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Evolution

A heterodimeric DNA polymerase: Evidence that members of Euryarchaeota possess a distinct DNA polymerase

(Archaea/DNA replication/DNA Polymerase δ/hyperthermophile/methanogen)

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ABSTRACT **We describe here a DNA polymerase family highly conserved in Euryarchaeota, a subdomain of Archaea. The DNA polymerase is composed of two proteins, DP1 and DP2. Sequence analysis showed that considerable similarity exists between DP1 and the second subunit of eukaryotic DNA polymerase δ, a protein essential for the propagation of Eukarya, and that DP2 has conserved motifs found in proteins with nucleotide-polymerizing activity. These results, together with our previous biochemical analyses of one of the members, DNA polymerase II (DP1 + DP2) from *Pyrococcus furiosus*, implicate the DNA polymerases of this family in the DNA replication process of Euryarchaeota. The discovery of this DNA-polymerase family, aside from providing an opportunity to enhance our knowledge of the evolution of DNA polymerases, is a significant step toward the complete understanding of DNA replication across the three domains of life.**

The DNA replication apparatus has been well characterized in Bacteria, with *Escherichia coli* serving as a model (1, 2). In this organism, chromosomal duplication is the function of the DNA polymerase III holoenzyme. The genes encoding all 10 subunits of the holoenzyme have been identified, and these proteins have been overproduced, purified, and reconstituted. Proteins with corresponding functions have been identified in Eukarya (3, 4). However, in both Bacteria and Eukarya, few of these similarly functioning proteins exhibit any meaningful amino acid conservation.

Two decades ago, biologists witnessed a landmark discovery by Woese and Fox (5), who announced the existence of a third form of life, currently referred to as Archaea (6). Even though members of this domain are dissimilar to the eukaryotes (6), archaeal information-processing systems (i.e., transcription, translation, and apparently replication systems) are more similar to the eukaryotic than to the bacterial versions. The study of archaeal information processing may, therefore, help us to understand the structure, function, and evolution of homologous eukaryotic systems and vice versa.

Previously, we cloned a DNA-polymerase gene that encodes an eukaryote-like family B (α-like) DNA polymerase from the euryarchaeote *Pyrococcus furiosus* (7). Furthermore, we showed that the crenarchaeote *Pyrodictium occultum* possesses at least two family B DNA polymerases (8). Including our results, every archaeal DNA polymerase sequenced before the first complete archaeal genome report of *Methanococcus jannaschii* (9) was a single-subunit member of family B (10–13).

An extremely puzzling observation from the complete genome sequence of *M. jannaschii* was the presence of what is apparently a single DNA polymerase sequence (9). This finding was inconsistent with the presence of multiple DNA polymerases serving different functions in other forms of life. In a recent report, Olsen and Woese (14) noted the possibility that the archaeal replicative polymerase may have eluded researchers. Edgell and Doolittle (15) also argued that nonhomologous proteins are likely recruited into a replication function in one of the lineages, thereby replacing cenancestral components.

We discovered a distinct DNA polymerase (Pol II) from a *P. furiosus* cell extract (16). During the purification of the native Pol II from *P. furiosus*, deoxynucleotide incorporation activity was detected from a protein (Pfu DP2) with an apparent molecular mass of 130 kDa and another protein with a larger molecular mass, as determined by gel filtration. The presence of the larger protein prompted three hypotheses: (*i*) multimerization of the 130-kDa protein, (*ii*) an interaction of an accessory protein with the 130-kDa protein, and (*iii*) the existence of an entirely different DNA polymerase in *P. furiosus.* This uncertainty was clarified when the gene encoding DP2 in *P. furiosus* was isolated (17). Arranged in tandem with this gene, which in actuality codes for a protein with a molecular mass of 143,161 Da, is a smaller gene encoding a protein (Pfu DP1) with a molecular mass of 69,294 Da. Nested deletion analyses of the corresponding genes indicated that DP1 regulates the level of DNA polymerase activity (17). Some biochemical properties of Pol II, such as an excellent primer-extension ability (which uses the single-stranded M13 DNA primed with an oligonucleotide) and the strong $3' \rightarrow 5'$ exonuclease activity (for proofreading), suggest that the DNA polymerase is a replicative enzyme (17).

