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Gene Cloning and Function Analysis of Replication Factor C from *Thermococcus kodakaraensis* KOD1

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Replication factor C (RFC) catalyzes the assembly of circular proliferating cell nuclear antigen (PCNA) clamps around primed DNA, enabling processive synthesis by DNA polymerase. The RFC-like genes, arranged in tandem in the *Thermococcus kodakaraensis* KOD1 genome, were cloned individually and co-expressed in *Escherichia coli* cells. *T. kodakaraensis* KOD1 RFC homologue (*Tk*-RFC) consists of the small subunit (*Tk*-RFCS: MW = 37.2 kDa) and the large subunit (*Tk*-RFCL: MW = 57.2 kDa). The DNA elongation rate of the family B DNA polymerase from *T. kodakaraensis* KOD1 (KOD DNA polymerase), which has the highest elongation rate in all thermostable DNA polymerases, was increased about 1.7 times, when *T. kodakaraensis* KOD1 PCNA (*Tk*-PCNA) and the *Tk*-RFC at the equal molar ratio of KOD DNA polymerase were reacted with primed DNA.

Key words: replication factor C; clamp loader; DNA polymerase; *Thermococcus kodakaraensis* KOD1; proliferating cell nuclear antigen

Replicative DNA polymerases synthesize DNA with high processivity in collaboration with two key accessory proteins: a circular clamp and a clamp loader complex.[1] These accessory proteins are conserved in structure and function among a variety of organisms including bacteriophage (e.g. T4 gp45 clamp and gp44/62 clamp loader),[2] bacteria (e.g. *Escherichia coli* β clamp and γ complex clamp loader),[3] archaea (e.g. *Pyrococcus furiosus* proliferating cell nuclear antigen (PCNA) clamp and replication factor C (RFC) clamp loader),[4–10] and eukaryotes such as *Saccharomyces cerevisiae*[11] and human[12,13] (PCNA and RFC). PCNA, a ring-shaped homotrimeric protein capable of encircling and sliding along duplex DNA, works as an elongation factor for DNA polymerases by tethering the polymerases to the DNA template. For the loading of PCNA onto DNA, a clamp loader consisting of four distinct small subunits and one large subunit is required. The clamp loader, commonly known as RFC, performs this function in an ATP-dependent manner by (i) recognizing the primer terminus, (ii) binding to and opening the donut-shaped PCNA, and (iii) linking the opened PCNA topologically to the DNA.

Except for the euryarchaeal heterodimeric DNA polymerase,[14] all archaeal DNA polymerases described to date are single subunit proteins with sequences similar to those of the family B (α-like) DNA polymerases, which include the chromosomal DNA replicases of *Eucarya*.[15] Most of the archaeal family B DNA polymerases have low processivity *in vitro*. Their processivities were improved by the addition of PCNA and RFC from the *Archaea*. For instance, the processivities of *Pyrococcus furiosus* DNA polymerase I (Pol BI) and DNA polymerase II (Pol D) are increased by the addition of *P. furiosus* PCNA (*Pfu*PCNA).[4] Furthermore, the *P. furiosus* RFC (*Pfu*RFC) stimulated *Pfu*PCNA-dependent DNA synthesis with both Pol BI and Pol D.[8]

We have reported a novel family B DNA polymerase from *T. kodakaraensis* KOD1 'KOD DNA polymerase' with the highest processivity (over 300b) and the highest elongation rate (about 140 bases/sec) among all archaeal DNA polymerases.[16] The processivity of KOD DNA polymerase was 10 times or

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more excellent than all archaeal family B DNA polymerases, and led to the highest PCR fidelity and PCR reliability among them. We reported in the previous research that the KOD DNA polymerase improved its processivity so that fewer templates could be used on PCR by addition of PCNA from *T. kodakaraensis* KOD1 (*Tk*-PCNA).[17] We aim to improve the PCR performance of KOD DNA polymerase.

