Biosci. Biotechnol. Biochem., **66** (10), 2194–2200, 2002

Gene Cloning and Polymerase Chain Reaction with Proliferating Cell Nuclear Antigen from *Thermococcus kodakaraensis* KOD1

Masao KITABAYASHI,[1,2,†] Yoshiaki NISHIYA,[1] Muneharu ESAKA,[2] Mitsuo ITAKURA,[3] and Tadayuki IMANAKA[4]

[1]*Tsuruga Institute of Biotechnology, Toyobo Co., Ltd., 10-24 Toyo-cho, Tsuruga, Fukui 914-0047, Japan*
[2]*Faculty of Applied Biological Science, Hiroshima University, Kagamiyama, Higashi-Hiroshima 739-8528, Japan*
[3]*Institute for Genome Research, University of Tokushima, 3-8-15 Kuramoto-cho, Tokushima 770-8503, Japan*
[4]*Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Yoshidahonmachi, Sakyo-ku, Kyoto 606-8501, Japan*

Received April 15, 2002; Accepted June 19, 2002

The gene encoding the proliferating cell nuclear antigen (PCNA), a sliding clamp of DNA polymerases, was cloned from an euryarchaeote, *Thermococcus kodakaraensis* KOD1. The PCNA homologue, designated *Tk*-PCNA, contained 249 amino acid residues with a calculated molecular mass of 28,200 Da and was 84.3% identical to that from *Pyrococcus furiosus*. *Tk*-PCNA was overexpressed in *Escherichia coli* and purified. This protein stimulated the primer extension abilities of the DNA polymerase from *T. kodakaraensis* KOD1 'KOD DNA polymerase'. The stimulatory effect of *Tk*-PCNA was observed when a circular DNA template was used and was equally effective on both circular and linear DNA. The *Tk*-PCNA improved the sensitivity of PCR without adverse effects on fidelity with the KOD DNA polymerase. This is the first report in which a replication-related factor worked on PCR.

Key words: PCNA; sliding clamp; DNA polymerase; *Thermococcus kodakaraensis* KOD1; PCR

DNA replication is an essential event for the maintenance of life. DNA polymerases used an elongation factor, called a sliding clamp, for processive DNA synthesis. Proliferating cell nuclear antigen (PCNA) plays this role in the eukaryotic and archaeal systems. PCNA is composed of three subunits and forms a torus-like structure with a central cavity that accommodates double-stranded DNA.[1] The eukaryotic clamp loader (replication factor C: RF-C), which is needed for the loading of PCNA onto the DNA duplex, is composed of five subunits.[2,3]

Recent genomic sequencing of archaea[4–9] has identified three genes encoding putative homologues of RF-C and PCNA. These genes have sequences similar to those of the RF-C large and small subunits and PCNA from eukaryotes. The PCNA from the euryarchaeote *Pyrococcus furiosus* (*Pfu*-PCNA) has been characterized recently.[10] In addition, two PCNA homologues have been reported for the crenarchaeotes *Sulfolobus solfataricus*[11] and *Pyrobaculum aerophilum*.[12] The PCNAs from *S. solfataricus* and *P. furiosus* increase the processivity of the respective family B DNA polymerases. However, a large amount (several times 10 at the molar ratio) of PCNA was needed to stimulate the processivity.

Many DNA polymerases have been cloned and sequenced from various organisms, and some of them have been studied for their scientific and industrial importance.[13,14] The PCR, which enables amplification of a specific DNA fragment *in vitro*, is one of the most important applications of DNA polymerase. In the conventional method of PCR, family A DNA polymerases isolated from thermophilic bacteria, *Thermus aquaticus* and *T. thermophilus*, were often used. Recently, family B DNA polymerases from hyperthermophilic archaea, *P. furiosus* and *Thermococcus litoralis*, have been widely used because they have higher fidelity in PCR based on their strong 3′-5′ exonuclease (proof-reading) activity.[15,16] However, these family B DNA polymerases had lower DNA elongation abilities than those of family A DNA polymerases. The improvement of long PCR techniques with high PCR fidelity is in strong demand, to make PCR more useful.[17]

We have reported a novel family B DNA polymerase from *T. kodakaraensis* KOD1 'KOD DNA polymerase' with the highest level in DNA elongation rate, processivity, and fidelity among many thermostable DNA polymerases used for PCR.[18] These advantages made it possible to carry out timesaving and high fidelity PCR.[19–23] However the PCR sen-

† To whom correspondence should be addressed. Fax: +81-770-22-7643; E-mail: masao_kitabayashi@bio.toyobo.co.jp

sitivity was insufficient. So we attempted to improve the PCR sensitivity by adding a replication-related factor to the system.

In this paper, we describes gene cloning and characterization of a PCNA homologue (*Tk*-PCNA) from the hyperthermophilic euryarchaeote *T. kodakaraensis* KOD1. On examining the interaction of KOD DNA polymerase and *Tk*-PCNA, we confirmed that *in vitro* primer extension abilities of the KOD DNA polymerase were increased with added the *Tk*-PCNA.