When the total genome sequence of *M. jannaschii* was reported (9), we found the homologs of Pfu Pol II and showed that these proteins (MJ0702 and MJ1630) have both DNA-polymerizing activity and $3' \rightarrow 5'$ exonuclease activity (18).

In this study, we show that polypeptides having sequences similar to Pfu Pol II exist in the genomes of three other euryarchaeotes, *Methanobacterium thermoautotrophicum* (19), *Archaeoglobus fulgidus* (20), and *Pyrococcus horikoshii* (ref. 21; for more information see www.bio.nite.go.jp). Furthermore, the archaeal–eukaryotic relationship is substantiated by the similarity of amino acid sequences of euryarchaeal DP1 and the small subunit of eukaryotic DNA polymerase δ (Pol δ), a protein essential for replication in Eukarya. In DP2 proteins, motifs, including invariant carboxylates (Asp) found in the palm subdomain of nucleotide polymerases, could be predicted. As further evidence that the two proteins constitute a heterodimeric DNA polymerase, we show here that DP1 and DP2 interact to make up a complex in *P. furiosus* cells.

We propose that euryarchaeotes have a different type of DNA polymerase from those found in eukaryotic enzymes and

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Abbreviations: Pol I, DNA polymerase I; Pol II, DNA polymerase II; Pol δ, DNA polymerase δ.

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that the DP2 proteins are likely to be the catalytic subunit of this DNA polymerase family.

MATERIALS AND METHODS

Protein Sequences. The protein sequences used in this study, together with their accession numbers and ORF numbers (from archaeal genome projects), are as follows: from *P. furiosus* (Pfu DP1 and Pfu DP2: D84670); from *M. jannaschii* (Mja DP1: F64387, MJ0702) and (Mja DP2: D64503, MJ1630); from *A. fulgidus* (Afu DP1: AE000979, AF1790) and (Afu DP2: AE000984, AF1722); from *M. thermoautotrophicum* (Mth DP1: AE000903, MTH1405) and (Mth DP2: AE000913, MTH1536); from *P. horikoshii* (Pho DP1: AB009468, PHBN023) and (Pho DP2: AB009468, PHBN021); from *Homo sapiens* Pol δ small subunit (Hsa PolD: U21090); from *Arabidopsis thaliana* Pol δ small subunit (Ath PolD: AC002561); from *Caenorhabditis elegans* Pol δ small subunit (Cel PolD: Z73425); from *Saccharomyces cerevisiae* (Sce HYS2: D50324); from *Schizosaccharomyces pombe* (Spo Cdc1: Y12561); from *S. cerevisiae* transposon TYI protein B (Sce TYI: P47098); from HIV DNA polymerase polyprotein (HIV RT: P05961); from *Euplotes aediculatus* telomerase subunit (Eau Tel: U95964); from bacteriophage T5 DNA polymerase (T5 Dpol: P19822); from bacteriophage KII DNA-directed RNA polymerase (KII Rpol: P18147); from Sendai virus RNA polymerase β subunit (Sen Rpol: P06829); from *E. coli* DNA polymerase I (Pol I; Eco DPolI: P00582); from *Thermus aquaticus* Pol I (Taq DPolI: D32013); from *H. sapiens* DNA polymerase α (Hsa DPolα: P09884); from *E. coli* Pol II (Eco DPolII: X54847); from *S. cerevisiae* DNA polymerase ε catalytic subunit A (Sce Epsi: P21951); from *S. pombe* DNA-polymerase ε catalytic subunit A (Spo Epsi: Z95397); and from *H. sapiens* DNA polymerase ε catalytic subunit A (Hsa Epsi: Q07864).

