

# Isolation and Characterization of a Split B-type DNA Polymerase from the Archaeon *Methanobacterium thermoautotrophicum* $\Delta$ H\*

(Received for publication, May 18, 1999)

Zvi Kelman<sup>‡§</sup>, Shmuel Pietrokovski<sup>¶</sup>, and Jerard Hurwitz<sup>‡¶</sup>

From the <sup>‡</sup>Department of Molecular Biology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 and the <sup>¶</sup>Department of Molecular Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel

**We describe here the isolation and characterization of a B-type DNA polymerase (PolB) from the archaeon *Methanobacterium thermoautotrophicum*  $\Delta$ H. 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)<sup>1</sup> can be classified into at least five distinct groups (2, 3). Type (or family) A polymerases are named for their homology to *Escherichia coli* polI and include eubacterial, mitochondrial (poly), and bacteriophage pols. Type B pols are

named for their homology to *E. coli* polIII. This family is more diverse than family A; they include eubacterial, bacteriophage, archaea, and viral pols and the catalytic subunits of eukaryotic pol $\alpha$ , pol $\delta$ , and pol $\epsilon$ . 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  $\beta$  repair pols with some members also identified in eubacteria and archaea. A new group of pols, 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 pols (11) and perhaps also a DP2 type. However, some archaea also contain other pols, and the role of each is presently unclear (7).

The archaeon *Methanobacterium thermoautotrophicum*  $\Delta$ H 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 pols, three putative pols 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 pols 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 this pol were studied *in vitro*.

## EXPERIMENTAL PROCEDURES

**Materials**—Labeled deoxy- and ribonucleoside triphosphates were obtained from Amersham Pharmacia Biotech. Unlabeled deoxynucleoside triphosphates were from Amersham Pharmacia Biotech. Single-stranded M13mp19 was from Life Technologies, Inc.; the various pET vectors used were from Novagene, 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).

\* This work was supported in part by National Institutes of Health Grant GM 38559 (to J. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AE000901.

§ Supported by a Helen Hay Whitney postdoctoral fellowship. To whom correspondence should be addressed: Dept. of Molecular Biology, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., Box 97, New York, NY 10021. Tel.: 212-639-5895; Fax: 212-717-3627; E-mail: z-kelman@ski.mskcc.org.

¶ Professor of the American Cancer Society.

<sup>1</sup> The abbreviations used are: pol, polymerase; mth, *Methanobacterium thermoautotrophicum*; RPA, replication protein A; RFC, replication factor C; PCNA, proliferating cell nuclear antigen; SSB, single-stranded DNA-binding protein; kb, kilobase pair; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; ssDNA, single-stranded DNA; IPTG, isopropyl-1-thio- $\beta$ -D-galactopyranoside.

Rabbit polyclonal antibodies were generated by Cocalico Biologicals 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 L which contained 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 10% glycerol.

**Computational Sequence Analysis**—Protein sequences were retrieved from the NCBI data bases and aligned across short conserved sequence regions using the BlockMaker (15) and MACAW (16) programs as described previously (17, 18). Dendrograms were calculated from the block alignments by standard methods described previously (19).

**Cloning of *M. thermoautotrophicum* Genes**—PolB1 and PolB2 genes (*MTH1208* and *MTH208*, respectively) were amplified by polymerase chain reaction from *M. thermoautotrophicum* DNA (kindly provided by John Reeve, Ohio State University) and were cloned, after sequencing, between the *Nde*I and *Bam*HI sites of the bacterial expression vector pET-16b (Novagene) (called pET16-PolB1 and pET16-PolB2). These two constructs contained a His<sub>10</sub> tag at the N terminus of their respective proteins. PolB2 was also cloned into pET-21a (Novagene) (called pET21-PolB2) using the same restriction sites. *mthRPA* was cloned between the *Bam*HI and *Sal*I sites of pET28a (Novagene) (called pET28-RPA) and contained a His<sub>6</sub> tag at the N terminus. A vector that expressed both subunits of PolB, PolB1 and PolB2, was generated as follows. A *Bgl*III-*Bam*HI fragment of pET16-PolB1 that contained the entire coding region and the upstream regulatory sequences (the T7 promoter and the ribosome-binding site) was cloned into the *Bam*HI site of pET21-PolB2. Thus, although the new vector (called pET21-PolB) expressed both subunits of PolB, only the large subunit, PolB1, contained a His<sub>10</sub> tag. The cloning of proliferating cell nuclear antigen (PCNA) and the two-subunit RFC complex will be described elsewhere.