Materials and Methods

Chemicals. M13 ssDNA, deoxyribonucleoside 5′-triphosphates, T4 DNA ligase, and the modification and restriction enzymes were prepared from Toyobo Co., Ltd. (Osaka, Japan). Molecular mass markers, HiTrap Q, Superdex-200, and oligonucleotides were all obtained from Amersham-Pharmacia Biotech (Uppsala, Sweden). All other chemicals were molecular-biology-grade reagents from Nacalai (Kyoto, Japan).

Bacterial strains. The transformation-competent *E. coli* strains, JM109 and BL21 (DE3), were obtained from Toyobo and Stratagene (La Jolla, CA), respectively.

Cloning of the PCNA gene from T. kodakaraensis KOD1. DNA amplifications for cloning the PCNA gene were done by PCR as follows. Each 50-μl reaction mixture containing chromosomal DNA (100 ng) as a template and sense and anti-sense primers (15 pmol each), was prepared with the KOD-Plus polymerase (Toyobo) according to the manufacturer's instructions. The PCR primers were based on DNA sequences encoding PCNA homologues found from the total genome sequences of *P. furiosus* and *P. horikoshii*. Two primers designed were as follows: PCNA-f1 (5′-CGGAATTCATGMGSGCYATGAY-CCVAGYAGRGT-3′) and PCNA-r1 (5′-GCTCTA-GATASTCCATYTGSAKSGGCATYTC-3′), which have an *Eco*RI and a *Xba*I site (underlined), respectively. After it was verified by DNA sequencing that the amplification fragment contained the PCNA homologue gene, its fragment was used as a probe for screening the KOD genomic λ10 library, constructed according to the manufacturer's protocol (Stratagene). The positive clones were obtained by plaque hybridization screening (1×10^4 multiplicity of infection).

The cloned fragments were sequenced in both orientations by the dideoxy chain termination method using a dye terminator cycle sequencing kit (Applied Biosystems, Forest City, CA) and an automated DNA sequencer ABI Prism 3700 Genetic Analyzer (Applied Biosystems). Computer analyses of the DNA sequence data and the deduced amino acid sequence were done with GENETYX (Software Development Co., Ltd., Tokyo).

Construction of PCNA expression plasmid. For expression of the PCNA gene in *E. coli*, the sense primer containing an *Nde*I site was designed as follows: 5′-GGAATTCCATATGCCGTTCGAAGTT-GTTTT-3′; the anti-sense primer containing *Xba*I sites was designed as follows: 5′-GCTCTAGATC-ACTCCTCAACGCGCGGAGCG-3′, and PCR was done with the positive phage clone found by plaque hybridization screening serving as a template. The amplified 750-bp fragment was digested with *Nde*I/*Xba*I and subcloned into the *Nde*I/*Nhe*I site of plasmid pET11c (Novagen, Madison, WI). The resultant PCNA-positive plasmid was designated pTKPCNA, and this plasmid was introduced into *E. coli* BL21 (DE3).

Overproduction and purification of the Tk-PCNA. *E. coli* BL21 (DE3) (pTKPCNA) was cultured at 37°C in 1 liter of Luria-Bertani (LB) medium containing 0.1 mg/ml ampicillin. The protein was induced with 1 mM of isopropyl-D-β-thiogalactopyranoside, and the cells were collected by centrifugation (12,000 g, 15 min, 4°C), suspended in 50 ml of buffer A (30 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 30 mM NaCl), and disrupted by sonication. The insoluble debris was removed by centrifugation after a heating program (80°C, 30 min). The resulting supernatant (about 50 ml) was put on an anion-exchange column (HiTrap Q, Amersham Pharmacia Biotech). The eluted fractions containing PCNA (400–500 mM NaCl gradient) were pooled, concentrated to a final volume of 9 ml and put onto a Superdex-200 pg column (ϕ5.0 × 28 cm; Amersham Pharmacia) previously equilibrated with buffer B (50 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 30 mM NaCl, 1 mM dithiothreitol, 5% glycerol). The *Tk*-PCNA fractions were pooled, concentrated, and finally dialyzed against buffer C (50 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 50 mM KCl, 1 mM dithiothreitol, 0.1% Tween-20, 0.1% NP-40, 10% glycerol).

A sample containing full-length PCNA was fractionated by electrophoresis on a SDS-10% polyacrylamide gel, electroblotted onto a polyvinylidene difluoride membrane (Immobilon-PSQ; Millipore, Bedford, MA), stained with Coomassie brilliant blue R250 (0.02% in 40% methanol), and destained with 5% methanol. The protein bands were excised and put through to automated Edman degradation in a PSSQ21 protein sequencer (Shimadzu, Kyoto).

