Isolation and Characterization of a Split B-type DNA Polymerase from the Archaeon *Methanobacterium thermoautotrophicum ΔH*

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We describe here the isolation and characterization of a B-type DNA polymerase (PolB) from the archaeon *Methanobacterium thermoautotrophicum ΔH*. Uniquely, the catalytic domains of *M. thermoautotrophicum* PolB are encoded from two different genes, a feature that has not been observed as yet in other polymerases. The two genes were cloned, and the proteins were overexpressed in *Escherichia coli* and purified individually and as a complex. We demonstrate that both polypeptides are needed to form the active polymerase. Similar to other polymerases constituting the B-type family, PolB possesses both polymerase and 3′→5′ exonuclease activities. We found that a homolog of replication protein A from *M. thermoautotrophicum* inhibits the PolB activity. The inhibition of DNA synthesis by replication protein A from *M. thermoautotrophicum* can be relieved by the addition of *M. thermoautotrophicum* homologs of replication factor C and proliferating cell nuclear antigen. The possible roles of PolB in *M. thermoautotrophicum* replication are discussed.

DNA replication is the basis for evolution and propagation of living organisms. DNA-dependent DNA polymerases replicate double-stranded DNA, utilizing each complementary strand as the template for the synthesis of the other (1). Most organisms possess several DNA polymerases that differ in their catalytic properties such as processivity, fidelity, and rate of chain extension. Different polymerases are used for replication, repair, and recombination and have distinct polypeptide compositions. They also vary between the different genomes present in organelles found in eukaryotic cells (nuclear, mitochondrial, and chloroplast). Based on their amino acid sequences, DNA polymerases (pol) can be classified into at least five distinct groups (2, 3). Type (or family) A polymerases are named for their homology to *Escherichia coli* polA and include eubacterial, bacteriophage, archaea, and viral polys and the catalytic subunits of eukaryotic polys, pols, and pol. Eubacterial replicative pol (polIII, DNAE) is the prototype of the type C group, and the type X group includes proteins with homology to the eukaryotic β repair pols with some members also identified in eubacteria and archaea. A new group of polys, with no strong homology to any of the above families, has recently been identified in archaea (3). This family is named after the first member identified, the DP2 pol from *Pyrococcus furiosus*. These five groups appear only distantly related, and members in each group can be further subdivided by their function and sequence similarities.

Archaea, the third domain of life (4), are believed to replicate DNA in a eukaryotic like fashion. This conclusion is based in large part on the amino acid sequences of several archaea (5–8). Homologs of proteins involved in eukaryotic DNA replication have been identified within these genomes (reviewed in Refs. 9 and 10), whereas only limited similarities have been observed for bacterial proteins involved in replication. All archaea studied to date contain one or more type B polys (11) and perhaps also a DP2 type. However, some archaea also contain other polys, and the role of each is presently unclear (7).

The archaeon *Methanobacterium thermoautotrophicum ΔH* is an obligatory anaerobic thermophilic microorganism with an optimal growth temperature of 65–70 °C and a generation time of about 5 h (12). Based on sequence similarities to known polys, three putative polys have been identified within its genome as follows: a type B, a type DP2, and a type X. The pol constituting the B-type is unique in being made up of two separate gene products, PolB1 and PolB2 (Fig. 1A), with calculated molecular masses of 68 and 25 kDa, respectively (their complex will be referred to hereafter as PolB). The two genes are 850 kb apart on the circular genome of *M. thermoautotrophicum* and are encoded on different strands (7). Whereas all other known B-type polys are coded as one contiguous protein, several such euryarchaeote (the major archaeal subdivision to which *M. thermoautotrophicum* belongs) proteins contain one to three inserts that are post-translationally removed by protein splicing (inteins) (13) (Fig. 1A).

In this study, we describe the isolation and the biochemical characterization of the split PolB from *M. thermoautotrophicum*. Recombinant proteins were expressed and purified from *E. coli* cells, and the properties of these pol were studied in vitro.