Here, we describe the gene cloning and characterization of an RFC complex homologue (*Tk*-RFC) from the hyperthermophilic euryarchaeote *T. kodakaraensis* KOD1. The *Tk*-RFC was able to stimulate the primer-elongation abilities of KOD DNA polymerase. The accessory proteins may improve the PCR performance of KOD DNA polymerase.

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, and oligonucleotides were all obtained from Amersham Biosciences (Uppsala, Sweden). An Econo-Pac CHT-II colum was obtained from Bio-Rad Laboratories (Hercules, CA). A TSKgel G3000SW colum was obtained from TOSOH (Tokyo, Japan). 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 RFC gene (Tk-RFC) from T. kodakaraensis KOD1. We guessed that *Tk*-RFC was composed of two polypeptides of a small subunit (*Tk*-RFCS) and a large subunit (*Tk*-RFCL) from the results for *P. furiosus*. Then we did two DNA amplifications for cloning the *Tk*-RFCS and *Tk*-RFCL genes with 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- DNA polymerase (Toyobo) according to the manufacturer's instructions. The PCR primers were based on DNA sequences encoding RFC-S and RFC-L homologues of *P. furiosus* and *P. horikoshii*. Two primers of the RFC-S were designed as follows: RFCS-f1 (5′-GAGCTCAACGCSAGYGATGAGAG-3′) and RFCS-r1 (5′- TCYCTBGCCTCCATGAASTTACC-3′). And two primers of the RFC-L were designed as follows: RFCL-f1 (5′-CGGAATTCGAGCTCAACGCSAGYGATGAGAG-3′) and RFCL-r1 (5′-GCTCTAGACTSKAGCYTACTCATGTGCATCTC-3′), which have an *Eco*RI and a *Xba*I site (underlined), respectively. After it was verified by DNA sequencing that each amplification fragment contained the RFC-S and RFC-L homologue genes, their fragment were used as a probe for screening the KOD genomic λ10 library, constructed according to the manufacturer's protocol (Stratagene). Each positive clone was 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, an ABI Prism 3700 Genetic Analyzer (Applied Biosystems). Computer analysis of the DNA sequence data and the deduced amino acid sequence were done with GENETYX (Software Development Co., Ltd., Tokyo).

Construction of RFC expression plasmid. For expression of the RFC-S and RFC-L genes in *E. coli*, the sense primers containing an *Nde*I site were designed as follows: RFCS-f2 (5′-GGAATTCCATATGTCCGAGGAAGTGAAGGAAG-3′), and RFCL-f2 (5′-GGAATTCCATATGTACGGAAGTCCCATGGGTTG-3′), respectively. The anti-sense primers containing *Xba*I sites were designed as follows: RFCS-r2 (5′-GCTCTAGATCACTTACCCATAATCGTGAAC-3′), and RFCL-r2 (5′-GCTCTAGATCACTTCTTGAGGAAGTCGAACAG-3′), respectively. The amplified 2601-bp and 1500-bp fragments were digested with *Nde*I/*Xba*I and subcloned into the *Nde*I/*Nhe*I site of plasmid pET11c (Stratagene).

Similar to its homolog in *P. furiosus*, the gene encoding the putative *Tk*-RFCS contained an intervening sequence composed of a 1620-bp intein encoding 540 amino acid residues. Therefore, four primers were designed to fuse the two exteins *via* PCR to obtain the entire RFC-S gene (see Fig. 1). The primers used were RFCS-f2, RFCS-f3 (5′-CGTCGGGAAGACAACCGCTGCACTGGCTTTAG-3′), RFCS-r2, and RFCS-r3 (5′-CAGCGGTTGTCTTCCCGACGCCGGGTGGC-3′). The DNA encoding the N-terminal (59 amino acid residues) and C-terminal (268 amino acid residues) exteins were amplified by the combinations RFCS-f2–RFCS-r3 and RFCS-f3–RFCS-r2, respectively. To fuse the two exteins together, portions of each PCR product served as templates in a second PCR with RFCS-f2 and RFCS-r2 as the primers. The PCR product (981 nucleotides), which contained a *Nde*I and a *Xba*I site at each 5′ terminus, respectively, was cloned into pET11c. The integrity of the nucleotide sequence was confirmed as described above. The PCR-amplified *Tk*-RFCS gene fragment was digested with *Nde*I and *Xba*I, and was cloned into *Nde*I/*Nhe*I recognition site of the pET11c vector. This construct was desig-

nated p*TK*RFCS. The resultant RFCL-positive plasmid was designated p*TK*RFCL. And these plasmids were introduced into *E. coli* BL21(DE3).

