequences from a plasmid using the developed methodology. (**A**) General strategy to remove DNA (green) from the original plasmid. The caged thymidine (blue squares) prohibit PCR extension and prevent hybridization until removed via UV irradiation. (**B**) Primers (red) aligned with the pGFPuv DNA sequence (black) demonstrating the deletion of the GFP gene, and the sequencing results confirming the deletion. T = NPOM caged thymidine.

(i.e. an NPOM group every five to six bases) efficiently inhibit DNA:DNA hybridization (13,15,29), thus large, single-stranded regions in PCR primers can be complementary but remain unhybridized until the caging groups are removed, enabling efficient amplification by PCR.

We further examined the scope of the methodology by designing primer sets to not only introduce DNA and make point mutations, but to remove DNA from the plasmid template. Specifically, we designed a primer set to completely remove the GFPuv gene (Figure 8). Primers were designed by selecting DNA sequences starting 30 bases upstream and downstream of the GFP gene, amplifying away from the GFP sequence. 15 bases were added to the 5′ end of one primer to be complementary to the 5′ end of the other primer. Moreover, this sequence of 15 bases possessed multiple thymidine residues to be caged for the formation of appropriate 5′ overhangs in the PCR product. Two sets of primers were used, a set with multiple caging groups (**P11**/**P12**) and a set with a single caging group (**P13**/**P14**). The primer set **P13**/**P14** possessing a single caging group produced the desired PCR product in low yield with other side products. This is most likely a result of primer-dimer formation or non-productive hybridization to the DNA template. In contrast, the primer set **P11**/**P12** containing three caged thymidines furnished a clean PCR product, as competing hybridizations were prevented. Based on the site-specific mutagenesis protocol, a second PCR was performed to further amplify the amount of PCR product relative to the initial template. The PCR product was purified (Wizard SV, Promega), followed by a brief UV irradiation (365 nm, 25 W, 8 min, transilluminator) and hybridization of the generated single-stranded overhangs. The nicked plasmid was then transformed into chemically competent Top 10 cells and plated on ampicillin supplemented LB agar. The removal of the GFP gene affords a visible assessment of the success of the mutagenesis, as successful deletions yield colonies capable of growth on ampicillin media, but which lack fluorescence. The deletion was confirmed via the sequencing of plasmids isolated from non-fluorescent colonies (Figure 8).

In each of the light-mediated mutagenesis experiments, a mutation frequency greater than 90% was achieved reproducibly (Table 3), by employing the caged oligonucleotides in accordance to the optimized protocol. While colony numbers are typically below one hundred, the approach does not require ultra-competent cells typically associated with mutagenesis kits. Additionally, both point-mutations and the insertion or deletion of long

sequences of DNA were achieved with similar mutation efficiency.

CONCLUSIONS

In summary, we investigated the effects of photocaged nucleosides on the DNA polymerization reaction, and discovered that most polymerases are unable to recognize and read-through the presence of a single caging group on the DNA template. This feature of caged DNA enabled the development of a new method of introducing mutations into plasmid DNA via a light-mediated mutagenesis protocol. This methodology is advantageous over other approaches in that it can be completed in a single day and requires the use of only two PCR primers. Most importantly, no restriction sites, restriction enzymes, or other enzymes than a DNA polymerase are necessary providing a high level of flexibility for DNA manipulations. Additionally, this approach enables not only site-directed mutations, but also the insertion of long DNA sequences into plasmids and the deletion of entire genes from plasmids.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

DNA synthesis was conducted in the Biomolecular Resource Facility of the Comprehensive Cancer Center of Wake Forest University.

FUNDING

ACS Medicinal Chemistry Division Graduate research fellowship (to D.D.Y.). A.D. is a Beckman Young Investigator and a Cottrell Scholar; NIH grant P30 CA-12197-30. Funding for open access charge: NCSU.

Conflict of interest statement. None declared.