EXPERIMENTAL PROCEDURES

Materials—Labeled deoxy- and ribonucleoside triphosphates were obtained from Amersham Pharmacia Biotech. Unlabeled deoxyribonucleoside triphosphates were from Amersham Pharmacia Biotech. Single-stranded M13mp19 was from Life Technologies, Inc.; the various pET vectors used were from Novagen, and oligonucleotides were synthesized by Gene Link (Hawthorne, NY). *E. coli* SSB and the bacteriophage T4 gene product 32 were from Amersham Pharmacia Biotech. *Schizosaccharomyces pombe* RPA was purified as described previously (14).
Rabbit polyclonal antibodies were generated by Cocalico Biologics Inc. (Reamstown, PA). The buffers used and their composition were as follows: buffer A which contained 20 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 0.5 mM EDTA, and 10% glycerol; buffer B which contained 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 10% glycerol.

**Expression and Purification of mthPolB**—To determine whether PolB is an active pol, its subunits (PolB1 and PolB2) were purified and charmed individually and together into E. coli expression vectors and expressed as fusion proteins containing N-terminal His	extsubscript{6} tags (see “Experimental Procedures”). The PolB complex, containing both subunits, was soluble and was purified to near homogeneity by affinity chromatography onto Ni	extsuperscript{2+} chelate and a HiTrap-Q column (Amersham Pharmacia Biotech) (Fig. 2A).

**RESULTS**

**Split Polymerase**—As originally reported, mthPolB is encoded by two separate genes that are 850 kb apart and located on different strands (7). The sequences of these two proteins together correspond jointly to the single contiguous protein characteristic of all other known type B pols and contain all their conserved motifs. The division of these two genes occurs in a non-conserved sequence region, unlike the intein integration sites of related pols that are all found in highly conserved regions (Fig. 1A). Within the archaea, the type B DNA pol can be divided into three subgroups of which the mthPolB falls within the archaeal group I of the type B DNA pols (11) (Fig. 1B).

**Expression and Purification of mthPolB**—To determine whether PolB is an active pol, its subunits (PolB1 and PolB2) were purified and charmed individually and together as the complex. The genes encoding PolB1 and PolB2 (open reading frame MTH1208 and MTH208, respectively) (7) were individually and together into E. coli expression vectors and expressed as fusion proteins containing N-terminal His	extsubscript{6} tags (see “Experimental Procedures”). The PolB complex, containing both subunits, was soluble and was purified to near homogeneity by affinity chromatography onto Ni	extsuperscript{2+} chelate and a HiTrap-Q column (Amersham Pharmacia Biotech) (Fig. 2A).
PolB1 alone was marginally soluble (few percent) and was purified by affinity chromatography on Ni²⁺ chelate (Fig. 2B), whereas PolB2 was completely insoluble and could be extracted from cells only in the presence of 6 M urea. PolB2 was purified to near homogeneity following chromatography on Ni²⁺ chelate (Fig. 2C). This protein fraction was used to generate polyclonal antibodies against PolB2. The observations that the two individual subunits were not soluble when each was expressed alone but were soluble as the heterodimeric complex support the idea that they work jointly together.

Glycerol gradient centrifugation of the pooled HiTrap-Q fractions of PolB yielded a single peak of DNA synthetic activity that sedimented between aldolase and BSA (Fig. 3). SDS-PAGE analysis of the gradient fractions revealed that the peak of pol activity sedimented coincidentally with both PolB proteins (Fig. 3).

Characterization of the PolB Replication Activity—M. thermoautotrophicum is a thermophile that grows optimally at 65–70 °C (12). For this reason, we examined the influence of temperature on DNA synthesis catalyzed by PolB. As shown in Fig. 4A, PolB, at the concentration used (50 fmol), was not appreciably active at temperatures below 50 and above 80 °C, observations consistent with the optimal growth conditions. Furthermore, under appropriate conditions, the addition of 228 fmol of PolB was sufficient to replicate the entire 7.25 kb of M13 between 10 and 20 min at 60 °C (Fig. 4B). In similar experiments, no replication activity was detected when the PolB1 subunit alone was used (data not shown).