A vector that co-expressed both subunits of *Tk*-RFCS and *Tk*-RFCL was generated as follows. PCR for the *Tk*-RFCS gene was done with RFCS-f2 and phosphorylated RFCS-r2 primers and p*TK*RFCS as a template. The amplified DNA fragment was cut with *Nde*I. Then, PCR for *Tk*-RFCL gene was done with phosphorated pET-f primer (5′-GCCAGGGTTTTCCCAGTCACGAC-3′) and RFCL-*Spe*I primer (5′-GACTAGTCACTTCTTGAGGAAGTCGAACAG-3′) and p*TK*RFCL as a template, and the amplified DNA fragment was cut with *Spe*I. This *Tk*-RFCL gene fragment contained a T7 promoter and the ribosome binding sites upstream. These two DNA fragments were ligated with pET11c vector, which was cut at the *Nde*I/*Nhe*I recognition site at the same time, and a co-expression vector was acquired. The cloned fragments were confirmed in both orientations by sequencing. We named this co-expressing vector p*TK*RFC.

Overproduction and purification of the Tk-RFC. *E. coli* BL21(DE3) (p*TK*RFC) was grown at 37°C in 1 liter of Luria-Bertani (LB) medium containing 0.1 mg/ml ampicillin to an optical density at 600 nm of 0.4. The cells in the culture were then induced by 1 mM of isopropyl-D-β-thiogalactopyranoside for 2 hr, and they were collected by centrifugation (15,000 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 Econo-Pac CHT-II column. The column was developed with a 50-ml linear gradient of potassium phosphate buffer from 25 to 300 mM in buffer B (25 mM potassium phosphate (pH 7.5), 50 mM KCl, 1 mM dithiothreitol, 10% glycerol). The eluted fractions containing *Tk*-RFC (100–170 mM potassium phosphate gradient) were pooled (12 ml) and put onto an anion-exchange column, a HiTrap Q column, previously equilibrated with buffer C (50 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, 10% glycerol). The column was developed with a 50-ml linear gradient of NaCl from 50 to 1000 mM in buffer C. The *Tk*-RFC fractions (420–540 mM NaCl gradient) were pooled and finally dialyzed against buffer C.

A sample containing *Tk*-RFC 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. Two protein bands guessed *Tk*-RFCS and *Tk*-RFCL were excised and put through to automated Edman degradation in a PSSQ21 protein sequencer (Shimadzu, Kyoto).

Analytical gel filtration. To estimate the molecular mass of the *Tk*-RFC complex, purified *Tk*-RFC fractions were put through gel-filtration analysis using an Amersham FPLC system. A TSKgel G3000SW colum was pre-equilibrated with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl. The flow-rate was 0.5 ml/minute. 4.0 mg/ml of *Tk*-RFC complex and 1.0 mg/ml of *Tk*-PCNA was analyzed respectively. Glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), and myokinase (32 kDa) were used as molecular mass markers.

Primer elongation analysis. The primer elongation abilities of KOD DNA polymerase in the absence or presence of *Tk*-PCNA and *Tk*-RFC were investigated by the same method as that used on *Tk*-PCNA alone.[17] The 1200 fmol of circular M13 single-stranded DNA (ssDNA) annealed biotin-5′-end-labeled primer, were used in this method. The KOD DNA polymerase and *Tk*-PCNA was prepared as described previously.[16,17] The reaction was started by adding 0.5U (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).

