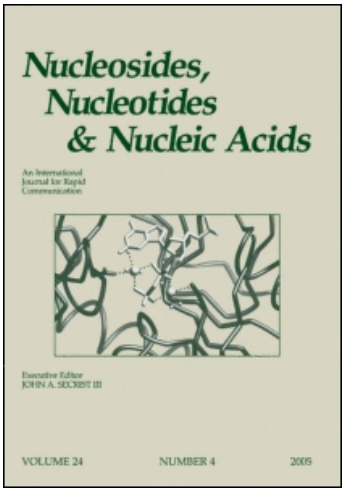


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# Comprehensive Search for DNA Polymerase in the Hyperthermophilic Archaeon, *Pyrococcus furiosus*

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# COMPREHENSIVE SEARCH FOR DNA POLYMERASE IN THE HYPERTHERMOPHILIC ARCHAEON, *Pyrococcus furiosus*

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□ *DNA polymerase activities were scanned in a* Pyrococcus furiosus *cell extract to identify all of the DNA polymerases in this organism. Three main fractions containing DNA polymerizing activity were subjected to Western blot analyses, which revealed that the main activities in each fraction were derived from three previously identified DNA polymerases. PCNA (proliferating cell nuclear antigen), the sliding clamp of DNA polymerases, did not bind tightly to any of the three DNA polymerases. A primer usage preference was also shown for each purified DNA polymerase. Considering their biochemical properties, the roles of the three DNA polymerases during DNA replication in the cells are discussed.*

**Keywords** DNA polymerase; DNA replication; DNA repair; Archaea

## INTRODUCTION

DNA replication is a fundamental phenomenon to maintain genetic information in living cells, and it is essential to elucidate the precise molecular mechanism of the process to understand the pathology of genetic diseases including cancer. Many protein factors involved in DNA replication have already been identified, and their functions have been deduced. Results from extensive research efforts have suggested that the principle of the DNA replication mechanism seems to be the same in the three biological domains, Bacteria, Eukarya, and Archaea,[1–4] although the protein factors

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This article is dedicated to Professor Eiko Ohtsuka on the occasion of her 70th birthday.

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with conserved functions are predicted to have evolved from different origins from the distinct diversity of the amino acid sequences.[5] In terms of the DNA polymerases in the cells, plural enzymes have been identified in each living organism, and the elucidation of the concrete functions of these DNA polymerases is essential to understand the molecular mechanisms of DNA replication and repair. DNA polymerases can be classified by the amino acid sequence similarity, and seven classes, the A, B, C, D, E, X, and Y families, are now widely recognized.[1,6,7]

Archaea, the third domain of life, inhabit places with extreme conditions, such as high temperature, high salt, and anaerobic environments.[8,9] The organisms in Archaea are now recognized as very interesting targets for life science research, from the aspects of molecular mechanism and evolution. Euryarchaeota and Crenarchaeota are the two major subdomains in Archaea.[9] We identified two family B DNA polymerases in the hyperthermophilic crenarchaeon, *Pyrodictium occlutum,* as the first example of a prokaryotic organism with plural family B DNA polymerases.[10] Furthermore, we identified two family B DNA polymerases in *Aeropyrum pernix,* another crenarchaeal organism.[11] These were interesting because all three replicative DNA polymerases, Pol$\alpha$, Pol$\delta$, and Pol$\varepsilon$, belong to family B. The total genome sequences of many archaeal organisms have revealed that two (or may be three) family B DNA polymerases exist in the crenarchaeal organisms. On the other hand, only one family B DNA polymerase exists in the genomes of euryarchaeal organisms.[12] We cloned the gene for a family B DNA polymerase from an euryarchaeon, *Pyrococcus furiosus*[13] and named it PolI.[14] Then, we identified the second DNA polymerase (PolII) from *P. furiosus.*[15,16] Because no sequence conservation between the PolII and any other DNA polymerases, we proposed Family D as a new DNA polymerase family.[1] Family D DNA polymerase has only been identified in the euryarchaeal organisms so far.[17,18] The two DNA polymerases of *P. furiosus* are now called PolBI and PolD, respectively.[19] *P. furiosus* Pol D has efficient strand extension activity and strong proof-reading activity.[16,19] Other family D DNA polymerases were also characterized by various groups.[20–24] After the identification of PolB and PolD in *P. furiosus*, we found a gene encoding a sequence homologous to that of the catalytic subunit of the eukaryotic primase, p48 protein, and characterized the gene product designated p41. Unexpectedly, the p41 protein did not catalyze the synthesis of short RNA by itself, but preferentially utilized deoxynucleotides to synthesize DNA strands up to several kilobases in length.[25] Then, we found that the gene next to the p41 gene encodes a protein with very weak similarity to the p58 subunit of the eukaryotic primase. The gene product, named p46, actually forms a stable complex with p41, and the complex can synthesize a short RNA primer in vitro, as well as DNA strands with several hundred nucleotide lengths *in vitro.*[26] An investigation of the primer in *Pyrococcus* cells showed that it is a short RNA.[27] Taken together, these results suggest that DNA replication

