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MINI-REVIEW

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Archaeal DNA replication: spotlight on a rapidly moving field

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Abstract The replication of DNA is a fundamental step in the cell cycle, which must be coordinated with cell division to ensure that the daughter cells inherit the same genomic material as the parental cell. The recently published complete genome sequences of some archaeal species together with preliminary biochemical studies suggest that the Archaea quite likely duplicate their chromosome by using replication machinery that seems to be a simplified version of the eukaryotic machinery, although their metabolic facets and their cellular morphology are prokaryotic-like. This review is focused on recent progress on the structural and functional analysis of proteins and enzymes involved in the initiation and elongation steps of DNA replication in Archaea. Differences between the genome replication apparatus of the Euryarchaea and the Crenarchaea (the two main phylogenetic divisions of the Archaea domain) are highlighted.

Key words Euryarchaea · Crenarchaea · DNA polymerase · DNA replication · Archaeal replication fork

Introduction

Since their definition as a monophyletic domain of life by the work of Woese et al. (1977), the Archaea have been the subject of intensive investigation. Several total genomic sequences from a number of archaeal strains have meanwhile been published. These include *Methanococcus jannaschii* (Bult et al. 1996), *Methanobacterium thermoautotrophicum* (Smith et al. 1997), *Archaeoglobus fulgidus* (Klenk et al. 1997), *Pyrococcus horikoshii* (Kawarabayasi et al. 1998), *Halobacterium* NRC-1 (Ng et al. 2000), *Pyrococcus furiosus* [Institute for Genomic Research (TIGR) microbial database, http://www.tigr.org/tdb/index.shtml], and *Pyrococcus abyssi* (Genoscope, http://genoscope.cns.fr/Pab/), all belonging to the subdomain Euryarchaea. The first completely sequenced genome of a crenarchaeon was that of *Aeropyrum pernix* (Kawarabayasi et al. 1999). Additional crenarchaeal genome projects recently completed include *Pyrobaculum aerophilum* (TIGR database, http://www.tigr.org/tdb/index.shtml) and *Sulfolobus solfataricus* (Sulfolobus genome project, http://niji.imb.nrc.ca/sulfolobus/super_pub/supers.html), making several sequences available for comparison. Despite this rapid increase in the amount of available sequence information, little is known about the duplication of genetic material in the third domain of life.

Meanwhile, it has been established that genes encoding components of the central metabolic pathways, e.g., those involved in energy formation, are similar between Archaea and Bacteria. On the other hand, archaeal genes that specify functions of information processing – replication, transcription, and translation – are most similar to their eukaryotic counterparts.

The genome of an archaeon is generally composed of a single, circular DNA molecule of 1×10^6 to 3×10^6 bp (Mitchell et al. 1979; Klein and Schnorr 1984). Clustered genes (operons) that are coexpressed as polycistronic transcripts are a bacterial feature of archaeal genomes, whereas introns, prominent in most eukaryotic nuclear genomes, have been reported only for a few archaeal lineages. Plasmids are found in all groups of hyperthermophilic Archaea, but viruses have not been described for all groups. Eukaryal-type histones and nucleosomes are present in the Euryarchaea but not in the Crenarchaea. The *M. jannaschii* genome, for example, contains five histone genes, two of which are located on the large extrachromosomal elements. The implied presence of histones suggests that, although the *M. jannaschii* gene map looks rather bacterial, the genome

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itself may actually be organized in vivo in a manner more typical of eukaryotic than of bacterial chromosomes (Gray 1996). The Crenarchaea, on the other hand, possess small, nonhistone double-stranded DNA (dsDNA)-binding proteins, such as Sso7d and Sso10a, found in *Sulfolobus solfataricus* (Wadsworth and White 2001). Usually a single replication origin is found, but the existence of multiple origins in some species cannot be excluded to date (Myllykallio et al. 2000; Bernander 2000).

The general cell cycle features of this thermoacidophilic crenarchaeon belonging to the genus *Sulfolobus* (Bernander 1998) differ considerably from those of the methanogenic euryarchaeon *Methanococcus jannaschii* (Malandrin et al. 1999; Bernander 2000). A strong coupling between nucleoid partition and cell division has been demonstrated in *Sulfolobus* species. *M. jannaschii*, on the other hand, displays several unusual features, including an apparent excess of copies of the chromosome, accompanied by asymmetric cell division. While the cell division mechanism in the Euryarchaea seems to resemble the prokaryotic *FtsZ*/*MinD* apparatus (as deduced from genome sequence data), the crenarchaeal cell division mechanism remains to be elucidated (Bernander 2000).

Before a cell divides into two identical daughter cells, the entire genome must be replicated faithfully. The mechanisms involved in the complex macromolecular process of DNA replication have been intensely investigated in Bacteria and Eukarya (Kornberg and Baker 1992; Hübscher and Sogo 1997; Waga and Stillman 1998). In all known living organisms, the following steps are common to DNA replication: (I) initiation of replication; (II) unwinding of the supercoiled DNA helix at the origin; (III) stabilization of the single-stranded DNA (ssDNA); (IV) generation of an RNA primer; (V) polymerization, involving assembly of a protein complex composed of clamp loaders and sliding clamps; and (VI) maturation of the Okazaki fragments (i.e., removal of the RNA-primer and gap-filling and ligation of the Okazaki fragments on the lagging strand). Table 1 compares the proteins involved in the bacterial, eukaryal, and archaeal replication machinery.

Since the first review of replication in Archaea by Cann and Ishino (1999) was published, a large number of new proteins involved in the replication process have been isolated from archaeal species. Here, we present an overview of the current state of the art of this rapidly moving field. The composition of an archaeal replication fork as deduced from the described proteins is depicted in Fig. 1. The roles of the DNA polymerases, especially, are still the subject of speculation. The latest findings, however, have shown that there is a clear difference between crenarchaeota and euryarchaeota regarding this point.

I. Initiation of replication

In Bacteria, initiation of replication occurs at a single locus, *oriC*, and is triggered by a single protein, DnaA (Kornberg and Baker 1992). In Eukarya, initiation takes place at multiple replication origins that are almost permanently occupied by the origin recognition complexes (ORC), composed of six subunits. These ORCs are made competent for initiation by the loading of minichromosome maintenance proteins (MCM) (Kearsey and Labib 1998; Pasero and Gasser 1998), an association that is triggered by the protein cdc6 (cell division cycle gene family, Liang and Stillman 1997). *CDC6* is an essential gene in *Saccharomyces cerevisiae*. By interacting with the ORC protein complex as an anchor, it enables loading of MCM proteins onto the replication origins (ARS sequences) of the chromosomes and thus makes the origins competent for replication (Liang et al. 1995; Rowles and Blow 1997). Cdc6/cdc18 (*CDC18* is the *Schizosaccharomyces pombe* homologue of *CDC6*) shares significant homology with regions of certain ORC subunits, particularly Orc1 (Bell et al. 1995; Quintana and Dutta 1999). In particular, this homology encompasses a nucleotide-binding domain of the Rossmann fold family. Together with Orc1, cdc6/cdc18 falls into the AAA^+ subclass of ATPases associated with various cellular activities (AAA proteins; Patel and Latterich 1998; Neuwald et al. 1999). Proteins showing sequence similarity to orc1p as well as to cdc6/cdc18, termed the cdc6p/orc1p family, have been found in *P. furiosus* (Uemori et al. 1997), *Methanobacterium thermoformicicum* (Edgell and Doolittle 1997), *Pyrococcus abyssi* (Myllykallio et al. 2000), and *Thermococcus aggregans* (K. Böhlke, unpublished observation). Some other archaeal species such as *Methanobacterium thermoautotrophicum*, *Archaeoglobus fulgidus*, *Aeropyrum pernix,* and *Thermoplasma acidophilum* possess two putative cdc6p/orc1p homologues in their genome sequence (Myllykallio and Forterre 2000). The cdc6p/orc1p orthologue from *Pyrobaculum aerophilum* has been isolated and the 45-kDa protein crystallized (Liu et al. 2000). The protein exists as a monomer in solution and exhibits ATPase activity. It contains a structure belonging to the winged helix family of folds, which is conserved between the cdc6/cdc18 and orc1p members, and it is structurally similar to several proteins known to bind DNA. These and other relations between the cdc6/cdc18 and Orc1 lead us to speculate that the Archaea might utilize a cdc6p/orc1p hybrid that acts both in origin recognition and MCM recruitment. In this scenario, the winged helix domains might act as a DNA localization factor (Liu et al. 2000).

