The Archaeal DNA Primase

BIOCHEMICAL CHARACTERIZATION OF THE p41-p46 COMPLEX FROM PYROCOCCUS FURIOUSUS

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The abbreviations used are: pol α-pri, polymerase α-primase complex; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; RPA, replication protein A; ORF, open reading frame; ss, single-stranded; ds, double-stranded; AGE, agarose gel electrophoresis; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

We characterized the primase complex of the hyperthermophilic archaeon, Pyrococcus furiosus. The two proteins, Pfuap41 and Pfuap46, have similar sequences to the p48 and p58 subunits, respectively, of the eukaryotic DNA polymerase α-primase complex. Unlike previously reported primases, the Pfuap41 preferentially utilizes deoxyribonucleotides for its de novo synthesis, and moreover, it synthesizes up to several kilobases in length in a template-dependent manner (Bocquier, A., Liu, L., Cann, I., Komori, K., Kohda, D., and Ishino, Y. (2001) Curr. Biol. 11, 452–456). The p41-p46 complex showed higher DNA binding activity than the catalytic p41 subunit alone. In addition, the amount of DNA synthesized by the p41-p46 complex was much more abundant and shorter in length than that by Pfuap41 alone. The activity for RNA primer synthesis, which was not detected with Pfuap41, was observed from the reaction using the p41-p46 complex in vitro. The in vitro replication of M13 single-stranded DNA by the P. furiosus proteins was stimulated by ATP. Observation of the labeled primers by using [γ-32P]ATP in the substrates suggests ATP as the preferable initiating nucleotide for the p41-p46 complex. These results show that the primer synthesis activity of Pfuap41 is regulated by Pfuap46, and the p41-p46 complex may function as the primase in the DNA replication machinery of P. furiosus, in a similar fashion to the eukaryotic polymerase α-primase complex.

DNA replication is the fundamental process for the maintenance of life and also for the evolution of extant organisms, by which species transfer genetic information to their offspring. In both the bacterial and eukaryotic DNA replication processes, DNA polymerases are incapable of de novo DNA synthesis, and it is well known that DNA primase works for the de novo synthesis of the short RNA/DNA oligonucleotide on the template DNAs for both the leading and lagging strands. The short oligonucleotide, called the primer, is then extended by DNA polymerases to synthesize the long DNA strand (reviewed in Refs. 1 and 2). In

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The archaeal genome, in a similar fashion to the eukaryotic DNA replication machinery of P. furiosus, contains an ORF that has some sequence similarity to the eukaryotic DNA primase subunit p48. The corresponding genes have been cloned from Methanococcus jannaschii and P. furiosus.
lysate was prepared by sonication in 70 ml of buffer A (50 mM Tris-HCl, pH 8.0, 0.3 M NaCl, and 1 mM dithiothreitol) and dialyzed against buffer A containing 100 mM imidazole. The protein fractions were pooled, dialyzed against buffer A containing 100 mM NaCl, and subjected to cation-exchange chromatography (HiTrap SP, 1 ml, Amersham Pharmacia Biotech) on an AKTA system (Amersham Pharmacia Biotech). The column was extensively washed with buffer A containing 10 mM NaCl. The concentration of purified Pfup46 was determined by using a molar extinction coefficient \( \varepsilon_{43950} = 1 \text{ cm}^{-1} \). For the preparation of the p41-p46 complex, the purified Pfup46 was combined with an excess amount of purified Pfup41 in 50 mM Tris-HCl, pH 7.0, and was subjected to cation-exchange chromatography (HiTrap SP) as described above for the Pfup46 purification. The excess, free Pfup46 was found in the flowthrough fraction, and the p41-p46 complex was eluted at a salt concentration of 0.5–0.7 M NaCl.