Pols from several archaea have been studied, and each has distinct salt, pH, and Mg²⁺ requirements for optimal activity. These parameters were determined for PolB. Optimal activity was observed in the presence of 100 mM NaCl (Fig. 4C), 7 mM Mg²⁺, and at pH 7.5 (data not presented). The effects of several pol inhibitors were also examined. N-Ethylmaleimide and aphidicolin, which inhibit eukaryotic pols, were, however, not effective against the activity of PolB. Antibodies generated against PolB2 inhibited PolB polymerase activity, further supporting the conclusion that the two subunits jointly participate in supporting DNA synthesis. These antibodies, however, did not inhibit the activity of E. coli pol I (data not presented).

Exonuclease Activity of PolB—The majority of enzymes in the B family of pols possess exonuclease activity. Several members, however, do not (e.g., pola). The amino acid sequence of
PolB includes a putative exonuclease domain located between amino acid residues 165 and 362 of the PolB1 subunit (Fig. 1A). The following experiments were designed to determine whether PolB possessed exonuclease activity.

As shown in Fig. 5A, PolB preparations contain a temperature-dependent 3' to 5' exonuclease activity when assayed in the presence of a singly primed M13 DNA template. Although no activity was observed at 30 °C, efficient removal of the 32P-labeled nucleotide from the 3' end of the primed DNA was observed at 70 °C (Fig. 5A). At 50 °C, the efficiency of exonuclease activity was lower than that observed at 70 °C but greater than that detected at 30 °C. These results are similar to Fig. 3.

**Fig. 3. Glycerol gradient sedimentation of PolB.** This step was carried out as described under “Experimental Procedures.” A, aliquots (20 μl) of the glycerol gradient fractions were subjected to 10% SDS-PAGE analysis followed by Coomassie Blue staining. Lane 1, molecular mass markers; lane 2, the load on material; lanes 3–15, glycerol gradient fractions. The peak positions of BSA (4.3 S), aldolase (7.3 S), and catalase (11.3 S) are marked at the top. B, elution profile of PolB activity determined by the replication assay described under “Experimental Procedures.”

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**Fig. 2. Purification of recombinant proteins.** All of the gels shown were stained with Coomassie Blue after 10% SDS-PAGE analysis. A, purification of PolB; lane 1, molecular mass markers; lane 2, extract from uninduced whole cells; lane 3, extract from IPTG induced whole cells; lane 4, soluble fraction of cell lysate (10 μg); lane 5, Ni2+ chelate column (2 μg); lane 6, Q-Sepharose eluate (2 μg). B, purification of PolB1; lane 1, molecular mass markers; lane 2, extract from uninduced cells; lane 3, extract from IPTG-induced cells; lane 4, soluble fraction of cell lysate (10 μg); lane 5, cell lysate solubilized in 6 M urea (10 μg); lanes 6, Ni2+ chelate column eluate (2 μg). C, purification of PolB2; lane 1, molecular mass markers; lane 2, extract from uninduced whole cells; lane 3, extract from IPTG-induced whole cells; lane 4, soluble fraction of cell lysate (10 μg); lane 5, cell lysate solubilized in 6 M urea (10 μg); lane 6, Ni2+ chelate column (2 μg).
the temperature effects observed for DNA synthesis (see Fig. 4A). No 5' to 3' exonuclease activity was detected when the enzyme was incubated with either the primed DNA at any temperature (30–70 °C; data not presented) or single-stranded polydeoxyoligonucleotide substrates (data not shown). When reactions were incubated for a longer length of time or when high levels of PolB were used, the length of the 5'-labeled oligonucleotide was reduced due to its digestion from the 3'-end as judged by its chromatographic properties on PEI plates (data not shown). Under the conditions used in the experiment described in Fig. 5B, 3' to 5' exonuclease activity was not observed at 30 °C. However, when the concentration of PolB was increased 300-fold, 3' to 5' exonuclease activity was detected (data not shown). Although PolB exhibited limited exonuclease activity at low temperatures on primed ssDNA, potent 3' to 5' exonuclease activity was evident even at low temperatures in the presence of the single-stranded polydeoxyoligonucleotide substrate (Fig. 5B).