**Expression and Purification of Recombinant Proteins**—PolB and *mthRPA* proteins were overexpressed as follows: 12 liters of *E. coli* cells BL21(DE3) pLysS (Novagene) harboring the different plasmids were grown at 37 °C in Luria-Bertani (LB) medium in the presence of appropriate antibiotics. When the culture reached an A<sub>600</sub> of 0.5, protein expression was induced by incubation in the presence of 2 mM IPTG for 3 h after which time the cells were harvested yielding 60 and 33 g (wet weight) of cells expressing PolB and *mthRPA*, respectively. PolB and RPA were purified from *E. coli* cells as follows: bacterial lysates were prepared by sonication in 75 ml of buffer L. After centrifugation for 20 min at 36,000 × *g*, extracts were mixed with 5 ml of Ni<sup>2+</sup> chelate (ProBound resin, Invitrogen) for 2 h at 4 °C with gentle shaking. The mixtures were then poured onto a column, washed with 25 ml of buffer L containing 10 mM imidazole, and eluted with 10 ml of buffer L containing 500 mM imidazole. The latter fraction was dialyzed overnight against 2 liters of buffer A containing 100 mM NaCl. The dialyzed material was loaded onto a 5-ml HiTrap-Q column (Amersham Pharmacia Biotech) equilibrated with buffer A containing 100 mM NaCl. The column was washed with 25 ml of buffer A containing 200 mM NaCl and developed with a 50-ml linear gradient of NaCl from 200 to 700 mM in buffer A. The pooled protein peaks (6 mg of PolB peaking at 450 mM NaCl and 12 mg of *mthRPA* peaking at 550 mM NaCl) were dialyzed overnight against 2 liters of buffer A containing 100 mM NaCl (see Fig. 2A). PolB1 was purified essentially as described for PolB but without the HiTrap-Q step from 2 liters of cells (8 g). PolB1, however, has limited solubility (see Fig. 2B). In contrast, PolB2 was not soluble under similar conditions (see Fig. 2C) and therefore was purified in the presence of urea as follows: bacterial lysates were prepared by sonication in 75 ml of buffer L containing 6 M urea. After centrifugation for 20 min at 36,000 × *g*, the extract was mixed with 5 ml of Ni<sup>2+</sup> chelate (ProBound resin, Invitrogen) for 2 h at 4 °C with gentle shaking. The mixture was then loaded onto a column, washed with 25 ml of buffer L containing 6 M urea and 10 mM imidazole, and eluted with 10 ml of buffer L containing 6 M urea plus 500 mM imidazole. The eluted protein fraction (10 mg) was dialyzed overnight against 2 liters of buffer A containing 6 M urea. Protein concentrations were determined by Bradford assay (Bio-Rad) using bovine serum albumin (BSA) as the standard. Proteins were stored at -70 °C. PolB activity was stable to repeated freezing and thawing.

**Elongation of a Singly Primed M13 DNA Template**—PolB catalyzed elongation of singly primed M13 DNA was carried out in reaction mixtures (20 μl) containing 40 mM Tris-HCl (pH 7.5), 0.5 mM dithiothreitol, 0.01% BSA, 7 mM magnesium acetate, 2 mM ATP, 100 μM each of dCTP, dGTP, and dTTP, 20 μM [ $\alpha$ -<sup>32</sup>P]dATP (0.5–2 × 10<sup>4</sup> cpm/pmol), 12 fmol of singly primed M13 DNA (primed with M13-1 primer, map position 5999–6033), and varying levels of PolB as indicated. Reaction mixtures were incubated as indicated in the legends, stopped with 10 mM EDTA, and separated by electrophoresis through an alkaline-aga-

rose gel (1.5%) followed by autoradiography. For quantitation, an aliquot (2 μl) of the reaction mixture was removed, and the amount of DNA synthesis was measured by adsorption to DE81 paper.