**Experimental Procedures**

**Cloning and Sequencing of the Genes for Pfup41 and Pfup46**—The two genes were amplified directly from *P. furiosus* genomic DNA, prepared as described earlier (11), by polymerase chain reaction using pPFPR41 plasmid and production of the Pfup41 protein for *E. coli*, and characterized the gene product, named Pfup46. We demonstrated that the Pfup46 protein modulates the activities of Pfup41 for the primase-polymerase. From the biochemical properties of the p41-p46 complex, we propose an archaeal replication model, in which the p41-p46 complex works as the primase-polymerase, like the eukaryotic pol alpha-prime complex.

**Expression and Purification of Pfup41, Pfup46, and the p41-p46 Complex**—The Pfup41 and Pfup46 proteins were overproduced as follows: *E. coli* BL21-codonPlus (DE3)-RIL (Novagen) Cells harboring pPFPR41 or pPFPR46 were grown at 37 °C in 1 liter of LB medium in the presence of ampicillin and chloramphenicol. When the culture reached an absorbance (A600) of 0.4, the expression of the target genes was induced by adding isopropyl-β-D-thiogalactopyranoside to 1 mM. After cultivation for a further 5 h, the cells were harvested by centrifugation, with yields of 3.8 and 4.0 g (wet weight), respectively, for Pfup41 and Pfup46. The Pfup41 protein was purified from *E. coli* pPFPR41 cells. The cell lysate was prepared by sonication in 70 ml of buffer A (50 mM Tris-HCl, pH 8.0, 2 mM β-mercaptoethanol, 0.3 mM NaCl containing 1 mM phenylmethylsulfonyl fluoride. After centrifugation for 20 min at 30,000 × g, the supernatant was incubated at 80 °C for 15 min to remove most of the *E. coli* proteins, and then the supernatant was loaded onto a chelating column charged with Co2+ ions (5 ml, TALON™, CLON-TECH), connected on an FPLC apparatus (Amersham Pharmacia Biotech). The column was extensively washed with buffer A containing 10 mM imidazole, and the bound proteins were eluted with buffer A containing 100 mM imidazole. The protein fractions were pooled, dialyzed against buffer B (50 mM Tris-HCl, pH 7.0, 0.3 mM NaCl), and subjected to cation-exchange chromatography (HiTrap SP, 1 ml, Amersham Pharmacia Biotech) on an AKTA system (Amersham Pharmacia Biotech). The chromotography was developed with a 20-ml linear gradient of 0.3–0.8 M NaCl in 50 mM Tris-HCl, pH 7.0, at a flow rate of 1 ml/min. The active fractions, which eluted at a salt concentration of 0.5–0.7 M NaCl, were pooled, dialyzed against 10% (v/v) glycerol, 50 mM Tris-HCl, pH 8.0, 0.3 mM NaCl, and 1 mM β-mercaptoethanol, and stored at 4 °C. The concentration of purified Pfup41 was determined by using a molar extinction coefficient \( \varepsilon_{425} = 1 \text{ cm}^{-1} \). The concentration of purified Pfup46 was determined by using a molar extinction coefficient \( \varepsilon_{43950} = 1 \text{ cm}^{-1} \), which was calculated from the amino acid sequence with the ExPASy-ProParam Tool program. The Pfup46 was purified from *E. coli* pPFPR46 cells. The cell lysate was prepared and heat-treated as described above. The supernatant was prepared by sonication with polyethyleneimine (Sigma) and NaCl to 0.2% (w/v) and 0.3 M, respectively, and the mixture was stirred for 30 min on ice to precipitate the DNA. The proteins were then precipitated with ammonium sulfate (80% saturation). The precipitate was resuspended in and dialyzed against buffer A. The dialyzed sample was diluted with 50 μM Tris-HCl, pH 7.0, and 8 M urea to a final concentration of 0.1 M and was applied immediately to an anion exchange column (HiTrap Q, 5 ml, Amersham Pharmacia Biotech). The chromatography was developed with a 50-ml linear gradient of 0.1–1.0 M NaCl at a flow rate of 1.0 ml/min. The Pfup46 protein, which eluted at a salt concentration of 0.1 to 0.25 M, was collected and applied to a HiTrap Heparin column (Amersham Pharmacia Biotech). The chromatography was developed with a 20-ml linear 0.05–0.8 M NaCl gradient, and the Pfup46 protein was eluted at a salt concentration of 0.3–0.5 M NaCl. The concentration of Pfup46 protein was determined by using a molar extinction coefficient \( \varepsilon_{43950} = 1 \text{ cm}^{-1} \). For the preparation of the p41-p46 complex, the purified Pfup46 was combined with an excess amount of purified Pfup41 in 50 mM Tris-HCl, pH 7.0, and was subjected to cation-exchange chromatography (HiTrap SP) as described above for the Pfup46 purification. The excess, free Pfup46 was found in the flowthrough fraction, and the p41-p46 complex was eluted at a salt concentration of 0.5–0.7 M NaCl.