Results and Discussion

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

We identified the *Tk*-RFCS and the *Tk*-RFCL genes separately with plaque hybridization screening from the KOD genomic λ10 library. These two genes are arranged in tandem on the *T. kodakaraensis* KOD1 chromosome. They form a 4106-bp operon and are separated by a short (5 bp) intergenic sequence (Fig. 1A). *Tk*-RFCS and *Tk*-RFCL are 2601 bp and 1500 bp long, respectively. The *Tk*-RFCS gene is only 981 bp long after the excision of an intein. Putative transcriptional and translational signals have been identified (Fig. 1A).

The deduced amino acid sequences of the *Tk*-RFCS and *Tk*-RFCL were compared together with the human RFC subunit (Fig. 1B). Similarities were found in seven regions known as RFC boxes II–VIII (Fig. 1B). When the intein was excised, *Tk*-RFCS was

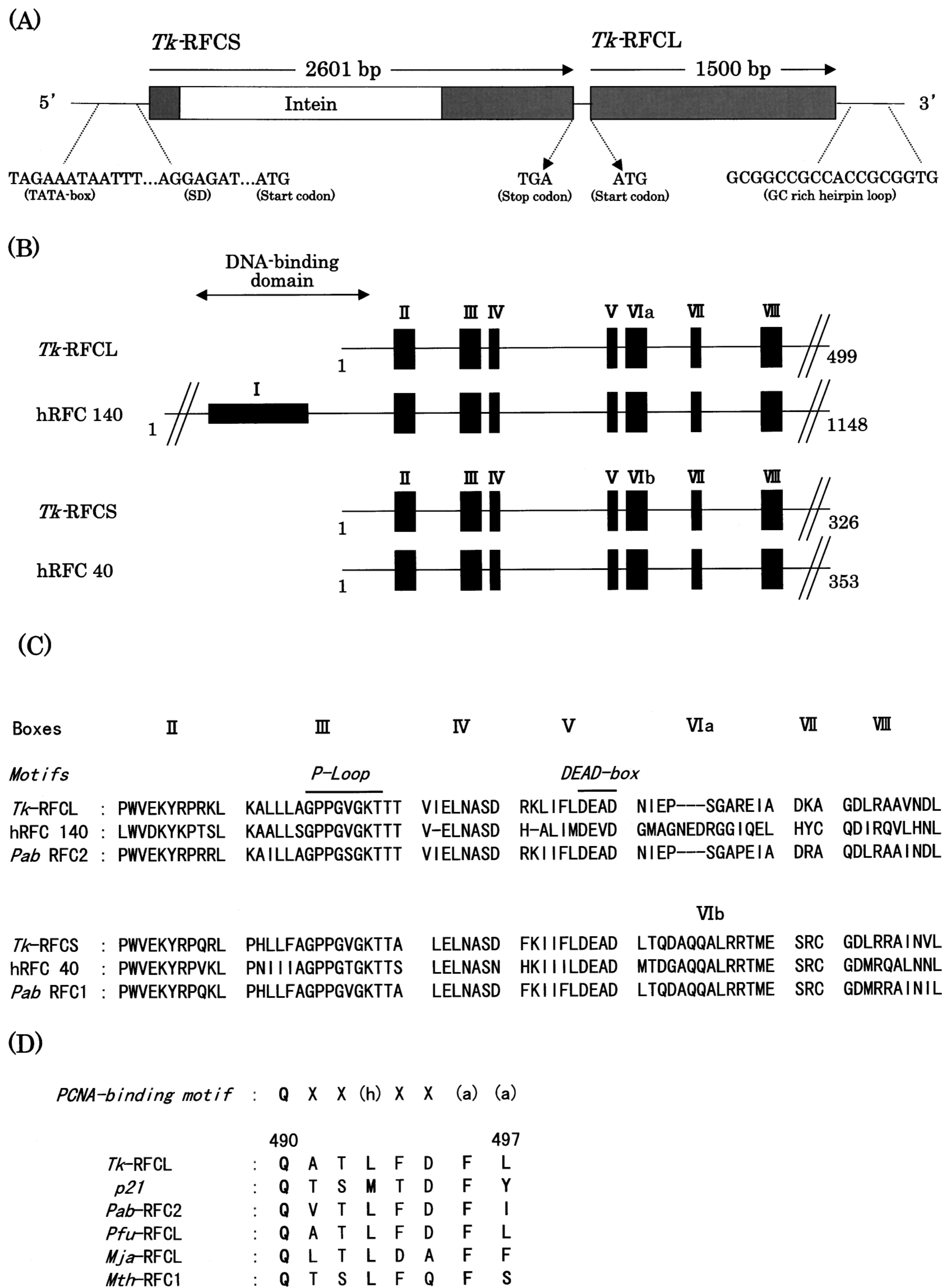

Fig. 1. Comparison of Archaeal and Eukaryotic RFC Subunits.

(A) Physical map of the RFC genes from *T. kodakaraensis*. *Tk*-RFCS, and *Tk*-RFCL are arranged in tandem, in an operon (4106 bp). The putative transcription and translation signals are shown at the bottom: from left to right, a putative eukaryotic-like TATA-box, a putative prokaryotic-like Shine-Dalgamo motif (SD), the *Tk*-RFCS gene start and stop codons, The *Tk*-RFCL gene start codon, and the putative eukaryotic-like transcription termination sequence (GC-rich heirpin roop). (B) Conserved RFC boxes among *Tk*-RFC and human RFC. The corresponding regions are numbered on top. The length (in amino acid residues) is shown on the right. The arrows map the DNA-binding domain (369–480) in the hRFC 140. (C) Amino acid sequence similarities within the RFC boxes. hRFC (from human) and PabRFC (from *P. abbysi*) were compared to the *Tk*-RFC. The P-loop and DEAD-box were indicated. (D) The conserved PCNA-binding motif of RFC