Primer extension analysis. The primer elongation abilities of KOD DNA polymerase in the absence or presence of *Tk*-PCNA were investigated by the same method as that used on those of *P. furiosus*.[10] The

600 fmol of linearized M13 single-stranded DNA (ssDNA) and circular M13 ssDNA annealed biotin-5′-end-labeled primer, were used in this method. The KOD DNA polymerase was prepared as described previously.[18] The reaction was started by adding 0.5 U (120 fmol) of the KOD DNA polymerase. Each assay mixture was 25 μl in volume, and the reaction was done at 70°C. Portions (7 μl) were taken at 0.5, 1, and 2 min after the reaction began and dispensed into 3 μl of stop solution (60 mM EDTA, 60 mM NaOH, 0.1% bromophenol blue), and 3 μl of each were analyzed on 1.0% alkaline agarose gel in 50 mM sodium hydroxide and 1 mM EDTA. After electrophoresis, DNA in the alkali agarose gel was transferred to the nylon filter and detected with a non-RI luminescent system kit (Toyobo).

PCR fidelity assay. The PCR fidelity was measured as the mutation frequency in PCR products with the full-length (4.0 kbp) of the plasmid pMOL21,[24] carrying the *bla* gene for ampicillin resistance and the *rpsL* gene for the streptomycin-sensitive phenotype, as a template. The plasmid pMOL21 was digested with *Sca*I at the site on the Amp^r gene, and PCR was done using two primers annealed at the ends of the digested plasmid, one of the two primers being biotnylated. The PCR products were collected by the streptavidin beads (Toyobo), which were attached at the end of one primer. After digestion by the *Mlu*I site in the primer region and self-ligation, the reacted products were used to transform the host cells.[24] Colonies formed on the LB plates containing ampicillin were counted as total colonies. The number of colonies formed on the LB plates containing both ampicillin and streptomycin were counted as mutated colonies, because the mutations in the *rpsL* gene on PCR would allow these cells to grow on the plates. The mutation frequency was calculated as the ratio of the mutated colonies to the total colonies (Table 1).

Results and Discussion

Gene cloning, expression, and purification of recombinant Tk-PCNA

The gene was subcloned into the vector pET11c and expressed in *E. coli* BL21 (DE3). About 6.0 mg of the *Tk*-PCNA protein was purified from a 1-liter culture of *E. coli* cells harboring the gene. The N-terminal amino acid sequence of the purified protein was confirmed to match that of the initiation region of the *Tk*-PCNA open reading frame (Fig. 1). The gene coded for a protein of 249 amino acids with an estimated molecular mass of 28.2 kDa. The G+C content of the gene was 55.2%, higher than that of *Pfu*-PCNA (40.1%) but close to that of the PCNA homologue from *T. fumicolans* (55.1%).[25] The amino acid sequence of *Tk*-PCNA is 84.3% and 91.2%, identical to those of *P. furiosus* and *T. fumicolans*, respectively. The *Tk*-PCNA, as well as *Pfu*-PCNA, had all the conserved region of the PCNA protein (Fig. 1). From crystal structure analysis of the *Pfu*-PCNA, its PCNA trimer is formed through intermolecular main chain amide-to-carbonyl hydrogen bonds between the anti-parallel β strands βI1 and βD2. There are only five hydrogen bonds, Thr108 O-Lys178 N, Thr110 N-Glu176 O, Thr110 O-Glu176 N, Arg112 N-Glu174 O, and Arg112 O-Glu174 N in them.[26] The *Tk*-PCNA trimer may be formed through four hydrogen bonds, Thr110 N-Glu176 O, Thr110 O-Glu176 N, Arg112 N-Glu174 O, and Arg112 O-Glu174 N, and an ion bond at the Lys108-Arg178 at the corresponding positions to *Pfu*-PCNA.

The molecular weight of *Pfu*-PCNA (28.2 kDa), anticipated from the amino acid sequence, agreed with that measured on SDS-PAGE.[10] Although the molecular mass of *Tk*-PCNA was calculated as 28.2 kDa from the amino acid sequence, it was detected as a protein of 37.6 kDa on SDS-PAGE (Fig. 2). This phenomenon could also be recognized in the PCNAs from other organisms. Although the calculated molecular mass of PCNA from eukaryotes (human, *Saccharomyces pombe*, and *Saccharomyces cerevisiae*) is about 29 kDa, these proteins migrate on SDS-PAGE as a 36-kDa protein. Similar observations have been reported with the PCNA isolated from archaea, *S. solfataricus*[11] and *Methanobacterium thermoautotrophicus* ΔH.[27] The *Tk*-PCNA amino acid sequence is nearly identical to the PCNA from *P. furiosus*, but they might have a large difference in the net charge of the proteins at pH 7.