**Expression and Activity Assays**—The p41-p46 complex was assayed for its synthesis of single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), and RNA. ssDNA synthesis was assayed by using a synthetic ssDNA template-primer of 30 nucleotides long as the primer. The reaction mixture contained primase assay buffer, 100 μM each of dGTP, dCTP, and dTTP, 10 μM dATP containing [α-32P]dATP (Amersham Pharmacia Biotech), and was incubated with 0.5 μM each of dGTP, dCTP, and dTTP in the presence of 8M urea. To analyze the lengths of DNA fragments, the reaction mixtures were fractionated by 1% alkaline gel electrophoresis (AGE) in 50 mM sodium hydroxide and 1 mM EDTA, followed by autoradiography. A laser-excited image analyzer (LAS-5000; Fuji Film, Tokyo, Japan) was used to quantify the synthesized strands.

**In Vitro Replication of M13 Single-stranded DNA by *P. furiosus* Primases**—DNA synthesis reaction was carried out in an assay mixture (20 μl) containing primase assay buffer (50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 1 mM β-mercaptoethanol), 1 μM M13 mp18 single-stranded DNA (ssDNA), and 0.14 μM p41-p46 DNA polymerase containing [α-32P]dATP (Amersham Pharmacia Biotech), 0.5 μg of M13mp18 single-stranded DNA, and 0.14 μM p41-p46 DNA polymerase in addition to 2.5 units of *P. furiosus* pol I (pol B1) (14) and pol II (pol D) (16), 0.05 μM PfupPCNA (17) and PfupRFC (18), and 0.8 μM PfupRPA (19) at 70 °C for 20 min except for some concrete descriptions. The products were analyzed by 10% polyacrylamide gel electrophoresis (PAGE) in the presence of 8 M urea. To analyze the lengths of DNA fragments, the reaction mixtures were fractionated by 1% alkaline AGE, followed by autoradiography. To compare the product amounts in the presence of different NTP concentrations, the band of the full-length product from each reaction on the autoradiogram was quantified as described above. As a control, a synthetic DNA 30 nucleotides long was annealed to M13 DNA and was used as the template-primer for the DNA synthesis reaction by *P. furiosus* proteins except p41-p46 as described earlier (17, 18).