large subunit from the archaeas. p21 is the cell cycle regulatory protein from human. *Pab*-RFC2 (from *Pyrococcus abbysi*), *Pfu*-RFCL (from *Pyrococcus furiosus*), *Mja*-RFCL (from *Methanococcus jannaschii*), and *Mth*-RFC1 (from *Methanobacterium thermoautotrophicum* ΔH) were compared to the *Tk*-RFCL. QXX(h)XX(a)(a) defined as follows. h, residues with moderately hydrophobic side chains, *e.g.*, leucine, isoleucine, or methionine (L, I, M); a, residues with highly hydrophobic, aromatic side chains, *e.g.*, phenylalanine and tyrosine (F, Y); X, any residue.

found to be 92.3% identical with the RFC small subunit from the *P. abyssi*. *Tk*-RFCL showed the conserved RFC boxes observed in the large human RFC p140 subunit, but all of the motifs are not present in the *Tk*-RFCL. An additional box (I), showing homology to prokaryotic DNA ligases and poly (ADP-ribose) polymerases,[18] is part of the N-terminal region of the human RFC p140, but is not in the *Tk*-RFCL (Fig. 1B). The motif I in the RFC-L is not admitted in all RFC-L from the archaea, although the DNA-binding domain is thought to exist in the motif I. However the PCNA-binding motif exist in the C terminus of all RFC-L (containing *Tk*-RFCL) from the archaea.[19] The identified boxes[20] shared by the five RFC polypeptides contain three well-defined motifs: two of these boxes (boxes III and V) contain putative ATP-binding sequences known as Walker A (P-loop) and B motifs (DEAD box), respectively (Fig. 1C). Also, the conserved p21-like PCNA-binding motif (QXX(h)XX(a)(a)) is present at the C-terminal part of the *Tk*-RFCL (Fig. 1D).[21] From structural studies of the p21 peptide co-crystallized with PCNA, the conserved glutamine (Q) is involved in a water-mediated contacts, while the hydrophobic residues methionine, phenylalanine, and tyrosine (M, F, Y) occupy a hydrophobic pocket on the surface of PCNA.