Effects of Tk-PCNA on the processivity of KOD DNA polymerases

One of the biological functions of the *Tk*-PCNA was investigated through its effect on the primer elongation abilities of KOD DNA polymerase. When the synthesized products were made visible by denaturing gel electrophoresis with linear and circular ssDNA of M13 phage as substrates, longer products were observed in the presence of the *Tk*-PCNA (Fig. 3). As shown in Fig. 3(A) for the linear DNA, KOD DNA polymerase in the absence of the *Tk*-PCNA synthesized products up to the approximate size of 3.7 kb in 1 min. The reactions in which 3 times more *Tk*-PCNA was added than KOD DNA polymerase at the molar ratio, resulted in completely replicated products (7.4 kb) in 1 min. Furthermore, the yields of full-size products were increased by increasing the amount of *Tk*-PCNA (10 times at the molar ratio). On the circular DNA as shown in Fig. 3(B), the additive effect of *Tk*-PCNA was also found. From these results, we proposed the idea that the *Tk*-PCNA might work as the sliding clamp of KOD DNA polymerases like PCNA in Eucarya and the β-subunit in

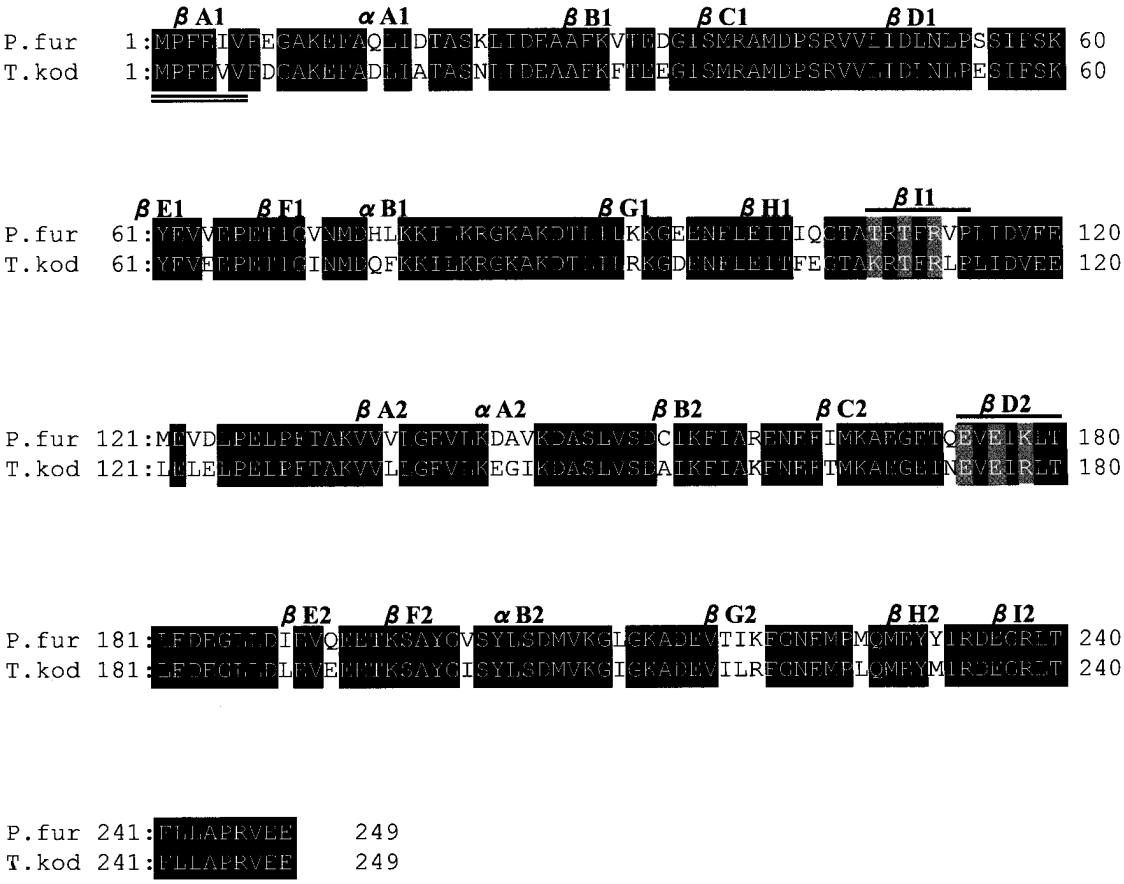

Fig. 1. Comparison of Amino Acid Sequences for PCNAs from Pfu and KOD.
The double underlining is the region checked by the amino acid sequencer. Black boxes indicate highly conserved regions and gray boxes indicate amino acids that seem to be concerned in intermolecular binding. Abbreviation: P.fur., PCNA from *Pyrococcus furiosus*; T.kod, PCNA from *Thermococcus kodakaraensis* KOD1.

bacteria.

The active form of *Tk*-PCNA seemed to be a trimer from results of the molecular weight estimation by gel chromatography. Interestingly, the *Tk*-PCNA promoted DNA elongation for linear and circular DNA as the template. Similarly, *Pfu*-PCNA promoted the DNA elongation of circular DNA by the DNA polymerase from *P. furiosus*, and the effects on circular DNA were greater than those on linear DNA. The PCNAs from archaea had fewer hydrogen bonds, which contributed to the intermolecular bonding, than the PCNAs from eukaryotes. So the archaeal PCNAs could work without the clamp-loader (RF-C), though the existence of the RF-C seemed to be essential to open the PCNA ring from the eukaryotes.

