CHEMBIOCHEM

DOI: 10.1002/cbic.200800285

Site-Selective Blocking of PCR by a Caged Nucleotide Leading to Direct Creation of Desired Sticky Ends in The Products

Keita Tanaka, Hitoshi Katada, Narumi Shigi, Akinori Kuzuya,* and Makoto Komiyama*[a]

In order to terminate the polymerase reaction at a desired position, a caged thymine derivative—4-O-[2-(2-nitrophenyl)propyl]thymine—was incorporated into PCR primers. In the PCR cycles, the elongation of the nascent strand (5′→3′ direction) by polymerase was site-selectively terminated at the 3′-side of T^{NPP}. Accordingly, predetermined protruding ends were obtained after the removal of the protecting group by short UVA irradiation. Recombinant vectors coding the GFP gene were successfully prepared by direct ligation of these light-assisted cohesive-ending PCR (LACE-PCR) products with scission fragments obtained by use either of restriction enzymes or of artificial restriction DNA cutters and were used for transformation of E. coli.

Introduction

Photocaging of oligonucleotides has become an attractive topic because of its potential to control genetic events simply through light irradiation without alteration either of chemical or of other physical conditions.[1,2] Various oligonucleotides protected with photocleavable groups (caged oligonucleotides) have been reported to control events such as transcription,[3–5] translation,[6] RNA interference,[7] DNAzyme reactions,[8,9] DNA replication,[10,11] aptamer binding,[12] higher-order structure formation,[13–15] and PCR.[16] In this study we have examined the use of a caged nucleotide as a site-selective terminator of the polymerase reaction in PCR.

In current molecular biology and biotechnology, vectors are constructed by digesting plasmid DNA with restriction enzymes, followed by connection of this vector with a predetermined gene fragment by use of a ligase. In many cases, those gene fragments are prepared by PCR from various sources, and the blunt ends of the PCR products are converted into cohesive ends by use of restriction enzymes for ligation with the vector.[17] Although this technology has been mostly successful, two problems still remain unsolved for further developments of the fields. Firstly, recognition sites of type II restriction enzymes, which are most often used in these fields, are mostly limited to certain palindromic sequences, and it is sometimes difficult to find an appropriate enzyme to digest DNA at (or near) the target manipulation site. Secondly, most of these enzymes recognize only four to eight DNA base sequences, so precise manipulation of large vectors such as adenovirus (30–38 kbp) with restriction enzymes is not very practical because digestion occurs at too many sites in such large DNA sequences.

As a solution to these problems, artificial restriction DNA cutters (ARCUT) have been prepared by combining the Ce^{4+}/EDTA complex (molecular scissors) and a pair of pseudocomplementary peptide nucleic acids (pcPNAs) showing double-duplex invasion.[18,19] The binding sites of these pcPNA strands are laterally shifted relative to one another, and so, in the invasion complex, single-stranded portions are formed in both strands of substrate DNA. These single-stranded portions are selectively hydrolyzed by the Ce^{4+}/EDTA, since they are more susceptible to the catalysis of this complex. Although the sticky ends formed by ARCUT are not directly compatible with differently prepared termini of other fragments (for example, restriction enzyme fragments), these fragments can be ligated by adding a joint oligonucleotide that fills the space between them, thus forming an "apparently" complementary structure.[19,20] However, these trimolecular processes could decrease the efficiency of ligation and also make separation/purification steps more difficult.

To date, several methods for providing desired termini to PCR products have been reported.[21–26] The "staggered reannealing" method uses two primer pairs and first involves the preparation of two staggered PCR products with additional bases at one of the ends corresponding to the sticky end.[21] Melting and reannealing of the two staggered products result in a quarter of the product bearing the desired sticky ends at both ends. The "Autosticky PCR" method utilizes an artificial abasic site in the primers, which will be repaired as a dT or simply deleted in *E. coli*.[22] Other methods involve post-treatment of the PCR products with unique enzymes (T4 DNA polymerase,[23] uracil DNA glycosylase,[24] RNase H,[25] and poxvirus DNA polymerase),[26] which digest elegantly designed terminal regions of the product in a required fashion. In particular, ligation-independent cloning of PCR products (LIC-PCR) is one of the most significant applications of artificial long sticky ends.[23]

[a] *K. Tanaka, H. Katada, Dr. N. Shigi, Dr. A. Kuzuya, Prof. Dr. M. Komiyama*
Research Center for Advanced Science and Technology
The University of Tokyo
4–6–1 Komaba, Meguro-ku, Tokyo 153-8904 (Japan)
Fax: (+81) 3-5452-5209
E-mail: komiyama@mkomi.rcast.u-tokyo.ac.jp

