Archaeal DNA Replication: Identifying the Pieces to Solve a Puzzle

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ABSTRACT

Archaeal organisms are currently recognized as very exciting and useful experimental materials. A major challenge to molecular biologists studying the biology of Archaea is their DNA replication mechanism. Undoubtedly, a full understanding of DNA replication in Archaea requires the identification of all the proteins involved. In each of four completely sequenced genomes, only one DNA polymerase (Pol BI proposed in this review from family B enzyme) was reported. This observation suggested that either a single DNA polymerase performs the task of replicating the genome and repairing the mutations or these genomes contain other DNA polymerases that cannot be identified by amino acid sequence. Recently, a heterodimeric DNA polymerase (Pol II, or Pol D as proposed in this review) was discovered in the hyperthermophilic archaeon, *Pyrococcus furiosus*. The genes coding for DP1 and DP2, the subunits of this DNA polymerase, are highly conserved in the Euryarchaeota. Euryarchaeotic DP1, the small subunit of Pol II (Pol D), has sequence similarity with the small subunit of eukaryotic DNA polymerase δ. DP2 protein, the large subunit of Pol II (Pol D), seems to be a catalytic subunit. Despite possessing an excellent primer extension ability *in vitro*, Pol II (Pol D) may yet require accessory proteins to perform all of its functions in euryarchaeotic cells. This review summarizes our present knowledge about archaeal DNA polymerases and their relationship with those accessory proteins, which were predicted from the genome sequences.

THE discovery of the Archaea (Woese and Fox 1977) ushered in the period after which life was classified into three domains, namely, the Archaea, Bacteria, and Eukarya (Woese *et al.* 1990). The Archaea and Bacteria are similar in cellular ultrastructure, and the two are often referred to as prokaryotes. However, some rooted-phylogenetic trees of life, based on some protein sequences, suggest that the Archaea and Eukarya are sister groups, and they branched out earlier from Bacteria (Gogarten *et al.* 1989; Iwabe *et al.* 1989; Brown and Doolittle 1995). A more comprehensive analysis involving four complete archaeal genome sequences (Bult *et al.* 1996; Klenk *et al.* 1997; Smith *et al.* 1997; Kawarabayasi *et al.* 1998) shows that Archaea are different from the other two domains. Archaea, despite having an information processing machinery that is similar to Eukarya (translation, transcription, and replication), possesses metabolic features that exhibit closer similarities to bacterial processes.

Elucidation of the molecular mechanism of DNA replication is one of the most exciting research topics in Archaeal biology, because it may contribute to the understanding of the basic mechanism of eukaryotic DNA replication. However, currently the essential components involved in DNA replication have not been identified (Edgell and Doolittle 1997; Bernander 1998), which hinders research aimed at unraveling the mechanism. DNA polymerases are pivotal to the molecular machinery driving the replication of the chromosome, and it is well known that in Bacteria and Eukarya, multiple DNA polymerases are required in the process (Kornberg and Baker 1992). The history of the research on archaeal DNA polymerases can be followed via the review articles by Forterre *et al.* (1994), Perler *et al.* (1996), and Ishino and Cann (1998). While almost every report published was on a single DNA polymerase, there were interesting findings suggesting that some crenarchaeotes contain two or three family B DNA polymerase genes (Prangishvili and Klenk 1994; Uemori *et al.* 1995; Edgell *et al.* 1997). In *Pyrodictium occultum*, two genes were confirmed to encode proteins having DNA polymerase activity (Uemori *et al.* 1995). Contrasting this finding, the analysis of the first complete genome sequence of an archaeon (Bult *et al.* 1996) suggested that *Methanococcus jannaschii* depended on a single family B DNA polymerase for its DNA metabolic functions, and this certainly was unusual (Edgell and Doolittle 1996; Gray 1996; Morell 1996). This puzzling observation was further confounded by other complete genome sequences from the Archaea (Klenk *et al.* 1997; Smith *et al.* 1997; Kawarabayasi *et al.* 1998).

To gain more insight into the archaeal DNA replication mechanism, it is necessary to isolate all of the fundamental proteins involved, and of cardinal importance are the DNA polymerases. Our finding of the novel DNA polymerase composed of the heterodimeric proteins in *Pyrococcus furiosus* (Uemori *et al.* 1997) and the conserva-

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tion of its components in Euryarchaeota (Ishino *et al.* 1998; Cann *et al.* 1998a) clearly show that the euryarchaeotic organisms also depend on at least two DNA polymerases. In this review, we summarize the recent findings on the archaeal DNA replication apparatus, especially on the discovery of a novel DNA polymerase in the euryarchaeotes. The current understanding of DNA replication in the other two domains of life is also discussed where appropriate.

DNA REPLICATION

DNA replication, a process that ensures the maintenance of the integrity of the genome while allowing mutations that confer selective advantage to the offspring, is a critical process in the evolution of all species. The fundamental nature of the DNA replication process is underscored by the conservation of the function of individual proteins in both Bacteria and Eukarya (Stillman 1994). To summarize the process, it involves (1) the recognition of a replicational origin by the origin recognition proteins, (2) melting and unwinding of the duplex parental DNA by a replicative DNA helicase and topoisomerase in cooperation with a single-stranded DNA-binding protein, (3) synthesis of an RNA/DNA primer for the leading strand and for each Okazaki fragment on the lagging strand by a primase, (4) the clamp loader's recognition of the primer/template and loading of the sliding clamp that forms a ring around the duplex DNA behind the primer/template junction, (5) loading of the polymerase onto the DNA, and (6) elongation following the presence of all four dNTPs (deoxyribonucleoside triphosphates: dATP, dCTP, dGTP, dTTP). The RNA primers attached to the 5′-end of each Okazaki fragment are removed by an endonuclease and a ribonuclease, and the gaps created are filled by a DNA polymerase. The adjacent Okazaki fragments are then joined by a DNA ligase.

It should be noted that most of what is known about DNA replication in Bacteria is derived from an *Escherichia coli* system. In Eukarya, the major experimental model is the Simian Virus 40 DNA origin of replication and cell extracts from mammalian cells. There is no report on an experiment analyzing the molecular mechanism of DNA replication in Archaea. However, many homologs of the proteins required for eukaryotic DNA replication have been identified in the total genome sequences of several archaeal strains. In Table 1, we compared the archaeal homologs to the proteins involved in DNA replication in the two other domains. As noted above, the majority of the proteins involved in replicating the archaeal chromosome are eukaryotic type. The eukaryotic and bacterial replication mechanisms have been reviewed elsewhere (Kelman and O'Donnell 1995; Waga and Stillman 1998).

BACTERIAL AND EUKARYOTIC DNA POLYMERASES

During the past decade, significant advances in gene cloning techniques aided scientists to clone and express many genes that code for DNA polymerases. Eventually, this led to a proposal to classify DNA polymerases into family A, B, C, or X based on their amino acid sequences (Ito and Braithwaite 1991). These families are represented by *E. coli* DNA polymerase I (family A), DNA polymerase II (family B), DNA polymerase III α-subunit (family C), and others such as DNA polymerase β and terminal transferase (family X). Most of the biochemical properties of DNA polymerases in the same family are similar. In Bacteria and Eukarya, several types of DNA polymerases have been isolated and characterized (Table 2). The most thoroughly studied bacterial DNA poly-

TABLE 1

Replication proteins of Eukarya, Bacteria, and Archaea

Function	Archaeal	Eukaryal	Bacterial
Origin recognition	ORC1-like protein	Origin recognition complex (ORC) proteins 1–6	DnaA
Single-stranded DNA-binding	RPA-like protein	Replication protein A (RPA, three subunits)	Single-stranded DNA-binding protein (SSB)
Primer synthesis	Eukaryotic-like primase	DNA Pol α	DnaG
Helicase	Dna2-like, MCM-like	Dna2, MCM	DnaB
Clamp loader	RFC-like proteins (small, large)	RFC	γ-complex
Clamp (elongation factor)	PCNA-like proteins	PCNA	Pol III β-subunit
DNA strand synthesis	Family B DNA Pol Family D DNA Pol	DNA Pol α, δ, ε	Pol III
DNA strand ligation (on lagging strand)	DNA ligase (ATPdependent)	DNA ligase (ATPdependent)	DNA ligase (NADdependent)
Removal of primers	FEN1, RNaseH	FEN1, RNaseH	Pol I, RNaseH

This table includes both identified and predicted proteins.

merases are the *E. coli* proteins. *E. coli* Pol I and Pol II are implicated in the repair of damaged DNA. Both polymerases are single polypeptide enzymes, while Pol III, which is the DNA replicase of this organism, is a multisubunit enzyme (10 different subunits). In Eukarya five DNA polymerases (α, β, δ, ε, and γ) have been characterized in detail (Nethanel *et al.* 1988; Tsurimoto *et al.* 1990; Kornberg and Baker 1992; Waga and Stillman 1994; Sugino 1995; Wang 1996; Zlotkin *et al.* 1996). In addition, Pol ζ and η were recently characterized from yeast (Nelson *et al.* 1996; Johnson *et al.* 1999). Both are involved in DNA repair and mutagenesis. One more different DNA polymerase, essential for growth and probably repair, has been reported as Pol V (Sugino 1995). Pol α, δ, and ε are the DNA replicases and have multisubunit structures. The catalytic subunits of these polymerases for DNA polymerizing activity belong to family B (Table 2). The major function of Pol β is DNA repair, while Pol γ is responsible for replicating the mitochondrial DNA. All known viral DNA polymerases belong to family A or B. The extensive phylogenetic analysis of viral DNA polymerases have been reported (Heringa and Argos 1994; Knopf 1998).

