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

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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. 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.

Recent genomic sequencing of archaea 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. In addition, two PCNA homologues have been reported for the crenarchaeotes *Sulfolobus solfataricus* and *Pyrobaculum aerophilum*. 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. 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 archaean, *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. 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.

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. These advantages made it possible to carry out timesaving and high fidelity PCR. However the PCR sen-
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 molecular-biology-grade reagents from Nacalai (Uppsala, Sweden). All other chemicals were all obtained from Amersham-Pharmacia Biotech HiTrap Q, Superdex-200, and oligonucleotides were Co., Ltd. (Osaka, Japan). Molecular mass markers, and restriction enzymes were prepared from Toyobo and this plasmid was introduced into E. coli JM109 and BL21 (DE3). All other chemicals were molecular-biology-grade reagents from Nacalai (Kyoto, Japan).

Materials and Methods

Chemicals. M13 ssDNA, deoxyribonucleoside 5'-triphosphates, T4 DNA ligase, and the modification and restriction enzymes were prepared by 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 Toyobo Co., Ltd., Tokyo).

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'-GGGAATTCCATATGCGGTTCGCAGGTT-GTITT-3') and PCNA-r1 (5'-GCTCTAGATC-ACTCCTCAACGCGGAGCGG-3'), which have an EcoRI and a XbaI 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 x 10^4 multiplicity of infection).

The cloned fragments were sequenced in both orientations by the dye terminator 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 NdeI site was designed as follows: 5'-GGGAATTCCATATGCGGTTCGCAGGTT-GTITT-3'; the anti-sense primer containing XbaI sites was designed as follows: 5'-GCTCTAGATC-ACTCCTCAACGCGGAGCGG-3', and PCR was done with the positive phage clone found by plaque hybridization screening acting as a template. The amplified 750-bp fragment was digested with NdeI / XbaI and subcloned into the NdeI / XbaI 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), 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 x 28 cm; Amersham Pharmacia) previously equilibrated with buffer B (50 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM NaCl, 1 mM dithiothreitol, 5% glycerol). The Tk-PCNA fractions were pooled, concentrated, and finally dialyzed against buffer C (25 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. The...
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 ScaI at the site on the Ampr gene, and PCR was done using two primers annealed at the ends of the digested plasmid, one of the two primers being biotinylated. The PCR products were collected by the digested plasmid, one of the two primers being biotinylated. The PCR products were used to transform the host cells.24) The mutation frequency was calculated as the ratio of the number of colonies formed on the LB plates containing ampicillin and streptomycin were counted as total colonies. The number of colonies formed on the LB plates containing ampicillin and streptomycin were counted as mutated colonies. 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. fumicola_ (55.1%).25 The amino acid sequence of Tk-PCNA is 84.3% and 91.2%, identical to those of _P. furiosus_ and _T. fumicola_, 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 βI 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.27 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_28 and _Methanobacterium thermoautotrophicus_ _ΔH_.29 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 kodakarenensis KOD1.

**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 PCNA.

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. kodakarenensis would lead all molecules of Tk-PCNA on the initiation point of DNA synthesis and decrease the requirement of Tk-PCNA.
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 brilliant 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. 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 HindIII-digested λDNA. ‘KOD: PCNA’ is the molar ratio of KOD DNA polymerase and Tk-PCNA.

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’-GGTGTTCCCTTGGATGTAGCACA-3’), and reverse, (5’-ACATGTATTTGCATGGAAAACATCTC-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 (αDNA digested with HindIII).
merase improved the processivity by the addition of to perform long PCR, because the KOD DNA polymerase. Then we will try PCR sensitivity without adverse effect on PCR fidelity of the DNA polymerase from 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.

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References