Since the exonuclease domain of the pol is located in the PolB1 subunit, we examined whether PolB1 by itself possesses exonuclease activity. As shown in Fig. 5B, PolB1 exhibited catalyzed elongation of singly primed M13 DNA was carried out in reaction mixtures (20 μl) described under “Experimental Procedures” in the presence of 12.8 fmol of DNA and 0.1 mM NaCl. Lane 1, no polymerase was added; lanes 2–4 contained 0.288 pmol of PolB. Reactions were incubated at 60 °C for 5, 10, and 20 min in lanes 2–4, respectively, and for 20 min in lane J. Reactions were processed as described for DNA synthesis and alkaline-agarose gel electrophoresis. Size markers (in kb) are shown at the left. C, effect of salt on the replication activity of PolB was performed as described in A at 70 °C at salt concentrations as indicated.

Fig. 4. DNA synthesis by PolB. A, effect of temperature on the replication activity of PolB was performed as described under “Experimental Procedures.” Reaction mixtures (20 μl) containing 8.3 fmol, singly primed M13 single-stranded DNA, 50 fmol of PolB and 0.1 mM NaCl were incubated for 30 min at the indicated temperatures and analyzed as described under “Experimental Procedures.” B, Pol B-

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)
exonuclease activity, but its activity was lower than that observed with the PolB complex (2-fold at 50 °C). Whether this is due to the limited solubility of PolB1 (and possible aggregation) or to the activation of the exonuclease activity of PolB1 through its association with PolB2 is presently unknown.

The Effects of Single-stranded DNA-binding Protein on PolB Activity—In mesophiles, a single-stranded DNA-binding protein (SSB) is an essential component of all replication systems (1). SSBs stimulate the activity of pols by removing DNA secondary structures that interfere with their movement. M. thermoautotrophicum grows at high temperatures, and thus the problems due to DNA secondary structure are likely to be reduced. However, a sequence search revealed the presence of RPA homologs in the M. thermoautotrophicum genome (mthRPA).

When the sequence of the M. thermoautotrophicum genome was first published, it was reported that RPA is encoded by two genes with partially overlapping sequences (7). The authors suggested that there might be a frameshift mutation in the sequence. The cloning and sequencing of the two putative mthRPA genes described in this study detected a single base insertion in the published sequence located in the overlap region. Correction of this error indicated that the nucleotide sequence of the gene encoding mthRPA is one continuous sequence leading to a single polypeptide chain of 792 amino acids with a calculated molecular mass of 90.2 kDa (Fig. 6A). In keeping with the sequence presented in Fig. 6A, the cloning, expression, and isolation of mthRPA (as described under “Experimental Procedures”) yielded a single protein band of the expected size (Fig. 6B) that contained strong ssDNA binding activity.

The experiments described in Fig. 4 were carried out in the absence of a SSB. In the following experiments the role of SSB on PolB replication activity was examined (Fig. 7). Surprisingly, DNA synthesis by PolB was inhibited by mthRPA. Reactions carried out at 60–70 °C in the presence of mthRPA were inhibited (Fig. 7, A and B); reactions carried out with SSBs from other organisms did not inhibit DNA synthesis by PolB and even slightly stimulated DNA synthesis compared with reactions carried out without SSB (Fig. 7A). Although S. pombe RPA and phage T4 gene product 32 bind weakly to DNA at 70 °C, E. coli SSB strongly binds DNA at 30 and 70 °C (data not presented). The inhibition of PolB-catalyzed DNA synthesis by mthRPA appears specific. mthRPA did not inhibit Thermus aquaticus (Tag) (Life Technologies, Inc.) and P. furiosus (Pfu) (Stratagene) DNA polymerases under similar assay conditions (data not presented).