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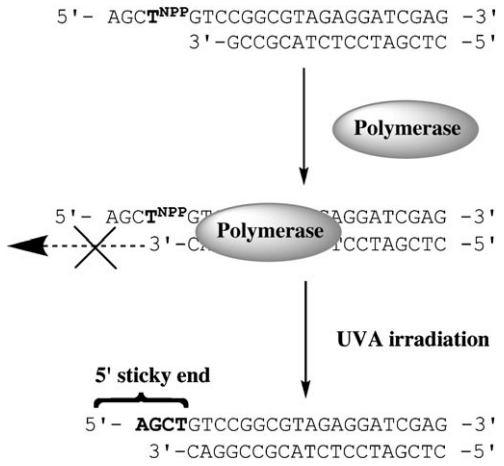
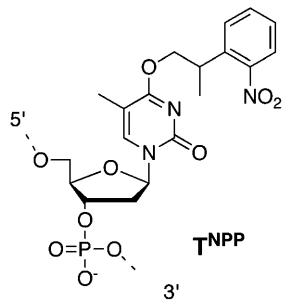
If a caged nucleotide were able to act as a site-selective terminator of a polymerase reaction in PCR, this should make it possible to fashion a new PCR system in which fully natural sticky ends of desired length and sequence are directly created in the PCR process with no additional enzyme treatment being required. An outline of the strategy of this light-assisted cohesive-ending PCR (LACE-PCR) is depicted in Scheme 1. A caged



Scheme 1. Outline of light-assisted-cohesive-ending PCR (LACE-PCR) with the caged nucleotide T^{NPP}. Here **1** is used as the template and the HindIII terminus is formed at the end of the product.

nucleotide—4-*O*-[2-(2-nitrophenyl)propyl]thymine—developed by Heckel et al. (T^{NPP} in Scheme 2)[4,13] is introduced into PCR primers. In the second and subsequent cycles of PCR, these primers will be incorporated into the templates for the polymerase reaction. The modified nucleotide T^{NPP} units in these templates cannot form Watson–Crick pairs with their counterpart dATP monomers, since their 4-*O* atoms are protected with the photoremovable 2-(2-nitrophenyl)propyl (NPP) groups.

1 5'- AGCT^{NPP}GTCCGGCGTAGAGGATCGAG -3'
2 5'- FAM-CTCGATCCTCTACGCCG -3'
3 5'- pAGCTCTTGAAGACG -3'
4 5'- AA$T^{NPP}T^{NPP}$CACCGTCACCCTGGATGCTG -3'
5 5'- ACGGGTT^{NPP}ACTGATGAT^{NPP}GTCCGGCGTAGAGG-ATCGAG -3'



Scheme 2. Structure of T^{NPP} and the sequences of oligonucleotides used in this work. Underlined portions correspond to the sticky ends in the PCR products.

Steric hindrance by the bulky NPP groups is also probable. The elongation of the nascent chain (5′→3′ direction) is therefore terminated by the T^{NPP}. After the cycles, the NPP groups are removed by UVA irradiation and the desired sticky ends are straightforwardly created at the ends of the PCR products. As shown below, we found that T^{NPP} is stable enough even under PCR conditions and indeed terminates the polymerase reaction site-selectively. LACE-PCR products coding the GFP gene were successfully and directly ligated to vectors digested either with naturally occurring restriction enzymes or with ARCUT, and were used to transform *E. coli*.

Results and Discussion

Site-selective termination of the polymerase reaction by a caged T^{NPP} nucleotide in the template under PCR conditions

Site-selective termination of the polymerase reaction by T^{NPP} under PCR conditions was examined by primer extension experiments as shown in Figure 1. Here, FAM-labeled primer **2**

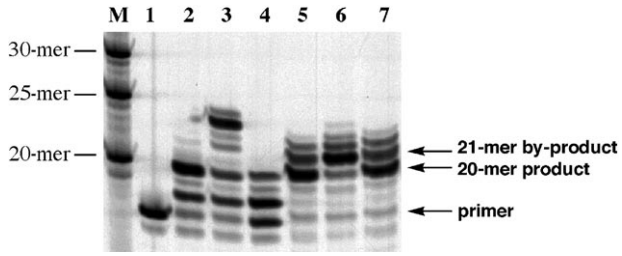


Figure 1. Denaturing PAGE (20%) patterns for primer extension with caged oligonucleotide **1** as template. Lane 1: unreacted **2**. Lane 2: Ex Taq. Lane 3: UV irradiation+Ex Taq. Lane 4: rTaq. Lane 5: KOD-Plus-. Lane 6: KOD-Dash-. Lane 7: PrimeSTAR HS. Details of the reaction conditions are shown in the Experimental Section.

(17 nt, FAM = 6-fluoresceinamide) was extended on the modified oligonucleotide template **1** (24 nt), containing one T^{NPP} unit at the fourth position from the 5′-end, by use of various polymerases commonly used for PCR (the sequences of **1** and **2** are presented in Scheme 2). When Ex Taq was used as the polymerase, the longest product formed after the predetermined thermal cycles was 20 nt, showing that primer extension was completely terminated at the 3′-side of $T^{NPP(4)}$ (lane 2 in Figure 1). No undesired removal of NPP was observed in the control HPLC analysis of the template **1** even after the corresponding PCR thermal cycles (Figure S1 in the Supporting Information). MALDI-TOF/MS analysis of the reaction mixture also confirmed that d(GAC) had been correctly added to the 3′-end of **2** (Figure S2). When the reaction mixture was irradiated with UVA light ($300 < \lambda < 400$ nm) for 30 min prior to the addition of Ex Taq, on the other hand, extension proceeded down to the end of the template **1** (the longest major product was 24 nt). What terminated the elongation is indeed, as expected, the NPP group on the template.

