Site-selective Termination of DNA Replication by Using a Caged Template

Keita Tanaka, Akinori Kuzuya,* and Makoto Komiyama*
RCAST, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8904

(Received February 28, 2008; CL-080225; E-mail: komiyama@mkomi.rcast.u-tokyo.ac.jp)

A caged DNA template, which bears a caged thymine {4-*O*-[2-(2-nitrophenyl)propyl]thymine, T^{NPP}} near its 5′-end, was prepared, and used for in vitro DNA replication. The photo labile 2-(2-nitrophenyl)propyl (NPP) group efficiently blocked DNA polymerase reaction, and site-selectively terminated the extension of complementary strand there. This NPP-group can be removed by simple 30-min UVA irradiation to the solution, to give restriction-enzyme-free sticky end on the template strand.

The key technology in the present biology-related research fields such as biochemistry, medicinal chemistry, and biotechnology is DNA manipulation, which is a combination of site-selective DNA cleavage using restriction enzymes and the reverse connecting reaction using DNA ligase.[1] To facilitate and ensure selective reaction, sticky-ended DNA fragments are very often used for ligation. Most of the sticky ends used in modern biological studies are products of restriction enzyme reaction, which usually recognizes 8 or less bases palindrome stretch and produces sticky ends of 4 or 2 bases long.

Although this strategy has fulfilled vast success so far, limited choice of recognition sequence of restriction enzyme sometimes makes researchers to take detour pass to obtain desired recombinant genes. On the other hand, demands for non-natural, restriction-enzyme independent sticky ends are also increasing today. Ligation independent cloning of PCR products (LIC-PCR)[2] is one of the most popular examples of such systems. It is shown that sufficiently long (12 bases or longer) sticky ends can tightly hold two DNA ends together even if they are not ligated to each other, stable enough to survive the uptake process into a competent cell. Inherent recovery system of the host cell then fixes the nicks in the joint to provide fully intact DNA duplex and complete transformation. A few systems have been proposed so far to obtain such long sticky ends for the purpose.[3] Most of them require additional reaction with natural enzymes such as T4 or *Pfu* polymerases to prepare single-stranded overhang. Artificial restriction DNA cutter (ARCUT),[4] which we developed recently, is another system that requires non-natural sticky end. ARCUT usually produces sticky ends of 10–15 bases long with completely non-natural sequence. For further manipulation of the product, i.e. ligation with another gene fragment amplified by PCR, complementary sticky end to the ARCUT product should be attached to the substrate. Therefore, simple and easy procedure to prepare restriction-enzyme-free sticky end is desirable.

In this study, we have focused on a caged nucleotide to develop a new restriction-enzyme-free method to prepare sticky ends (Figure 1). The caged nucleotide 4-*O*-[2-(2-nitrophenyl)-propyl]thymine (T^{NPP}), which was developed by Kröck and Heckel[5] bears photocleavable protecting group, 2-(2-nitrophenyl)propyl (NPP), on the 4-O-position, and this NPP-group efficiently hinders complementary base-pairing with dA. When T^{NPP} is introduced in a template for DNA polymerase reaction, we found that the NPP-group efficiently blocks the catalysis of DNA polymerase there, and site-selectively terminates the elongation of the nascent strand.

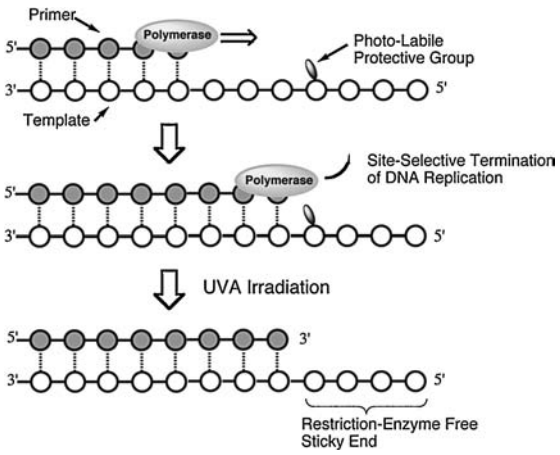

Figure 1. Schematic representation of the present strategy for site-selective termination of DNA replication.

Structures of DNA strands used in this study are shown in Figure 2. The caged template **1** bears a T^{NPP} residue as the 4th residue from the 5′-end. The 17-mer primer **2** is complementary to the 3′-portion of **1**, and it is fluorescently labeled with 6-fluoresceinamide (FAM) dye at the 5′-end. If NPP-group successfully and site-selectively terminates DNA polymerase reaction, only 3 nucleotides are added to the 3′-end of **2** to give a 20-mer product. If not, full elongation results in a 24-mer product.

All the oligonucleotides used in this study were synthesized on an automated DNA synthesizer using standard phosphoramidite chemistry, except for the caged template **1** which was synthesized by using a set of ultramild cyanoethyl phosphoramidites (phenoxyacetyl-protected dA and 4-isopropylphenoxyacetyl-protected dG monomers from Glen Res., USA). The phosphor-

1 5'- AGCT^{NPP}GTCCGG CGTAGAGGAT CGAG -3'
2 3'- GCC GCATCTCCTA GCTC-**FAM** -5'

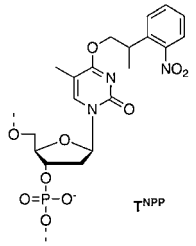

Figure 2. Structures of DNA strands used in this study.