To determine whether the inhibition of DNA synthesis was dependent on the concentration of RPA, the effects of increased levels of mthRPA were examined. As shown in Fig. 7, B and C, mthRPA inhibited DNA synthesis in a concentration-dependent manner. These results also demonstrated that the inhibition was predominantly due to the ssDNA binding activity of mthRPA and not to its interaction with the polymerase (described below). At the lowest levels of mthRPA added, mthRPA was present to a large molar excess over PolB (Fig. 7, B and C). At the highest concentration added, enough RPA was present to coat the entire DNA template. This value was calculated assuming that mthRPA and the RPA from Methanococcus jan-naschii bind to DNA in an identical manner. RPA from this archaea was shown to bind 15–20 nucleotides of ssDNA per molecule of RPA (20).

We next examined the effect of SSB on the 3’ to 5’ exonuclease activity of PolB. Both primed ssDNA and single-stranded polydeoxyoligonucleotide were used as substrates to determine whether mthRPA affected the 3’ to 5’ exonuclease activity. As shown in Fig. 8A, exonuclease activity with singly primed M13 DNA was not detected at 30 °C in the absence of SSB. This low activity was stimulated by the addition of SSBs (Fig. 8A). This may be due to a reduction in the nonspecific binding of the pol to the extensive ssDNA region. At higher temperatures (50 and 70 °C), exonuclease activity was detected without SSBs and their presence stimulated the exonuclease activity (Fig. 8A). When the single-stranded polydeoxyoligonucleotide substrate (71-mer) was used, the 3’ to 5’ exonuclease activity of PolB was detected at all temperatures (Fig. 8B). The exonuclease activity was slightly reduced by the presence of SSBs at all temperatures. These results demonstrate that in contrast to the polymerase activity of PolB, its 3’ to 5’ exonuclease activity was hardly affected by mthRPA.

PolB Interacts with RPA—In several replication systems, pols have been shown to interact directly with their corresponding SSBs. For example, eukaryotic pol interacts with RPA (21). E. coli polII interacts with E. coli SSB (22). T4 gene product 43 (the pol of phage T4) interacts with gene product 32 (phage T4 SSB) (23), and the T7 phage gene 2.5 protein (phage T7 SSB) interacts with the phage T7 pol (24). For this reason, we tested whether PolB interacted with mthRPA.

The interaction between mthRPA and the polymerase was studied by gel retardation centrifugation and co-immunoprecipitation experiments. PolB and RPA individually and in combination were sedimented through a 15–35% glycerol gradient. The proteins (1.5 nmol of each) alone, or in combination, were applied to a 5-ml glycerol gradient as described under “Experimental Procedures.” After centrifugation, the distribution of the proteins across the gradient was analyzed by SDS-PAGE. As shown in Fig. 9, PolB alone sedimented as a homogeneous protein, peaking at fraction 17. RPA alone behaved identically and peaked at fraction 19. These proteins alone sedimented to a position between BSA (4.3 S) and aldolase (7.3 S). When the two proteins were mixed together, they co-sedimented as a complex that peaked at fraction 15. Furthermore, the presence of the RPA-PolB complex was evident even in fraction 11. This trailing may indicate that the complex is not completely stable under the condition used (4 °C and in the presence of 0.5 M NaCl) and partially dissociated during the sedimentation period.

Direct interaction between PolB and mthRPA was also detected using co-immunoprecipitation of the complex. For these studies, either labeled mthRPA generated by in vitro transcription/translation or purified mthRPA was used for immunoprecipitation with antibodies against PolB. Only when purified PolB was combined with mthRPA was mthRPA detected in the immunoprecipitates. No RPA was observed in control reactions carried out in the absence of PolB (data not presented). These results demonstrate that PolB and mthRPA directly interact to form a complex.

RFC and PCNA Relieve the Inhibitory Effect of mthRPA—In bacteria and eukaryotes, replicative pols have low processivity unless they are associated with a ring-shaped accessory protein, a DNA sliding clamp (reviewed in Refs. 25 and 26). The sliding clamp is assembled around DNA by a protein complex called the clamp loader. By encircling the DNA and interacting with the polymerase, the clamp tethers the pol to the DNA template for processive DNA synthesis (25, 26). Homologs of the eukaryotic clamp (proliferating cell nuclear antigen (PCNA)) and its clamp loader (replication factor C (RFC)) have been identified in M. thermoautotrophicum (7). Both proteins were cloned in E. coli and purified to homogeneity (data not presented). We studied whether PolB can work in conjunction with mthRFC and mthPCNA and whether these accessory proteins could relieve the inhibition of DNA synthesis by
mthRPA. As shown in Fig. 10, the presence of RFC and PCNA not only relieved the inhibitory effects of mthRPA on PolB activity but also stimulated DNA synthesis compared with the activity observed in reactions containing PolB alone.