In the *M. jannaschii* genome, initially no homologue of eukaryotic cdc6p/orc1p was found, but four *MCM* genes were detected, instead of one as in the other known archaeal genomes. It has been suggested that the cdc6p/orc1p function can be replaced by another protein (Bernander 1998). This has been demonstrated by the inactivation of the *dnaA* gene in the bacterium *Synechocystis* sp. strain PCC 6803, which had no phenotypic effect (Richter et al. 1998). Meanwhile, one of the four putative MCM proteins of *M. jannaschii* (ORF MJ 0774) has been recognized as a possible cdc6p/orc1p homologue (Liu et al. 2000; Aravind and Koonin 1999). According to these authors, it is likely that *M jannaschii* possesses three putative MCM homologues and one putative cdc6p/orc1p homologue.

Recently, a single origin of bidirectional replication was identified in *Pyrococcus abyssi* by means of cumulative oligomer-skew in silico analyses and the identification of an

Table 1. Comparison of replication in Bacteria, Eukarya, and Archaea

Function	Bacteria[a]	Eukarya[a,b]	Archaea	
			Crenarchaea	Euryarchaea
Ori	1	Multiple	?	1[c]
Initiation	DnaA	6 ORC proteins	Cdc6p/orc1p-like[d]	
Unwinding: helicase	DnaB (hexamer)	Dna2, 6 MCM (heteromultimer)	Dna2-like, MCM-like (double hexamer)[e]	
Stabilization of ssDNA	SSB (monomer)	RPA (heterotrimer)	SSB/RPA[f] (monomer)	RPA-like[f]
Creation of RNA primer	DnaG (primase)	Initiating pol (subunit of pol α)	Eukaryotic-like primase[g]	
Polymerization	Pol III (10 subunits) Core: α ε θ Dimer $(\alpha\ \varepsilon\ \theta)_2 + 2\ \tau$	3 family B pols: α, δ, ε initiation: pol α leading and lagging strand: pol δ and ε	2 (3) family B pols[h]	1 family B pol[h] 1 family D pol (dimer)[i] (DP1~pol δ subunit; DP2 = catalytic subunit)
Proofreading 3′–5′ exonuclease	Subunits α and ε of Pol III	Subdomains in pol δ and pol ε	Subdomains in both B pols	Subdomains in B pol and DP2 subunit
Sliding clamp	2 β subunits	1 PCNA (homotrimer)	2 PCNA-like[k] (homotrimer)	1 PCNA-like[l] (homotrimer)
Clamp loader	γ (5 different subunits)	RFC (5 different subunits)	RFC-like (4 + 1 subunits)[m]	RFC-like (4 + 2/4 + 1 subunits)[n]
Length Okazaki fragment	ca. 1,000 Nt.	100–200 Nt.	120–160 Nt.[o]	
Removal of RNA primers	Pol I 5′–3′ exonuclease, RNase H	FEN-1, RNase H	FEN-1[p], RNase H	
Ligation of Okazaki fragment[q]	Ligase (NAD-dependent)	Ligase (ATP-dependent)	Ligase (ATP-dependent)	

cdc, cell division cycle (yeast gene nomenclature); FEN, flap endonuclease; MCM, minichromosome maintenance; NAD, nicotinamide adenine dinucleotide; Nt, nucleotides; ORC, origin recognition complex; Ori, origin of replication; PCNA, proliferating cell nuclear antigen; pol, polymerase; RFC, replication factor C; RPA, replication protein A

[a] combined from Kornberg and Baker (1992), Kelman and O'Donnell (1995), and reviews Edgell and Doolittle (1997), Cann and Ishino (1999)

[b] adapted from review Hübscher and Sogo (1997)

[c] a single origin of replication was identified in three *Pyrococcus* genomes (Myllykallio et al. 2000) and in *Methanobacterium thermoautotrophicum* (Lopez et al. 1999)

[d] identified in *Pyrococcus furiosus* (Uemori et al. 1997), *T. aggregans* (K. Böhlke, unpublished observation), and *Methanobacterium thermoformicicum* (Edgell and Doolittle 1997); cdc6 is an ATPase as is DnaA, but displays sequence similarities to eukaryal ORC1

[e] MCM protein was investigated in *Methanobacterium thermoautotrophicum* (Kelman et al. 1999a; Chong et al. 2000); Dna2-like proteins were identified (Cann and Ishino 1999)

[f] euryarchaeal RPA was investigated in *Methanococcus jannaschii* (Kelly et al. 1998) and *M. thermoautotrophicum* (Kelman et al. 1999b); crenarchaeal SSB/RPA was from *Sulfolobus solfataricus* (Wadsworth and White 2001)

[g] investigated in *M. jannaschii* (Desogus et al. 1999)

[h] the roles of the archaeal DNA polymerases in replication remain to be established. Crenarchaea generally have a B1 and a B3 DNA polymerase, the Euryarchaea a B3 polymerase. *Sulfolobus* spp., *Archaeoglobus fulgidus,* and *Halobacterium* NRC1 additionally contain the gene for a B2 DNA polymerase (see the text). It is not clear if this protein is functional

[i] investigated in *P. furiosus* (Uemori et al. 1997) and *M. jannaschii* (Ishino et al. 1998); identified in *T. aggregans* (K. Böhlke, unpublished observation)

[k] investigated in *S. solfataricus* (De Felice et al. 1999); both PCNA-like factors interact with the B1 pol

[l] investigated in *P. furiosus* (Cann et al. 1999a); PCNA interacts with both family B and family D pols (subunit DP2)

[m] investigated in *S. solfataricus* (Pisani et al. 2000)

[n] investigated in *Methanobacterium thermoautotrophicum* (Kelman and Hurwitz 2000; Ishino et al. 1998)

[o] proposed by H. Myllykallio (personal communication)

[p] investigated in *M. jannaschii* (Rao et al. 1998)

[q] adapted from Cann and Ishino (1999)

early replicating chromosomal segment (Myllykallio et al. 2000). The replication origin in three *Pyrococcus* species investigated was found to be highly conserved, and several eukaryotic-like DNA replication genes are clustered around it. As in Bacteria, the chromosomal region containing the replication terminus was a hot spot of genome shuffling. The authors infer that although bacterial and archaeal replication proteins differ profoundly, they are used to replicate chromosomes in a similar manner in both prokaryotic domains (Myllykallio et al. 2000).