Use of other polymerases commonly used in biochemical researches was also examined (lanes 4–7). The reactions with

rTaq (lane 4), KOD-Plus- (lane 5), and PrimeSTAR HS (lane 7) were also reasonably terminated by T^{NPP} under appropriate thermal cycles, although some minor introduction of extra bases was observed for KOD-Plus- and PrimeSTAR HS. The yields of the desired 20-mer product were 70% for KOD-Plus- and 60% for PrimeSTAR HS. For KOD-Dash- (lane 6), on the other hand, the major product was the 21-mer with one extra base; the yield of the desired 20-mer for KOD-Dash- was only 7%. According to MALDI-TOF/MS analysis of the reaction mixtures (Figure S2), all of these 21-mer byproducts in lanes 5–6 have a mass number 328 higher than that of the desired 20-mer. It is clear that the additional monomer is dG, although those polymerases are known to add extra dA. This finding is quite reasonable, because the hydrogen-bonding pattern of T^{NPP} is almost identical to that of dC except for the protected 4-*O* atom. Similar undesired addition of extra dG was also observed for other polymerases such as Klenow Fragment, T4 DNA polymerase, and DNA polymerase I in isothermal reactions at 37 °C (Figure S3).

Photoremoval of the NPP group for further manipulation

To examine whether or not the sticky ends with T^{NPP} at the junction were ligatable with other fragments, ligation of the 20-mer extension product obtained with Ex Taq to a short oligonucleotide was examined. The outline of the experiment is shown in Figure 2A. The 14-mer oligonucleotide **3** has the d(AGCT) sequence at the 5′-end, and it is complementary to the sticky end (underlined bases shown in Figure 2A) created in the 20-mer product/**1** complex. Without UVA irradiation, no ligation product was observed in a denaturing PAGE analysis of the reaction mixture (lane 2 in Figure 2B). It was thus found that the presence of the NPP group on the template completely inhibits ligation at the opposite site. Quite on the contrary, the 34-mer ligation product was efficiently obtained when the reaction mixture was irradiated with UVA before addition of ligase to the solution (lane 3). According to HPLC analysis, the deprotection yield of the NPP group under these conditions (pH 7.5, 30 min) is about 54% (Figure S1). The estimated yield of 20-mer-to-34-mer conversion in lane 3 is 58%, and this figure is in good accordance with the deprotection yield. Ligation of successfully deprotected complex seems to proceed almost quantitatively.

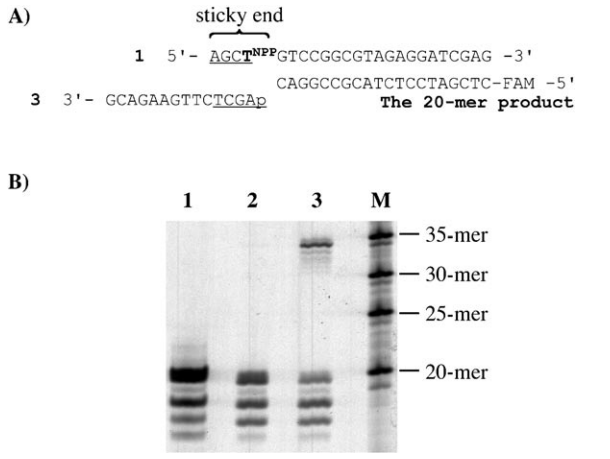


Figure 2. A) Structures of the oligonucleotides used in the ligation experiment. Underlined portions are complementary to each other. B) Denaturing PAGE (20%) patterns for ligation of primer extension product with Ex Taq and **3**. Lane 1: 20-mer product. Lane 2: ligation without UVA irradiation. Lane 3: ligation with prior UVA irradiation.

The deprotection yield obtained here is significantly small in comparison with the yield of nearly 90% at pH 8.8 reported in the literature.[4] In view of the mechanism of NPP removal, which proceeds via an anionic nitronic acid intermediate,[27] this low yield might be because of pH dependence of NPP removal. However, a high pH is not favorable for this system, because it elevates the risk of oxidative base damage.[28]

Use of LACE-PCR to provide products possessing restriction enzyme termini

To check whether site-selective termination would occur practically and whether desired sticky ends could be obtained after LACE-PCR, PCR products bearing sticky ends complementary to EcoRI and HindIII termini were prepared with T^{NPP}. As shown below, the products were successfully inserted into vectors, produced by digesting pUC18 with EcoRI and HindIII, and cloned into *E. coli*.