Computer Analysis. Database searches were carried out with FASTA (22) and BLAST (23) at GenomeNet (www.genome.ad.jp) and with PSI-BLAST (24) at the web site of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-psi_blast). Multiple alignments were constructed with CLUSTALW, version 1.7 (25). The percentage of identity between two sequences was calculated after removing the gaps caused by the alignment. The statistical significance of sequence similarity was verified by the jumbling test (26) with 1,000 randomizations.

Immunoprecipitation Experiment. *P. furiosus* cells were grown under anaerobic conditions in 500 ml of medium (8) overnight at 95°C. The cells were harvested by centrifugation at 5,000 × *g* for 15 min, and the pellet was resuspended in 10 ml of buffer A (50 mM Tris·HCl, pH 8.0/2 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride/10% glycerol). Cells were disrupted by sonication on ice, followed by centrifugation for 10 min at 10,000 × *g*. The supernatant was kept on ice until used. All subsequent steps were carried out at room temperature. Aliquots (30 μl) of protein A-Sepharose (Pharmacia) were washed three times with PBS (10 mM sodium phosphate, pH 7.5/0.15 M NaCl). The sepharose in each tube was mixed with one of the following polyclonal antisera that were raised independently by immunizing rabbits: anti-Pfu Pol I (family B DNA polymerase), anti-Pfu DP1, anti-Pfu Pol II (DP1 + DP2), or anti-PI-PfuI [a *P. furiosus* intein protein (K.K., N. Fujita, K. Ichiyanagi, H. Shinagawa, K. Morikawa, and Y.I., unpublished work) as a control]. The mixtures were incubated for 1 h on a rotary shaker, followed by two washes with PBS and one with buffer A. The sepharose in each tube was then mixed with 300 μl of supernatant from the *P. furiosus* cell extract and incubated for 30 min on a rotary shaker.

Western Blotting. The immunoprecipitates, prepared as described above, were washed twice with buffer A and mixed with 240 μl of buffer A and 60 μl of 5× sample buffer [0.25 M Tris·HCl (pH 6.8)/5% (vol/vol) glycerol/5% 2-mercaptoethanol/0.2% bromophenol blue]. The mixture was boiled for 5 min, followed by microcentrifugation at 10,000 × *g* for 5 min. Supernatant (3 μl) was electrophoresed on an SDS/7.5% PAGE, transferred onto a poly(vinylidene difluoride) membrane (0.2 μm; Bio-Rad) and reacted with each antiserum. The blots were analyzed with the enhanced chemiluminescence system (Amersham) incorporated with horseradish peroxidase-linked anti-rabbit IgG (Amersham), according to the manufacturer's instructions.

RESULTS

Search for Sequences Homologous to DP1 and DP2 of *P. furiosus*. We searched for the homologs of both DP1 and DP2 in the complete genome sequences of three euryarchaeotes, *M. thermoautotrophicum*, *A. fulgidus, and P. horikoshii*, and found that DP1 and DP2 are highly conserved in these euryarchaeotes. Amino acid sequence identities of DP1 and DP2 within five euryarchaeotes are shown in Table 1. A comparison of DP1s yielded sequence identity values ranging from 38% (Pfu DP1 and Mja DP1) to 44.1% (Afu DP1 and Mja DP1). The DP2 amino acid sequences showed a higher conservation within these euryarchaeotes. The identity values were greater than 50% (Table 1).

Our examination of the gene arrangement in *Pyrococcus woesei*, through PCR amplification (data not shown), and in *P.*

Table 1. Percentage of identity of DP1 and DP2 amino acid sequences among five euryarchaeal strains