In the eukaryotic DNA replication, DNA polymerase α forms a complex with DNA primase to synthesize RNA/DNA primers for initiation of leading strand synthesis and for each Okazaki fragment during lagging strand replication. Biochemical studies using plasmids containing the Simian Virus 40 origin of replication suggest that DNA polymerase δ replicates the leading strand and also completes the lagging strand in eukaryotic cells. Therefore, the DNA polymerase α/primase complex switches to DNA polymerase δ sometime after initiation. On the contrary, in *E. coli* a primase (DnaG) synthesizes the initial RNA primer, which is then elongated by the DNA polymerase III core enzymes. Therefore, the switch is from a primase to a DNA polymerase. Recently, it has been shown that this switch in *E. coli* requires the disruption of the primase-SSB (single-stranded DNA-binding protein) contact, which is triggered by the clamp loader complex (Yuzhakov *et al.* 1999). A remarkable feature of the *E. coli* replication apparatus is that the same protein complex synthesizes the leading and lagging strands simultaneously. The coordinated synthesis is made possible through the dimerization of the polymerase catalytic core (α, ε, θ), which is promoted by the τ subunit (Onrust *et al.* 1995b).

Pol δ and ε in Eukarya as well as low G + C Gram-positive bacteria (having class II Pol III) contain separate domains for DNA polymerizing and 3′ → 5′ exonucleolytic activities in the same polypeptide. In contrast, *E. coli* and many other bacteria have a class I Pol III, where the subunit for polymerase activity (α-subunit) differs from that for exonuclease activity (ε-subunit) as described by Huang and Ito (1998). The exonuclease activity preferentially excises a mismatched nucleotide from the primer terminus (Baker and Bell 1998). This activity increases the fidelity of DNA replication by three orders of magnitude (Kornberg and Baker 1992).

ARCHAEAL FAMILY B DNA POLYMERASES

Halophilic archaea were the first subjects in the study of archaeal DNA polymerases. Aphidicolin, a tetracyclic diterpenoid antibiotic, which is a specific inhibitor of DNA polymerase α from eukaryotic cells (Huberman 1981), was found to inhibit the growth of *Halobacterium halobium* (Forterre *et al.* 1984; Schinzel and Burger 1984). Therefore, it was hypothesized that the DNA replicase of Archaea was similar to that of Eukarya. Subsequently, α-like DNA polymerases were purified from *H. halobium* (Nakayama and Kohiyama 1985), *M. vannielii* (Zabel *et al.* 1985), and *Sulfolobus solfactaricus* (Rossi *et al.* 1986). Interestingly, there were other reports that, in contrast, described aphidicolin-resistant DNA polymerase activities in *S. acidocaldarius* (Klimczak *et al.* 1985), *Methanobacterium thermoautotrophicum* (Klimczak *et al.* 1986), *H. halobium* (Nakayama and Kohiyama 1985), and *Thermoplasma acidophilum* (Hamal *et al.* 1990). A few years after these reports, DNA polymerase genes were cloned from *S. solfataricus* (Pisani *et al.* 1992), *Thermococcus litoralis* (Perler *et al.* 1992), and *P. furiosus* (Uemori *et al.* 1993), which were all hyperthermophiles. Later, DNA polymerase genes were cloned

TABLE 2

Distribution of DNA polymerases in Archaea, Bacteria, and Eukarya

	Family					
Domain	A	B	C	D	X	?
Archaea						
Euryarchaeota		Pol BI		Pol D		
Crenarchaeota		Pol BI, Pol BII				
Bacteria	Pol I	Pol II	Pol III			
Eukarya	γ	α, δ, ε, ζ, V			β	η[a]

[a] This is a DNA polymerase yet to be assigned a family.

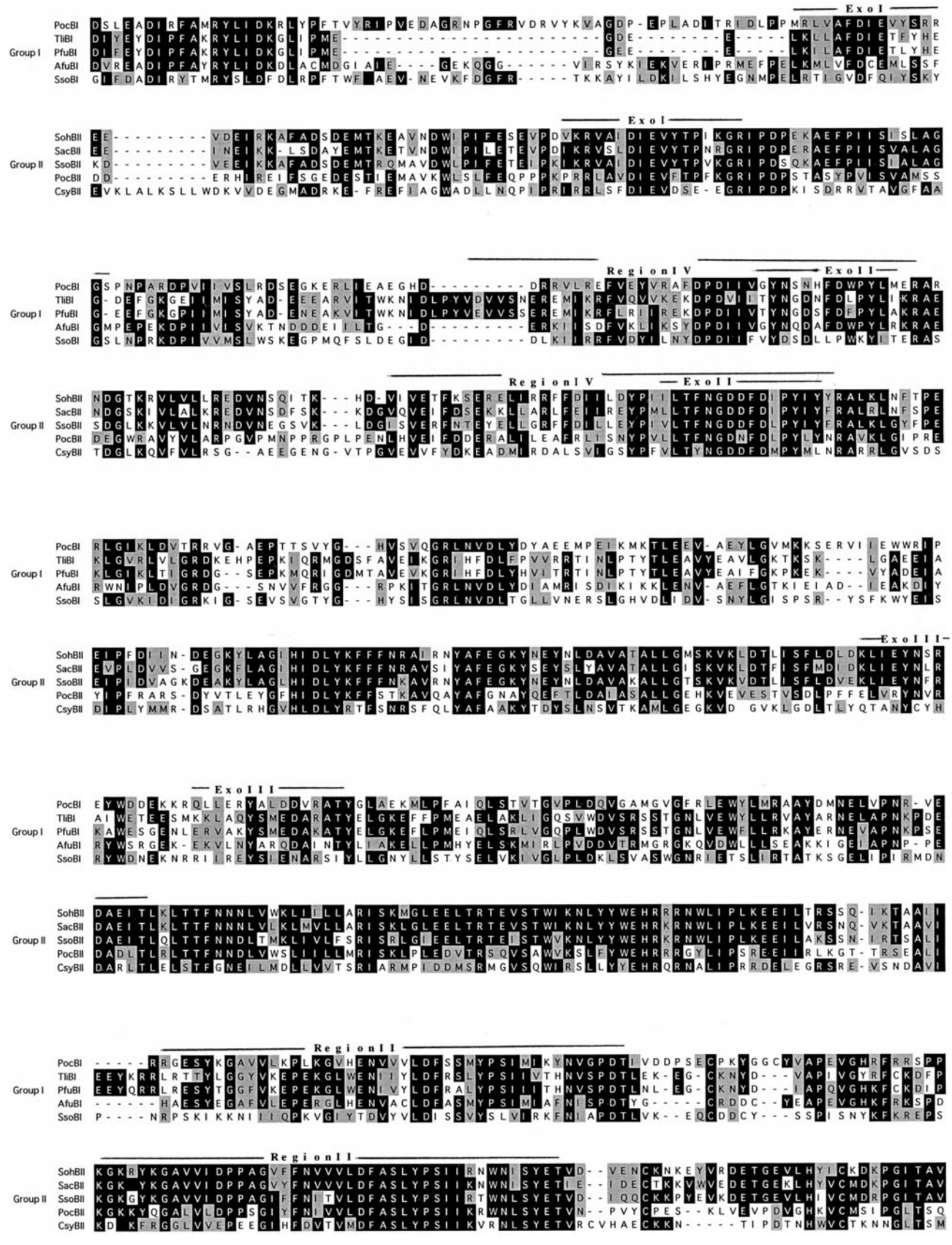
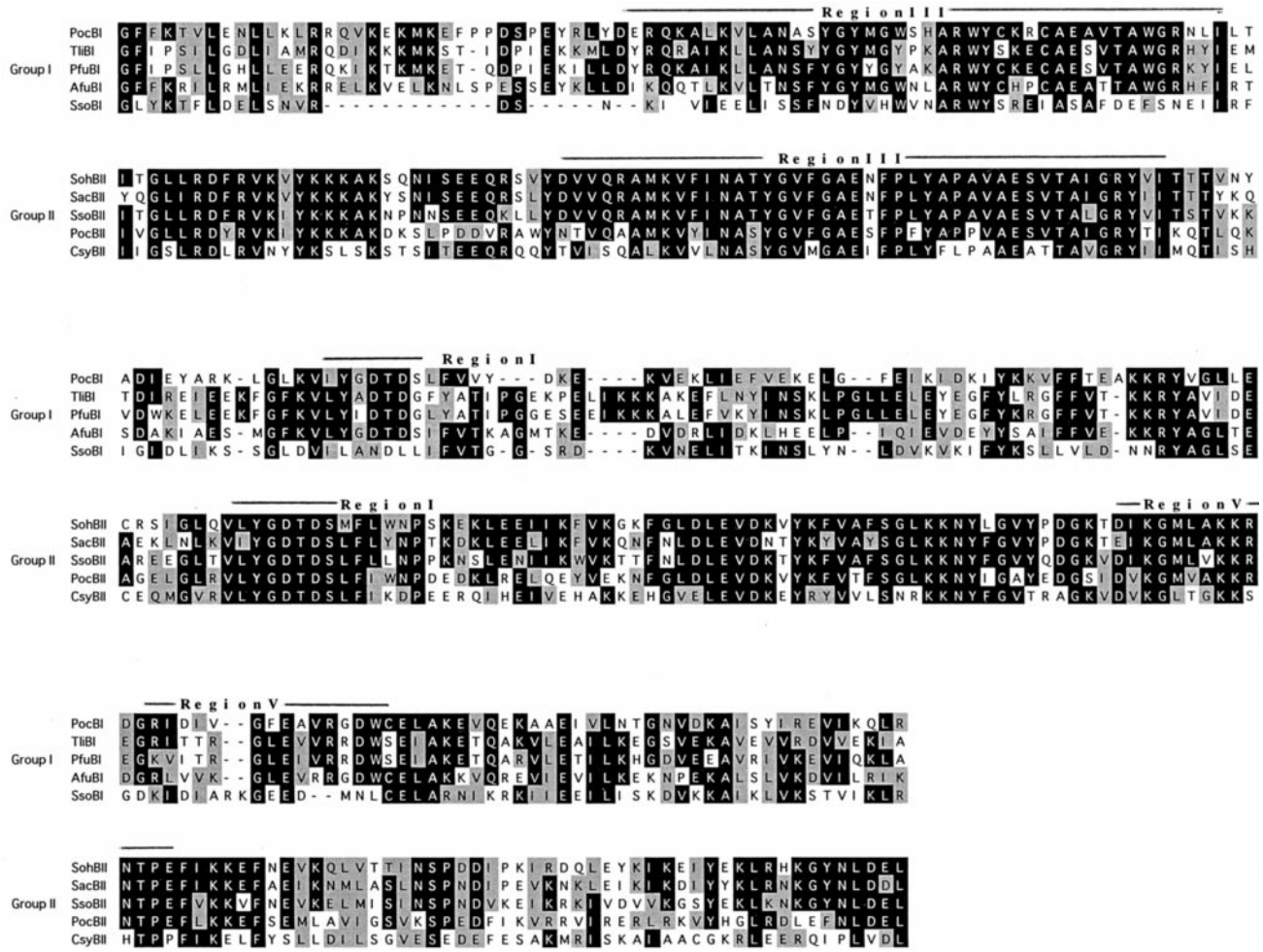