1) Preparation of LACE-PCR products with EcoRI/HindIII termini: The 121–1170 bp region of pQBI T7-GFP plasmid was amplified by LACE-PCR. This portion of the plasmid includes both GFP gene (GFP = green fluorescence protein) and T7 promoter. For this experiment, KOD-Plus- was used because of its high fidelity and reasonable termination by T^{NPP} observed in the primer extension experiment above. In one of the primers used in the LACE-PCR (**1** in Scheme 2), one T^{NPP} unit was incorporated at the fourth position from its 5′-end (5′-AGCT^{NPP}GT-). The resulting sticky end is complementary to the HindIII terminus. Another end of the product of LACE-PCR (5′-AATTCA-/3′-GT-) was prepared by using the caged primer **4** (Scheme 2). In this primer, two T^{NPP} units were incorporated at the third and the fourth positions from the 5′-end to minimize undesired primer dimer formation. After the NPP groups had been removed, the EcoRI terminus was formed here. As shown in Figure 3, a product of desired length (1 kbp) was successfully and selectively amplified (lane 1).

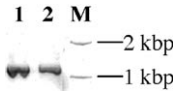


Figure 3. Agarose gel electrophoresis patterns for the LACE-PCR product. Lane 1: LACE-PCR product before UV irradiation. Lane 2: LACE-PCR product after 1 h UV irradiation.

Removal of the NPP groups in the PCR product was performed with UVA irradiation on the purified product solution. To remove as much of the NPP as possible, irradiation time here was extended to 1 h. From the yield of NPP removal (> 54% for each NPP) and the yield of successfully terminated elongation (70% for KOD-Plus-), approximately 10% of the product would be expected to have the fully deprotected, desired sticky ends at both ends. Recent studies have shown that both UVA radiation and UVB cause various forms of oxidative damage on DNA, which

result in DNA degradation or mutation.[29–34] From the dose dependencies for each type of base damage caused by UVA irradiation,[34] the estimated frequencies of cyclobutane pyrimidine dimer (CPD) formation, purine oxidative damage, pyrimidine oxidative damage, and abasic damage in the 1 kbp product after the irradiation would be estimated to be 0.013, 0.014, 0.006, and 0.008 per clone, respectively. About 4% of the population might thus contain one of the above damaged bases overall, and the remaining 96% would be expected to be intact. No significant alteration of the band pattern in agarose gel was observed even after 1 h UVA irradiation (lane 2 in Figure 3). Moreover, these forms of DNA damage would be repaired in vivo, so the apparent frequency of damage should be much smaller. These arguments are further supported by the sequencing experiments (vide infra).

2) Construction of recombinant vector from LACE-PCR product: The insert obtained above was ligated to a vector prepared by digesting pUC18 with EcoRI and then with HindIII. Taking into account that the estimated yield of LACE-PCR product bearing completely ligatable sticky ends was 10%, 50 equiv of insert was added to the vector for ligation, although normal vector to insert ratios range from 1:3 to 1:10. JM109 was then transformed with the ligation product.

Precise construction of the recombinant vector was directly confirmed by sequencing the extracted plasmid from one of the colony-PCR positive clones. The sequences near the EcoRI site (Figure 4A) and the HindIII site (Figure 4B) are both completely consistent with the expected sequences. In addition, no mutation was found in the range corresponding to the LACE-PCR product for this clone (Figure S4). These results show that the strategy proposed in Scheme 1 is fairly reasonable. According to Dmochowski et al.,[10] DNA polymerization by the Klenow fragment is more efficiently blocked when photocleavable DABSYL and fluorescein are bound to dC residues in a template next to each other. However, the promoting effect of $T^{NPP(3)}$, the second T^{NPP} in **4**, is not very explicit in the present LACE-PCR, since only one T^{NPP} in the primer (and thus in the template for polymerase reaction) is able to stop elongation to provide the HindIII terminus, at least under the conditions employed here.