DP1 and Pol δ	*Pfu*	*Pho*	*Mja*	*Afu*	*Mth*	*Ath*	*Sce*	*Spo*	*Cel*	*Hsa*
P. furiosus	—									
P. horikoshii	84.8	—								
M. jannaschii	38.0	39.7	—							
A. fulgidus	42.4	42.1	44.1	—						
M. thermoautotrophicum	42.4	41.3	40.5	43.5	—					
A. thaliana	19.3	19.6	17.9	18.7	18.5	—				
S. cerevisiae	17.4	17.6	19.0	17.4	16.3	32.8	—			
S. pombe	14.0	14.6	12.9	16.5	14.3	29.8	34.4	—		
C. elegans	17.1	16.8	17.4	19.3	17.4	19.6	23.7	20.9	—	
H. sapiens	17.1	17.4	15.4	18.2	17.9	42.4	31.1	32.5	25.1	—
DP2	*Pfu*	*Pho*	*Mja*	*Afu*	*Mth*					
P. furiosus	—									
P. horikoshii	90.4	—								
M. jannaschii	56.2	56.2	—							
A. fulgidus	54.2	54.7	53.1	—						
M. thermoautotrophicum	54.6	53.9	53.1	50.4	—					

The region used in calculating the % identities is demarcated by two vertical lines in Fig. 1.

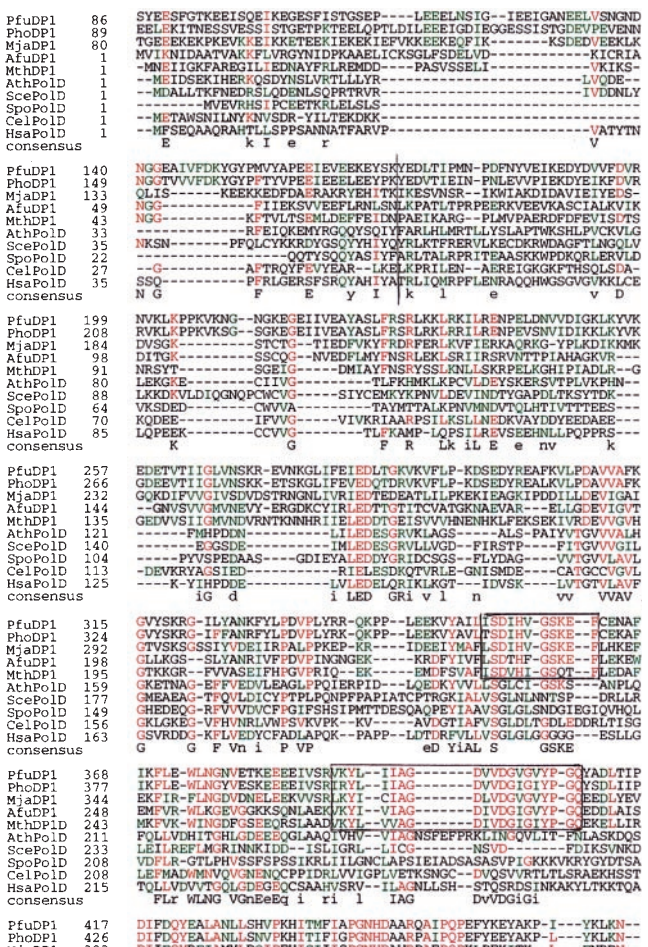

FIG. 1. Amino acid sequence alignment of the euryarchaeal DP1 and eukaryotic Pol δ small subunit. The sequences were aligned with CLUSTALW, version 1.7. The polymerases shown are from *P. furiosus* (Pfu), *P. horikoshii* (Pho), *M. jannaschii* (Mja), *A. fulgidus* (Afu), *M. thermoautotrophicum* (Mth), *A. thaliana* (Ath), *S. cerevisiae* (Sce), *S. pombe* (Spo), *C. elegans* (Cel), and *H. sapiens* (Hsa). The ORF names of the archaeal proteins obtained from their genome projects were Mja DP1 (MJ0702), Afu DP1 (AF1790), Mth DP1 (MTH1405), and Pho DP1 (PHBN023). Amino acid residues that are identical (red) or similar (green) in ≥50% of the positions are indicated. Upper case letters indicate consensus residues with identities at ≥50% positions, including, at least, one from each domain. Lowercase letters indicate the most frequent residues including, at least, one from each domain. The highly conserved regions from the euryarchaeal group are boxed. Amino acids with similar properties are grouped as LIMV, AG, YWF, DEQN, KRH, and ST.

horikoshii, from the reported genome sequence, indicates that in the genus *Pyrococcus*, but not in the other genera, the genes for DP1 and DP2 are arranged in tandem, as observed in *P. furiosus* (17). Furthermore, the genes are adjacent to the genes for a homolog of Cdc6 and Orc1 (proteins essential to the initiation of eukaryotic DNA replication) and to the genes for a homolog of Rad51 (a protein essential to eukaryotic recombination and repair) in their genome.