The *Tk*-PCNA worked well with the excess in comparison with KOD DNA polymerase at the molar ratio. Products of various lengths were observed with the DNA elongation, because all molecules of *Tk*-PCNA could not participate in the DNA elongation. The clamp loader from *T. kodakaraensis* would lead all molecules of *Tk*-PCNA on the initiation point of DNA synthesis and decrease the requirement of *Tk*-PCNA.

Effects of Tk-PCNA on PCR

In the thermostable α-like DNA polymerase used for PCR, KOD DNA polymerase has the highest level of DNA elongation rate, processivity, and PCR fidelity. The supplemental effect of the *Tk*-PCNA on PCR was investigated by the amplification of 3.6 kb of β-globin cluster from human genomic DNA (Fig. 4). In the absence of the *Tk*-PCNA, the faint amplification of target DNA in the smear could be shown on PCR used 3 ng of human genomic DNA as a template. In the presence of the *Tk*-PCNA, the amplification of target DNA became clear on PCR used the same quantity (3 ng) as a template. Also in 30 fmol of the *Tk*-PCNA, clear amplification of target DNA was observed on PCR used only 1 ng of human genomic DNA as a template. The PCR sensitivity was increased over 3 times by the *Tk*-PCNA addition.

In the experiment on single extension by DNA polymerase (Fig. 3), all DNA contained in the reaction system was used as the substrate (primer-template), which were in excess over the quantity of the

KOD DNA polymerase. Therefore, the amount of *Tk*-PCNA needed might have been more than the quantity of KOD DNA polymerases. On the other hand, in the experiment on PCR (Fig. 4), only a part of the DNA in the reaction system was used as the substrates (primer-template), which were scarce compared with the KOD DNA polymerase. There may be only several hundreds of copies as the substrates in the initial reaction stage of PCR cycle. Then less than 30 fmol of the *Tk*-PCNA would be enough on PCR. The requirement of *Tk*-PCNA might depend on the

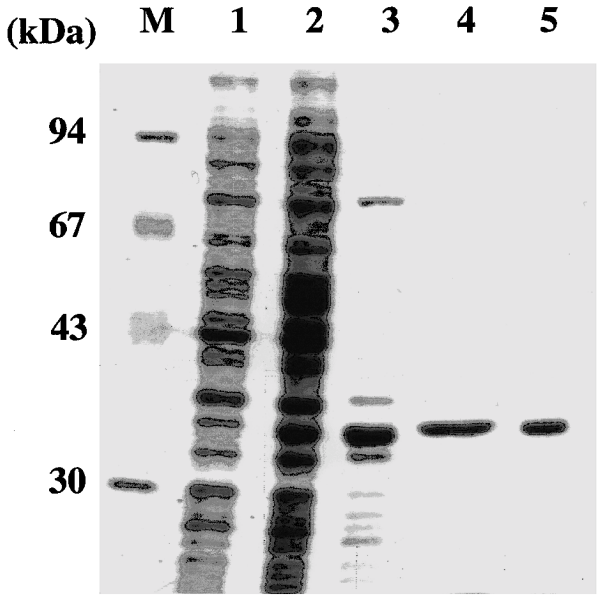

Fig. 2. SDS-PAGE of *Tk*-PCNA Protein during Purification.
Recombinant *Tk*-PCNA was purified, put onto an SDS-10% polyacrylamide gel, and stained with Coomassie brilllant blue. Lanes: M, molecular mass markers (97 kDa, phosphorylase *b*; 66 kDa, albumin; 45 kDa, ovalbumin; 30 kDa, carbonic anhydrase; 20 kDa, trypsin inhibitor). Lanes: 1, crude extract before induction; 2, crude extract after induction by IPTG; 3, supernatant after being heated program; 4, fraction from the HiTrap Q column; 5, fraction from the Superdex-200 pg column. The sizes of the molecular mass markers are indicated on the left.

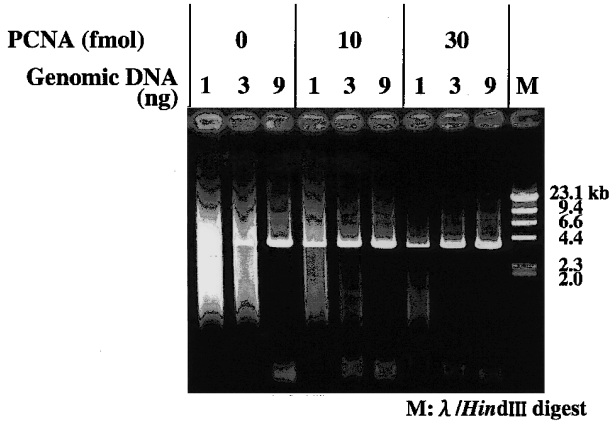

Fig. 4. Effects of Recombinant *Tk*-PCNA on PCR with KOD DNA Polymerase.
With the presence or absence of *Tk*-PCNA, PCR was done in 50-μl reaction mixtures containing human genomic DNA as the template, 1 unit of KOD DNA polymerase, 15 pmol of each primer, designed on the basis of part of the human globin gene (3.6 kbp) [forward, (5′-GGTGTTCCCTTGATGTAGCACA-3′), and reverse, (5′-ACATGTATTTGCATGGAAAACAA-CTC-3′)]. Reaction conditions were 94°C for 2 min and then 35 cycles of 94°C for 20 s, 60°C for 30 s, and 68°C for 4 min. Lane M, size markers (λDNA digested with *Hin*dIII).