in *Pyrococcus* cells starts with short RNA primers produced by the primase complex, p41-p46. To advance DNA replication research in *Pyrococcus*, we searched for DNA polymerase activities in *P. furiosus* cell extracts. Anion-exchange chromatography fraction was carefully investigated.

## MATERIALS AND METHODS

### Strain and Culture Condition

*P. furiosus* Vc1 strain was grown at 95°C in broth without shaking, as described earlier.[13] The *P. furiosus* cells were harvested after 16 h of growth at an optical density of 0.8 at 600 nm. Seven hundred mg of the cells were suspended with 10 ml of buffer A (50 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 0.5 mM DTT, 10% glycerol) including Complete (Roche Diagnostics GmbH, Tokyo, Japan), and were disrupted by sonication. The supernatant was applied to an anion-exchange column (HiTrap Q HP, 5 ml; GE Healthcare Bio-Science, Piscataway, WI, USA), and the chromatography was developed with 0 to 1.0 M NaCl-gradient in 50 ml of buffer A.



### DNA Polymerase Assay

The nucleotide incorporation assay was performed to detect the DNA-polymerizing activity in the chromatographic fractions. The reaction mixtures contained, in 40 $\mu$l, 20 mM Tris-HCl, pH 8.8, 5 mM $MgCl_2$, 14 mM $\beta$-mercaptoethanol, 8 $\mu$g of calf thymus activated DNA, 0.2 mM deoxynucleoside triphosphate (dNTP) containing 400 nM [methyl-$^3$H]TTP, and 2 $\mu$l of each fraction, were incubated at 72°C for 15 min. The acid-insoluble radioactivity bound to DE 81 filter was detected by a scintillation counter. To detect the strand extension activity, $^{32}$P-labeled oligodeoxyribonucleotide (dAGCTATGACCATGATTA) was annealed to the 49-mer oligodeoxyribonucleotide (dAGCTACCATGCCTGCACGAATTAAGCAATTCGTAATCATGGTCATAGCT) and used as a primer-template. The reaction mixture contained, in 20 $\mu$l, buffer B (50 mM Tris-HCl, pH 8.8, 5 mM $MgCl_2$, 2 mM $\beta$-mercaptoethanol), 0.1 mM primer-template DNA, and 0.25 mM dNTP, and the reaction was carried out by adding 5 micro liter of each fraction at 72°C for 8 min. The reaction products were analyzed by 8% PAGE containing 8 M urea followed by autoradiography.

### Western Blot Analysis

To obtain the elution profile of the three known DNA polymerases in the anion exchange chromatography, each fraction was subjected to Western blotting using anti-PolBI, anti-PolD (DP1+DP2), anti-primase (p46), and anti-PCNA, respectively. Two $\mu$l of each fraction was applied to the

electrophoresis to detect PolBI, PolD (DP1+DP2), and primase (p46), and 0.5 $\mu$l of each fraction was used for the detection of PCNA. The protein bands were detected by using an enhanced chemiluminescence system (ECL Plus, GE Healthcare Bio-Science, Piscataway, WI, USA) according to the supplier's recommendations.