**Synthesis of RNA Primers and Their Elongation by DNA Polymerases from *P. furiosus*—RNA primer synthesis was carried out at 55 °C for 20 min in an assay (20 μl) containing primase assay buffer, 100 μM each of dATP, dCTP, and dTTP, 10 μM dGTP containing 10 μM dCTP (Amersham Pharmacia Biotech), 0.5 μg of M13 mp18 single-stranded DNA, and 0.14 μM p41-p46 DNA polymerase containing [α-32P]dATP (Amersham Pharmacia Biotech), 0.5 μg of M13mp18 single-stranded DNA, and 0.14 μM p41-p46 DNA polymerase in addition to 2.5 units of *P. furiosus* pol I (pol B1) (14) and pol II (pol D) (16), 0.05 μM PfupPCNA (17) and PfupRFC (18), and 0.8 μM PfupRPA (19) at 70 °C for 20 min except for some concrete descriptions. The products were analyzed by 10% polyacrylamide gel electrophoresis (PAGE) in the presence of 8 M urea. To analyze the lengths of DNA fragments, the reaction mixtures were fractionated by 1% alkaline AGE, followed by autoradiography. To compare the product amounts in the presence of different NTP concentrations, the band of the full-length product from each reaction on the autoradiogram was quantified as described above. As a control, a synthetic DNA 30 nucleotides long was annealed to M13 DNA and was used as the template-primer for the DNA synthesis reaction by *P. furiosus* proteins except p41-p46 as described earlier (17, 18).
dNTPs were added to the reaction for RNA primer synthesis. The reaction products were analyzed by PAGE on a 10% gel containing 8 M urea, followed by autoradiography. Each product signal was quantified as described above. The concentration ratios of the substrates (dNTPs/ NTPs) were no NTPs, 1/1000, 1/100, 1/10, 1/1, and no dNTP. In reverse, increasing concentrations (1, 10, and 100 μM) of ATP or NTPs were added to the reaction for DNA primer synthesis, containing 1 μM concentration of each dNTP ([32P]dCTP) at 55 °C 30 min. To see the effect of high concentrations of NTPs, dNTP concentration was lowered to 1 μM in this experiment.

Interaction of p41 with p46 in Vivo—Rabbit polyclonal antibodies were raised against homogenates of *Pfu* p41 and *Pfu* p46, respectively. The antibodies for pol I (pol BI) were as described previously (15). Immunoprecipitation experiments were done as follows at room temperature: 30 μl of protein A-Sepharose (Amersham Pharmacia Biotech) were dispensed into each of four Eppendorf tubes, and the resin was washed three times with PBS (10 mM sodium phosphate, pH 7.5, 150 mM NaCl). The protein A-Sepharose in each tube was then mixed with one of the above antisera (10 μl) and incubated for 1 h on a rotary shaker. Each mixture was washed twice with PBS and once with buffer D (50 mM Tris-HCl, pH 8.0, 10% glycerol, 0.1 mM EDTA, 0.5 mM dithiothreitol). The contents of each tube were mixed with 400 μl of *P. furiosus* cell extract (from 40 μg of cells) and were incubated for 30 min on a rotary shaker. Precipitates were washed three times with buffer D, and the immunoprecipitating products were eluted by boiling them in 30 μl of 1 × loading buffer (0.05 M Tris-HCl, pH 6.8, 10% glycerol, 1% 2-mercaptoethanol, 0.04% bromphenol blue, 1% SDS), followed by Western blot analysis. The blots were analyzed with the enhanced chemiluminescence system (Pierce), using peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch).

**RESULTS**

Identification of the p46 Protein as the Subunit of the *P. furiosus* Primase—In the *P. furiosus* genome, there is one ORF with an amino acid sequence similar to that of the p48 subunit of the eukaryotic pol α-primi complex. We characterized the protein, named *Pfu* p41, as a primase *in vitro* (22). One remarkable characteristic is that this enzyme has *de novo* activity to synthesize long DNA strands. To further characterize the function of the primase in *P. furiosus* cells, we sought to identify additional primase subunits corresponding to those of Eukarya. We found that an ORF overlapping that of *Pfu* p41 and *Pfu* p46, respectively. The antibodies for pol I (pol BI) were as described previously (15). Immunoprecipitation experiments were done as follows at room temperature: 30 μl of protein A-Sepharose (Amersham Pharmacia Biotech) were dispensed into each of four Eppendorf tubes, and the resin was washed three times with PBS (10 mM sodium phosphate, pH 7.5, 150 mM NaCl). The protein A-Sepharose in each tube was then mixed with one of the above antisera (10 μl) and incubated for 1 h on a rotary shaker. Each mixture was washed twice with PBS and once with buffer D (50 mM Tris-HCl, pH 8.0, 10% glycerol, 0.1 mM EDTA, 0.5 mM dithiothreitol). The contents of each tube were mixed with 400 μl of *P. furiosus* cell extract (from 40 μg of cells) and were incubated for 30 min on a rotary shaker. Precipitates were washed three times with buffer D, and the immunoprecipitating products were eluted by boiling them in 30 μl of 1 × loading buffer (0.05 M Tris-HCl, pH 6.8, 10% glycerol, 1% 2-mercaptoethanol, 0.04% bromphenol blue, 1% SDS), followed by Western blot analysis. The blots were analyzed with the enhanced chemiluminescence system (Pierce), using peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch).