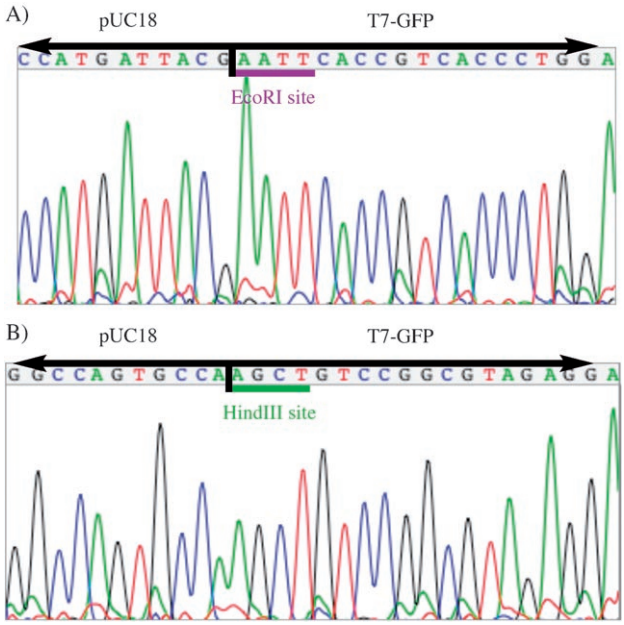
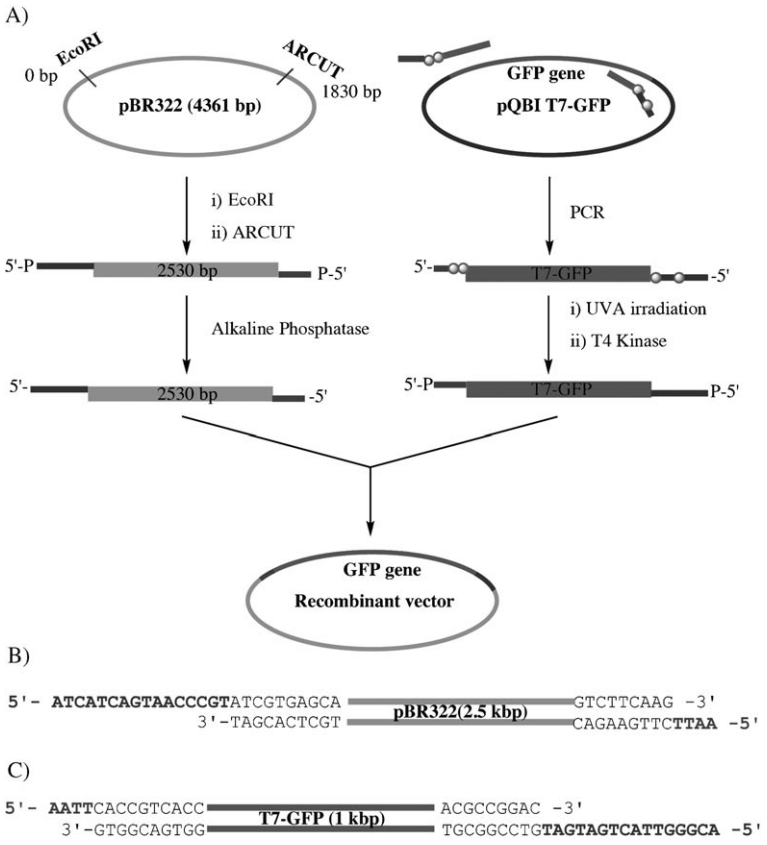


Figure 4. Sequencing analysis of cloned recombinant vector at A) the EcoRI site conjunction, and B) the HindIII site conjunction.



Scheme 3. A) Construction of recombinant vector by use of the EcoRI/ARCUT system (the red circles refer to T^{NPP} units). B) Structure of the vector formed by cutting pBR322 with EcoRI (right-side end) and ARCUT (left-side end). C) Structure of the insert prepared by LACE-PCR.

LACE-PCR to provide products possessing the scission termini of the man-made tool ARCUT

Next, a vector was prepared by cutting pBR322 with man-made tool ARCUT (the digestion at another site was achieved with EcoRI; see Scheme 3A). The insert for this experiment also involves both GFP gene and T7 promoter. In the terminus formed by ARCUT employed here (Figure S5), 16 nucleotides at the 5′-end of one strand are protruding beyond the 3′ terminus of another strand (see the left-side end of the vector in Scheme 3B). Through the use of LACE-PCR, the sticky end, which is complementary with this terminus and required for efficient ligation, can be straightforwardly created at the end of the PCR product (right-side end of the insert in

Scheme 3C). Note that the plasmid, the polymerase, and the competent cells used here are all different from those in the previous section, so the wide applicability of the LACE-PCR method is further substantiated.

1) Preparation of the vector by cutting pBR322 with EcoRI and ARCUT: The plasmid vector pBR322 (4361 bp) was linearized with EcoRI (Figure 5, lane 1) and then treated with ARCUT. Site-

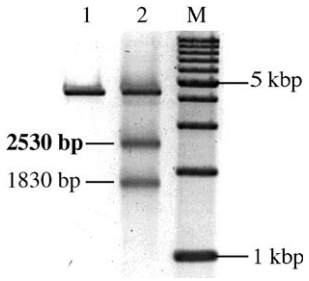


Figure 5. Agarose gel electrophoresis patterns for the scission of pBR322 by EcoRI and ARCUT. Lane 1: EcoRI only. Lane 2: EcoRI and then ARCUT. Lane M: 1 kbp ladder.

selective scission at the two sites gave two major bands at 2530 bp and 1830 bp in the gel electrophoresis (lane 2). The band of 2530 bp size was extracted from the gel and used as the vector. It has been reported that scission by ARCUT takes place at several phosphodiester linkages in a hot-spot in the single-stranded region (Figure S5). This band should thus contain several DNA fragments, possessing different end structures. As shown below, however, the vector DNA possessing the end structure in Scheme 3B is selectively picked up from the mixture in the ligation step, since both of the ends of the insert, formed by LACE-PCR, are complementary with the two ends of this vector.