Figure 1.—Amino acid sequence alignment of family B DNA polymerase homologs (exonuclease and polymerase regions) found in both Crenarchaeotes and Euryarchaeotes (Group I) and only in Crenarchaeotes (Group II). The sequences were aligned with CLUSTAL W at a website (http://www.genome.ad.jp/SIT/CLUSTAL W.html). The conserved regions (regions I–V) in family B DNA polymerases originally proposed (Wong *et al.* 1988) and the motifs (Exo I–III) for 3′ → 5′ exonuclease

from the mesophilic methanogen *M. voltae* (Konisky *et al.* 1994) and the psychrophilic crenarchaeote *Cenarchaeum symbiosum* (Schleper *et al.* 1997). The deduced amino acid sequences of these genes contained the signatures of family B DNA polymerases.

It was significant that two different genes were cloned from *S. solfataricus* P2 (Prangishvili and Klenk 1994) and from *P. occultum* (Uemori *et al.* 1995), both of which encoded family B DNA polymerases. The genes from *P. occultum* were expressed in *E. coli* and both products actually exhibited DNA polymerase and exonuclease activities (Uemori *et al.* 1995). In addition, in the course of sequencing the genome of *S. solfataricus* P2, one more gene that is supposed to code for a family B DNA polymerase was identified (Edgell *et al.* 1997). Despite being conserved in the Sulfolobales, the amino acid sequence similarity of the third one (designated B2) to that of other family B members is weak, and there is still some uncertainty as to whether the gene product has DNA polymerase activity. If *S. solfataricus* contains three family B DNA polymerases, it would be a very interesting finding, because it suggests that the Crenarchaeota and Eukarya, which has three family B DNA polymerases (α, δ, and ε) in the nucleus for DNA replication, may share a similar molecular mechanism of DNA replication. *Pyrobaculum aerophilum* has open reading frames (ORFs) coding for proteins with similar amino acid sequences to *P. occultum* Pol I and Pol II (Fitz-Gibbon *et al.* 1997), and very recently, we cloned two family B DNA polymerase genes from *Aeropyrum pernix* (I. Cann, S. Ishino, N. Nomura, Y. Sako and Y. Ishino, unpublished results), an aerobic hyperthermophilic crenarchaeote (Sako *et al.* 1996). All of these organisms described as having two or three family B DNA polymerases belong to Crenarchaeota.

After we cloned a family B DNA polymerase gene



(Bernad *et al.* 1989) are shown. The conserved motifs in the catalytic domain, *i.e.*, motifs A, B, and C, are found in regions II, III, and I, respectively. The proteins and their accession numbers are as follows: PocBI (*P. occultum* Pol II, D38574), TliBI (*T. litoralis* Pol, M47198), PfuBI (*P. furiosus* Pol, D12983), AfuBI (*A. fulgidus* Pol, AE001070), SsoBI (*S. Solfataricus* Pol II, X71597), SohBII (*S. ohwakuensis* Pol I, AB008894), SacBII (*S. acidocaldarius* Pol I, U33846), SsoBII (*S. solfataricus* Pol I, U92875), PocBII (*P. occultum* Pol I, D38573), CsyBII (*C. symbiosum* Pol I, AF028831). The sequences of *A. pernix* Pol BI and Pol BII are not shown here. Positions of identical and similar amino acid residues are indicated by black and gray, respectively. Amino acids with similar properties are grouped into LIMV, AG, YWF, DEQN, KRH, and ST.

from *P. furiosus*, we attempted to clone another gene for a member of this family from *P. furiosus*, but we did not succeed. When the total genome sequence of *M. jannaschii* was published, several startling findings were reported (Bult *et al.* 1996). One of the inconsistencies concerned the DNA polymerase. Only one family B DNA polymerase was found in the whole genome as described above, and no more sequences likely to encode DNA polymerase were found. Accessibility to the complete genome sequences of *Archaeoglobus fulgidus*, *M. thermoautotrophicum*, and *P. horikoshii* enabled us to search for their genes encoding family B DNA polymerases. Each of these euryarchaeotes possesses one recognizable family B DNA polymerase gene as well.

The archaeal family B DNA polymerases are, overall, similar in amino acid sequence; however, they can be divided into two groups. The euryarchaeotic family B DNA polymerases are very similar to one of the crenarchaeotic homologs and can be placed under one group (Group I), whereas the other group contains only crenarchaeotic members (Group II in Figure 1). The amino acid sequence identities within the groups are over 35%; however, they are about 20% in the case of intergroup comparisons. Regions I, II, and III, which are important to the formation of the catalytic domain, are not strictly conserved between the two groups. This may affect the difference of sensitivity to aphidicolin as described below. At this point, the nomenclature of archaeal DNA polymerase has to be considered. We have been using I and II as the order of discovery. However, distribution of DNA polymerases is different between Euryarchaeota and Crenarchaeota and, therefore, it happens that the two DNA polymerases called Pol I or Pol II sometimes belong to the different families as described below. To avoid this problem, we propose here that the two family B DNA polymerases found in Crenarchaeota be called Pol BI and Pol BII, and the one that is common with Euryarchaeota should be called Pol BI to signify the first enzyme found in this subdomain (Table 2). Then, *P. furiosus* Pol I, *P. occultum* Pol II, and *A. pernix* Pol II become Pol BIs and belong to Group I. *P. occultum* Pol I, *A. pernix* Pol I, and *Sulfurisphaera ohwakuensis* Pol I are Pol BIIs and belong to Group II. From our knowledge, Group I and Group II enzymes are sensitive and resistant, respectively, to aphidicolin at a concentration of 2 mm (the resistance of *S. ohwakuensis* Pol I is a personal communication from N. Kurosawa). An exception is SsoBII from *S. solfataricus*, which is sensitive to aphidicolin (Y. Taguchi and Y. Ishino, unpublished results), even though its sequence is more similar to Group II (BII) as shown earlier (Forterre *et al.* 1994). The difference between the two family B DNA polymerases, in terms of their biological roles in the crenarchaeotic cells, is a very important and interesting subject and should be investigated.

Some DNA polymerases from hyperthermophilic archaea are commercially available as PCR enzymes. One of the remarkable advantages pertaining to the use of archaeal DNA polymerases for PCR, instead of Thermus DNA polymerases such as *Taq* polymerase, is the high fidelity of DNA strand synthesis derived from their associated strong $3' \rightarrow 5'$ exonuclease activity (Lundberg *et al.* 1991; Mattila *et al.* 1991; Takagi *et al.* 1997). Archaeal family B DNA polymerases can elongate primers *in vitro* by themselves, even though they generally have very low processivities, for example, seven dNTP/binding for *T. litoralis* DNA polymerase (Perler *et al.* 1996). The limitation of the archaeal enzymes for PCR may be this low elongation ability.

The three-dimensional structures of several nucleotide polymerases using DNA or RNA as a template have been solved as described below. However, the structure of family B DNA polymerases was not known until recently. The three-dimensional structure of the family B DNA polymerase from *E. coli* bacteriophage RB69 was solved in 1997. The structure of its catalytic palm domain was found to be basically the same shape as that of family A DNA polymerase, reverse transcriptase, and RNA polymerase (Wang *et al.* 1997). However, the structures of the fingers and thumb domains are unrelated to all other known polymerase structures. Further analyses of the structure–function relationship of the DNA polymerases of this family are still necessary. The family B DNA polymerases from the hyperthermophilic archaea are useful for this purpose, because of the excellent stability of these proteins. Crystal formation of three DNA polymerases and preliminary diffraction analysis from *P. furiosus* (Goldman *et al.* 1998), *S. solfataricus* (Nastopoulos *et al.* 1998), and *Thermococcus* sp. 9°N-7 (Zhou *et al.* 1998) were published at the end of last year. We will be able to see and compare the structures of these DNA polymerases soon.