**DNA Binding Ability of the Primase Complex**—We tried primase and polymerase reaction assays using the purified *Pfu* p46 protein, as measured for *Pfu* p41 in our previous study (22). However, neither activity was detected (data not shown). To investigate the role of *Pfu* p46 in the primase complex, we first measured the DNA binding activity of the proteins using a gel-mobility shift assay (Fig. 4). The *Pfu* p41 protein did not have a strong DNA binding activity (Fig. 4A, lane 2). In contrast, with the same amount of the *Pfu* p46 protein, two clear shifted bands were observed in the cases of single-stranded (ss) and primed (pri) DNAs, probably derived from the complexes of the DNA with one and two *Pfu* p46 molecules, respectively (Fig. 4A, lane 3). The double-stranded DNA (ds) was less appropriate for *Pfu* p46 binding, as expected. When the p41-p46 complex was used for the binding assay, one shifted band with a different mobility, showing the complex formation of (p41-p46)-DNA, was observed (Fig. 4A, lane 4). To measure the difference of the DNA binding abilities among the primase molecules more precisely, a titration analysis was carried out. However, as shown in Fig. 4B, *Pfu* p41 seems to aggregate at higher concentrations. This characteristic of *Pfu* p41 made it difficult to obtain the quantified data suitable for calculating the DNA binding affinity of the primase to DNA by forming a complex with the catalytic *Pfu* p41 subunit.

**De Novo Synthesis of DNA Strands by p41-p46**—We previously found that the *Pfu* p41 protein synthesizes long DNA segments (up to several kilobases) on the template DNA without a tag (22). Therefore, we tried this reaction using the p41-p46 complex and found that it also synthesizes DNA strands *de novo*. The amount of the synthesized products was drastically increased than that from *Pfu* p41 alone. Quantification of the signals of these products showed ten times difference. However, the sizes of the DNA strands were less than 700 bases, which are notably shorter than those from *Pfu* p41 (Fig. 5A). No increase of the product size was observed with longer reaction times (Fig. 5B). To investigate whether the synthesized primers can be extended by DNA polymerases from *P. furiosus*, pol I and pol II were added with their auxiliary proteins to the primer reaction mixtures containing M13 mp18 single-stranded DNA, dNTP (containing [α-32P]dCTP), and p41-p46 complex. As shown in Fig. 5C, in the presence of *Pfu*PCNA and *Pfu*RFC, pol I and pol II extended the primers. DNA (primer) synthesis activity of p41-p46 complex was not stimulated by *Pfu*PCNA and *Pfu*RFC. Furthermore, no product was observed when the p41-p46 complex was omitted from the reaction mixtures. These results indicate that the p41-p46 complex synthesized the primers, which can be extended in the combination with other replication proteins from *P. furiosus*.