### Primer Extension Analysis

Highly purified PolBI, PolD, and primase were obtained as described earlier.[14,19,26] The elongation abilities of the DNA primer and the RNA primer by Pol BI, Pol D, and primase (p41-p46) were investigated by using M13 single-stranded DNA (Takara Bio, Shiga, Japan) as a template. As a DNA primer and RNA primer, oligodeoxyribonucleotide (dTGTAAAACGACGGCCAG) and oligoribonucleotide (rGUUUUCCCAGUCACGACGUUGUA) were used after labeling at 5′-terminus with $^{32}$P. The reaction was carried out using 0.2 units of each DNA polymerase in 20 $\mu$l buffer B with 50 nM of the template-primer and 0.125 mM dNTP at 70°C for 5 min. The reaction products were analyzed by 9% PAGE containing 8 M urea followed by autoradiography.



### Exonuclease Assay

To investigate the 3′-5′ exonuclease activity, $^{32}$P-labeled oligodeoxyribonucleotide (dAGCTATGACCATGATTACGAATTGCTT) annealed to the oligodeoxyribonucleotide (dAGCTACCATGCCTGCACGAATTAAGCAATTCGTAATCATGGTCATAGCT) was used at 0.3 $\mu$M. The reaction was carried out using 1 unit of each DNA polymerase in 10 $\mu$l of buffer B in the absence of dNTP at 70°C for 10 min. The reaction products were analyzed by 9% PAGE containing 8 M urea followed by autoradiography.

## RESULTS

### Identification of Three DNA Polymerases in the Chromatographic Fractions

To identify all of the major DNA polymerases in *P. furiosus* cell extracts, DNA polymerase assays were performed with all of the fractions after anion exchange chromatography developed with a linear gradient of 0–1 M NaCl. As shown in the upper panel of Figure 1, three peaks of DNA polymerizing activity were obtained, indicating that the DNA polymerase activity was separated into three major fractions. Our previous studies showed that the pass-through fraction (fr. I) and the tightly bound fraction (fr. III) contain PolBI and PolD, respectively, and thus it was most interesting to determine the source of the activity in the weakly bound fraction (fr. II). To address this