A unique finding is that the archaeal family B DNA polymerases often contain inteins, which are the intervening sequences spliced out as proteins and not as mRNAs (Cooper and Stevens 1993; Perler *et al.* 1994). After the production of the precursor protein, the intein is excised from the protein and the external protein regions, which are termed exteins, are joined together. Archaeal DNA polymerases contain hot spots for insertion of inteins. The regions that contain inteins are regions I, II, and III (originally proposed by Wong *et al.* 1988, these contain the most conserved motifs, *i.e.*, motifs A, B, and C, proposed by Delarue *et al.* 1990). These three regions are actually important for the formation of the catalytic center of DNA polymerizing activity (Table 3). Inteins are also inserted into the conserved motifs in the archaeal homologs of replication factor C (RFC) as described below. The archaeal homologs of the proliferating cell nuclear antigen (PCNA) found to date do not contain inteins. An interesting observation is that, so far, all intein-containing DNA polymerases come from the euryarchaeotes. Two inteins each were found in the precursor proteins from *T. litoralis* (Perler

TABLE 3

Inteins found in some proteins involved in archaeal DNA replication

Protein	Origin	Number of intein	Insertion site
Family B DNA Pol	*P. horikoshii*	1	Motif B
	Pyrococcus sp. *KOD*	2	Motif A, B
	Pyrococcus sp. *GB-D*	1	Motif B
	M. jannaschii	2	Motif A, B
	T. litoralis	2	Motif B, C
	T. fumicolans	2	Motif A, C
Family D DNA Pol, DP2	*P. horikoshii*	1	Motif ND
RFC small subunit	*P. horikoshii*	1	Box III
	P. furiosus	1	Box III
	M. jannaschii	3	Box III, IV, ND

ND, a conserved region without a designated name (see Figures 3 and 5).

et al. 1992), *M. jannaschii* (Bult *et al.* 1996), *T. fumicolans* (Cambon and Querellou 1996), and *Pyrococcus* sp. KOD1 DNA polymerase (Takagi *et al.* 1997). One intein was found in each of the precursors from *Pyrococcus* sp. GB-D (Xu *et al.* 1993) and *P. horikoshii* (I. Matsui, personal communication).

EURYARCHAEOTIC Pol II (Pol D)

Identification of a novel DNA polymerase activity in *P. furiosus*: *P. furiosus* cell extract was fractionated by an anion exchange chromatography, and each of the fractions was analyzed for DNA polymerase (deoxynucleotide incorporation) activity. Three different DNA polymerase activities (I, II, and III) were detected in the cell extracts (Imamura *et al.* 1995). The activities in fractions I and II were sensitive to aphidicolin, which suggested the presence of family B DNA polymerases in these fractions. The enzyme eliciting DNA polymerizing activity in fraction I was purified, and its N-terminal amino acid sequence matched that of *Pfu* Pol I (Pol BI), which we previously cloned (Uemori *et al.* 1993). To purify the protein responsible for the DNA polymerase activity in fraction III, the cell extracts were passed through several purification steps. An *in situ* (in SDS-PAGE) DNA polymerase assay (Wernette and Kaguni 1986) suggested that the DNA polymerase activity originated from a protein of molecular mass 130–135 kD (Imamura *et al.* 1995). We designated the enzyme as *P. furiosus* DNA polymerase II (*Pfu* Pol II) as the second enzyme discovered in this organism. The activity in fraction II, which has not yet been identified, could be a third DNA polymerase in *P. furiosus*, or *Pfu* Pol I (BI) might have formed a complex with some other proteins and eluted at a different place in the chromatography. Further analyses are necessary to clarify this finding.

Gene cloning: A cosmid library, which contained *P. furiosus* genomic DNA inserts ranging in size from 35 to 50 kb, was screened for the gene encoding *Pfu* Pol II. Out of 500 heat-treated cell extracts prepared from independent transformants carrying each recombinant cosmid, 9 produced heat-stable DNA polymerases. Five clones contained inserts originating from the same region of *P. furiosus* genomic DNA; however, note that the restriction enzyme digestion pattern was different from that of the region that contained the gene for *Pfu* Pol (BI) previously cloned (Uemori *et al.* 1993). Within the cloned 10-kb *Xba*I fragment, which contained the genes producing the protein or proteins responsible for the novel heat-stable DNA polymerase activity, there were five continuous ORFs transcribed together as a single operon as shown in Figure 2 (Uemori *et al.* 1997). Nested deletion analysis of the corresponding genes indicated that the DNA polymerase activity originated from the products of the second and third genes in the operon. The proteins produced by the second and third ORFs were named DP1 and DP2, respectively, as the subunits of Pol II (Figure 2). The estimated molecular mass of DP1 was 69 kD, while that of DP2 was 143 kD. The deduced N-terminal amino acid sequence of DP2 from the nucleotide sequence matched that of the 130- to 135-kD protein that was purified from *P. furiosus* (Imamura *et al.* 1995), and therefore, the name *Pfu* Pol II was given to the heterodimeric DNA polymerase constituted by DP1 and DP2. Although the 130-kD protein band was detected in our previous activity gel assay, purified DP2 protein from recombinant *E. coli* strains possessed very little DNA polymerase activity by itself in a conventional solution assay of [^{3}H]TTP incorporation. The distinct activity was detected only in the presence of both proteins (DP1 and DP2) in a reaction mixture, and the activity from DP2 was only 2% of the full activity from DP1-DP2 complex. No activity was detected from DP1 (Uemori *et al.* 1997). Thus, *Pfu* Pol II actually comprises two proteins, a small subunit (DP1) and a large subunit (DP2). *Pfu* Pol II possesses a very strong

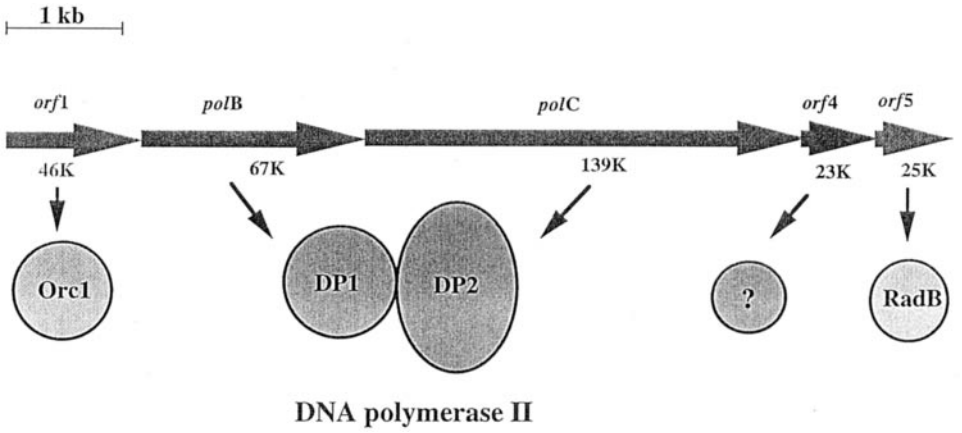


Figure 2.—An operon containing the genes for Pol II (Pol D) found in *P. furiosus*. Five structural genes are indicated by the large arrows with the encoded products. The Rad51-/Dmc1-like protein in this operon was named RadB to distinguish it from RadA, another homolog found in this organism (DiRuggiero and Robb 1998).

3′ → 5′ exonuclease activity. This proofreading property is also detected only in the presence of its two components. An immunological analysis showed that DP1 and DP2 interact with each other to form a complex in *P. furiosus* cells (Cann *et al.* 1998a).

Conservation of euryarchaeotic Pol II: Upon publication of the genome sequence of *M. jannaschii*, ORFs coding for homologs of *Pfu* DP1 (40% identity) and *Pfu* DP2 (60% identity) were found. The genes for these two ORFs were expressed in *E. coli*, and both DNA polymerase and 3′ → 5′ exonuclease activities were confirmed (Ishino *et al.* 1998). Subsequently, homologs of both DP1 and DP2 were found in the complete genome sequences of *M. thermoautotrophicum*, *A. fulgidus*, and *P. horikoshii* (Cann *et al.* 1998a). In each of these three euryarchaeotes, DP1 and DP2 are highly conserved (Table 4). In addition, using primers based on conserved motifs in DP2, the presence of a homolog has been demonstrated in *Methanopyrus kandleri* (I. Cann and Y. Ishino, unpublished results), which is one of the most ancient of known hyperthermophilic archaea, according to the phylogenetic tree based on 16S rRNA.

So far, every archaeon that has been shown to contain DP1 and DP2 belongs to Euryarchaeota. There is no evidence suggesting the presence or absence of DP1 and DP2 homologs in crenarchaeotic cells. In most of the euryarchaeotes investigated, the genes coding for DP1 and DP2 occur at different regions of the genome as described earlier (Ishino *et al.* 1998). However, they are arranged in tandem in the genus Pyrococcus (*P. furiosus*, Uemori *et al.* 1997; *P. woesei*, I. Cann and Y. Ishino, unpublished results; *P. horikoshii*, Kawarabayasi *et al.* 1998; *P. abyssi*, J. Querellou, personal communication). The significance of the different gene arrangements is not known. However, we expect the arrangement to regulate the production level of Pol II in the cells.