Expression and subunit composition of the Tk-RFC complex

Tk-RFCS and *Tk*-RFCL genes were cloned separately into a pET expression vector. The *Tk*-RFCS was expressed as a soluble product, but the *Tk*-RFCL remained insoluble (data not shown). To overcome this problem, we decided to co-express both *Tk*-RFCS and *Tk*-RFCL into *E. coli*. The expressed *Tk*-RFC complex consisting of *Tk*-RFCS and *Tk*-RFCL was found in the soluble extract. The molecular weight of *Tk*-RFCS (37 kDa) anticipated from the amino acid sequence agreed with that measured on SDS-PAGE of *Tk*-RFC (Fig. 2). But the molecular weight of *Tk*-RFCL (57 kDa) anticipated from the amino acid sequence didn't agree with that (71 kDa) measured on SDS-PAGE of *Tk*-RFC. Therefore their N-terminal sequencing confirmed the identity of the subunits; the N-terminal sequences were MSEEVK for *Tk*-RFCS, and MTEVPW for *Tk*-RFCL, as expected. About 9.0 mg of the *Tk*-RFC protein was purified from a 1-liter culture of *E. coli* cells harboring the gene.

The molecular weight of *Tk*-RFC was measured by gel filtration analysis to clarify the association ratio of the *Tk*-RFCS and *Tk*-RFCL. It was already reported that recombinant proteins from the hyperthermophile were mature by the heat effect.[22] The molecular mass of *Tk*-RFC was about 150 kDa, when *Tk*-RFC alone was analyzed after heating for 10 min at 70°C (Fig. 3(A)). The molecular mass of the *Tk*-

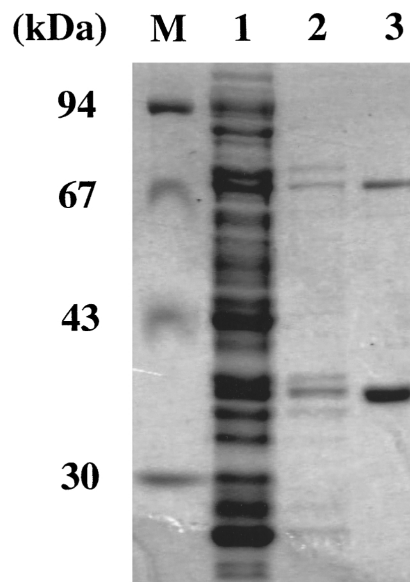

Fig. 2. SDS-PAGE of *Tk*-RFC Protein during Purification.
Co-expression of the *Tk*-RFC subunits in *E. coli* produced a stable complex. Purification of *Tk*-RFC subunits was done as outlined in Materials and Methods. Recombinant *Tk*-RFC was purified, put onto an SDS-10% polyacrylamide gel, and stained with Coomassie brilllant blue. Lanes: M, molecular mass markers (97 kDa, phosphorylase *b*; 66 kDa, albumin; 45 kDa, ovalbumin; 30 kDa, carbonic anhydrase). Lanes: 1, crude extract after induction by IPTG; 2, fraction concentrated the Econo-Pac CHT-II column; 3, fraction concentrated the HiTrap Q column. The sizes of the molecular mass markers are indicated on the left.

RFCS and *Tk*-RFCL peptides calculated from the amino acid sequences were 37.2 kDa and 57.2 kDa, respectively. The association ratio of *Tk*-RFCL and *Tk*-RFCS would be 1:2 or 1:3 from this measurement result. Then, when the molecular weight of *Tk*-RFC and the *Tk*-PCNA mixture was analyzed after heating for 10 min at 70°C, the peak position of molecular mass changed to about 230 kDa and 87 kDa (Fig. 3(B)). We could not confirm each composition because some were below the detection-limit, though we analyzed each peak fraction with SDS-PAGE. We proposed the idea that *Tk*-RFC bound with *Tk*-PCNA or the association ratio of *Tk*-RFC was changed to 1:4 by the existence of PCNA. It was suggested that the *Tk*-RFC was interactive with the *Tk*-PCNA without the DNA heteroduplex.