Sequence Comparison of Archaeal DP1 Proteins with Eukaryotic DNA Pol δ. The euryarchaeal DP1s showed considerable amino acid sequence similarities to homologs of the Pol δ small subunit from various eukaryotes, including fungi, nematodes, plants, and animals, even though the amino acid identities between the proteins ranged from 12.9% to 19.6% (Table 1). To verify the significance of such weak identities, every pair of aligned sequences was subjected to the jumbling test (26). The *Z* scores obtained showed statistical significance in the similarity between euryarchaeal DP1s and the eukaryotic Pol δ small subunit (data not shown). Fig. 1 shows the sequence alignment, including five euryarchaeal DP1s and five eukaryotic Pol δ small subunits. As shown in the alignment, a considerable sequence conservation between the euryarchaeal and eukaryotic families exists in all the proteins. However, the C-terminal half is much more conserved than the N-terminal half. DP1 proteins from *P. furiosus*, *P. horikoshii*, and *M. jannaschii* are larger than the other DP1s and all of the eukaryotic Pol δ small subunits, which may signify additional function in their N-terminal regions. We also noted some sequences conserved only in the euryarchaeal DP1s but not in the eukaryotic Pol δ group (Fig. 1).

Evidence That DP2 Proteins Constitute the Catalytic Subunit of the Heterodimeric DNA Polymerase. It is believed that the larger subunit of the heterodimeric core of the eukaryotic Pol δ has both DNA-polymerizing and 3′ → 5′ exonucleolytic activities (27, 28). The larger subunit shares amino acid sequence similarity with the catalytic subunit of family B DNA polymerases. In the case of euryarchaeal heterodimeric DNA polymerase, we could detect only a weak DNA-polymerizing activity in DP2 protein of *P. furiosus* (16, 17). However, a database search with FASTA, BLAST, or PSI-BLAST did not detect proteins with global sequence similarity to DP2 proteins. The accumulation of sequence and structural data of various polymerases has established the consensus patterns existing in the amino acid sequence and structure of polymerases. Crystal structures of nucleotide polymerases from four categories, DNA-dependent DNA polymerase (29–31), DNA-dependent RNA polymerase (32), RNA-dependent DNA polymerase (reverse transcriptase) (33), and RNA-dependent RNA polymerase (34), share a common folding pattern that resembles a right hand composed of the finger, thumb, and palm subdomains. Although the topological relationship between the finger and thumb subdomains is different from polymerase to polymerase, the structures of the palm subdomains are highly conserved. The palm subdomains include two sequence motifs (Fig. 2). The sequences of the motifs are highly diverged among the polymerases, which explains why the database search that used DP2 as a query failed to predict this domain. Therefore, we searched for the motif sequences in the aligned sequences of five euryarchaeal DP2s by visual inspection and found the two invariant carboxylates (Asp) present in motifs A and C, which are thought to constitute part of the polymerase active site in the palm subdomain (Fig. 2).

Interaction of DP1 with DP2 but Not with Pol I (Family B DNA Polymerase) in *P. furiosus* Cells. To examine whether DP1 and DP2 interact in the cells of *P. furiosus*, we performed immunoprecipitation experiments with anti-DP1 antibody.