Comparison of euryarchaeotic DP1 with known DNA polymerases: Euryarchaeotic DP1s exhibited weak but significant similarities on the amino acid level to the small subunit of eukaryotic DNA polymerase δ (Cann *et al.* 1998a). It has been shown that the subunits from mammalian cells are required for efficient stimulation of the polymerase processivity of Pol δ by PCNA (Sun *et al.* 1997; Zhou *et al.* 1997). A homolog of this protein is not found in published bacterial genomes (Kaneko *et al.* 1996; Blattner *et al.* 1997; Tomb *et al.* 1997). Thus, this finding further strengthens the archaeal-eukaryotic relationship. The amino acid sequence alignment of the archaeal and eukaryotic proteins showed a high similarity from the central to the C-terminal region. Within this region, several conserved motifs that are likely to play important roles in the function of this protein were noted. In the central region, there were conserved motifs that were found only in the euryarchaeotic homologs. These motifs may be involved in the interaction of DP1 with DP2 and perhaps with other accessory proteins. A recent report shows that the DP1s also contain in their central and C-terminal regions the four conserved motifs that define the superfamily of calcineurin-like phosphatases (Aravind and Koonin 1998). The motifs contain conserved histidine and aspartate residues that are known to be involved in metal coordination and catalysis (Goldberg *et al.* 1995). Two hypotheses have been proposed for the high conservation of these motifs in DP1 as follows: (i) pyrophosphate hydrolysis, which will increase DNA polymerization rate and (ii) PCNA binding. It is of interest to note that in the second subunit of eukaryotic Pol δ these motifs are missing. The diverged N-terminal regions among the archaeal and eukaryotic subunits are expected to be involved in species-specific interactions. *In vitro* deletion analyses showed that the C-terminal two-thirds of DP1 is important for its interaction with DP2 to elicit DNA polymerase activity (I. Hayashi, I. Cann, S. Ishino, K. Morikawa and Y. Ishino, unpublished results). DP1 proteins from the Pyrococci and *M. jannaschii* are significantly larger than the second subunit of the eukaryotic Pol δ and also the euryarchaeotic DP1s from *A. fulgidus* and *M. thermoautotrophicum*. Comparison of homologous proteins shows that hyperthermophilic proteins tend to be shorter than their mesophilic counter-

TABLE 4

A comparison of euryarchaeotic DP1 and DP2 homologs

	% identity		
	P. furiosus	*A. fulgidus*	*M. thermoautotrophicum*
DP1			
A. fulgidus	38.4		
M. thermoautotrophicum	36.9	42.6	
M. jannashii	32.6	41.4	35.8
DP2			
A. fulgidus	52.6		
M. thermoautotrophicum	53.4	50.2	
M. jannaschii	54.3	52.7	53.1

parts (Russell *et al.* 1997; Cann *et al.* 1998b). The opposite seemed to be true for DP1 proteins. In our opinion, the longer hyperthermophilic DP1s may harbor additional functions.

It has been published very recently that the second subunits of eukaryotic Pol α, δ, ε, and euryarchaeotic DP1 constitute a family (DNA polymerase-associated B subunits) by the sequence similarity (Makiniemi *et al.* 1999). The second subunit of Pol α has been implicated in cell-cycle control and regulation (Nasheuer *et al.* 1991; Foiani *et al.* 1994), and a stable Pol ε complex essential for chromosomal replication requires the second subunit (Araki *et al.* 1991). Therefore, it becomes more interesting to understand the roles of DP1 in the euryarchaeotic cells. The large subunits of eukaryotic Pol α, δ, and ε all belong to family B. By analogy, DP1 might be expected to interact with *Pfu* Pol I (BI), a family B DNA polymerase. However, Pol I (BI) activity was not affected by the addition of DP1 *in vitro* and an interaction was not detected *in vivo* by immunological analysis (Cann *et al.* 1998a). Hence, it is possible that DP1 and DP2 are specific partners in the formation of Pol II in euryarchaeotic cells. It is not known if any other protein that has sequence similar to that of the proteins in the family of DNA polymerase-associated B subunits, which interacts with Pol I (BI), exists in the euryarchaeotes.

Comparison of euryarchaeotic DP2 with known DNA polymerases: A database search, using the computer-assisted homology search facility on the World Wide Web (http://www.ncbi.nlm.nih.gov/) and the BLAST algorithm (Altschul *et al.* 1990) to scan GenBank and other nonredundant databases, did not yield any proteins of significant similarity to euryarchaeotic DP2. However, the protein is highly conserved in euryarchaeotes (Table 4). The homologs that are known share more than 50% amino acid conservation.

The three-dimensional structure of the polymerase domain of nucleotide polymerases is suggestive of a right hand, in which the palm, fingers, and thumb form the DNA-binding crevice (Ollis *et al.* 1985; Kohlstaedt *et al.* 1992; Sousa *et al.* 1993; Kim *et al.* 1995; Hansen *et al.* 1997; Kiefer *et al.* 1997; Li *et al.* 1998). Within the palm subdomain are two motifs (motifs A and C) containing two invariant carboxylates, which are thought to constitute part of the polymerase active site. Amino acid sequences resembling motifs A and C were found in DP2s (Figure 3), but not in DP1s, by visual inspection of four euryarchaeotic DP2s (Cann *et al.* 1998a). In addition to the detection of weak activity from *Pfu* DP2 protein by itself as described above, the DP2s have been proposed as the catalytic subunit of the euryarchaeotic heterodimeric DNA polymerase by these motifs. The genes coding for inteins tend to invade the regions encoding indispensable motifs in proteins as discussed in archaeal family B DNA polymerases. An intein is found in the DP2 of *P. horikoshii* within a highly conserved motif (GYAHYFHAAKRRNCDGDED) in all known DP2 proteins (Table 3). The function of the motif in which the intein occurs (Figure 3) in *P. horikoshii* DP2 is not known, but it is very close to the putative catalytic residues of Pol II (Cann *et al.* 1998a). It may, therefore, be crucial for maintaining the integrity of active site conformation. Site-directed mutagenesis within this motif is required to examine this hypothesis.

In the middle and C-terminal regions of DP2s, zinc-finger motifs that are likely to be involved in interactions with other proteins, in addition to DNA binding, are conserved. At the C-terminal region of all known DP2s are two conserved motifs with amino acid sequences similar to the so-called PIP (PCNA interacting protein)-box (Warbick 1998). Euryarchaeotic DP2s were, therefore, expected to interact with PCNA homologs found in Euryarchaeota, and this is discussed later.

The amino acid sequence of the catalytic subunit of Pol II does not belong to any family proposed so far as described, and therefore we propose here family D for euryarchaeotic Pol IIs, and Pol IIs are renamed Pol D by following our proposal that gives Pol BI and BII for family B DNA polymerases as described above (Table 2).

Biochemical characterization of euryarchaeotic Pol II (Pol D): *Mja* Pol D, produced in *E. coli* cells, was

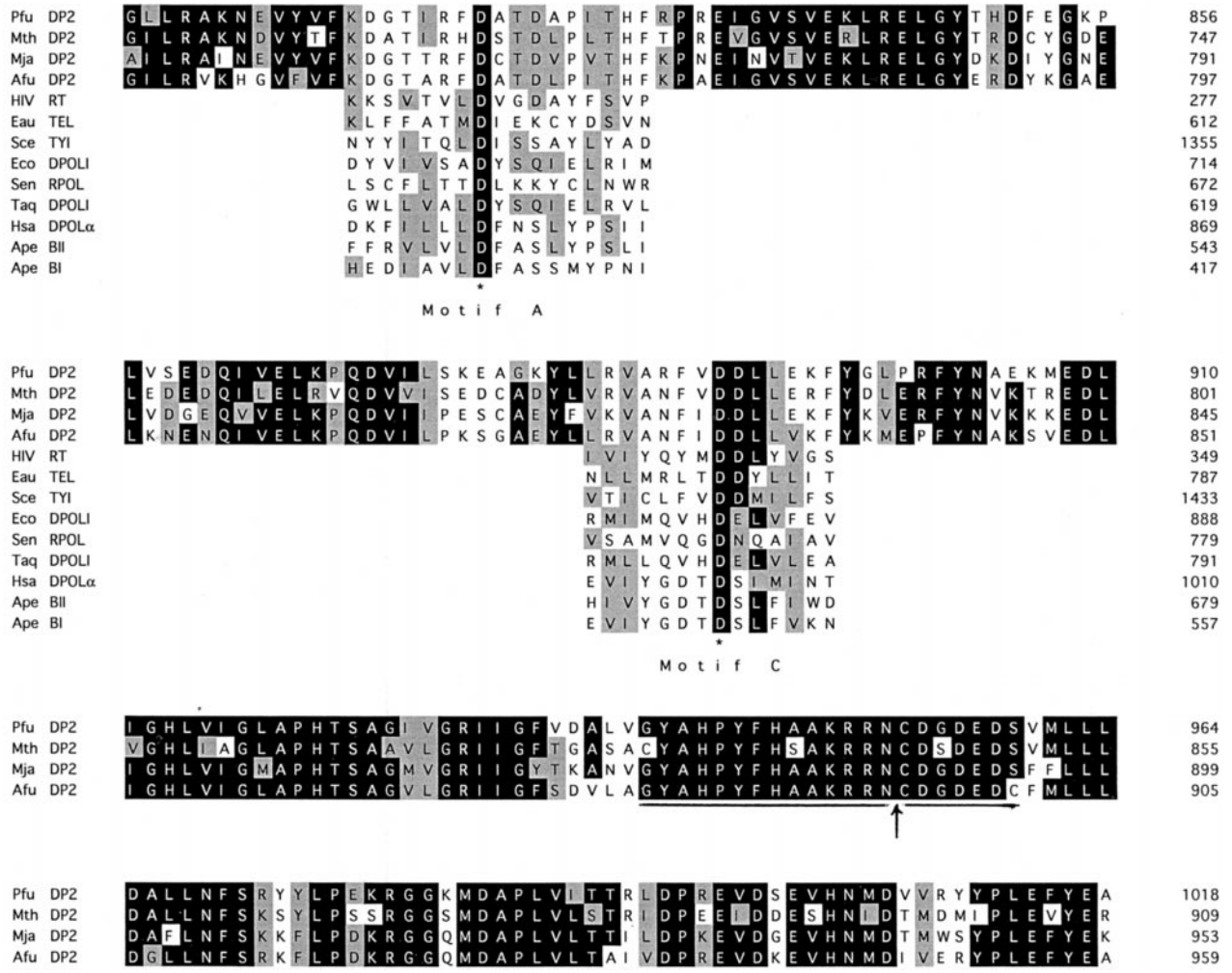


Figure 3.—Amino acid sequence alignment of the region containing the putative DNA polymerase motif A and motif C of euryarchaeotic DP2. The DP2s shown and their accession numbers are *P. furiosus* (Pfu, D84670), *M. jannaschii* (Mja, D64503), *M. thermoautotrophicum* (Mth, AE000913), and *A. fulgidus* (Afu, AE000984). The motifs A and C are aligned with those of other polymerases and the asterisks indicate invariant carboxylates probably essential for catalysis. The intein-insertion site within the DP2 from *P. horikoshii* is indicated by an arrow. To avoid two homologs from the same genus, the *P. horikoshii* DP2 is not included in the alignment. Positions of identical and similar amino acid residues are indicated by black and gray, respectively.

biochemically characterized, and its properties were compared with those of *Pfu* Pol D (Ishino *et al.* 1998). A polyclonal antibody raised against *Pfu* Pol D reacted with both *Mja* DP1 and DP2. Similar to *Pfu* Pol D, *Mja* Pol D possesses an extremely active 3′ → 5′ exonuclease activity. The DNA polymerase activity of each Pol D is sensitive to N-ethylmaleimide (NEM). On the other hand, they are resistant to aphidicolin. Both Pol Ds are more sensitive to ddTTP and salt (KCl) than *Pfu* Pol BI. These reactions of the euryarchaeotic Pol D to the above reagents are different from that of DNA polymerases referred to in the book by Kornberg and Baker (1992).