The function analysis of Tk-RFC

The function of *Tk*-RFC was examined as follows. We reported in the previous research that the elongation rate of KOD DNA polymerase was increased over 3 times by an excessive amount of *Tk*-PCNA against it. The elongation rate of KOD DNA polymerase was not increased but the amount of synthesized DNA was increased as well as the result on *Pfu*-RFC,[8] when the effect of *Tk*-RFC had been examined under an excessive amount of *Tk*-PCNA

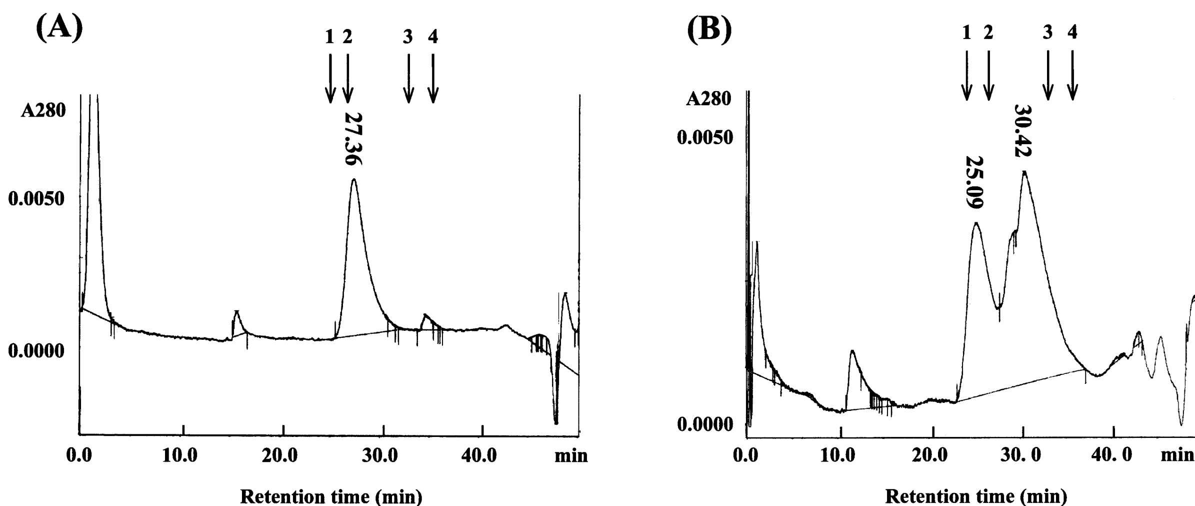

Fig. 3. Analytical Gel-permeation Chromatography of the Purified *Tk*-RFC Complex, and *Tk*-RFC and *Tk*-PCNA Mixture.

These samples were subjected to FPLC on a TSKgel G3000SW column as described in Materials and Methods. (A) *Tk*-RFC alone analyzed after heating for 10 min at 70°C. (B) After the mixture of *Tk*-RFC and the *Tk*-PCNA was heated for 10 min at 70°C, this mixture was analyzed. The molecular mass standards were: 1, Glutamate dehydrogenase (290 kDa); 2, Lactate dehydrogenase (142 kDa); 3, Enolase (67 kDa), 4, Myokinase (32 kDa).

(data not shown). Then the elongation ratio of the KOD DNA polymerase was examined under an equal molar ratio of the *Tk*-PCNA and the *Tk*-RFC. These experiments were done without rATP. It was reported that the RFC from *P. furiosus* was able to use dATP to the same extent as rATP.[8] Furthermore the RFC from *P. abyssi* did not require ATP hydrolysis for PCNA-loading *in vitro*.[10] So we experimented without addition of rATP. When only *Tk*-PCNA was added in the DNA synthesis, KOD DNA polymerase synthesized 2200 nucleotides and its DNA elongation rate was not improved in this case (Fig. 4). When both *Tk*-PCNA and *Tk*-RFC were added in the DNA synthesis, KOD DNA polymerase synthesized only 1800 nucleotides.