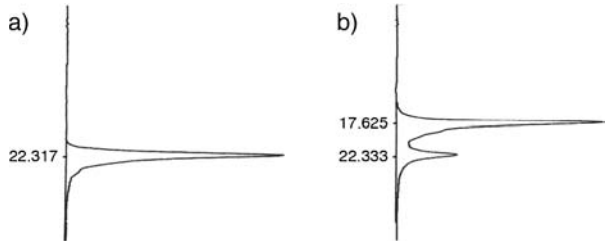

Figure 3. Reversed-phase HPLC charts of **1** before (a) and after (b) 30-min UVA irradiation. A linear gradient of 2.5–22.5% acetonitrile in 50 mM ammonium formate over 40 min was made for the measurement.

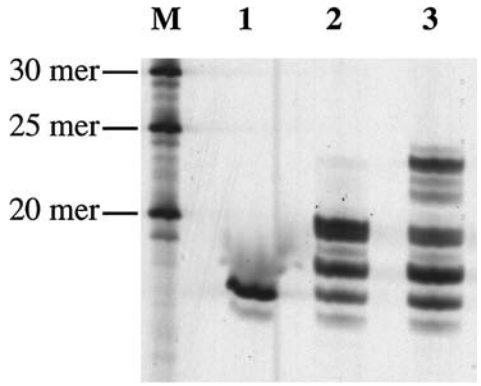

Figure 4. Denaturing PAGE analysis of extension reaction using the caged template. Lane 1, intact **2**; lane 2, the reaction mixture without UVA irradiation; lane 3, the reaction mixture with prior 30-min UVA irradiation. Lane M, size markers. Reaction conditions; [**1**] = 4 μM, [**2**] = 2 μM, [dNTPs] = 100 μM, [Ex Taq polymerase] = 0.2 U/μL at 37 °C for 30 min.

amidite monomer for T^{NPP} was synthesized from thymidine and 2-ethylnitrobenzene according to the literatures.[5–8]

Photoremoval of NPP-group in the template was confirmed by reversed-phase (RP) HPLC analysis of **1**. The UV irradiation of $300 < \lambda < 400$ nm was achieved with a UV Spot Light Source (Hamamatsu Photonics, 200 W) and UV-D36C filter (Asahi Technoglass) at 2.5 mW/cm^2. Figure 3 shows RP-HPLC charts of **1** before (a) and after (b) 30 min irradiation to the solution of **1** at pH 8.0 (10 mM Tris-HCl). The intensity of the peak at 22 min, which corresponds to intact **1** bearing NPP, significantly decreased after UVA irradiation, and in exchange, the intensity of the peak at 17 min increased. Co-injection with an authentic sample of unmodified **1** and MALDI-TOFMS analysis of the solution after irradiation clearly confirmed that the former peak is deprotected **1** (data not shown). The yield of the removal was nearly 70% after 30 min irradiation.[9]

Figure 4 is a typical denaturing PAGE pattern of DNA replication experiment using **1** as the template and **2** as the primer. As in lanes 2 and 3, **2** was successfully elongated by DNA polymerase in the presence of **1**, showing that NPP-modification does not nonselectively inhibit polymerase reaction. However, the length of the product clearly depended on whether the reaction mixture was irradiated with UVA prior to the polymerase addition or not. When the reaction mixture was irradiated with UVA for 30 min, fully elongated 24-mer product was obtained (lane 3). This result strongly suggests that the dT residue formed by NPP-removal recovers native properties such as hydrogen-bonding ability, and can be recognized by enzymes as normal dT. Without UV irradiation, on the other hand, the band corresponding to fully elongated product was not detected at all, and only shorter ($\leq$ 20 nt) products were obtained (lane 2). MALDI-TOFMS analysis of the mixture showed that the major and the longest product was the 20 mer (calcd. m/z, 6547.9; found, 6546.7; see Supporting Information for the spectrum). As the original design indicates, elongation of **2** was completely terminated just before T^{NPP}, and the last 4 nucleotides in the 5′-end of **1** was kept single stranded.

By using caged oligonucleotide as a template, we have successfully achieved the first light-switchable and site-selective termination of DNA replication catalyzed by DNA polymerase. The resulting protruding end protected by photocleavable NPP group can be turned into a ligation-ready sticky end simply after UVA irradiation. The final product does not contain any unnatural nucleotide, thus it is expected to be highly compatible with further biological processing such as cloning in *E. coli*. One of the feasible applications of the present system is in situ restriction-enzyme-free preparation of desired sticky ends on PCR amplicon. An attempt to establish such system is now under way in our laboratory.

This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References and Notes

1 *Molecular Cloning: A Laboratory Manual*, 3rd ed., ed. by J. Sambrook, D. W. Russel, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., **2001**.
2 C. Aslanidis, P. J. de Jong, *Nucleic Acids Res.* **1990**, *18*, 6069.
3 Q. Lu, *Trends Biotechnol.* **2005**, *23*, 199.
4 M. Komiyama, Y. Aiba, Y. Yamamoto, J. Sumaoka, *Nat. Protoc.* **2008**, *3*, 655.
5 L. Kröck, A. Heckel, *Angew. Chem., Int. Ed.* **2005**, *44*, 471.
6 A. Heckel, G. Mayer, *J. Am. Chem. Soc.* **2005**, *127*, 822.
7 Y. Tsuji, S. Kotachi, K. T. Huh, Y. Watanabe, *J. Org. Chem.* **1990**, *55*, 580.
8 R. Saladino, C. Crestini, F. Occhionero, R. Nicoletti, *Tetrahedron* **1995**, *51*, 3607.
9 This yield is small compared to the yield of nearly 90% for homo-T oligomer employed in ref. 5, and did not much increase even after 1 h irradiation. Assumedly, there is some sequence dependency in NPP removal.
10 Supporting Information is also available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.

Published on the web (Advance View) April 26, 2008; doi:10.1246/cl.2008.584