II. Unwinding of the supercoiled DNA helix

The helicase for unwinding the DNA helix in *Escherichia coli* is an ATP-dependent hexamer encoded by the *dnaB* gene. In Eukarya, two proteins have been related to this function: Dna2 and the minichromosome maintenance protein (MCM). Six highly related *MCM* genes have been identified (Chong et al. 1996; Kearsey et al. 1996). Protein complexes of six MCM proteins (Chong et al. 1995; Adachi

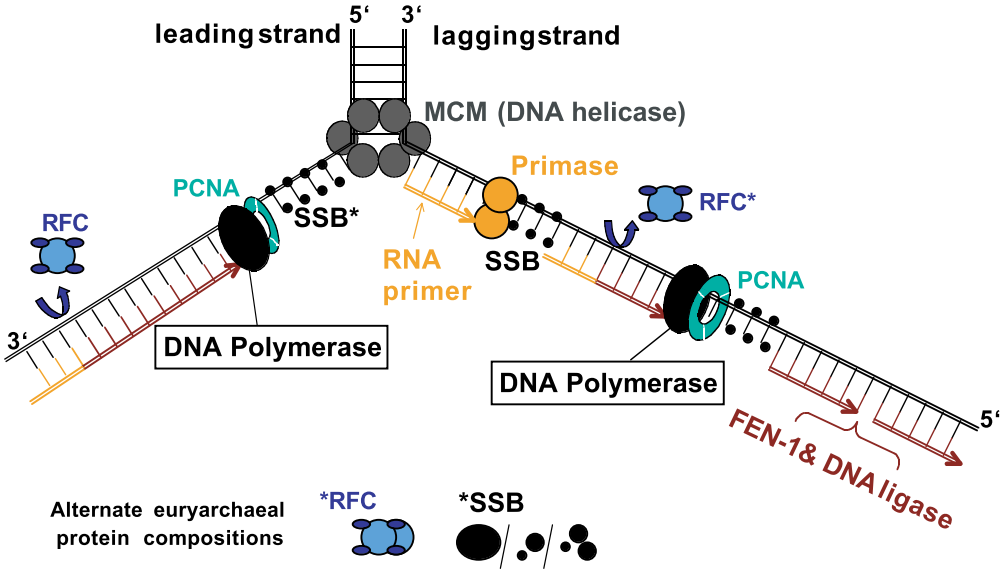

Fig. 1. Schematic drawing of an archaeal replication fork. The overall arrangement of the archaeal replicational proteins resembles the eukaryal replication fork. The parental DNA is depicted by *black lines*; it is unwound by the minichromosome maintenance protein (*MCM*)-like helicase (*gray*). The newly synthesized DNA is drawn in *red* and the primer RNA in *yellow*. The main difference between the Euryarchaea and Crenarchaea consists in the DNA polymerases involved. Pol B1 and, probably, pol B3 are supposed to be replicative DNA polymerases in the Crenarchaea; pol B3 and/or pol D seem to be involved in the euryarchaeal replicative process. Single-stranded DNA-binding proteins (*SSB, black*) have been shown to be monomers with one OB fold in the crenarchaeon *Sulfolobus solfataricus*. The euryarchaeal SSBs are more diverse, being monomers with 4 oligonucleotide/oligosaccharide-binding (OB) folds in *Methanococcus jannaschii,* heterotrimers in *P. furiosus,* and heterodimers in *A. fulgidus* (compare text). The archaeal DNA primase (*yellow*) seems to be a heterodimer composed of subunits similar to the eukaryotic p50 and p60 polypeptides, as judged from findings in *M. jannaschii* (Pisani and Onesti, unpublished results). In the genome of the crenarchaeon *Sulfolobus solfataricus,* homologues of the two *M. jannaschii* genes have been found, but no protein has been isolated yet. The archaeal replication factor C (*RFC, blue*) loads the proliferating cell nuclear antigen (*PCNA, greenblue*) onto DNA in an ATP-dependent manner, as in eukaryotes. For this scheme, we have arbitrarily assumed that this process takes place when DNA pol begins to elongate the RNA primer, whereas at the eukaryotic replication fork, the above process is preceded by a switch from the DNA pol α–primase complex to DNA pol δ. Okazaki fragments on the *lagging strand* are joined by an ATP-dependent ligase after the RNA has been removed by flap endonuclease (FEN-1)

et al. 1997; Richter and Knippers 1997) as well as subcomplexes with fewer than six different subunits have been purified (Ishimi et al. 1996; Kimura et al. 1996; Kubota et al. 1997; Thömmes et al. 1997), but biochemical analysis has been limited. For both Dna2 and MCM, homologous genes have been identified in Archaea (Cann and Ishino 1999). In *Methanobacterium thermoautotrophicum*, as in the majority of archaeal genomes, only one copy of an MCM-like gene exists. This MCM protein has been purified (Kelman et al. 1999a; Chong et al. 2000) and proposed to be a double hexamer (Chong et al. 2000). The protein binds to single-stranded DNA in an ATP-independent manner. It contains 3′–5′ ATP-dependent helicase activity, and its ATPase is stimulated by DNA, preferably double-stranded DNA. The N terminus of the protein is proposed to function in the multimerization of the complex. It remains to be determined whether MCM proteins promote unwinding of the DNA molecule only at origins of replication (as suggested for eukaryotes, Ishimi 1997; You et al. 1999) or whether the helicase also functions at the DNA replication fork as the main replicative helicase. The high processivity of *M. thermoautotrophicum* MCM helicase activity suggests that it might also function in the DNA replication fork (Chong et al. 2000). It is not known whether the archaeal MCM-like proteins play a role in cell cycle regulation as has been shown for eukaryotic proteins. These proteins dissociate and reassociate to the chromatin in a process termed replication licensing (Ishimi et al. 1996; Thömmes et al. 1997; Kubota et al. 1997; Kearsey et al. 1996). As reported above, in contrast to other archaeal genomes, *M. jannaschii* has three *MCM* genes instead of one (Bernander 1998).

III. Stabilization of the single-stranded DNA

In *E. coli*, single-stranded DNA (ssDNA) is bound and stabilized by ssDNA binding protein (SSB), a homotetramer with a molecular mass of 75.6 kDa (Kornberg and Baker 1992). In eukaryotes, this task is performed by replication protein A (RPA), a heterotrimer composed of subunits of 70, 32, and 14 kDa (referred to as RPA 70, RPA 32, and RPA 14, respectively). RPA 70 contains 2 ssDNA-binding sites characterized by the so-called OB fold (oligonucleotide/oligosaccharide-binding fold, about 100 amino acids), and additionally it mediates interactions with cellular proteins. RPA 32 and RPA 14 each possess one OB-fold

domain, but their DNA-binding activity is rather weak (Bochkarev et al. 1999). Thus, in combination, eukaryal RPA has four ssDNA-binding sites. In several euryarchaeal genomes, a novel type of RPA, possessing four conserved DNA-binding sites within a single polypeptide, has been found (Chédin et al. 1998). This finding and the analysis of the crystal structures of the three eukaryotic RPA proteins suggest that the mechanism of ssDNA binding via tetramers of OB folds has been conserved during evolution (Chédin et al. 1998; Bochkarev et al. 1997, 1999). In Euryarchaea, SSBs/RPAs also exist as heterodimers (e.g., *Archaeoglobus fulgidus*) and heterotrimers (e.g., *P. furiosus*; Chédin et al. 1998). A monomeric 80-kDa protein having four tandem repeats of the RPA-like ssDNA-binding site has been isolated from *Methanococcus jannaschii* (Kelly et al. 1998). This protein is able to bind ssDNA as a monomer with an association constant similar to the one measured for eukaryotic RPA (Kelly et al. 1998). The binding site of the *M. jannaschii* ssDNA-binding protein (mjaSSB) consists of about 20 nucleotides. This is in agreement with the observation that in the eukaryotic RPA crystal structure, the DNA-binding channel of each of the two OB folds contacts three nucleotides of the DNA chain, with two nucleotides bridging the space between (Bochkarev et al. 1997). This leads to a prediction of 12–20 occluded nucleotides for 4 OB folds in tandem. In contrast to the eukaryal RPA 70 protein, the mjaSSB lacks N- and C-terminal regions through which RPA 70 interacts with several other cellular proteins. These domains seem to have evolved after the divergence of the Archaea and Eukarya.