**RNA Primer Synthesis by the p41-p46 Complex**—Primases from Bacteria and Eukarya synthesize ribonucleotides as
the primer. However, in our previous study little activity to synthesize the ribooligomer was detected in *Pfu*<sub>p41</sub> in vitro (22). To investigate whether the p41-p46 complex can synthesize an RNA primer, we performed the assay using M13 mp18 single-stranded DNA and an NTP mixture containing [α-<sup>32</sup>P]UTP and found that the p41-p46 complex synthesized 12–40-base-long RNA primers. The RNA primers were extended by pol I, pol II, and also by the p41-p46 complex itself, when the dNTP mixture was added after the synthesis reaction of the RNA primers (data not shown).

In an attempt to determine which type of primer (DNA or RNA) is synthesized *de novo* in the *P. furiosus* cells, we tried the primer synthesis assay in the presence of both dNTPs and NTPs. The primase complex synthesized RNA segments only when NTPs containing [α-<sup>32</sup>P]UTP, but not dNTPs, were added as the substrates, as described above. However, we detected a dose-dependent decrease in labeled RNA products with increasing dNTP concentrations of the reactions. When the product amount from the reaction with only NTPs is 100%, the products were decreased to 57%, 41%, and less than 15% in the presence of dNTPs at 1/1000, 1/100, and 1/10, respectively, relative to the amount of NTPs. When both dNTPs and NTPs existed at equal concentrations, no RNA products were observed from the reaction containing [α-<sup>32</sup>P]UTP (data not shown). This result indicates that the p41-p46 primase preferentially uses dNTPs. However, in the opposite case of detecting a DNA primer using

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**Fig. 1.** A, five regions with similarities in the archaeal ORFs and the eukaryotic DNA primase p58 subunit. Identical and similar amino acid residues are indicated in black and gray, respectively. The ORF names from each data base were shown. The organisms are: *MTH*, *M. thermoautotrophicus*; *MJ*, *M. jannaschii*; *Ta*, *Thermoplasma acidophilum*; *AF*, *A. fulgidus*; *VNG*, *Halobacterium* sp. NRC-1; ORF-, *S. solfataricus*; the yeast (*Sce*) and human p58 proteins were aligned at the bottom. B, gene organization of the primase complex on the *P. furiosus* genome. The genes encoding the amino acid sequences similar to those of the p48 and p58 subunits, respectively, of the eukaryotic primase complex were found in tandem with overlapping of their ORF on the *P. furiosus* genome. The coding regions of p41 and p46 overlap with each other, as shown on the bottom.
[\alpha^{32}P]dCTP, the DNA strand synthesis was stimulated by the addition of NTPs. By addition of NTPs to 1, 10, and 100 \muM, the amounts of the reaction products were increased by 1.7-, 4.0-, and 5.3-fold, respectively, relative to the product from the reaction without NTPs. This stimulation of DNA strand synthesis was also observed with ATP to the same level as NTPs (data not shown). Furthermore, the in vitro replication assay of the M13 mp18 single-stranded DNA using \textit{P. furiosus} replication proteins, p41-p46, pol I, \textit{Pfu}PCNA, and \textit{Pfu}RFC, as described above, revealed that the amount of full-length product was increased by the addition of ATP, but not by the other three nucleotides (Figs. 6, A and B). When a synthetic DNA primer was annealed to M13 DNA template instead of de novo primer synthesis by p41-p46 in the reaction, no enhancement of production was observed by either ATP or NTPs (Fig. 6C). These results suggest that the primer synthesis possibly starts by reaction with the primase in the initiation step of DNA replication in \textit{P. furiosus}, as found in other organisms of the different biological domains. To investigate the initiation step of the primer synthesis more directly, the reactions using [\gamma^{32}P]ATP and cold dNTPs were performed. As shown in Fig. 7, synthesized strands of 700 bases in length were detected by autoradiography. The amount of the labeled products was clearly decreased with increased additions of cold ATP. The labeled products were slightly decreased by addition of cold GTP, and no effect was observed by cold CTP and UTP. The amount of the ATP-labeled products was not decreased by addition of increasing amount of cold dATP in the reaction mixtures (data not shown). These results suggest that the p41-p46 primase can discriminate ATP from other NTPs to start primer synthesis.