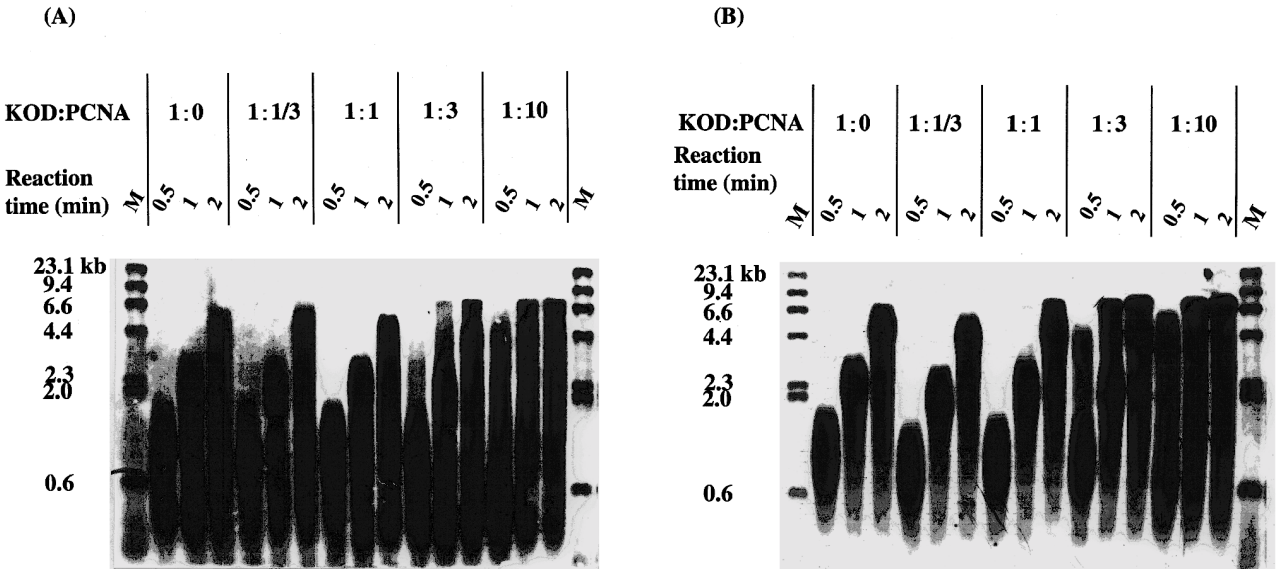

Fig. 3. Effects of Recombinant *Tk*-PCNA on Primer Extension by KOD DNA Polymerase.
The primer extension abilities of KOD DNA polymerase were compared with linear DNA (A) or circular DNA (B) as the template in the presence or absence of *Tk*-PCNA. Equal volumes of reaction mixtures were taken at 0.5, 1, and 2 min after the start of reaction. The products were separated by 1.0% alkaline agarose gel electrophoresis and made visible by autoradiography. The sizes indicated on the left (Lane M) are from *Hin*dIII-digested λDNA. 'KOD: PCNA' is the molar ratio of KOD DNA polymerase and *Tk*-PCNA.

Table 1. Comparison of PCR Fidelity

	Colonies		Mutant frequency (%)
	Total	Mutant	
KOD	46,424	46	0.099
KOD + *Tk*-PCNA*[a]	42,743	42	0.098
Pfu*[2]	34,961	54	0.15
Taq	45,175	2168	4.8

*[1] KOD DNA polymerase with 30 fmol of *Tk*-PCNA.
*[2] The DNA polymerase from *P. furiosus*.

quantity of DNA substrates rather than that of the KOD DNA polymerases.

In addition, the PCR fidelity of KOD DNA polymerase with *Tk*-PCNA was examined as described in Materials and Methods. The PCR fidelity of KOD DNA polymerase was about 1.5 times better than that of the DNA polymerase from *P. furiosus*. And no significant difference could be observed between the absence and the presence of *Tk*-PCNA (Table 1).

We confirmed that the *Tk*-PCNA improved the PCR sensitivity without adverse effect on PCR fidelity with the KOD DNA polymerase. Then we will try to perform long PCR, because the KOD DNA polymerase improved the processivity by the addition of *Tk*-PCNA. In the future, we will make a new DNA polymerase system that has higher PCR efficiency by adding another replication-related factor cloned from *T. kodakaraensis* KOD1.

Acknowledgments

We thank Dr. Masayuki Machida, Dr. Yousuke Takahama, Ms. Sonoko Ishino, and Dr. Yoshizumi Ishino for their valuable discussions. This work was supported by a grant from the New Energy and Industrial Technology Development Organization (NEDO).