Very recently, the first crenarchaeal SSB protein was described for *Sulfolobus solfataricus* (Wadsworth and White 2001). It is an abundant protein (2%–5% of the total soluble protein) with a unique structural organization, existing as a monomer in solution and multimerizing (probably to a tetramer) upon binding to DNA. While the *Sulfolobus* SSB OB fold is related to other (eury-)archaeal SSB/RPA domains, the C-terminal region of the protein has unexpected similarities to bacterial SSBs. This flexible C-terminal tail is sensitive to trypsin digestion, and it is not needed for DNA binding but probably for protein–protein interactions, proposed by analogy with the *E. coli* SSB. The large subunits of both eukaryal and euryarchaeal RPA/SSB contain a putative zinc finger domain that is absent in the crenarchaeal, eubacterial, and mitochondrial SSBs.

IV. Generation of an RNA primer

A primase initiates the actual DNA replication by synthesizing short oligoribonucleotides on single-stranded DNA templates, which serve as primers both for the initiation of replication at the origin and for the initiation of the Okazaki fragments on the lagging strand. DNA primases from various viral, bacterial, and eukaryotic sources have been isolated and characterized, and the structure of the catalytic domain of the *E. coli* primase (the product of the *DnaG* gene) has been recently determined (Keck et al. 2000). No significant sequence similarity can be detected between the eukaryotic primases and primases purified from bacteriophages and bacteria.

In eukaryotes, the DNA primase activity copurifies with DNA polymerase α. The polymerase α-primase complex is a heterotetramer consisting of subunits with molecular masses of about 180, 70, 60, and 50 kDa, which are highly conserved in a wide range of organisms (for a recent review see Arezi and Kuchta 2000). The large p180 polypeptide is responsible for the polymerase activity (Plevani et al. 1985), while the DNA primase consists of 60- and 50-kDa polypeptides. In a number of species, the p50 subunits were found to be sufficient for RNA primer synthesis, although in some cases both the p50 and p60 subunits were reported to be necessary for the primase activity (Foiani et al. 1997). The function of the p60 subunit is still poorly understood, although it appears to stabilize the enzyme activity and is required for the binding of the primase heterodimer to the p180 polymerase α polypeptide (Longhese et al. 1993). The 70-kDa protein species has no known enzymatic activity, but it is phosphorylated in a cell-cycle dependent manner and is possibly involved in the interaction with other proteins bound to the origin of replication (Foiani et al. 1997).

In the complete genome sequence of several archaeal species, open reading frames (ORFs) were identified that potentially coded for homologues of the eukaryotic DNA primase p50 and p60 subunits. Nevertheless, only one archaeal DNA primase activity has been characterized to date, that belonging to the euryarchaeon *Methanococcus jannaschii* (Desogus et al. 1999). The gene coding for the homologue of the eukaryotic DNA primase p50 subunit was cloned from this species and overexpressed in *E. coli* as a His-tagged polypeptide. The purified recombinant protein (Mjpri, M_r 42 kDa) was shown to be able to synthesize oligoribonucleotides on pyrimidine single-stranded DNA templates [poly(dT) and poly(dC)]. This activity requires divalent cations (such as Mg^{2+}, Mn^{2+}, or Zn^{2+}) and is additionally stimulated by K^+. The *M. jannaschii* homologue of the eukaryotic DNA primase p60 subunit (ORF MJ 0701) has been recently produced as recombinant protein in *E. coli* and purified; its structural and functional interaction with the p50 homologue is currently under investigation (Pisani and Onesti, unpublished results). An important issue to be addressed with respect to the initiation of DNA replication in Archaea is whether DNA primase associates with a DNA polymerase to form a bifunctional complex like the DNA pol α-primase complex in eukaryal species or whether the DNA primase acts as an independent enzyme as in the other prokaryotes.

V. Polymerization involving moving platform proteins

In *E. coli*, DNA polymerization is carried out by a deca-protein complex called the DNA polymerase III holoenzyme (*E. coli* Pol III; Kornberg and Baker 1992) during chromosomal replication. In this hetero-oligomeric complex, the polymerase catalytic core (formed by the α, ε, and

Table 2. DNA polymerase families

Family	Organism	Known as	Sliding clamp[a]	Function	Subunits (catalytic/associated)
A	Bacteria	Pol I	No	Gap filling in replication; repair	1/2
	Bacteriophages (e.g. T5, T7)		No[b]	Replication	1/-
	Mitochondria	γ	No	Replication of mitochondrial genome	1/-
B	Bacteria	Pol II	Yes	Repair	1/2
	Bacteriophages (e.g., T4, M2)		Yes	Replication	1/-
	Eukaryal viruses		N.I.	Replication	1/-
B1	Crenarchaea		Yes	Replication[c]	1/-
B2	*Sulfolobus*		N.I.	N.I.	1/-
	A. fulgidus		N.I.	N.I.	1/-
	Halobacterium NRC1		N.I.	N.I.	1/-
B3	Euryarchaea		Yes	Replication[d]	1/-
	Crenarchaea		N.I.	Replication[e]	1/-
	Eukarya	α	No	Initiating pol	1/3
		δ	Yes	Leading and lagging strand pol; repair	1/1
		ε	Yes	Leading and lagging strand pol; gap filling; repair; recombination	1/1
C	Bacteria	Pol III	Yes	Replication	1/many
D	Euryarchaea		Yes	Replication[f]	1/1
X	Eukarya	β	No	Repair	1/-
	Methanobacterium thermoautotrophicum		N.I.	N.I.	N.I.

pol, polymerase; N.I., no information found. *A. fulgidus*, *Archaeoglobus fulgidus*

[a] interaction of polymerase with sliding clamp to gain processivity (Bacteria, protein β; Eukarya and Archaea, PCNA)

[b] T7 polymerase uses *E. coli* thioredoxin as a processivity factor

[c–f] these polymerases have been proposed to take part in replication: [c]De Felice et al. 1999; [d]Cann et al. 1999a; [e]Uemori et al. 1995; [f]Cann et al. 1998

θ subunits) is present as a dimer to copy the leading and lagging strands simultaneously. Three family B DNA polymerases with one or three accessory subunits are known to work at the eukaryotic replication fork (Table 2): DNA pol α initiates the Okazaki fragments on the lagging strand and also initiates the synthesis on the leading strand. Elongation of the Okazaki fragments on the lagging strand and synthesis on the leading strand is then continued by DNA pol δ (or probably ε). This replacement of polymerases is also known as the "DNA polymerase switch" (Waga and Stillman 1998). DNA pol ε, has also been implicated in the filling of the gaps after primer removal and in repair processes (Hübscher and Sogo 1997).