**DISCUSSION**

We identified the DNA primase complex in the hyperthermophilic archaean \textit{P. furiosus}. \textit{Pfu}p41 and \textit{Pfu}p46, which have some sequence similarity to the eukaryotic primase subunits, exist as a stable complex and may work as the primase in the replication machinery of \textit{P. furiosus}. The eukaryotic primases exist as a complex with pol \textalpha. By analogy with this case, we investigated whether the \textit{P. furiosus} p41-p46 complex directly interacts with \textit{P. furiosus} pol I (pol B1), because pol I is the only DNA polymerase that belongs to the family B DNA polymerases, including eukaryotic pol \textalpha, in \textit{P. furiosus} (8, 11). No coprecipitation of \textit{Pfu}p41 and pol I was observed with either anti-\textit{Pfu}p41 or anti-pol I (Fig. 3). The results suggest that pol I is not the functional counterpart of the eukaryotic pol \textalpha in \textit{P. furiosus}. In the eukaryotic replication machinery, it is believed that the de novo synthesis of RNA primer is performed by p48, and then the primers are extended by pol \textalpha by translocation of the active sites from p48 to p180 in the pol \textalpha-pri complex. Based on the evidence that the \textit{P. furiosus} p41-p46 complex can syn-
thesize short RNA segments and long DNA segments by itself in vitro, the p41-p46 complex may have dual functions in Archaea: de novo primer synthesis and its elongation. The synthesized DNA strands are then presumably further extended by the replicative DNA polymerases, pol I and/or pol II. The interactions of the PfuRPA with other replication proteins (pol I, pol II, PfuPCNA, and PfuRFC), including the primase, as we showed previously by the immunoprecipitation experiments (19, 22), support the idea that the p41-p46 complex is involved in the replisome in P. furiosus cells. In eukaryotes, both pol I/H9254 and pol II/H9280 have proofreading activity and express high processivity when combined with PCNA. Our preliminary results showed that neither Pfu p41 nor the p41-p46 complex has 3′→5′ exonuclease activity, which suggests that the archaeal primase has very low fidelity.2 It would be detrimental to the cells that an enzyme without proofreading activity synthesizes long DNA strands by itself. The Pfu p41 subunit synthesizes long DNA

2 S. Ishino and Y. Ishino, unpublished results.

Fig. 5. A, de novo DNA synthesis activity of Pfu p41 and the p41-p46 complex. The reaction mixture, containing M13 single-stranded circular DNA, dNTP including [γ-32P]dATP, and Pfu p41 or the p41-p46 complex, was incubated at 70 °C for 20 min, and the reaction products were analyzed by 1% alkaline AGE followed by autoradiography. Lane M, the size marker was prepared by 5′ labeling by 32P of the BstPI-digested lambda DNA. B, a time course experiment of the primer synthesis on M13 single-stranded DNA by the p41-p46 complex in vitro. The reaction mixture indicated in A was incubated at 70 °C, and aliquots were taken after 3, 10, 30, 45, and 60 min, respectively, and the reaction products were analyzed by 1% alkaline AGE followed by autoradiography. C, DNA synthesis reactions on M13 single-stranded DNA were carried out using p41-p46, pol I, and pol II at 70 °C for 20 min. PfuPCNA and PfuRFC were added to stimulate the reactions of pol I and pol II as described previously (18).

Fig. 6. ATP stimulates the in vitro replication of M13 single-stranded DNA by P. furiosus proteins. The full-length product (7.2 kilobases) of the reactions containing different concentrations of NTPs were separated by 1% alkaline AGE (A), and the protein bands were quantified from the autoradiogram (B). The reaction product did not increase by addition of NTP when a synthetic DNA oligomer was annealed to M13 single-stranded DNA as a primer (C).