The subunits of *Pfu* Pol D can complement those of *Mja* Pol D to yield DNA polymerase activity and vice versa (Ishino and Cann 1998). However, there seems to be a perceptible incompatibility between *Pfu* DP1 and *Mja* DP2. *Pfu* Pol D is more heat stable than *Mja* Pol D. Incubations at 94° for 20 min did not affect DNA polymerase activity of *Pfu* Pol D. In fact, DNA polymerase activity improved with longer periods of preincubation at temperatures close to the optimum for growth. In contrast, preincubating *Mja* Pol D at temperatures above 65° resulted in significant loss of DNA polymerase activity. When *Mja* DP1 was complemented with *Pfu* DP2, temperature stability was significantly improved (Y. Ishino, K. Komori, S. Ishino and Y. Koga, unpublished results). The optimum temperatures for growth of *P. furiosus* and *M. jannaschii* are 100° and 88°, respectively. While the high thermostability of *Pfu* Pol D was expected, the significant instability of *Mja* Pol D at a temperature well below its optimum was very surprising. These observations were, however, made *in vitro.* Thus, we hypothesize that under *in vivo* conditions there are factors that aid in the stabilization of *Mja* Pol D.

Purified *Pfu* Pol BI and Pol D investigated under the same conditions suggested several distinct differences in their characteristics (Uemori *et al.* 1997). Pol D preferred the single-primed template DNA to gapped double-stranded DNA, such as DNase I-activated DNA. This is in contrast to *Pfu* Pol BI. Moreover, Pol D can utilize RNA primers, whereas Pol BI cannot. Because of its strong primer elongation ability, we are tempted to hypothesize that Pol D is the replicase of the euryarchaeotes. To confirm or refute this proposal, further work is warranted.

ACCESSORY FACTORS

The sliding clamp: The basic function of a replicase is to accurately duplicate the chromosome at a very high speed. Studies on *E. coli* and eukaryotic replicases have shown that the replicases comprise three functional components: (i) a DNA polymerase, (ii) a processivity factor, which is also called the clamp, and (iii) a multisubunit clamp loader (Turner *et al.* 1999). Despite the inherent ability of the polymerase to synthesize DNA, it is able to attain the high processivity required to duplicate the genome only in the presence of the processivity factor. A doughnut-shaped structure, referred to as the sliding clamp, is formed by a homodimer of the β-subunit of the *E. coli* holoenzyme, while in eukaryotes it is formed by a homotrimer of the PCNA. The clamps are topologically linked to the DNA duplex without physical contact and through their interaction with the DNA polymerase, they ensure proximity and tracking along the DNA template (Stukenberg *et al.* 1994). The human PCNA migrates on polyacrylamide gels to a position corresponding to a mass of 36 kD, which is larger than the molecular mass estimated from its amino acid sequence (Zhang *et al.* 1995). Despite the fact that they are not very similar on the amino acid level, the human and yeast PCNAs portray an almost identical three-dimensional shape (Krishna *et al.* 1994; Gulbis *et al.* 1996; Kelman 1997). The structure is similar to that of the β-subunit of the holoenzyme and the gp45 protein, which are the functional homologs in *E. coli* and T4 phage, respectively. In addition to its stimulation of pol δ processivity (Bauer and Burgers 1988), PCNA has also been reported to stimulate pol ε processivity (Lee *et al.* 1991) under certain conditions. PCNA has also been reported to interact with several proteins involved in DNA repair and cell-cycle control such as the nucleotide excision repair protein XPG (Gary *et al.* 1997), FEN1 (Warbick *et al.* 1997), the cyclin-dependent kinase (CDK) inhibitor protein p21 (Gulbis *et al.* 1996), the p53-regulated protein Gadd45 (Smith *et al.* 1994), and the mismatch repair proteins MLH1 and MSH2 (Umar *et al.* 1996).

The amino acid sequences similar to the eukaryotic PCNAs were found in all completely sequenced archaeal genomes (Figure 4). The amino acid sequence identities between the eukaryotic PCNA and euryarchaeotic homologs are about 23%, with the highest value of 28% occurring between the human and *M. thermoautotrophicum* homologs. Using the sequence of the PCNA homolog from the *P. horikoshii* genome, we cloned the homolog from *P. furiosus* and expressed and characterized the protein (I. Cann, S. Ishino, I. Hayashi, H. Toh, K. Morikawa and Y. Ishino, unpublished results). Our preliminary results show that in solution, the PCNA homolog from *P. furiosus* (*Pfu* PCNA) exists in an oligomeric state, as observed with other PCNA homologs (Zhang *et al.* 1995). We also detected interactions of *Pfu*-PCNA with both *Pfu* Pol BI and *Pfu* Pol D, even though the exact roles of these DNA polymerases in euryarchaeotic cells are not yet known. The β-subunit of Pol III enhances the processivity of the *E. coli* replicase and has also been reported to increase the processivity of *E. coli* Pol II, an enzyme implicated in DNA repair (Bonner *et al.* 1992). Therefore, it was not surprising to find an interaction of PCNA with both DNA polymerases. The conserved motifs, resembling the PIP-box, are found in the extreme C-terminal regions of euryarchaeotic DP2, thus suggesting the universality of this motif in proteins interacting with PCNA (Table 5). The significance of the presence of two PIP-boxes in DP2s remains to be clarified. A convincing PIP-box-like sequence was not found in *Pfu* Pol BI.

Recently, three proteins with homologies on the amino acid level to PCNA were found in the genome of the crenarchaeote *A. pernix* (Y. Kawarabayasi, personal communication). From this organism we have already cloned and expressed two family B DNA polymerase (BI and BII) genes, as incidated above. Others (Edgell *et al.* 1997) have suggested the presence of three family B DNA polymerases in *S. solfataricus* P2. While a single PCNA homolog is found in the euryarchaeotes, the foregoing findings seem to suggest the presence of multiple PCNA homologs in the Crenarchaeota. An interesting question then arises. Do these three proteins exist in cells as individual PCNA homologs or do they come together to form a heterotrimer? As more research is carried out in this area, this cryptic observation will be clarified.

The clamp loader: Because the sliding clamps or processivity factors are ring shaped, an initial opening of the ring prior to loading onto the DNA is required, and this task is accomplished by the clamp loader. Amino acid sequence comparisons indicate that a family of related proteins capable of performing this function is present in both Eukarya and Bacteria (Cullmann *et al.* 1995). In humans and other eukaryotes, the clamp loader is referred to as the replication factor C (RFC), while in *E. coli* the functional homolog is the γ-complex of Pol III. The RFC and the γ-complex are each composed of five subunits, and each possesses an ATPase activity that is stimulated when bound to DNA and also