Next, after *Tk*-PCNA alone or both *Tk*-PCNA and the *Tk*-RFC were reacted beforehand with primed DNA as the substrate, the KOD DNA polymerase was added to the reaction mixture and the DNA synthesis reaction was started. When *Tk*-PCNA alone was added, KOD DNA polymerase synthesized only 2200 nucleotides in the same 2 minutes, but consolidated products were observed (Fig. 4). The length of DNA synthesis would be steadied with only the *Tk*-PCNA, because some of *Tk*-PCNA molecules were able to exist in the primed DNA part by reacting beforehand with DNA substrate. Further, when *Tk*-RFC was added with *Tk*-PCNA, KOD DNA polymerase could synthesize 3800 nucleotides in the same 2 minutes, and its DNA elongation rate was increased about 1.7 times (Fig. 4). The DNA elongation rate will be improved further by the existence of such as the DNA helicase and the replication protein A and so on.

It was reported in the previous research that the excess PCNA from archaea increased the DNA elongation rate, even if only PCNA was added to the DNA synthetic reaction. And in archaea, the effect of RFC was shown as the increase of synthesized DNA with an excess of PCNA. In this case, it was difficult to explain how RFC worked to load PCNA.

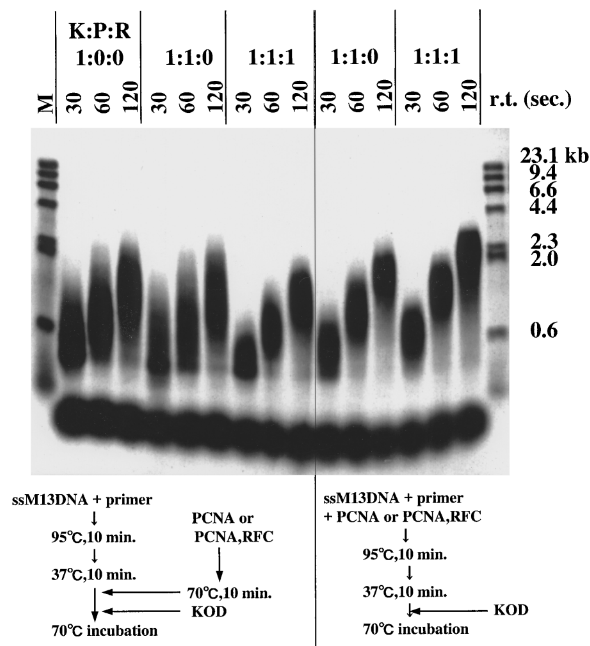

Fig. 4. Effects of *Tk*-RFC on Primer Extension by KOD DNA Polymerase.

The primer extension abilities of KOD DNA polymerase were measured with circular DNA as the template. Equal volumes of reaction mixture samples were taken at 30, 60, and 120 sec 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*d III-digested λDNA. 'K: P: R' is the molar ratio of KOD DNA polymerase, *Tk*-PCNA and *Tk*-RFC.

However, we experimented with an excess of primed DNA. In this case, KOD DNA polymerase could not turn over the DNA heteroduplex. So we could suggest the possibility that *Tk*-RFC led *Tk*-PCNA to the primed DNA part *in vitro*.

Tk-RFC does not have a DNA-binding domain. Even if *Tk*-PCNA and *Tk*-RFC were simply added to the reaction mixture, the extra assembly time would be required so that this complex could bind with primed DNA. And the addition of *Tk*-PCNA and *Tk*-RFC might not be consequently reflected by the DNA elongation rate. But most KOD DNA polymerase might bind and be available with the *Tk*-PCNA, when the *Tk*-RFC led most *Tk*-PCNA to the primed DNA part by giving assembly time beforehand. Consequently, the elongation rate of KOD DNA polymerase would be increased. The necessary assembly time for binding the PCNA-RFC complex and DNA heteroduplex would be decreased and the DNA elongation rate might be improved without pre-incubation, if the RFC from archaea had a DNA-binding domain.

We aim to improve the PCR performance of the KOD DNA polymerase that had the highest processivity and the highest elongation rate. Recently it was reported that a chimeric *Taq* DNA polymerase that had a PCNA-interaction domain increased the PCR performance by PCNA.[23] Also we reported that the *Tk*-PCNA improved the PCR sensitivity on the previous research.[17] And we are planning to search for the application to PCR, which can amplify the low copy molecule at higher elongation rate in the future.

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