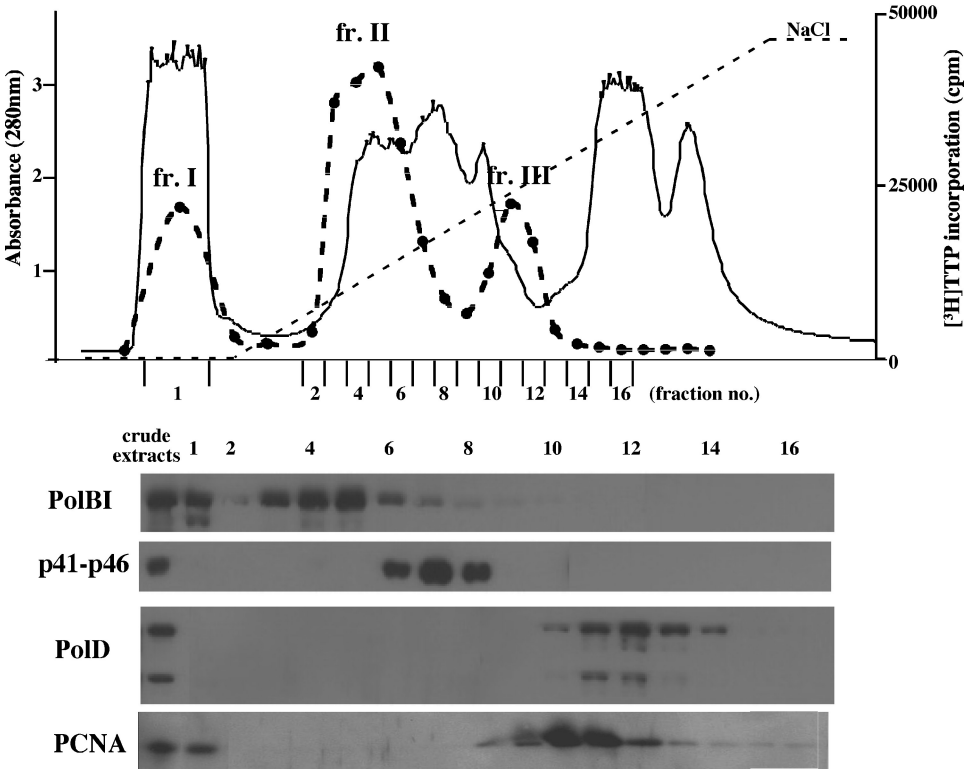


**FIGURE 1** Fractionation of the DNA polymerase activities in the *P. furiosus* cell extract. *P. furiosus* cell extract, prepared by sonication, was fractionated by anion exchange chromatography. The eluted proteins were monitored by the absorbance at 280 nm, and the DNA polymerase activity was measured by a nucleotide incorporation assay using a reaction mixture containing [methyl-$^3$H]TTP. A linear dotted line in the elution profiles indicates NaCl concentration from 0 to 1 M. Each fraction was subjected to Western blot analyses using the antisera indicated on the left side of each panel.



question, Western blot analyses were performed using anti-PolBI, anti-PolD (DP1+DP2), and anti-primase (p46), respectively. As shown in the lower panel of Figure 1, fr. I and fr. III mainly contain PolBI and PolD, respectively, as predicted. In addition, fr. II contained both PolBI and primase. From the elution profile, the primase was bound more tightly than PolBI to the resin. PolBI was present in both fr. I and fr. II and, therefore, there is some difference in the protein structure of the PolBI in fr. I and fr. II. In addition to the reproducibility of this anion-exchange chromatographic profiles, the fact that neither PolD nor primase was detected in the fr. I by the Western blot analysis would exclude the possibility of overloading of the proteins onto the column. One idea was that some of the PolBI molecules in the cells form a complex with PCNA, a ring-shaped protein to clamp DNA polymerase on the DNA strand for the processive strand synthesis and, therefore, we searched for the elution profile of PCNA in the anion exchange chromatography.

However, unexpectedly, PCNA was present in different fractions from either PolBI or PolD, which were bound to the anion exchange column (Figure 1). Some PCNA was detected in fr. I. PCNA is an acidic protein, and if a stable PCNA-DNA polymerase complex existed, then it would bind to the anion exchange column. Therefore, it would be interesting to investigate the presence of some replisome complex with other protein factors, including PolBI and PCNA, in fr. I.

## Primer Usage Preference for Each DNA Polymerase

Our search for DNA polymerases in the *P. furiosus* cells did not reveal any distinct activity other than the three known DNA polymerases. To predict the DNA replication mechanism in the *P. furiosus* cells with these three DNA polymerases, we investigated the primer usage preference for each DNA polymerase. Using M13 ssDNA as a template, an oligodeoxyribonucleotide or an oligoribonucleotide was annealed to work as a primer, and the primer-extended products from each reaction by the highly purified enzymes were detected by denaturing gel electrophoresis followed by autoradiography. As shown in Figure 2, PolD, but not PolBI, synthesized DNA strands from the RNA primer. The primase complex also extended the RNA primer with either