		Motif A			Motif C	
Pfu DP2	815	KDGTIRFDATDAPITHF	831	882	LRVARFVDDLLEKF	895
Pho DP2	817	KDGTIRFDATDAPITHF	833	884	LKVAKFVDDLLEKF	897
Mja DP2	750	KDGTTRFDCTDVPVTHF	766	817	VKVANFIDDLLEKF	830
Afu DP2	756	KDGTARFDATDLPITHF	772	823	LRVANFIDDLLVKF	836
Mth DP2	706	KDATIRHDSTDLPLTHF	722	773	VRVANFVDDLLERF	786
Sce TYI	1339	NYYITQLDISSAYLYAD	1355	1420	VTICLFVDDMILFS	1433
HIV RT	261	KKSVTVLDVGDAYFSVP	277	336	IVIYQYMDDLYVGS	349
Eau Tel	596	KLFFATMDIEKCYDSVN	612	774	NLLMRLTDDYLLIT	787
T5 DPol	494	GYRVIAWDLTTAEVYYA	510	701	KIVMLVHDSVVAIV	714
KII RPol	553	CSLPLAFDGSCSGIQHF	569	828	DSFALIHDSSGTIP	841
Sen RPol	656	LSCFLTTDLKKYCLNWR	672	766	VSAMVQGDNQAIAV	779
Eco DPolI	698	DYVIVSADYSQIELRIM	714	875	RMIMQVHDELVFEV	888
Taq DPolI	603	GWLLVALDYSQIELRVL	619	778	RMLLQVHDELVLEA	791
Hsa DPolα	853	DKFILLLDFNSLYPSII	869	997	EVIYGDTDSIMINT	1010
Eco DPolII	411	YDSVLVLDYKSLYPSII	427	539	DVIYGDTDSTFVWL	552
		*			*	

FIG. 2. Amino acid sequence alignment showing two major conserved regions of polymerases, including the DP2 of Euryarchaeota. The sequences were aligned with CLUSTALW, version 1.7. The asterisks indicate invariant residues. Amino acid residues that are identical (red) or similar (green) in ≥50% of the positions are indicated. The polymerases shown are from *P. furiosus* (Pfu), *P. horikoshii* (Pho), *M. jannaschii* (Mja), *A. fulgidus* (Afu), *M. thermoautotrophicum* (Mth), *S. cerevisiae* transposon TYI protein B (Sce TYI), HIV DNA polymerase polyprotein (HIV RT), *E. aediculatus* telomerase subunit (Eau Tel), bacteriophage T5 DNA polymerase (T5 DPol), bacteriophage KII RNA polymerase (KII Rpol), Sendai virus RNA polymerase β subunit (Sen Rpol), *E. coli* DNA polymerase I (Eco DPolI), *T. aquaticus* Pol I (*Taq* DPolI), *H. sapiens* DNA polymerase α (Hsa), and *E. coli* Pol II (Eco DpolII). The ORF names of the archaeal proteins obtained from their genome projects are Mja DP2 (MJ1630), Afu DP2 (AF1722), Mth DP2 (MTH1536), and Pho DP2 (PHBN021). Amino acids were classified as in Fig. 1.

DP2 was coprecipitated with DP1 from the cell extract of *P. furiosus* (Fig. 3*C*, lane 4).

Because DP1 is homologous to the small subunit of Pol δ, we predicted that there may be another subunit in *P. furiosus* homologous to the large (catalytic) subunit of Pol δ. The catalytic subunit of the eukaryotic Pol δ is a family B DNA polymerase (35), and Pol I of *P. furiosus* is also a family B DNA polymerase (7). Given these facts, we hypothesized that *P. furiosus* Pol I, a homolog of the catalytic subunit of the eukaryotic Pol δ, would interact with DP1 in *P. furiosus* cells and investigated accordingly. The immunological analysis showed no coprecipitation between Pol I and DP1 (Fig. 3 *A*, lanes 3 and 4, and *B*, lanes 3 and 4). These immunological experiments indicate that DP1 forms a complex with DP2 but not with Pol I in *P. furiosus* cells. The fact that the nucleotide incorporation activity of Pol I was not enhanced by the addition of DP1 (data not shown) also supports the idea that no interaction exists between Pol I and DP1 in the cells. These results suggest that Pol I is not the ortholog of the large subunit of Pol δ and that DP1 specifically interacts with DP2 to constitute Pol II in *P. furiosus*, although the existence of an unrecognized subunit cannot be excluded as discussed below.