References

1) Krishna, T. S. R., Kong, X. P., Gary, S., Burgers, P. M., and Kuriyan, J., Crystal structure of the eukaryotic DNA polymerase processivity factor PCNA. *Cell*, **79**, 1233–1243 (1994).
2) Mossi, R., and Hubscher, U., Clamping down on clamps and clamp loaders: The eukaryotic replication factor C. *Eur. J. Biochem.*, **254**, 209–216 (1998).
3) Hubscher, U., Maga, G., and Podust, V. N., DNA replication accessory proteins. In ''DNA Replication in Eukaryotic Cells'' Vol. 31, ed. DePamphilis, M. L., Cold Spring Harbor Laboratory Press, New York, pp. 525–543 (1996).
4) Bult, C. J., White, O., Olsen, G. J., Zhou, L., Fleischmann, R. D., Sutton, G. G., Blake, J. A., FitzGerald, L. M., Clayton, R. A., Gocayne, J. D., Kerlavage, A. R., Dougherty, B. A., Tomb, J. F., Adams, M. D., Reich, C. I., Overbeek, R., Kirkness, E. F., Weinstock, K. G., Merrick, J. M., Glodek, A., Scott, J. L., Geoghagen, N. S., and Venter, J. C., Complete genome sequence of the methanogenic Archaeon, *Methanococcus jannaschii*. *Science*, **273**, 1058–1073 (1996).
5) Klenk, H. P., Clayton, R. A., Tomb, J. F., White, O., Nelson, K. E., Ketchum, K. A., Dodson, R. J., Gwinn, M., Hickey, E. K., Peterson, J. D., Richardson, D. L., Kerlavage, A. R., Graham, D. E., Kyrpides, N. C., Fleischmann, R. D., Quackenbush, J., Lee, N. H., Sutton, G. G., Gill, S., Kirkness, E. F., Dougherty, B. A., McKenney, K., Adams, M. D., Loftus, B., Peterson, S., Reich, C. I., Macneil, L. K., Badger, J. H., Glodek, A., Zhou, L., Overbeek, R., Gocayne, J. D., Weidman, J. F., Mcdonald, L., Utterback, T., Cotton, M. D., Spriggs, T., Artiach, P., Kaine, B. P., Sykes, S. M., Sadow, P. W., D'andrea, K. P., Bowman, C., Fujii, C., Garland, S. A., Mason, T. M., Oisen, G. J., Fraser, C. M., Smith, H. O., Woese, C. R., and Venter, J. C., The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*. *Nature*, **390**, 364–370 (1997).
6) Kawarabayasi, Y., Sawada, M., Horikawa, H., Haikawa, Y., Hino, Y., Yamamoto, S., Sekine, M., Baba, S., Kogushi, H., Hosoyama, A., Nagai, Y., Sakai, M., Ogura, K., Otsuka, R., Nakazawa, H., Takamiya, M., Ohfuku, Y., Funahashi, T., Tanaka, T., Kudoh, Y., Yamazaki, J., Kushida, N., Oguchi, A., Aoki, K., and Kikuchi, H., Complete sequence and gene organization of the genome of a hyperthermophilic archaebacterium, *Pyrococcus horikoshii* OT3. *DNA Res.*, **5**, 147–155 (1998).
7) Sensen, C. W., Charlebois, R. L., Chow, C., Clausen, I. G., Curtis, B., Doolittle, W. F., Duguet, M., Erauso, G., Gaasterland, T., Garrett, R. A., Gordon, P., de Jong, I. H., Jeffries, A. C., Kozera, C., Medina, N., De Moors, A., van der Oost, J., Phan, H., Ragan, M. A., Schenk, M. E., She, Q., Singh, R. K., and Tolstrup, N., Completing the sequence of the *Sulfolobus solfataricus* P2 genome. *Extremophiles*, **2**, 305–312 (1998).
8) Smith, D. R., Doucette-Stamm, L. A., Deloughery, C., Lee, H., Dubois, J., Aldredge, T., Bashirzadeh, R., Blakely, D., Cook, R., Gilbert, K., Harrison, D., Hoang, L., Keagle, P., Lumm, W., Pothier, B., Qiu, D., Spadafora, R., Vicaire, R., Wang, Y., Wierzbowski, J., Gibson, R., Jiwani, N., Caruso, A., Bush, D., Safer, H., Patwell, D., Prabhakar, S., Mcdougall, S., Shimer, G., Goyal, A., Pietrokovski, S., Church, G. M., Daniels, C. J., Mao, J., Rice, P., Lling, J., and Reeve, J. N., Complete genome sequence of *Methanobacterium thermoautotrophicum* deltaH: Functional and comparative genomics. *J. Bacteriol.*, **179**, 7135–7155 (1997).
9) Forterre, P., Archaea: What can we learn from their sequences? *Curr. Opin. Genet. Dev.*, **7**, 764–770 (1997).
10) Cann, I. K. O., Ishino, S., Hayashi, I., Komori, K., Toh, H., Morikawa, K., and Ishino, Y., Functional interactions of a homologue of proliferating cell nuclear antigen with DNA polymerases in Archaea. *J. Bacteriol.*, **181**, 6591–6599 (1999).