Crenarchaeal DNA polymerases

Crenarchaeal DNA polymerases were first isolated from the hyperthermophilic species *Sulfolobus acidocaldarius* (Klimczak et al. 1985) and *S. solfataricus* (Rossi et al. 1986). These enzymes were purified as single-subunit proteins, sensitive to proteolytic degradation, having an optimal temperature for polymerization of around 70°–75°C. It has been pointed out that this value is mainly dependent on the DNA substrate helix stability and not on the protein structural integrity at temperatures above 80°C (Rossi et al. 1986). The DNA polymerase from *S. solfataricus* showed sensitivity to aphidicolin (a tetracyclic diterpenoid antibiotic and a specific inhibitor of eukaryal α-type DNA polymerases). The *S. solfataricus* (Pisani et al. 1992) and *S. acidocaldarius* (Elie et al. 1989) DNA polymerases have been shown to be endowed with a 3′–5′ exonuclease activity. A few years after these studies, the first archaeal DNA polymerase-encoding genes were cloned and sequenced. These include those from the crenarchaeon *S. solfataricus* (MT4 strain; Pisani et al. 1992) and from the euryarchaeon *Thermococcus litoralis* (Perler et al. 1992). Analysis of their amino acid sequences indicated that both enzymes are related to the B family of DNA polymerases (Braithwaite and Ito 1993). *S. solfataricus* and *S. shibatae* have been demonstrated to possess genes encoding for two additional family B DNA polymerases (Prangishvili and Klenk 1993; Edgell et al. 1997) and it was proposed that they be called DNA pol B1, B2, and B3, following the chronological order of their discovery. In contrast, only two DNA polymerase-encoding genes (similar to the B1 and B3 DNA pols of *S. solfataricus*) were isolated from other crenarchaeal species (*Pyrodictium occultum,* Uemori et al. 1995; *S. acidocaldarius*, Datukishvili et al. 1996; *Pyrobaculum aerophilum*, Fitz-Gibbon et al. 1997). On the other hand, genes coding for all three B1-, B2-, and B3-type DNA polymerases were identified in the crenarchaeal species *Aeropyrum pernix* (Cann et al. 1999b; Kawarabayasi et al. 1999) and *Sulfurisphaera ohwakuensis* (Iwai et al. 2000).

The primary structure analysis revealed that (1) crenarchaeal B1- and B3-type DNA polymerases possess the three Exo and the five Pol sequence motifs highly conserved (according to Blanco et al. 1991) with the exception of the B3 DNA polymerase sequences from *S. solfataricus* and *S. shibatae* (Edgell et al. 1997); and (2) the B2-type DNA polymerase sequences do not contain the Exo I and Exo III boxes, have critical substitutions in the Pol motif IV

(TxDTDS → HxI/L, I/V, DS), do not possess the Y-GG/A sequence that is located between the exonuclease and the polymerase domains, and are believed to play a critical role in coordinating the degradative and synthetic functions of family B DNA polymerases (Truninger et al. 1996; Pisani et al. 1998b; see below).

Two distinct DNA polymerase activities (one sensitive and the other resistant to aphidicolin) were partially purified from *P. occultum* (Uemori et al. 1995) and *A. pernix* (Cann et al. 1999b) cell extracts. In addition, the corresponding genes were cloned by polymerase chain reaction (PCR) using degenerate primers, and the recombinant enzymes were purified and characterized. Thus, it was possible to demonstrate that in both species the aphidicolin-sensitive activity corresponds to a B1-type DNA polymerase, whereas the aphidicolin-resistant enzyme is a B3-type DNA polymerase. Interestingly, the biochemical characterization of another enzyme, the B3 DNA polymerase from *Pyrobaculum islandicum* has shown that its exonuclease activity is strongly pH dependent (Kähler and Antranikian 2000). Moreover, this enzyme is the only crenarchaeal DNA polymerase that to date has been described to perform in PCR analyses. There are no reports in which the archaeal B2-type DNA polymerases are characterized biochemically. Although a certain number of *Sulfolobus* species have been demonstrated to possess three family B DNA polymerase genes, only that coding for the B1-type orthologue from *S. solfataricus* (strain MT4, Sso DNA pol B1) has been overexpressed in *E. coli* and the corresponding protein characterized in detail (for a review, see Pisani et al. 1998a). The Sso DNA pol B1 possesses a modular organization of its two catalytic activities, being composed of an amino-terminal domain responsible for the 3′–5′ exonuclease function and a carboxy-terminal domain containing the synthetic capability (Pisani and Rossi 1994). These two domains are connected by a linker region that is quite sensitive to proteolytic attack. The above linker polypeptide (referred to as Region 1) contains the sequence motif Y-GG/A, which is highly conserved among the family B DNA polymerases. Indeed, limited proteolysis studies carried out in the presence and absence of DNA molecules have shown that Region 1 is subjected to conformational changes upon DNA binding to the enzyme (Pisani et al. 1996). A site-specific mutational analysis of the Y-GG/A sequence motif demonstrated that Region 1 directly interacts with the primer/template molecule and is responsible for the processivity of the 3′–5′ exonuclease function in Sso DNA pol B1 (Pisani et al. 1998b). A B1-type polymerase has also been characterized from the uncultivated psychrophilic crenarchaeon *Cenarchaeum symbiosum* (Schleper et al. 1997).

A DNA replication mechanism similar to that of eukaryotic cells, in which two (Prelich and Stillman 1988) or three (Morrison et al. 1990) different family B DNA polymerases have been suggested to participate in leading strand and lagging strand synthesis, has been proposed for the Crenarchaea (Uemori et al. 1995). To date, the physiological role of the crenarchaeal DNA polymerase has not been elucidated. However, *S. solfataricus* DNA polymerase B1 is highly stimulated in vitro by the *Sulfolobus* homologues of the eukaryotic processivity factors PCNA (proliferating cell nuclear antigen) and RFC (replication factor C) (see below). This finding strongly suggests that DNA polymerase B1 could be involved in leading or lagging strand elongation in the crenarchaeal replication fork, as found in eukaryotes for DNA polymerase δ (Waga and Stillman 1998).

Euryarchaeal DNA polymerases

The investigation of euryarchaeal DNA polymerases has long been focused on B3 DNA pols from the order *Thermococcales*, which are interesting because of their applications in polymerase chain reaction (PCR) procedures. Many biochemical details on these enzymes have been collected in the excellent review by Perler et al. (1996). Only two euryarchaeal family B DNA polymerase proteins from other euryarchaeal orders have been described in the literature: those from *Thermoplasma acidophilum* (Hamal et al. 1990), and *Methanobacterium thermoautotrophicum* (Klimczak et al. 1985; Kelman et al. 1999b). Surprisingly, the latter protein is encoded by two genes, the split lying in the C-terminal half containing the polymerization domain. Both peptides are needed to form a functional enzyme. Genes for euryarchaeal family B3 DNA polymerases have been reported from many additional organisms, but they will not be discussed in this review.

Due to their relevance in the PCR technique, B3 DNA pols from *Thermococcales* strains have been the subject of intensive research, including mutational analyses. Gardner and Jack (1999) determined the amino acid residues that are involved in ribonucleotide exclusion and nucleotide binding in Vent DNA pol (source *T. litoralis*). In the case of *P. furiosus* DNA pol, the dideoxynucleotide utilization has been improved (Evans et al. 2000). In the case of the B3 DNA pol of *T. aggregans* (Niehaus et al. 1997), the PCR performance was improved by mutagenesis of the Y-GG/A motif located between the N-terminal exonuclease domain and the C-terminal polymerase domain (Böhlke et al. 2000). This motif was shown to balance the catalytic activities of the polymerase versus the exonuclease domain (Truniger et al. 1996). Five mutations of Tyr in the motif revealed that an aromatic ring system is crucial for the synthetic activity of the enzyme. When amino acids in this position lacked the ring system (Ser, Asn), a significant decrease in polymerase activity accompanied by improved enzyme fidelity (proofreading capability) was observed. Tyr to Phe, Trp, or His substitutions led to phenotypes with wild-type-like fidelity but enhanced PCR performance, which has been related to an increased Vmax of the synthetic activity. Analysis of the modeled structure of *T. aggregans* DNA pol suggests that the conformation of the flexible loop containing the Y-GG/A motif is a deciding factor that determines the equilibrium state between DNA polymerization and exonucleolysis. Sequence alignment of the *T. aggregans* DNA pol with thermostable DNA pols from other Thermococcales strains suggests that these results may also be valid for other DNA pols commercially available for use as PCR enzymes. In contrast to similar mutations made in the *Sulfolobus solfataricus* DNA polymerase (Pisani et al. 1998b), no significant change

in the exonuclease processivity was detected (K. Böhlke, unpublished results).