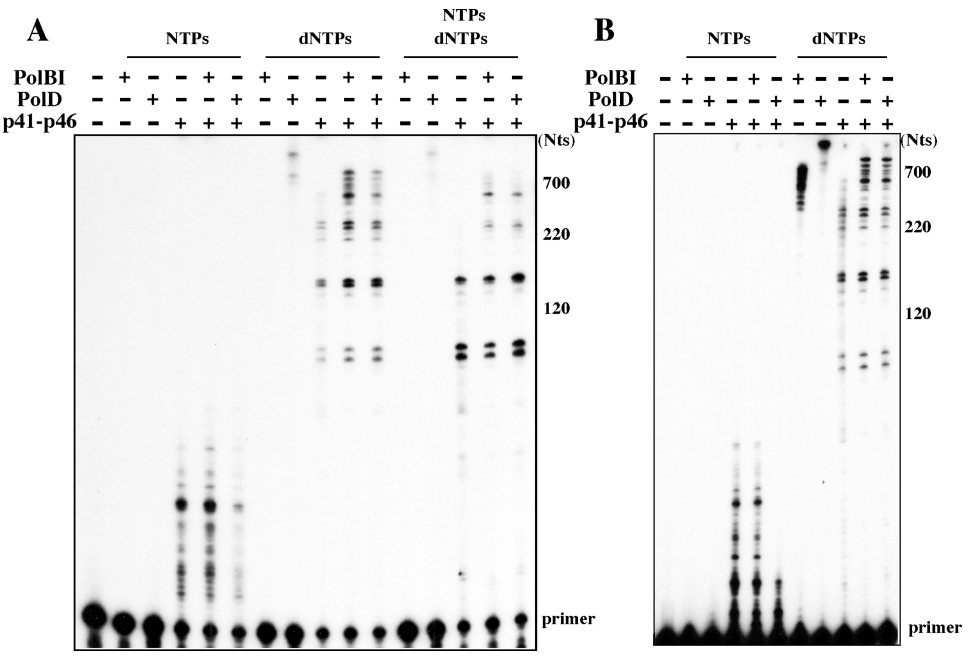


**FIGURE 2** Primer usage preference. Primer extension assays were performed for PolBI, PolD, and primase. An RNA primer (A) or DNA primer (B), described in Methods, was labeled with $^{32}P$ and annealed with M13 ssDNA. The primer extension reactions using a RNA primer (A), or a DNA primer (B) were performed for each DNA polymerase using either rNTP, dNTP, or both of them as substrates. The reaction mixtures were separated by denaturing PAGE, and the products were visualized by autoradiography.

dNTP or rNTP as the substrate. Furthermore, PolBI and PolD extended the DNA strand from the terminus of the DNA strand produced by the primase.

### The Primase Lacks 3′-5′ Exonuclease Activity

The 3′-5′ exonuclease activity associated with the 5′-3′ polymerizing activity is very important especially for the replicative DNA polymerases to have proofreading activity. Both PolBI and PolD from *P. furiosus* have very strong 3′-5′ exonuclease activity as presented previously.[14,16,19] Our preliminary results showed that the *P. furiosus* primase lacks exonuclease activity. Here, we used the most sensitive detection method to demonstrate that the highly purified p41-p46 primase complex lacks the activity. As shown in Figure 3,

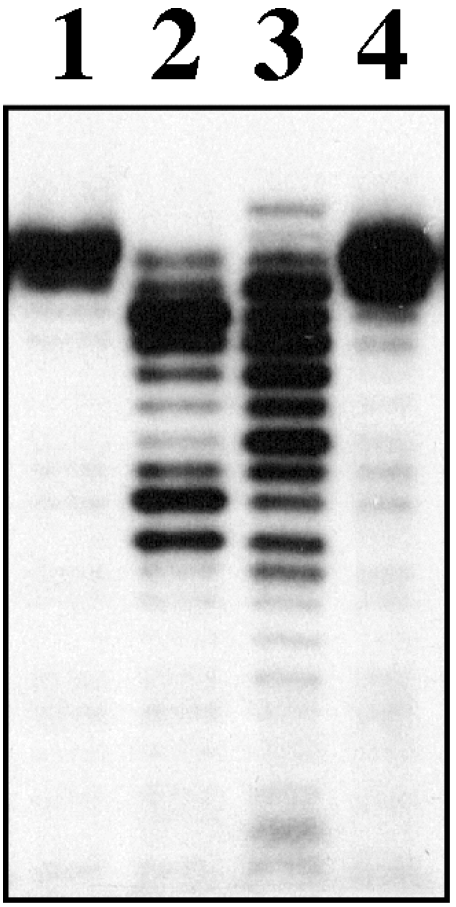


**FIGURE 3** Exonuclease activity of the three DNA polymerases. The 3′-5′ exonuclease activity of each DNA polymerase was measured by the oligonucleotide template-primer as described in the methods section. The primer was labeled at the 5′-terminus with $^{32}$P. The reaction mixtures were subjected to denaturing PAGE, and the products were visualized by autoradiography. Lanes: 1, p41-p46; 2, PolBI; 3, PolD; 4, no protein.

the $^{32}$P-labeled DNA was degraded in the presence of PolBI or PolD, but it was not degraded by the primase complex. These results suggest that the strand synthesis by the primase is very error prone, and that the primase is not suitable for the replication of long DNA strands. PolBI and PolD are likely to be involved in the strand elongation process after primer synthesis.