2) LACE-PCR to prepare the insert possessing ARCUT/EcoRI termini: One of the ends of the LACE-PCR product (5′-ACGGGTTACTGATGATGT-/3′-CA-) should be complementary with the ARCUT scission terminus (left-side end of Scheme 3B). However, this complicated terminus can be easily created by using the caged primer **5** (Scheme 2) in which two T^{NPP} units are incorporated at the seventh and 16th positions. As shown in the next section, elongation by PrimeSTAR HS DNA polymerase (at 72 °C) was successfully terminated by the caged nucleotide $T^{NPP(16)}$, which the nascent-chain elongation first encounters in LACE-PCR.

In **5**, $T^{(7)}$ was also replaced with $T^{NPP(7)}$, because the 5′-$G^{(3)}GGTT^{(7)}$-3′ sequence of this primer is, by chance, pseudo-complementary with 3′-$C^{(5)}T^{NPP}T^{NPP}AA^{(1)}$-5′ sequence in the primer **4** [Scheme 2; the 2-(2-nitrophenyl)propyl in T^{NPP} does not directly inhibit wobble pairing with G]. The possibility of undesirable interactions between these two primers (and also between the corresponding PCR products) can be minimized by this $T^{NPP(7)}$, since it cannot form a Watson–Crick pair with $A^{(1)}$ in the primer **4**.

The other end of the LACE-PCR product, which is complementary with the EcoRI terminus, was prepared by use of the caged primer **4** as described above. Agarose gel electrophoresis of the LACE-PCR product showed that the amplification was fairly selective and efficient (lane 2 in Figure 6).

3) Formation of recombinant vector and its expression in cells: DH5α cells were transformed with the ligation product of the

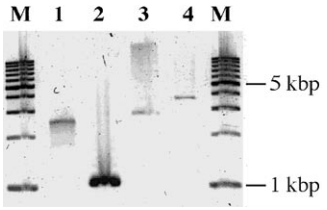


Figure 6. Agarose gel electrophoresis patterns for the LACE-PCR product and recombinant vectors. Lane 1: ARCUT product. Lane 2: LACE-PCR product. Lane 3: extracted plasmid. Lane 4: extracted plasmid linearized with EcoRI. Lane M: 1 kbp ladder.

vector prepared in Part 1 of this Section and the insert in Part 2. The size of the extracted plasmid (supercoiled, lane 3 in Figure 6; linearized, lane 4) was consistent with the expected 3590 bp. As shown by the sequencing experiments of the plasmid in Figure 7, the sequences near the ARCUT scission site (a) and the EcoRI digestion site (b) are again both completely consistent with the argument. Successful formation both of the ARCUT terminus and of the EcoRI terminus in the insert by LACE-PCR has been corroborated.

When the extracted recombinant vector was introduced into BL21-Gold (DE3), emission of green fluorescence from the expressed GFP was clearly observed (Figure 8). This is further evidence that no undesired critical DNA mutation was induced into the gene in this clone by the UVA irradiation that was carried out to remove the protecting group from T^{NPP}.

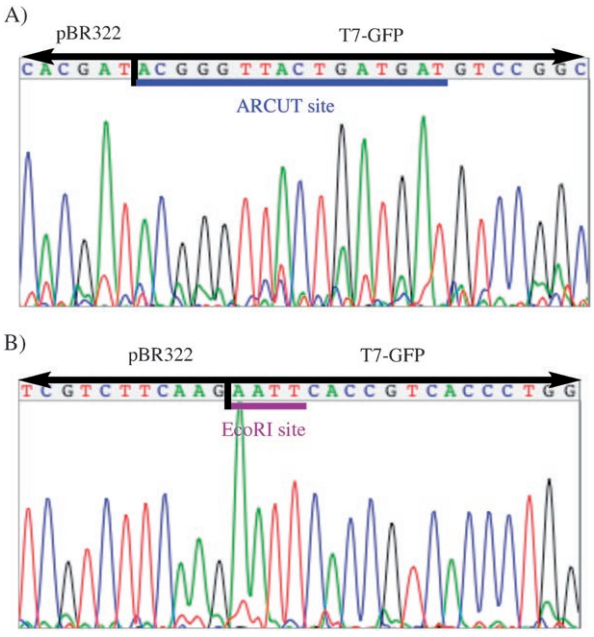


Figure 7. Sequencing analysis of cloned recombinant vector at A) the ARCUT site conjunction, and B) the EcoRI site conjunction.