In these reactions, the rate of elongation of singly primed M13 DNA by PolB alone was decreased by reducing the temperature of the reaction to 50 °C and by the presence of 0.25 M NaCl (see Fig. 4). The effects of mthRPA, RFC, and PCNA on chain elongation were examined at three different concentrations of PolB. As shown (Fig. 10), increasing levels of PolB alone under these conditions resulted in the synthesis of low levels of DNA of short chain length (Fig. 10, lanes 1, 4, and 7). Addition of mthRPA reduced both the level and size of DNA synthesized (Fig. 10, lanes 2, 5, and 8). The addition of mthRFC and mthPCNA markedly increased both the amount of DNA synthesized as well as the chain length of the products formed.
FIG. 7. Effect of various SSBs on PolB polymerase activity. A, the effect of different SSBs on PolB pol activity was analyzed using singly primed M13 ssDNA as described under “Experimental Procedures.” Reaction mixture (20 μl) contained 8.3 fmol of DNA and either no SSB, 1 pmol of *E. coli* SSB, 6.2 pmol of phage T4 gene 32, 7.25 pmol of mthRPA, or 3.8 pmol of *S. pombe* RPA. The reaction mixtures were incubated for 30 min at different temperatures, as indicated, and analyzed as described under “Experimental Procedures.” B, PolB-catalyzed elongation of singly primed M13 DNA was carried out in reaction mixtures (20 μl) described under “Experimental Procedures” in the presence of 12 fmol of DNA, 0.1 M NaCl, and either 48 fmol (lanes 2–5) or 288 fmol (lanes 6–9) of PolB and either 15 pmol (lanes 3 and 7), 7.5 pmol (lanes 4 and 8), or 3 pmol (lanes 5 and 9) of mthRPA. Reactions were incubated at 60 °C for 20 min, and an aliquot (2 μl) was removed to measure the extent of DNA synthesis. The remaining reaction mixtures were subjected to alkaline-agarose gel electrophoresis. Size markers (in kb) are shown on the left. C, inhibition of DNA synthesis as a function of mthRPA concentration. Reaction mixtures, as described in B, containing 48 fmol of PolB were incubated with the indicated amounts of mthRPA. After 20 min, an aliquot was used to measure nucleotide incorporation.
**Discussion**

The complete genomic sequence of several archaea (5–8), together with the isolation and identification of individual genes from other members of this domain, suggests that the processes leading to replication, transcription, and translation in all archaea studied to date are more similar to those in eukaryotes than those in bacteria (eubacteria) (10, 27). Although there are striking similarities in the DNA replication processes leading to replication, transcription, and translation in all archaea studied to date are more similar to those in eukaryotes than those in bacteria (eubacteria) (10, 27). Although there are striking similarities in the DNA replication processes leading to replication, transcription, and translation in all archaea studied to date are more similar to those in eukaryotes than those in bacteria (eubacteria) (10, 27).

These results demonstrate that PolB is stimulated by the processivity auxiliary factors RFC and PCNA which are likely to contribute to the replication of *M. thermoautotrophicum* DNA (see “Discussion”). They further demonstrate that these accessory proteins are capable of overcoming the inhibition of DNA synthesis by mthRPA.