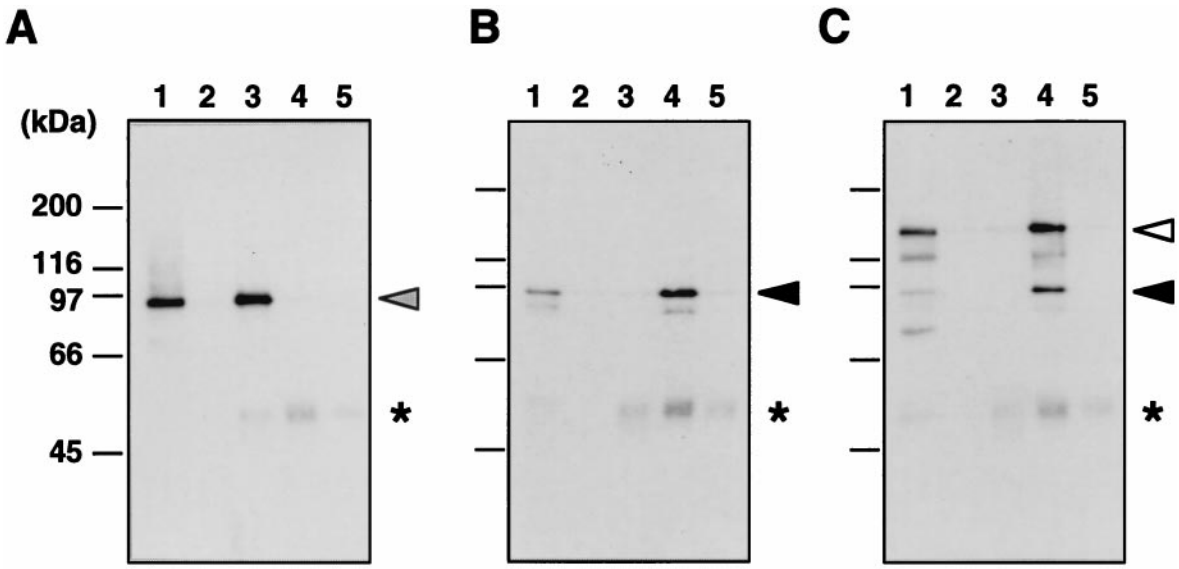

FIG. 3. Immunoprecipitation analysis of *P. furiosus* DNA polymerases. The total cell extracts were immunoprecipitated with three kinds of antiserum, anti-Pfu Pol I, anti-Pfu DP1, and anti-Pfu Pol II (DP1 + DP2). Immunoprecipitated fractions were separated by SDS/7.5% PAGE and then analyzed by Western blotting with anti-Pfu Pol I antiserum (*A*), anti-Pfu DP1 antiserum (*B*), and anti-Pfu Pol II antiserum (*C*). In each panel, lane 1 shows total cell extracts without immunoprecipitation; lane 2 shows total cell extracts precipitated with PBS; lane 3 shows total cell extracts precipitated with anti-Pfu Pol I; lane 4 shows total cell extracts precipitated with anti-Pfu DP1; and lane 5 shows total cell extracts precipitated with anti-PI-PfuI (negative control). The closed, open, and shaded arrowheads correspond to DP1, DP2, and Pol I, respectively. A nonspecific band (*) found in all of the immunoprecipitated samples probably corresponds to IgG judging by its molecular size.

DISCUSSION

The recent analyses of the complete genome sequences of three euryarchaeotes have further substantiated the evidence that archaeal replication proteins are more closely related to their eukaryotic homologs than to those of Bacteria. Despite this knowledge, our understanding of DNA replication in Archaea is still fragmentary.