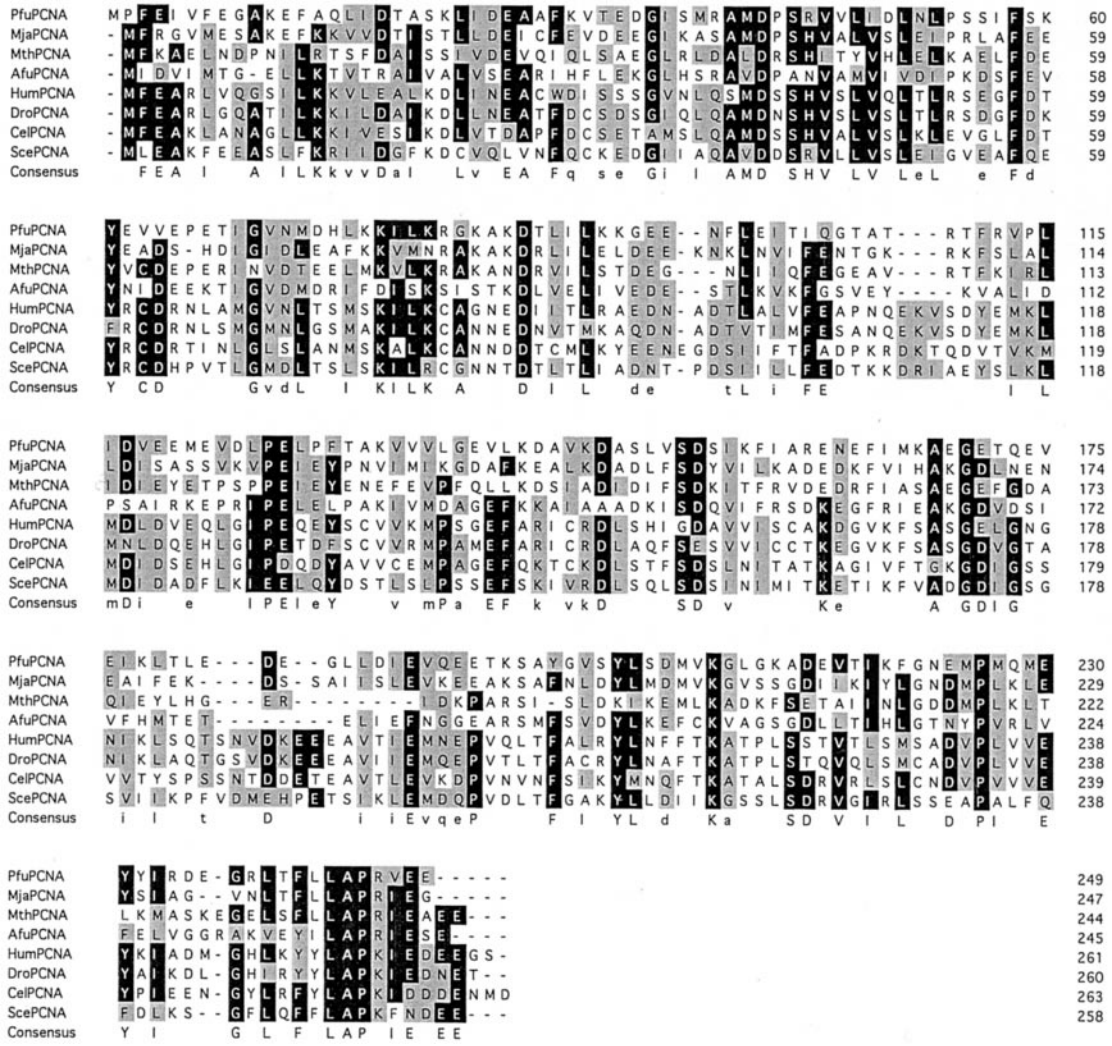


Figure 4.—Amino acid sequence alignment of euryarchaeotic and eukaryotic PCNA homologs. Four homologs from each group were used. The euryarchaeotic proteins and their accession numbers are *P. furiosus* (Pfu, AB017486), *M. jannaschii* (Mja, Q57797), *M. thermoautotrophicum* (Mth, sp027367), and *A. fulgidus* (Afu, AE001081), while those of the eukaryotic proteins are *Homo sapiens* (Hum, P12004), *Drosophila melanogaster* (Dro, P17917), *Caenorhabditis elegans* (Cel, sp002115), and *Saccharomyces cerevisiae* (Sce, P15873). Amino acid residues that are identical (black) or similar (gray) in >50% of the positions are indicated. Uppercase letters indicate consensus at residues with identities at >50% positions, including at least one from each domain.

by the clamp (PCNA or the β-subunit). To assemble the γ-complex (γ, δ, δ′, χ, ψ), the δ′- and ψ-subunits bind directly to the γ-subunit, followed by the binding of δ and χ to the δ′- and ψ-subunits, respectively (Onrust *et al.* 1995a). In a previous study, it was shown that a complex of γδδ′ was sufficient to load the clamp onto DNA (Onrust *et al.* 1991). Recently, however, the γ-complex has been shown to facilitate the disruption of the primase-SSB contact, which results in a switch from the primase to the holoenzyme during clamp loading on the lagging strand (Yuzhakov *et al.* 1999). This occurs through the binding of the χ-subunit to SSB (Turner *et al.* 1999). The RFC subunits are named according to their apparent sizes on SDS-PAGE (Cullmann *et al.* 1995); hence the human subunits are hRFC140, hRFC36, hRFC37, hRFC38, and hRFC40. The similarity between the γ-complex and the eukaryotic RFC suggests similarity in the mechanism by which they load the clamp onto DNA. However, note that while the RFC subunits appear to be present in equimolar amounts in the complex, two copies of the γ-subunit are found in the *E. coli* clamp-loader (Mossi and Hubscher 1998), which may suggest some differences in their mechanisms.

As shown in Figure 5, all of the RFC subunits share some conserved amino acid sequences, and two subunits of RFC are found in each of the completely sequenced archaeal genomes. We have cloned both subunits (small, *Pfu* RFCS, 37.4 kD; and large, *Pfu* RFCL, 55.3 kD) from *P. furiosus* and expressed them in *E. coli* (I. Cann, S. Ishino and Y. Ishino, unpublished results). Four archaeal RFCS share 60% identity and are most

TABLE 5

Conserved sequences in Archaea resembling PCNA-binding motifs in Eukarya

Domain	Protein	PIP sequence	Position	Size (amino acids)
Archaea	Pfu DP2	EtiLNshl	(1096–1103)	1263
	Pfu DP2	visLDdFF	(1253–1261)	
	Mja DP2	EkvIQshF	(1031–1038)	1139
	Mja DP2	QvkLsdFF	(1129–1136)	
	Mth DP2	EgvLmshF	(987–994)	1092
	Mth DP2	QssLDvFl	(1085–1092)	
	Afu DP2	ErvINvhF	(1037–1044)	1143
	Afu DP2	QvsISdFv	(1136–1143)	
	Pfu RFCL	QatLfdFl	(470–476)	479
	Mja RFCL	QltLDaFF	(508–515)	516
	Afu RFCL	NltLDsFF	(471–478)	479
	Mth RFCL	QtsLfqFs	(472–478)	479
Eukarya	Human XPG	QlrIDSFF	(990–997)	1186
	Human Fen1	QgrLDdFF	(337–344)	380
	Human p21	QtsMTdFy	(144–151)	164
	Human MCMT	QttITshF	(164–171)	1616
Consensus		QXXhXXaa		

The PIP-box or consensus minimal motif (Warbick 1998) is defined as: h, residues with moderately hydrophobic side chains; a, any residue with a highly hydrophobic, aromatic side chain; X, any residue. p21, cyclin-dependent kinase inhibitor 1, accession no. P38936; Fen 1, flap endonuclease 1, accession no. P39748; XPG, Xeroderma pigmentosum group G-complementing protein, accession no. P28715; MCMT, DNA (cytosine-5) methyltransferase, accession no. X63692.

similar to hRFC40 and hRFC37 (about 40% identity). They are the least identical (about 23%) to hRFC38. Four archaeal RFCL share 34% identity and are about 20% identical to hRFC140. RFC subunits contain eight highly conserved motifs numbered as box I to box VIII (Cullmann *et al.* 1995). Box I, with homology to ligase, comprises about 90 amino acids in the N terminus and is found only in the large subunit of the eukaryotic homologs. The box I region is, therefore, eliminated from the sequence comparisons (Figure 5). The deletion of hRFC140 box I is reported to have enhanced replication activity and PCNA loading (Uhlmann *et al.* 1997). Since the archaeal RFC large subunits do not contain this region, one may expect them to possess similar properties. As shown in the alignment, the homology between the archaeal and eukaryotic homologs is striking, and obviously the most conserved regions are the previously described RFC boxes. Among the motifs, the phosphate-binding loop (box III) is the most conserved followed by the DEAD-box motif, which is also found in the so-called DEAD-box proteins, a family of putative RNA helicases possessing also P-loops and ATPases (Pause and Sonenberg 1992; Mossi and Hubscher 1998). RFC homologs, however, do not possess helicase activity (Cullmann *et al.* 1995). Diversification, which may signify differences in function, was found at the C-terminal region of the alignment. Indeed, results from our laboratory indicate that *P. furiosus* RFC large subunit (*Pfu*RFCL) binds to PCNA (I. Cann, S. Ishino and Y. Ishino, unpublished results), and at the extreme C-terminal region of each archaeal homolog is a highly conserved PIP-box (Table 5). According to the complex forming of the eukaryotic RFC (Mossi and Hubscher 1998), archaeal RFC may form a complex of one RFCL and four RFCS. As expected, *Pfu* RFCS existed in oligomeric form in solution (I. Cann, S. Ishino and Y. Ishino, unpublished results), which suggests that, as in the T4 clamp loader (the T4 gene 44/62 complex), the *Pfu* RFC small subunit exists as multi-protomers interacting with the large subunit. The structure–function analyses, in addition to biochemical analyses of these accessory proteins, are now under way.

CONCLUDING REMARKS

In comparison with research on Bacteria and Eukarya, the molecular biology of Archaea is still far behind; however, the number of reports in this field has greatly increased. The discovery of the novel DNA polymerase family, which is probably involved in the DNA replication machinery of the Euryarchaeota, will greatly contribute to the understanding of the mechanism. In addition, the finding serves as a further confirmation of the archaeal organisms being truly different from those in Bacteria and Eukarya.

Currently, archaeal homologs involved in the eukaryotic DNA replication, other than those described above, such as minichromosome maintenance (MCM) proteins (Kearsey and Labib 1998), replication protein A (RPA; Chedin *et al.* 1998; Kelly *et al.* 1998), and 5′ →