**Fig. 8. Effect of SSBs on the 3′-5′ exonuclease activity of PolB.** A, exonuclease assays were performed as described under “Experimental Procedures” in reaction mixture (20 μl) containing 20 fmol of 3′-labeled singly primed M13 ssDNA, 50 fmol of PolB, and either no SSB, 7.55 pmol of mthRPA, or 1 pmol *E. coli* SSB. Reaction mixtures were incubated for 10 min at the indicated temperatures and analyzed as described under “Experimental Procedures.” B, exonuclease assays were performed as described under “Experimental Procedures” in reaction mixtures (20 μl) containing 20 fmol of 3′-labeled oligonucleotide, 5 fmol of PolB, and either no SSB, 200 fmol of mthRPA, or 200 fmol of *E. coli* SSB. Reaction mixtures were incubated for 10 min at the indicated temperatures and analyzed as described under “Experimental Procedures.”

(Fig. 10, lanes 3, 6, and 9). No synthesis was detected in reactions containing mthRPA, RFC, and PCNA but lacking PolB (lane 10). Furthermore, the marked stimulation required the presence of both RFC and PCNA (data not presented).

These results demonstrate that PolB is stimulated by the processivity auxiliary factors RFC and PCNA which are likely to contribute to the replication of *M. thermoautotrophicum* DNA (see “Discussion”). They further demonstrate that these accessory proteins are capable of overcoming the inhibition of DNA synthesis by mthRPA.

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2 S. Pietrokovski, unpublished results.
The latter pol is thought to be involved exclusively in DNA repair processes; thus, it is not clear which of the other two pols is responsible for the replication of *M. thermoautotrophicum* chromosome. To date, DP2-like pols have been identified in all fully sequenced euryarchaeota (36). Furthermore, based on the characterization of DP2 pol isolated from *P. furiosus* (processivity, 39 to 59 exonuclease activity), it has been suggested that this pol functions as the replicative pol (3, 38). It was not demonstrated, however, that DP2 pol activity is stimulated by *P. furiosus* PCNA and RFC. Stimulation by these factors is the hallmark of replicative polymerase in other systems. The stimulation of mthPolB by RFC and PCNA suggests that it may be the replicative pol in *M. thermoautotrophicum*. MthPolB may also act in conjunction with the DP2-like pol. One pol may replicate the leading strand whereas the other replicates the lagging strand.

PolB may also be involved in post-replicative processes. This may be the reason for its relatively low processivity and inhibition by mthRPA. For example, PolB may be a functional homolog of Polɛ, a eukaryotic member of the B-type pols. Polɛ was suggested to play a role in Okazaki fragment maturation by filling the gaps left on the lagging strand (39). PolB may also play a role in post-replicative DNA repair since it contains a potent 3' to 5' exonuclease activity. Pols also play important roles in recombination, and PolB may be involved in this process as well.

The pols of many organisms have been shown to interact with their respective SSBs. Such interactions have been observed with eukaryotic pola (21) and pold,3 *E. coli* polII (22), and bacteriophages T4 and T7 pols (23, 24). The interactions between these enzymes and their cognitive SSBs play different roles. For example, Pola does not bind stably to the DNA template unless supported by its interaction with RPA, whereas in other cases, the SSBs stimulate the pol activity. The role of the interactions between PolB and mthRPA, described here, is currently under investigation.

An interesting observation is the effect of mthRPA on DNA synthesis by PolB. mthRPA inhibits the replication activity of PolB in a concentration-dependent manner suggesting that the inhibition is, at least in part, due to the coating of the DNA and not exclusively through its interaction with the polymerase. The inhibition of DNA replication by mthRPA may have a specific function. If the DP2 pol of *M. thermoautotrophicum* is the replicative pol, then mthRPA may prevent PolB from acting at the replication fork. If PolB were to act solely in the repair and/or maturation of Okazaki fragments, which normally occurs over a relatively short region of DNA, little or limited

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3 A. Yuzhakov, Z. Kelman, J. Hurwitz, and M. O’Donnell, submitted for publication.
amounts of RPA should be present and therefore RPA would have little or no effect on PolB activity. Alternatively, if PolB is a part of the replicative pol, it would need to associate with PCNA to become processive. PCNA relieves the inhibitory effects of mthRPA and thus ensures that only in the right context of a polymerase-clamp complex, PolB will work at the replication fork. The isolation and characterization of the M. thermoautotrophicum DP2 pol may help to answer these possibilities.

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