DNA polymerases from the *Thermococcales* strains are often invaded by inteins. Inteins are intervening sequences spliced out of proteins instead of mRNAs (Perler et al. 1994). Inteins preferentially enter conserved sequence motifs important for catalysis. In the case of family B polymerases, these are motifs A, B, and C. One intein was found in the precursors of *Pyrococcus* sp. GB-D (Xu et al. 1993) and *P. horikoshii* DNA polymerases (Cann and Ishino 1999). Two inteins each were found in the precursor proteins of *T. litoralis* (Perler et al. 1992), *T. fumicolans* (Cambon and Querellou, GenBank direct submission 1996, Acc No. Z69882), and *Pyrococcus* sp. KOD1 (Takagi et al. 1997). The family B DNA polymerase of *Thermococcus aggregans* is the only protein known to date that harbors three inteins (Niehaus et al. 1997). The enzymatic properties of the intein-coded endonucleases derived from the *Thermococcus fumicolans* DNA polymerase have been investigated in detail (Saves et al. 2000).

Another feature that makes this group of DNA polymerases interesting is the presence of a so-called read-ahead function that detects promutagenic template-strand uracil. The deamination of cytosine to uracil is an inherent property of the chemistry of DNA, and it is greatly increased at the elevated growth temperatures of hyperthermophilic Archaea. It produces C-G => A-T transition mutations. The DNA polymerases from *T. litoralis* and *P. furiosus* have been shown to recognize specifically the presence of uracil in the template strand and to stall DNA synthesis before mutagenic misincorporation of an adenine (Greagg et al. 1999). Repair of cytosine deamination is achieved by base-excision repair by mismatch-specific glycosylases in Bacteria, animals, plants, and some double-stranded viruses (Greagg et al. 1999). No homologues corresponding to the known family of uracil-DNA glycosylases responsible for initiating base-excision repair have yet been found in archaeal genomes. The specific template-checking function of those archaeal DNA polymerases could represent the first step in an alternate pathway for the repair of cytosine deamination in Archaea.

As another noteworthy feature, *T. litoralis* B-type DNA polymerase has been reported to create genetic information in the absence of primer and template DNAs (Ogata and Miura 1997, 1998a). The sequence of the created DNA fragments is dependent on temperature, pH, and ionic strength. The fact that genetic information can be potentially created by a protein has implications relevant to the early stages of the evolution of life. A comparable reaction has recently been found also in *Thermus thermophilus* DNA polymerase (Ogata and Miura 1998b).

DNA polymerase D

The first complete archaeal genome from the euryarchaeon *Methanococcus jannaschii* (Bult et al. 1996) gave the astonishing impression that this organism harbors only a single family B DNA polymerase. Reports of two DNA polymerase activities in the euryarchaeon *Pyrococcus furiosus*, distinguished by their sensitivity to aphidicolin, ddTTP, and *N*-ethylmaleimide, and by the size of the partially purified protein (Imamura et al. 1995) encouraged Ishino and colleagues to search for further family B polymerases in a cosmid library of this euryarchaeon.

Instead of a second family B DNA polymerase, they found a novel type of DNA polymerase composed of two different subunits (Uemori et al. 1997) and proposed to call these novel type of polymerases family D DNA polymerases, a nomenclature that we have adopted in this review (Cann and Ishino 1999). The larger subunit, DP2, with a molecular weight of 140 kDa, was shown to be the catalytically active subunit, with respect to both DNA polymerization and 3′–5′ exonucleolysis (Uemori et al. 1997). Nevertheless, the catalytic reaction occurs only in the presence of the small subunit, DP1. The sequence of DP2 did not show any significant similarity to other database entries. This explains why only one DNA polymerase was previously identified in the *M. jannaschii* genome. Interestingly, the novel heterodimeric DNA polymerase was found to be able to use RNA primers and to prefer primed instead of gapped DNA as a substrate. Moreover, it has a higher intrinsic processivity than the family B DNA polymerase from the same organism. Accordingly, a role in DNA replication was proposed for this enzyme (Uemori et al. 1997).

The DP1 subunit is a 70-kDa protein with slight similarities to the small subunit of pol δ from eukaryotes (Table 2). Both seem to mainly mediate contacts between the polymerases and accessory subunits. The DP1 protein contains four conserved motifs in its central and C-terminal region that have been described as belonging to the vast superfamily of calcineurin-like phosphoesterases (Aravind and Koonin 1998). The motifs contain conserved histidine and aspartate residues that are thought to be involved in metal-dependent catalysis of phosphoester bond hydrolysis. It has been proposed that these motifs may be responsible for hydrolysis of the pyrophosphate released during nucleotide polymerization (Aravind and Koonin 1998). This reaction would drive the polymerization reaction forward and hence increase the DNA polymerization rate. In eukaryotic DNA polymerases, the sequence motif is disrupted, suggesting that a cellular phosphatase might have taken over this function (Aravind and Koonin 1998). Phosphatase domains from a distinct superfamily were found in bacterial DNA Pol III and two family X DNA polymerases.

The DP1 proteins from *Pyrococcus* sp., *M. jannaschii*, and *T. aggregans* (Cann and Ishino 1999; K. Böhlke, unpublished observation) are significantly larger than the second subunit of eukaryotic pol δ and also larger than the euryarchaeal DP1 subunit from *A. fulgidus* and *M. thermoautotrophicum*. Comparison of homologous proteins shows that the primary sequence of hyperthermophilic proteins tend to be shorter than their mesophilic counterparts (Russell et al. 1997; Hopfner et al. 1999; Rodriguez et al. 2000). The longer DP1 sequences from thermophilic organisms may harbor additional functions yet to be discovered. In vitro deletion analysis has shown that the C-terminal two-thirds of DP1 are important for the interaction with DP2 to elicit DNA polymerase activity (Cann and Ishino 1999). The dissimilar N-terminal regions among the

archaeal DP1 and eukaryal δ subunits are expected to be involved in species-specific interactions. Recently, the *DP1* genes were grouped into a novel family of DNA polymerase-associated B subunits together with the noncatalytic subunits of the eukaryotic family B polymerases α, δ, and ε (Mäkiniemi et al. 1999; Table 2). Given that the B subunits are conserved in the whole eukaryotic–archaeal branch of the phylogenetic tree, their function would also be conserved and fundamental. Mäkeniemi et al. proposed three possible functions for the B subunits: (i) regulation of DNA polymerase activity during replication; (ii) guidance of the polymerases to the replication fork; or (iii) maintenance of the structural integrity of the catalytic subunits. DP1 has also been shown to interact with the RadB protein, an enzyme involved in DNA recombination, suggesting that this heterodimeric polymerase might also be involved in this kind of DNA transaction (Hayashi et al. 1999).