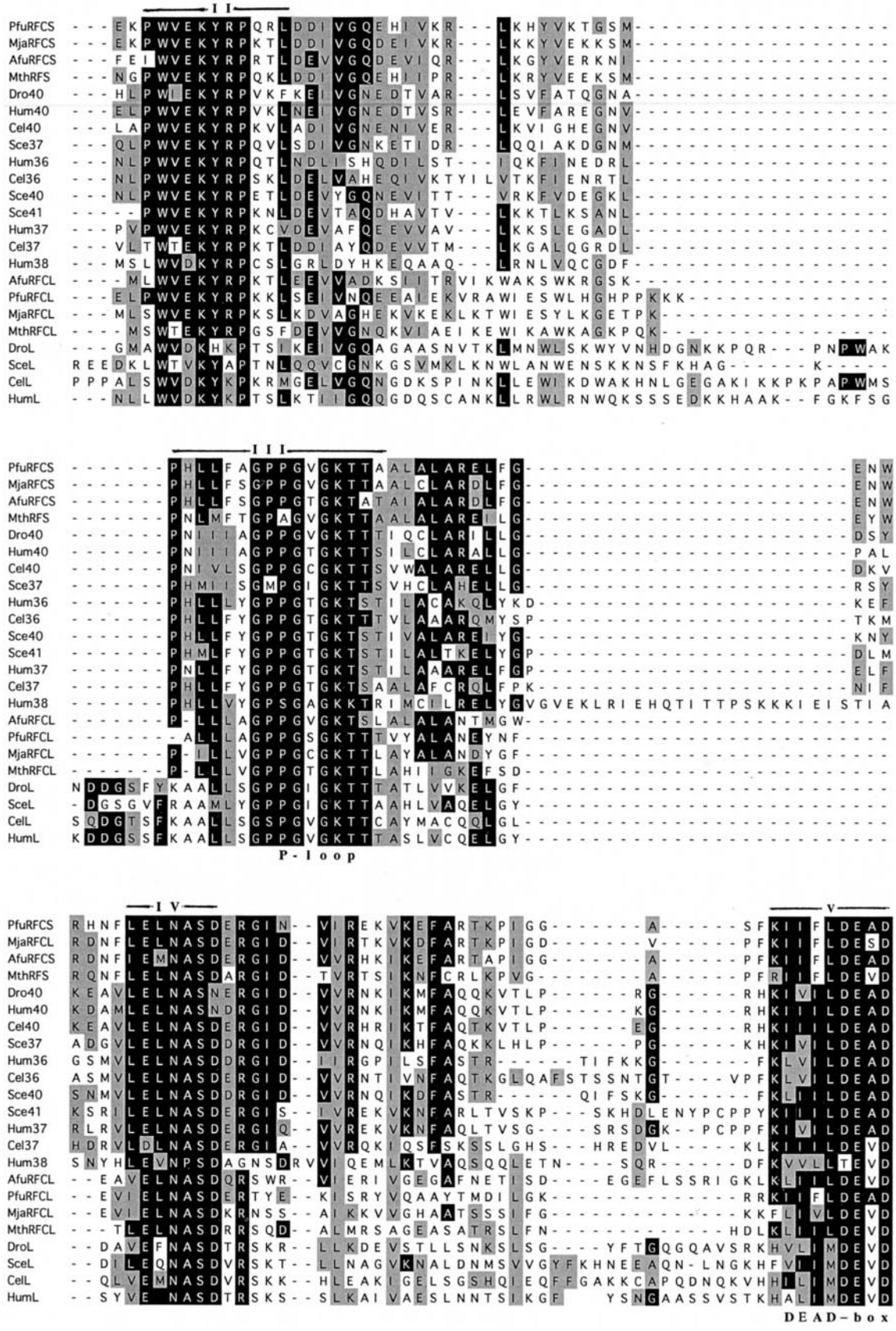
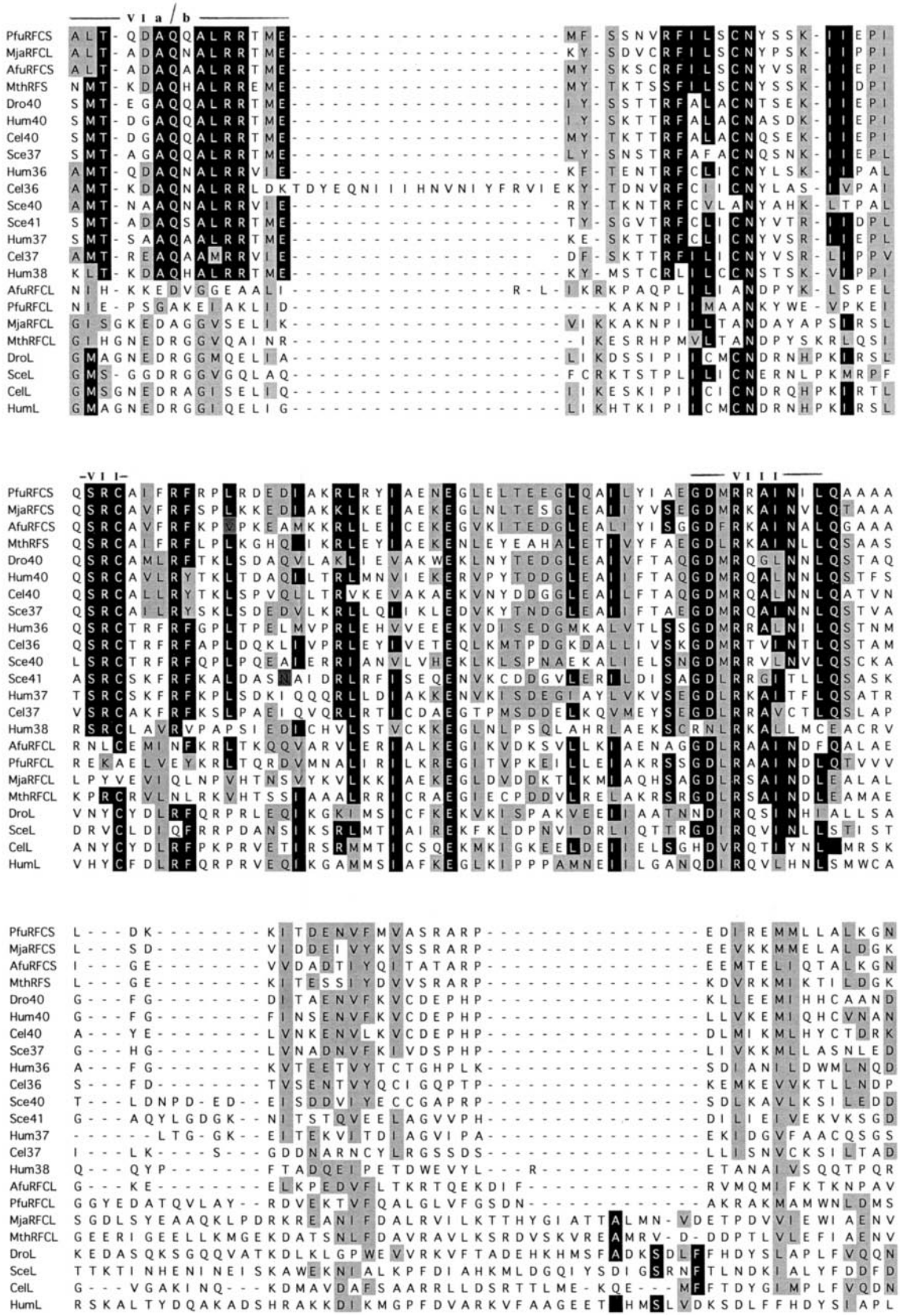


Figure 5.—Amino acid sequence alignment showing seven conserved regions of RFC subunits, including the putative homologs from Euryarchaeota. The alignment begins at RFC box II and ends immediately after box VIII. The sequences beyond this region are highly diverged and hence were not shown. RFCS indicates the euryarchaeotic small subunit, while RFCL indicates large subunits in both domains. The eukaryotic small subunits are named according to their respective molecular mass (kD). The



sequences and their locations are PfuRFCS (unpublished), MjaRFCS (U67583), AfuRFCS (AE000961), MthRFCS (AE000811), Dro40 (*D. melanogaster*, P53034), Hum40 (*H. sapiens*, P35250), Cel40 (*C. elegans*, AF036699), Sce37 (*S. cerevisiae*, P40339), Hum36 (P40937), Cel36 (P34429), Sce40 (P38629), Sce41 (P40348), Hum37 (P35249), Cel37 (P53016), Hum38 (P40938), AfuRFCL (AE001022), PfuRFCL (unpublished), MjaL (U67532), MthRFCL (AE000811), DroL (U97685), SceL (U26027), and CelL (Z75532).

3′ exo/endonuclease (FEN1; Hosfield *et al.* 1998a,b; Rao *et al.* 1998), are being studied at several laboratories in the world. We found the genes encoding archaeal homologs of Orc1/Cdc6 (Orp1/Cdc18) and Rad51/Dmc1 of yeast, respectively, in the operon containing *Pfu* Pol II (Pol D) as shown in Figure 2 (Uemori *et al.* 1997). These yeast homologs play key roles in the initiation of DNA replication and homologous DNA recombination, respectively. Thus, the proximity of these genes to *Pfu* Pol D may signify their indispensability to similar processes in *P. furiosus* cells. It is very interesting that Lopez *et al.* (1999) have recently predicted the replicational origins at the 5′-end of the orc1/cdc6-like genes in the genomes of *M. thermoautotrophicum*, *P. horikoshii*, and *P. furiosus* by three kinds of cumulative skew diagrams. These regions are most likely to be the origins because of the existence of the direct repeats of the AT-rich elements. We are currently biochemically investigating the roles of the Orc1/Cdc6-like protein. This interesting protein in *P. furiosus* will help to identify the replicational origin in the genome soon. The existence in archaeal cells of proteins similar to the essential factors for eukaryotic DNA replication heightens our expectation that unraveling the archaeal mechanism will contribute to the understanding of the mechanism, which became very complicated in the Eukarya as a result of evolution. We expect many exciting findings in Archaea related to DNA replication and also to repair and recombination in the very near future.

Some of our studies on DNA polymerases from *P. furiosus* cited in this article were carried out in Biotechnology Research Laboratories, Takara Shuzo, in collaboration with T. Uemori, I. Kato and other members. We thank Dr. K. Morikawa, K. Komori, and I. Hayashi for discussions and for providing some unpublished data. We also thank S. Ishino for providing some results. We thank Dr. S. Tsutakawa for critical reading of the manuscript. We acknowledge Drs. C. R. Woese, Y. Shimura, H. Shinagawa, A. Sugino, P. Forterre, F. Perler, F. Robb, D. Søll, and W. Whitman for discussions and continuous encouragement.

Note added in proof: The 2.5 Å resolution crystal structure of a family B DNA polymerase from *Thermococcus gorgonarius* has been published (K. P. Hopfner, A. Eichinger, R. A. Engh, F. Laue, W. Ankenbauer, R. Huber and B. Angerer, 1998, Proc. Natl. Acad. Sci. USA **96:** 3600–3605).

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