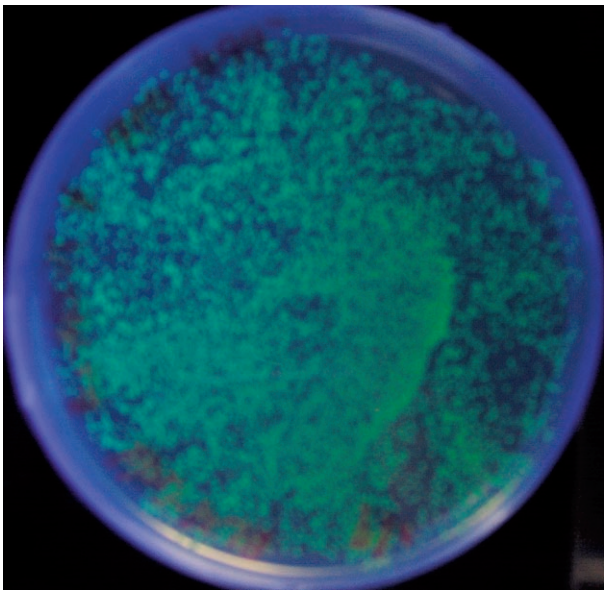


Figure 8. Emission of fluorescence from the GFP expressed in BL21-Gold (DE3). These cells were transformed with the recombinant vector prepared with the EcoRI/ARCUT system.

Conclusion

The caged thymine T^{NPP} site-selectively blocks the polymerase reaction under PCR conditions, although some minor misincorporation of dG in front of T^{NPP} was observed for some of the polymerases examined in this study. The resulting 5′-protruding end in the primer/caged template complex can be used for ligation with other complementary fragments after the NPP group has been removed by brief UVA irradiation. This finding made possible a new direct PCR system to prepare products bearing desired sticky ends. After PCR cycles using caged primers bearing T^{NPP} units, the product solution is briefly irradiated with UVA, and the protecting groups of T^{NPP} are removed. By this simple procedure, desired sticky ends have been successfully created at the ends of 1 kbp PCR products coding the GFP gene. There is almost no limitation in the sequence of the protruding 5′-end. Moreover, the choice of target gene is also almost unlimited, since LACE-PCR does not involve a digestion step with restriction enzymes, so there is no fear of undesired cleavage inside the gene. This finding should widen the scope of DNA manipulation. To achieve more precise termination of elongation, improved caged nucleotide design might be desirable. The recently reported T^{NPOM} residue,[9] in which the 3-N atom is protected by a photoremovable group, might be a good candidate for this purpose. Development of caged monomers for A, G, and C would also further extend the scope of applications. Investigations in this area are currently underway.

Experimental Section

Materials: The primers containing caged thymine T^{NPP} were prepared by standard phosphoramidite chemistry through the use of a set of ultramild cyanoethyl phosphoramidites [phenoxyacetyl-protected dA and (4-isopropylphenoxy)acetyl-protected dG monomers from Glen Res., USA], purified by reversed-phase HPLC, and characterized by MALDI-TOF/MS (Bruker AutoFLEX). Their sequences are shown in Scheme 2. The phosphoramidite monomer of T^{NPP} was synthesized from thymidine and 2-ethylnitrobenzene as described in the literature.[4,35,36] Water was deionized with a Millipore Water Purification System, and sterilized immediately before use. The Ce^{4+}/EDTA solution was prepared by mixing an aqueous solution of $Ce(NH_4)_2(NO_3)_6$ (20 mM, Nacalai Tesque) and 4Na·EDTA (20 mM, Tokyo Kasei Kogyo) in HEPES buffer, and then adjusting the pH to 7.0 with a small amount of NaOH.[19] The synthesis, purification, and characterization of pcPNA strands has been described elsewhere.[18] Photoirradiation was performed with a UV Spot Light Source (Hamamatsu Photonics; 200 W) and a UV-D36C filter (Asahi Technoglass) at 2.5 $mW\,cm^{-2}$. Imaging and quantification of gel electrophoresis were carried out with a FLA-3000G fluorescent imaging analyzer (Fujifilm).

Primer extension reaction with caged oligonucleotide as a template: A mixture containing the caged oligonucleotide substrate **1** (4 μM), the primer oligo **2** (2 μM), and dNTPs (1.35 mM for each) was prepared and divided into two portions. Unintended light exposure was carefully avoided throughout the process. One portion was irradiated with UVA light ($300 < \lambda < 400$ nm) for 30 min at room temperature to remove the protecting group from the T^{NPP}, whereas another portion was directly used for the primer extension reaction. After addition of Ex Taq polymerase (Takara) to the solution (0.2 $U\,\mu L^{-1}$), the following cycles—denaturation (94 °C, 15 s), annealing (50 °C, 30 s), elongation (60 °C, 70 s)—were repeated 30 times on a thermal cycler. The product was then analyzed by denaturing PAGE (20%). Extension with other polymerases was also examined at the following concentrations and without UV irradiation: [rTaq polymerase (Takara)] = 0.1 $U\,\mu L^{-1}$, [KOD-Plus- polymerase (Toyobo)] = 0.02 $U\,\mu L^{-1}$, [KOD-Dash- polymerase (Toyobo)] = 0.05 $U\,\mu L^{-1}$, and [PrimeSTAR HS DNA Polymerase (Takara)] = 0.025 $U\,\mu L^{-1}$. The thermal cycle for KOD-Plus- and KOD-Dash- was the same as for Ex Taq. The thermal cycle for rTaq was: denaturation (94 °C, 30 s), annealing (55 °C, 30 s), and elongation (72 °C, 70 s). The thermal cycle for PrimeSTAR HS was: denaturation (98 °C, 10 s), annealing (55 °C, 5 s) and elongation (72 °C, 70 s).