DP2 is the larger and more conserved subunit of the heterodimeric protein. All other known DNA polymerase families have conserved sequence motifs A and C (Delarue et al. 1990). These motifs contain two invariant carboxylates that are indispensable for the polymerase activity owing to their function in metal coordination. In DP2, amino acid sequence motifs resembling motifs A and C have been found (Cann and Ishino 1999). An intein found in *P. horikoshii* DP2 within motif (GYAHYFHAAKRRNCDGDED) is highly conserved in all DP2 proteins (Cann and Ishino 1999). Because inteins tend to enter the regions encoding motifs crucial for enzyme function, the relevant conserved region is likely to play a role in the catalysis of the DP2 protein. At the C-terminal end of all known DP2 proteins, there are two conserved motifs with amino acid sequences similar to that known as the PCNA interacting protein box (PIP-box) (Warbick 1998). Indeed, the DP2 protein from *P. furiosus* was recently shown to interact directly with PCNA from this organism (Cann et al. 1999a).

From the results described above and from the comparative analysis of the recently published complete genome sequences of several archaeal species, it is now clearly established that (1) the heterodimeric family D DNA polymerase is present only in the Euryarchaea subkingdom; (2) the crenarchaeal species possess two (B1 and B3) or three (B1, B2, and B3) family B DNA polymerase genes; and (3) the Euryarchaea possess only one B-type DNA polymerase (B3), with the exception of *A. fulgidus* (Edgell et al. 1998) and *Halobacterium* NRC1 (Ng et al. 2000), which also have a B2-type DNA polymerase gene. Thus, the Crenarchaea and Euryarchaea do not have the same set of DNA polymerases. This underlines their early phylogenetic separation. The situation concerning DNA polymerases in the archaeal replication fork remains unsolved.

Moving platform proteins (PCNA, RFC)

Processivity is conferred on *E. coli* Pol III by a sliding clamp consisting of two β subunits. This doughnut-shaped sliding clamp is loaded onto the DNA strand in an ATP-dependent reaction by the clamp loader protein γ (Kelman and O'Donnell 1995). The function of the so-called moving-platform formed by the sliding clamp and clamp loader is structurally and functionally conserved throughout the three domains of life (Kornberg and Baker 1992). In the Eukarya, the clamp is a heterotrimer called the proliferating cell nuclear antigen (PCNA). It is loaded onto DNA by replication factor C (RFC), a heteropentamer. In eukaryotic organisms, PCNA is involved in a number of metabolic processes, including cell cycle control, DNA replication, nucleotide excision repair, and postreplicational mismatch repair (Jonsson and Hübscher 1997; Kelman 1997; Tsurimoto 1998). Here, we will report on its role as a processivity factor of archaeal DNA polymerases. PCNA-like and RFC-like proteins have been isolated from both crenarchaeal and euryarchaeal species as described below. Interestingly, for the PCNA-like protein, two genes were found in *S. solfataricus* and three genes in *Aeropyrum pernix*, both crenarchaeal species.

Crenarchaeal moving platform proteins

Recent biochemical studies have demonstrated that the processivity of crenarchaeal family B DNA polymerases can be noticeably enhanced in vitro by the presence of protein factors similar to the eukaryotic PCNA. The genes coding for two PCNA-like sliding clamps (referred to as Sso 039p and Sso 048p) have been identified in the *S. solfataricus* genome sequence, cloned, and overexpressed in *E. coli* (De Felice et al. 1999). The recombinant proteins were purified to homogeneity and shown to form highly stable trimers in solution. They have been reported to noticeably increase the Sso DNA pol B1 synthetic activity by enhancing its processivity on linear primer/templates [such as poly(dA)/oligo(dT) and linearized primed ssM13 DNA]. By immunoblot analysis using specific antisera it was also demonstrated that both processivity factors are present in *S. solfataricus* cells taken from the logarithmic and stationary phase of the culture. More recently, Pisani et al. (2000) reported the biochemical characterization of a clamp-loader complex homologous to eukaryotic replication factor C (RFC) from *S. solfataricus* (SsoRFC). The *Sulfolobus* clamp-loader system offers a striking example of the reduced complexity of the archaeal DNA replication apparatus in comparison with the eukaryal apparatus. It has been established that eukaryotic RFC is a heteropentamer composed of five subunits of different sizes (in *H. sapiens* they are referred to as p36, p37, p38, p40, and p140; Waga and Stillman 1998). The higher complexity of the eukaryotic clamp-loader assembly may have originated during evolution by diversification of functions among the various subunits. In fact, SsoRFC is a heteropentamer composed of polypeptides of 37 kDa (small subunit, 40% identical to the human RFC p40 subunit) and 46 kDa (large subunit, 23% identical to the human p140 subunit). The two SsoRFC subunits were demonstrated to form a complex with a native M_r of about 200 kDa and a 4 : 1 (small to large) stoichiometric ratio. The small subunit has been individually expressed in *E. coli* and demonstrated to form a homotetramer (SsoRFC-small; native M_r 156 kDa). SsoRFC-small and -complex are both endowed with ATPase activity. However,

only the ATPase activity of the holoenzymatic assembly is stimulated by primed DNA molecules such as poly(dA)/oligo(dT). Only the SsoRFC-complex has been shown to possess DNA-binding capability and to stimulate highly the synthetic activity of Sso DNA pol B1 in reactions containing primed circular ssM13 DNA, ATP, and either of the two *S. solfataricus* PCNA-like processivity factors (Sso 039p or Sso048p).

Euryarchaeal moving platform proteins

The first PCNA-like factor isolated from a euryarchaeon was that from *Pyrococcus furiosus* (Cann et al. 1999a). The PCNA-like factor interacts with both family B and D polymerases in vivo and in vitro, shown by immunological procedures, with PfuPCNA mainly binding to the DP2 subunit of the family D polymerase. The stimulation of both family B and D DNA polymerases was observed by using a circular template without a clamp loader (that is, without RFC). This differs from the eukaryotic proteins, which require RFC to open the ring structure of PCNA prior to loading it onto circular DNA. The sensitivity of family D DNA pol to PCNA stimulation is rather low: with 0.3 μg of PfuPCNA, only a 1.2-fold stimulation on linear DNA and a 1.1-fold stimulation on circular DNA were observed. These data were interpreted to indicate that both polymerases might be needed for processive DNA synthesis as it occurs during replication (Cann et al. 1999a).

An interesting observation was reported for the PCNA from *Thermococcus fumicolans* (*Tfu* pol). To stimulate the *Tfu* B3 pol, large amounts (0.3 μg) of *Tfu* PCNA were required for a three- or fourfold stimulation of the polymerase activity/processivity, while calf thymus DNA pol δ was stimulated in the same manner by only 10–30 ng *Tfu* PCNA (Hennecke et al. 2000). These results indicate that PCNA has been functionally conserved from hyperthermophilic Euryarchaea to mammals.