## DISCUSSION

We searched for DNA polymerase activity in the fractions of a total cell extract of *P. furiosus,* separated by an anion-exchange chromatography, and identified the elution positions of the three major DNA polymerases in this organism (Figure 1). Some possibilities of the presence of other DNA polymerases still remain to be investigated. Another DNA polymerase with very weak activity may also be present within the three major fractions described in this study. It is also possible that some DNA polymerase activity in the cell extracts disappeared, because plural subunits of the DNA polymerase were separated by the anion exchange chromatography. In this study, we demonstrated that at least the three known DNA polymerases in *P. furiosus* can be detected in different anion exchange chromatography fractions, and that there is no other fraction that has a distinct activity derived from some unknown protein.



One of the major issues of the DNA replication mechanisms is the functional share of DNA strand synthesis of the plural DNA polymerases. It is very difficult to predict the responsible DNA polymerase in DNA replication for the synthesis of leading strand and lagging strand, respectively, only from their biochemical properties analyzed in vitro. If we presume this issue from the primer usage preferences of PolBI and PolD presented in this study, a possible discussion may be as follows. It is already known that strand synthesis in DNA replication in *Pyrococcus* cells starts with an RNA primer, as also found in bacterial and eukaryotic cells.[27] The RNA primer is probably synthesized by a primase (p41-p46 complex) in the cells as shown in vitro.[26] Since PolD, but not PolBI, can extend the DNA strand from the RNA primer terminus (Figure 2), PolD may be suitable for the lagging strand synthesis using each RNA primer. On the other hand, for the leading strand synthesis, the RNA primer must be extended only once, at the beginning of DNA replication. The *P. furiosus* primase can also synthesize a DNA strand by an RNA primer *in vitro* and, therefore, once the DNA terminus is made by the primase or PolD in the leading strand synthesis in the cells, then PolBI can take over the role of leading strand synthesis. We previously demonstrated that the DNA strand synthesis activities of both PolBI and PolD were stimulated by the physical interaction with PCNA. The activity of the primase for DNA strand synthesis was not stimulated by PCNA. PCNA probably functions as a clamp for both PolBI and PolD, to provide high processivity in the strand

elongation process of DNA replication. Primer usage preference of these polymerases may be changed in vivo by the effect of other protein factors, and further progress is necessary to obtain more convincing scheme of DNA strand synthesis.

The family Y DNA polymerases have been the center of attention recently in the field of DNA replication and repair, as responsible enzymes for translesion synthesis at a stalled replication fork.[28,29] Switching of the DNA polymerase from Pol$\delta$ or Pol$\varepsilon$ to Pol$\eta$, for example, at the stalled replication fork by the control of the ubiqitination of PCNA is a very exciting model of the molecular process of translesion repair in eukaryotic cells. However, the genomes of the *Pyrococcus* strains have no gene encoding a family Y DNA polymerase-like sequence. Therefore, it is not known whether a translesion repair system exists in these archaeal cells, and if it does, which DNA polymerase is responsible for this system. To answer these questions, more detailed searches for DNA polymerases are required. Our preliminary investigation indicated that a translesion synthesis activity exists in the fractions around fr. 6–8 of the chromatography shown in Figure 1 (Tori K et al., unpublished). Further analyses may provide a novel DNA polymerase, which gene expression may be damage-inducible in the cells.

We previously identified the replication origin (*oriC*) of the *Pyrococcus* strains.[30] In addition, we characterized the replication proteins, including Orc1/Cdc6, Mcm,[30] Primase,[25,26] Replication protein A (RPA),[31]Topo III (Yoshimochi et al., to be submitted), PCNA,[32] RFC,[33] DNA Ligase (Nishida et al., submitted), and FEN1 (Komori et al., to be submitted) from *P. furiosus*, besides these three DNA polymerases. Further investigations will provide important insights toward understanding the archaeal DNA replication mechanism, and will contribute to the elucidation of the principle of DNA replication as a fundamental phenomenon of life.



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