In this report, we show that DP1 and DP2, which constitute a distinct DNA polymerase, are highly conserved in Euryarchaeota, a subdomain of Archaea. This finding suggests that these proteins play an important role in these organisms. We also show the significant similarities between DP1 and the small subunit of eukaryotic Pol δ, thereby providing confirmation of the eukaryotic–archaeal relationship. The eukaryotic Pol δ is a heterodimer of a 125-kDa and a 50-kDa polypeptide, and both are essential for DNA replication (36, 37). Thus far, the function of the small subunit remains unknown. However, because both the polymerase and the $3' \rightarrow 5'$ exonuclease activities are located in the large subunit, some important function must be conserved in the small subunit. The large subunit (DP2) of Pol II, on the other hand, exhibits no similarity to the catalytic subunit of Pol δ or to any protein in public databases. However, we show that DP2 proteins contain the motifs conserved in the polymerase superfamily. Therefore, we propose that the large subunit of the euryarchaeal Pol II is the catalytic subunit.

Immunoprecipitation experiments indicated that DP1 and DP2 form a complex in *P. furiosus* cells. Pol I (a family B DNA polymerase of *P. furiosus*) was not coprecipitated with DP1 (Fig. 3). It is possible that another family B DNA polymerase that can interact with DP1 exists in *P. furiosus*. However, the complete genome sequence of *P. horikoshii* contains only one family B DNA polymerase homolog. Therefore, we may be able to conclude that DP1 is a specific partner of DP2 for the formation of the euryarchaeal Pol II, which, according to our previous results (17), is likely to be the euryarchaeal replicative DNA polymerase.

The eukaryotic Pol δ requires the accessory factor proliferating cell nuclear antigen (PCNA) for maximal processing, and the assembly of the Pol δ–PCNA complex on nascent DNA-strand ends requires replication factor C (RF-C; refs. 38–41). Several reports suggest that the small subunit of Pol δ is necessary for the interaction of the catalytic subunit with its auxiliary proteins (42–44). Homologs of the eukaryotic RF-C and PCNA are found in all completely sequenced euryarchaeal genomes (9, 19, 20). In the *P. horikoshii* genome, a single RF-C homolog is listed (PHBN012). However, we located the other subunit 7 bases downstream (PHBN013). Note that these RF-C homologs are only 4.2 kb downstream of the operon containing *P. horikoshii* Pol II. It would be interesting to investigate the interaction of DP2 with the euryarchaeal PCNA and the RF-C homologs in the presence or absence of DP1.

The comparison of DP1 sequences also raises some interesting questions; the high degree of conservation of the C terminus suggests the location of major functional components. The diverged N-terminal regions, in contrast, may be involved in species-specific interactions. Clonal deletion studies may confirm this hypothesis. The conserved regions found only in DP1s (not in the Pol δ small subunit) may be important for their specific interaction with DP2s and perhaps with other proteins required for the maximal processivity of Pol II. In DP2 proteins, zinc finger motifs, which could be involved in interaction with other proteins in addition to DNA binding, were found in the middle and C-terminal regions.

The homologs of the large subunit of eukaryotic Pol δ exhibit high conservation (50% identity on average; data not shown). This conservation is similar to that of euryarchaeal DP2s. The small subunits of eukaryotic Pol δ are, however, less conserved than the euryarchaeal DP1s. Although most of the euryarchaeotes we analyzed thrive under similar conditions (hyperthermophiles), the eukaryotes are adapted to diverse conditions. The constraints on these euryarchaeotes to conserve the DP1 proteins may be more pronounced. Isolation of Pol II homologs from mesophilic euryarchaeotes may shed some light on this hypothesis.

The phylogenetic relationship of the euryarchaeal heterodimeric DNA polymerase to other DNA polymerases is not known. When genome sequences of organisms from Crenarchaeota are reported, we should know whether this heterodimeric DNA polymerase commonly exists in the archaeal domain and should be able to discuss the relationship between DNA polymerase and phylogeny. Evolution is driven by the replication apparatus, and at the center of this process are the DNA polymerases. Our discovery, together with the identification of all DNA polymerases in the Crenarchaeota, will provide an opportunity to discuss the evolution of this indispensable protein thoroughly.

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