Fig. 7. Synthesis of the 5′-terminal labeled primers by [γ-32P]ATP was clearly inhibited by addition of cold ATP, but not other NTPs. The reaction mixture containing M13 mp18 ssDNA, p41-p46 complex, [γ-32P]ATP, and dNTPs was incubated at 70 °C for 45 min with increasing amount of NTPs (0.5, 5, and 50 μM, respectively). The reaction products were analyzed by 8% PAGE containing 8 M urea followed by autoradiography. Lane − indicates the reaction without cold NTPs.
strands up to several kilobases in vitro, and therefore, Pfup46 may have an essential function to suppress this long DNA synthesis. In the eukaryotic system, after the primase synthesizes a primer, pol α and primase become coordinated such that further primer synthesis is negatively regulated by the formation of the stable primer-template formation, which is likely to be associated with pol α-primase (25, 26). Moreover, it has been suggested that the eukaryotic p58 subunit mediates the transfer of primers from the primase active site to pol α (26). The results of our study imply that the archaeal p46 subunit has similar functions as those of the eukaryotic p58, although the archaeal primase may extend the strand by using dNTPs from the RNA primer by itself without switching to any other polymerase, as described above.

It would be very interesting and important to determine which primer (RNA or DNA) is synthesized de novo in the DNA replication process in P. furiosus cells. Our in vitro studies could not reach this conclusion. It is known that the eukaryotic primase forms a primase-ssDNA-NTP-NTP quaternary complex to start the RNA primer on the template, and purine nucleotides are more preferable than pyrimidines to form this initiation complex. This initiation of primer synthesis is the rate-determining step (27). In the case of the human primase, the p48-p58 complex, but not p48 alone, can initiate the dinucleotide synthesis using ribonucleotide triphosphates (24). Our data showing that the p41-p46 complex, but not p41 alone, can synthesize RNA primer are consistent with the idea that the role of the second subunit is to assist in primer initiation. Furthermore, the fact that ATP stimulated the strand synthesis of the p41-p46 primase (Figs. 6 and 7) implies that the archaeal primer starts with ribonucleotide(s) in the cells. Isolation and characterization of the Okazaki fragments from the archaeal cells are necessary for further clarification.

The sequence search for the primases in the total genomes of several archaeal organisms has yielded some interesting aspects. All of the euryarchaeal organisms, including M. jannaschii, Methanothermobacter thermautotrophicus (Methanothermobacter thermautotrophicus), Archaeoglobus fulgidus, and Halobacterium sp. NRC-1, as well as Pyrococcus, have open reading frames homologous to Pfup41 and Pfup46. The two ORFs are located in tandem in the genomes of the Pyrococcus and the M. thermautotrophicus and probably form an operon. It is very interesting that M. thermautotrophicus, A. fulgidus, and Halobacterium sp. NRC-1 have two p41-like ORFs in their genomes. In the crenarchaeal organisms, one ORF similar to p41 was found in the total genome sequences of Aeropyrum pernix, Sulfolobus solfataricus, and Sulfolobus tokodaii, respectively. On the other hand, a p46-like ORF is found in Sulfolobus, but not in A. pernix from our analyses. The p46 subunit of A. pernix DNA primase may be highly divergent from other archaeal primases. The sequence analysis of the eukaryotic type DNA primases are also shown on a website (chem-mgrip2.unl.edu/repli/EukPri1AA.html). The COG (clusters of orthologous groups of proteins) data base (www.ncbi.nlm.nih.gov/COG/) includes one ORF from A. pernix in the multiple alignment of the eukaryotic p58-like proteins. Further analyses are necessary to understand the conservation and the diversity of the archaeal primases.

The isolation of the novel Pfup46 protein and demonstration of archaeal primase function presented in this study significantly contributes to understanding of the DNA priming process in archaeal DNA replication. Furthermore, due to the similar structure and function of the primase proteins, detailed studies on the archaeal primase function will give further insights into the molecular mechanisms of more complicated eukaryotic DNA replication.

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