REFERENCES

1. Ling,M.M. and Robinson,B.H. (1997) Approaches to DNA mutagenesis: an overview. *Anal. Biochem.*, **254**, 157–178.
2. Shen,B. (2002) PCR approaches to DNA mutagenesis and recombination. An overview. *Methods Mol. Biol.*, **192**, 167–174.
3. Hubscher,U., Maga,G. and Spadari,S. (2002) Eukaryotic DNA polymerases. *Annu. Rev. Biochem.*, **71**, 133–163.
4. Nagashima,J., Minezaki,S., Obika,S., Imanishi,T., Kuwahara,M. and Sawai,H. (2007) Polymerisation of a DNA strand using oligo-DNA template with modified bases, sugars and phosphates. *Nucleic Acids Symp. Ser.*, **51**, 55–56.
5. O'Connor,D. and Stohrer,G. (1985) Site-specifically modified oligodeoxyribonucleotides as templates for Escherichia coli DNA polymerase I. *Proc. Natl Acad. Sci. USA*, **82**, 2325–2329.
6. Tang,X. and Dmochowski,I.J. (2007) Regulating gene expression with light-activated oligonucleotides. *Mol. Biosyst.*, **3**, 100–110.
7. Young,D.D. and Deiters,A. (2007) Photochemical control of biological processes. *Org. Biomol. Chem.*, **5**, 999–1005.
8. Mayer,G. and Heckel,A. (2006) Biologically active molecules with a "light switch". *Angew. Chem. Int. Ed. Engl.*, **45**, 4900–4921.
9. Lawrence,D.S. (2005) The preparation and in vivo applications of caged peptides and proteins. *Curr. Opin. Chem. Biol.*, **9**, 570–575.
10. Curley,K. and Lawrence,D.S. (1999) Caged regulators of signaling pathways. *Pharmacol. Ther.*, **82**, 347–354.
11. Adams,S.R. and Tsien,R.Y. (1993) Controlling cell chemistry with caged compounds. *Annu. Rev. Physiol.*, **55**, 755–784.
12. Lusic,H., Lively,M.O. and Deiters,A. (2008) Light-activated deoxyguanosine: photochemical regulation of peroxidase activity. *Mol. Biosyst.*, **4**, 508–511.
13. Lusic,H., Young,D.D., Lively,M.O. and Deiters,A. (2007) Photochemical DNA activation. *Org. Lett.*, **9**, 1903–1906.
14. Lusic,H. and Deiters,A. (2006) A new photocaging group for aromatic N-heterocycles. *Synthesis-Stuttgart*, **8**, 2147–2150.
15. Young,D.D., Edwards,W.F., Lusic,H., Lively,M.O. and Deiters,A. (2008) Light-triggered polymerase chain reaction. *Chem. Commun.*, **4**, 462–464.
16. Tanaka,K., Katada,H., Shigi,N., Kuzuya,A. and Komiyama,M. (2008) Site-selective blocking of PCR by a caged nucleotide leading to direct creation of desired sticky ends in the products. *Chembiochem*, **9**, 2120–2126.
17. Tang,X., Richards,J.L., Peritz,A.E. and Dmochowski,I.J. (2005) Photoregulation of DNA polymerase I (Klenow) with caged fluorescent oligodeoxynucleotides. *Bioorg. Med. Chem. Lett.*, **15**, 5303–5306.
18. Mattila,P., Korpela,J., Tenkanen,T. and Pitkanen,K. (1991) Fidelity of DNA synthesis by the Thermococcus litoralis DNA polymerase – an extremely heat stable enzyme with proofreading activity. *Nucleic Acids Res.*, **19**, 4967–4973.
19. Kunkel,T.A., Loeb,L.A. and Goodman,M.F. (1984) On the fidelity of DNA replication. The accuracy of T4 DNA polymerases in copying phi X174 DNA in vitro. *J. Biol. Chem.*, **259**, 1539–1545.
20. Brautigam,C.A. and Steitz,T.A. (1998) Structural and functional insights provided by crystal structures of DNA polymerases and their substrate complexes. *Curr. Opin. Struct. Biol.*, **8**, 54–63.
21. Hogg,M., Wallace,S.S. and Doublie,S. (2005) Bumps in the road: how replicative DNA polymerases see DNA damage. *Curr. Opin. Struct. Biol.*, **15**, 86–93.
22. Hsu,E.C., Sarangi,F., Iorio,C., Sidhu,M.S., Udem,S.A., Dillehay,D.L., Xu,W., Rota,P.A., Bellini,W.J. and Richardson,C.D. (1998) A single amino acid change in the hemagglutinin protein of measles virus determines its ability to bind CD46 and reveals another receptor on marmoset B cells. *J. Virol.*, **72**, 2905–2916.
23. Kunkel,T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl Acad. Sci. USA*, **82**, 488–492.
24. Taylor,J.W., Ott,J. and Eckstein,F. (1985) The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. *Nucleic Acids Res.*, **13**, 8765–8785.
25. Weiner,M.P., Costa,G.L., Schoettlin,W., Cline,J., Mathur,E. and Bauer,J.C. (1994) Site-directed mutagenesis of double-stranded DNA by the polymerase chain reaction. *Gene*, **151**, 119–123.
26. Chiu,J., March,P.E., Lee,R. and Tillett,D. (2004) Site-directed, Ligase-Independent Mutagenesis (SLIM): a single-tube methodology approaching 100% efficiency in 4 h. *Nucleic Acids Res.*, **32**, e174.
27. Tillett,D. and Neilan,B.A. (1999) Enzyme-free cloning: a rapid method to clone PCR products independent of vector restriction enzyme sites. *Nucleic Acids Res.*, **27**, e26.
28. Ailenberg,M. and Silverman,M. (1997) Site-directed mutagenesis using a PCR-based staggered re-annealing method without restriction enzymes. *BioTechniques*, **22**, 624–626, 628, 630.
29. Young,D.D., Lusic,H., Lively,M.O., Yoder,J.A. and Deiters,A. (2008) Gene silencing in mammalian cells with light-activated antisense agents. *Chembiochem*, **9**, 2937–2940.