A euryarchaeal clamp loader and clamp were isolated from *Methanobacterium thermoautotrophicum* (*Mth*RFC, *Mth*PCNA; Kelman and Hurwitz 2000). As RFC isolated from eukaryotic cells, *Mth*RFC possesses a DNA-dependent ATPase activity that is stimulated by *Mth*PCNA. In contrast to observations made with the eukaryotic system, the *Mth*RPA inhibited the ATPase activity of *Mth*RFC, probably owing to different ssDNA binding. This inhibition was lifted by the addition of *Mth*PCNA. *Mth*RFC binds preferentially to DNA primer ends and loads *Mth*PCNA onto singly nicked circular DNA. The loading of *Mth*PCNA onto DNA by *Mth*RFC is temperature dependent, being optimal at 70°C, close to the growth temperature of the host organism. Together with *Mth*PCNA, *Mth*RFC stimulates the activity of the family B polymerase of *M. thermoautotrophicum* (Kelman and Hurwitz 2000). On the basis of gel filtration, glycerol gradient centrifugation, and comparison of Coomassie blue band intensity on a sodium dodecyl sulfate (SDS) gel, an unusual 4 + 2 heterohexamer organization has been described (Kelman and Hurwitz 2000). It is similar to the composition of the *E. coli* clamp loader γ complex, which is composed of five different subunits, of which one is present in two copies, leading to a hexameric structure of the protein complex (Kelman and O'Donnell 1995). On the other hand, the RFC-like factor from *Pyrococcus furiosus* has a 4 + 1 structure (Ishino et al. 2000). Further investigations are needed to show whether the *Mth*RFC composition is exceptional.

VI. Maturation of the Okazaki fragments

In *E. coli*, RNA primers are removed by the 5′–3′ exonuclease function of Pol I or by an RNase H. Eukarya and Archaea have flap endonucleases for this purpose (Rao et al. 1998; Cann and Ishino 1999). "Flaps" are DNA structures with single-stranded branches, as they occur, for example, during lagging strand synthesis, when the DNA polymerase displaces the primer. While bacterial DNA polymerases (such as *E. coli* or *Thermus aquaticus* DNA polymerase I) remove flaps with their 5′–3′ exonuclease function, all known eukaryotic DNA polymerases lack this activity. Eukaryotic flap endonucleases (FENs) are 5′ exonuclease as well. The acronym FEN refers to both enzymatic activities. FEN-1 is an essential enzyme for chromosomal stability, and also plays a crucial role in DNA repair. Mammalian FEN enzymes are known to interact with PCNA, being stimulated more than 10-fold by this interaction (Rao et al. 1998). The first isolated archaeal FEN was from *M. jannaschii* (MjFEN-1), which has a rather low in vitro cleavage rate. It is presumed to increase upon the interaction of FEN with DNA polymerase, PCNA, or both (Rao et al. 1998). On the other hand, MjFEN-1 retains activity after preincubation at 95°C for 15 min. The pH optimum is 6 to 7, in contrast to 8 for murine FEN-1. This is unexpected because proteins from thermophilic organisms tend to have higher pH optima than their mesophilic homologues (Rao et al. 1998). MjFEN-1 exhibits activity over high concentrations of monovalent cations (Bae et al. 1999). The crystal structure of MjFEN-1 reveals a compact protein composed of two domains (Hwang et al. 1998). There is a large, deep cleft in the center of the protein harboring two metal ions coordinated with several conserved acidic residues. Although the primary sequence similarity is limited, the MjFEN-1 backbone structure can be superimposed on T5 exonuclease (Ceska et al. 1996), *Taq* 5′-exonuclease (Kim et al. 1995), and T4 RNase H (Mueser et al. 1996). As an unusual feature, MjFEN-1 structure contains a long, flexible loop that recognizes the substrate DNA and an active site with different ligand–metal coordination (Hwang et al. 1998). An extensive investigation of the cleavage pattern of the thermostable flap endonuclease from *Pyrococcus horikoshii* (PhFEN) has been performed. The original purpose was to find out whether the supposed γ-radiation resistance of *P. horikoshii* was related to this enzyme, which is involved in DNA repair (Matsui et al. 1999). phFEN can cleave replication fork-like structures and double-stranded flap structures, whereas mammalian FEN cleaves only single-stranded flap structures. The authors suggest that phFEN might play a role in the generation of a new replication fork

by releasing it from an arrest caused by, for example, a defect in a replicative DNA helicase.

The ligation of the Okazaki fragments is catalyzed by ATP-dependent DNA ligases in Eukarya and Archaea and by a nicotinamide adenine dinucleotide (NAD^+)-dependent DNA ligase in Bacteria. The first ligase isolated from Archaea was that from *Methanobacterium thermoautotrophicum* (Sriskanda et al. 2000). It is a monomeric 561-amino-acid protein catalyzing strand-joining on a nicked DNA in the presence of a divalent cation and ATP. It is smaller than ATP-dependent ligases from fungi and metazoa, but larger than the virus-encoded ligases of T bacteriophages. The protein is tightly folded; only a linker region between the conserved amino acid motifs III and IIIa is protease sensitive, a feature shared with vaccinia and T7 ligases.

Evolution of DNA replication

The DNA replication machinery in Bacteria, compared to that of Archaea-Eukarya is built from a patchwork of orthologous proteins and a core of polymerases that seem to be unrelated (compare Table 1). Orthologous means that the proteins are homologous but have apparently been recruited for replication independently, so they are sometimes highly diverged. On the basis of detailed sequence comparisons of the proteins that fulfill indispensable functions in DNA replication, Leipe et al. (1999) concluded that DNA replication may have evolved twice independently. They proposed that the last universal common ancestor (LUCA) had a genetic system that contained both RNA and DNA, the latter being produced by reverse transcription. This model would explain the universal conservation of the core transcription machinery, of the enzymes for DNA-precursor biosynthesis, and of the RNA polymerase subunits in the three domains of life. The replicational cycle of the LUCA was proposed to be mirrored in today's retroid viruses, especially caulimoviruses and hepadnaviruses (Leipe et al. 1999).

With a similar set of sequences, Edgell and Doolittle (1997) ruled out this possibility and hypothesized that the LUCA had a DNA-based genome. They proposed three possible explanations for the situation observed at present: (1) bacterial and archaeal-eukaryotic replication proteins are homologous, but often have been so radically changed in sequence that they have become unrecognizable as homologues; (2) the LUCA contained both bacterial- and archaeal-eukaryotic-type replication systems, perhaps one being responsible for repair, and different components of these systems were lost in the two lineages after divergence; and (3) new nonhomologous proteins have been recruited into the replication machinery of one or the other of the lineages, replacing cenancestral components.

It has also been proposed that the original cellular DNA informational proteins have often been replaced by proteins of viral or plasmid origin. Because viral and plasmid-encoded proteins are usually very divergent from their cellular counterparts, this would explain the puzzling phylogenies and distribution of many DNA informational proteins among the three domains of life (Forterre 1999).

Concluding remarks

Although intensive research efforts have been made regarding the mechanisms involved in DNA replication in the Bacteria, Archaea, and Eukarya, further efforts are still needed to understand these complex protein machineries. In this review, the steps that are common to all life forms have been discussed with a focus on the Archaea, comparing their replicational proteins with their bacterial and eukaryal counterparts. Generally, the archaeal proteins involved in DNA replication are more similar to those of the higher organisms (the Eukarya) rather than to those from the Bacteria. One notable difference is that in the Archaea, fewer proteins participate in each stage of chromosomal DNA replication. Examples include the presence of only a single MCM homologue in most archaeal species (Bernander 1998), compared with six in eukaryotic cells (Dutta and Bell 1997), and the existence of three distinct RPA subunits in eukaryotes, compared with a single subunit in most (Eury)archaea (Chédin et al. 1998). Also in both crenarchaeal and euryarchaeal species, only two subunits homologous to the heteropentameric eukaryotic RFC have been identified (Pisani et al. 2000; Kelman and Hurwitz 2000). Another point to stress is that the Crenarchaea and Euryarchaea display differences, especially with respect to the set of DNA polymerases found in their genomes, but also with respect to SSB proteins. These differences in the DNA replication process suggest an early phylogenetic separation of these two archaeal subdomains.

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