Ligation of primer extension product and short oligonucleotide: The product of primer extension with Ex Taq was purified by phenol/chloroform extraction and then on MicroSpin™ G-25 columns (GE Healthcare). This purified product was mixed with the ligation substrate **3** (4 equiv), and the mixture was divided into two portions. One portion was directly used for ligation. Another portion was irradiated with UVA light for 30 min at room temperature to remove the protecting group from the T^{NPP}. The ligation reaction was initiated by addition of the same volume of DNA Ligation Kit "Mighty Mix" (Takara) solution to the mixture (final concentrations of the purified product and **3** were 5 μM and 20 μM, respectively), allowed to proceed for 2 h at 16 °C, and analyzed on 20% denaturing PAGE after ethanol precipitation.

Light-assisted cohesive-ending PCR (LACE-PCR): With pQBI T7-GFP (Wako, 20 $pg\,\mu L^{-1}$) as the template, PCR was performed under the following conditions: [each primer] = 300 nM, [dNTPs] = 200 μM, and [KOD-Plus- polymerase] = 0.02 $U\,\mu L^{-1}$ (or [PrimeSTAR HS DNA polymerase] = 0.025 $U\,\mu L^{-1}$). Thermal cycles were the same as in the primer extension experiments.

After purification with a QIAquick PCR Purification kit (Qiagen), the product was irradiated with UVA light for 1 h at room temperature. The product was then phosphorylated with ATP (1 mM) and T4

polynucleotide kinase (0.22 U μL^{-1}, Toyobo) at 37 °C for 1 h, and was again purified with a QIAquick PCR Purification kit.

Scission of pBR322 with EcoRI and ARCUT: The pBR322 plasmid (4361 bp) was first linearized with EcoRI, and treated with ARCUT as reported previously;[19,20] the details on ARCUT used here are presented in the Supporting Information. DNA hydrolysis was achieved under the following conditions—[linearized pBR322] = 8 nM, [each PNA] = 100 nM, [Ce^{4+}/EDTA] = 100 μM, and [NaCl] = 100 mM at pH 7.0 (5 mM HEPES buffer), 37 °C for 72 h—and was stopped by addition of ethylenediaminetetramethylenephosphonic acid. After treatment with calf intestinal alkaline phosphatase, desired fragments of about 2530 bp were purified by agarose gel electrophoresis (0.8%) and extracted from the gel with a Get pureDNA Kit-Agarose (DOJINDO).

Construction of recombinant vectors: The vector for the LACE-PCR product containing EcoRI/HindIII digestion termini was prepared by cutting pUC18 with EcoRI and HindIII. The same volume of DNA Ligation Kit "Mighty Mix" was added to a 1:50 mixture of this vector DNA and the PCR product. After incubation for 3 h at 16 °C, JM109 (Toyobo) was transformed with the ligation product and cultured on LB-agar media. After the insertion was confirmed by colony PCR, positive colonies were picked up and cultured in LB media. The plasmid DNA was purified with a QIAprep spin Miniprep Kit (Qiagen), and its sequence starting 200 bp away from each conjunction was determined on a 3130x Genetic Analyzer (Applied Biosystems). As the primers, 5′-TCGCCATTCAGGCTGCGCAAC-3′ (243–263 bp region of pUC18 plasmid), 5′-CCTCTCCCCGCGCGTTGGC-3′ (647–665 bp region of pUC18 plasmid), 5′-GTTGAATACTCATACTCTTCC-3′ (4141–4161 bp region of pBR322 plasmid) and 5′-AAGCTCATCAGCGTGGTCGTG-3′ (2058–2038 bp region of pBR322 plasmid) were used both for the colony PCR and the sequencing.

In the EcoRI/ARCUT system, ligation was achieved at a 1:40 vector/insert ratio with 2 h incubation, and DH5α (Toyobo) was transformed with the ligation product. In order to observe the emission of the fluorescence from expressed GFP, the recombinant vector was introduced into BL21-Gold (DE3) (Stratagene®). Note that BL21-Gold (DE3) intrinsically possesses T7 RNA polymerase but DH5α does not. Otherwise, the experimental procedures were the same as those for the EcoRI/HindIII system.

Acknowledgements

This work was partially supported by a Grant-in-Aid for Specially Promoted Scientific Research from the Ministry of Education, Science, Sports, Culture and Technology, Japan and by the Global COE Program for Chemistry Innovation.

Keywords: caged compounds · DNA · PCR · recombination · sticky ends

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Received: April 25, 2008
Published online on August 7, 2008