11) De Felice, M., Sensen, C. W., Charlebois, R. L., Rossi, M., and Pisani, F. M., Two DNA polymerase sliding clamps from the thermophilic archaeon *Sulfolobus solfataricus*. *J. Mol. Biol.*, **291**, 47–57 (1999).
12) Fitz-Gibbon, S. T., Choi, A., Miller, J. H., Stetter, K. O., Simon, M. I., Swanson, R., and Kim, U. J., A fosmid-based genomic map and identification of 474 genes of the hyperthermophilic Archaeon *Pyrobaculum aerophylum*. *Extremophiles*, **1**, 36–51 (1997).
13) Ito, J., and Brainthwaite, D. K., Comparison and alignment of DNA polymerase sequences. *Nucleic Acid Res.*, **19**, 4045–4057 (1991).
14) Perler, F. B., Kumar, S., and Kong, H., Thermostable DNA polymerase. *Adv. Protein Chem.*, **48**, 377–435 (1996).
15) Lundberg, K. S., Shoemaker, D. D., Adams, M. W. W., Short, J. M., Sorge, J. A., and Mathur, E. J., High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*. *Gene*, **108**, 1–6 (1991).
16) Mattila, P., Korpela, J., Tenkanen, T., and Pitkanen, K., Fidelity of DNA synthesis by *Themococcus litoralis* DNA polymerase: an extremely heat-stable enzyme with proofreading activity. *Nucleic Acids Res.*, **19**, 4967–4973 (1991).
17) Barnes, W. M., PCR amplification of up to 35-kb DNA with high fidelity and high yield from λ bacteriophage templates. *Proc. Natl. Acad. Sci. USA*, **91**, 2216–2220 (1994).
18) Takagi, M., Nishioka, M., Kakihara, H., Kitabayashi, M., Inoue, H., Kawakami, B., Oka, M., and Imanaka, T., Characterization of DNA polymerase from *Pyrococcus* sp., strain KOD1 and its application to PCR. *Appl. Environ. Microbiol.*, **63**, 4504–4510 (1997).
19) Mizuguchi, H., Nakatsuji, M., Fujiwara, S., Takagi, M., and Imanaka, T., Characterization and application for hot start PCR of neutralizing monoclonal antibodies against KOD DNA polymerase. *J. Biochem.*, **126**, 762–768 (1999).
20) Hashimoto, H., Matsumoto, T., Nishioka, M., Yuasa, T., Takeuchi, S., Inoue, T., Fujiwara, S., Takagi, M., Imanaka, T., and Kai, Y., Crystallographic studies on family B DNA polymerase from hyperthermophilic archaeon *Pyrococcus kodakaraensis* strain KOD1. *J. Biochem.*, **125**, 983–986 (1999).
21) Nishioka, M., Mizuguchi, H., Fujiwara, S., Komatsubara, S., Kitabayashi, M., Uemura, H., Takagi, M., and Imanaka, T., Long and accurate PCR with a mixture of KOD DNA polymerase and its exonuclease deficient mutant enzyme. *J. Biotechnol.*, **88**, 141–149 (2001).
22) Hashimoto, H., Nishioka, M., Fujiwara, S., Takagi, M., Imanaka, T., Inoue, T., and Kai, Y., Crystal structure of DNA polymerase from hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1. *J. Mol. Biol.*, **306**, 469–477 (2001).
23) Imanaka, T., and Takagi, M., Evolution of PCR enzymes: Towards a better PCR system based on a KOD DNA polymerase. *J. Chin. Inst. Chem. Eng.*, **32**, 277–288 (2001).
24) Fujii, S., Akiyama, M., Aoki, K., Sugaya, Y., Higuchi, K., Hiraoka, M., Miki, Y., Saitoh, N., Yoshiyama, K., Ihara, K., Seki, M., Ohtsubo, E., and Maki, H., DNA replication errors produced by the replicative apparatus of *Escherichia coli*. *J. Mol. Biol.*, **289**, 835–850 (1999).
25) Henneke, G., Raffin, J., Ferrari, E., Jonsson, Z., Dietrich, J., and Hubscher, U., The PCNA from *Thermococcus fumicolans* functionally interacts with DNA polymerase δ. *Biochem. Biophy. Res. Commun.*, **276**, 600–606 (2000).
26) Matsumiya, S., Ishino, Y., and Morikawa, K., Crystal structure of an archaeal DNA sliding clamp: proliferating cell nuclear antigen from *Pyrococcus furiosus*. *Prot. Sci.*, **10**, 17–23 (2001).
27) Kelman, A., and Hurwitz, J., A unique organization of protein subunits of the DNA polymerase clamp loader in the Archaeon *Methanobacterium thermoautotrophicum* ΔH. *J. Biol. Chem.*, **275**, 7327–7336 (2000).