**Exonuclease Activity**—Exonuclease activity was determined using two different DNA substrates. One assay involved the use of a singly primed M13 single-stranded DNA, and the other involved the use of a labeled single-stranded oligonucleotide. In the first assay, a 34-mer oligonucleotide (M13-1 map position 5999–6033) was hybridized to ssM13mp19 DNA and then labeled at its 5'-end with T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP or at its 3'-end using Klenow and [ $\alpha$ -<sup>32</sup>P]dATP. In the second assay, a 71-mer oligonucleotide (Z18; 5'-CTTGC-CCCCAAAATTGGTGC GCGGGCT GCGGCGTAGATTACGGAATGCA-TATCTCCTAGGAATCTCTTTGC-3') was labeled at the 5'-end with T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP or at the 3'-end using calf thymus terminal transferase and [ $\alpha$ -<sup>32</sup>P]dATP. Unincorporated nucleotides were removed using the QIAquick nucleotide removal kit (Qiagen). Exonuclease assays (20 μl) were performed at different temperatures in the presence of 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 50 μg/ml BSA, 20 fmol of either the 3'- or 5'-end-labeled DNA substrate, and protein concentrations as indicated in the figure legends. The removal of <sup>32</sup>P from either the 3'- or 5'-end of the labeled substrates was analyzed using DE81 paper or by thin layer chromatography on polyethyleneimine (PEI) Cellulose F plates (EM Sciences, Gibbstown, NJ) using the solvent 0.5 M LiCl plus 1 M HCOOH, which readily separated mononucleotides from oligonucleotides.

In order to examine the exonuclease activity of PolB in the presence of different SSBs, 20 fmol of either the 3'-labeled singly primed M13 DNA or the 3'-labeled oligonucleotide was incubated for 10 min with 50 fmol of PolB using the standard exonuclease assay. In reactions containing singly primed M13 DNA, no SSB or 15 pmol of *E. coli* SSB or *mthRPA* was added. In reactions containing the 71-mer oligonucleotide, no SSB or 200 fmol of *E. coli* SSB or *mthRPA* was added.

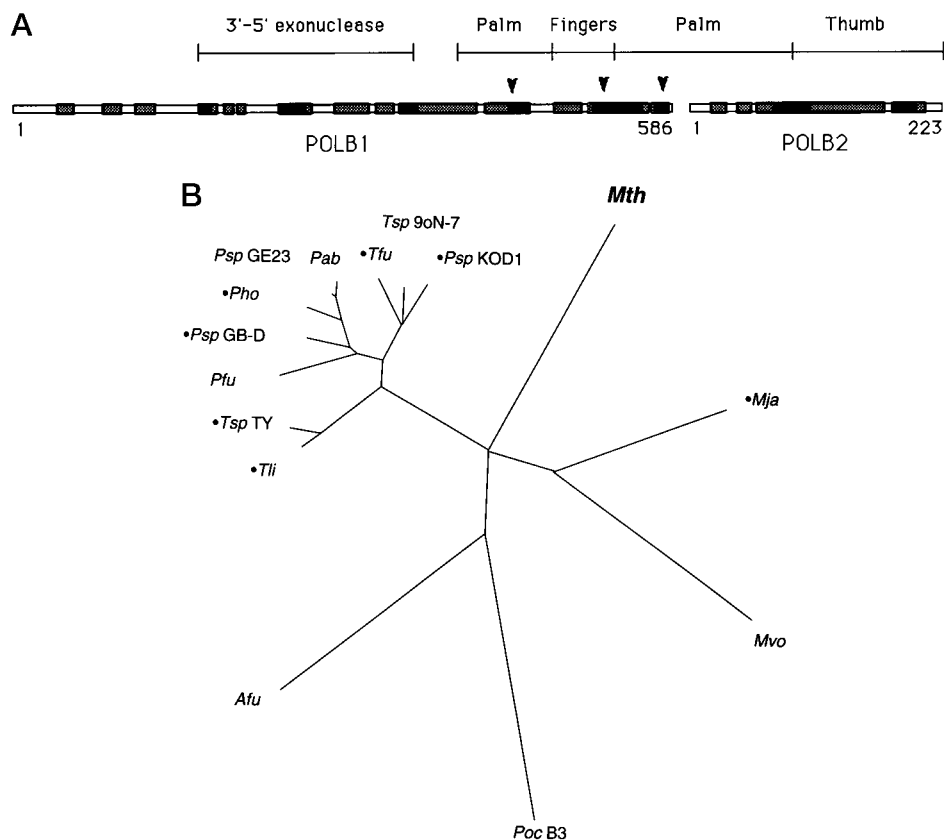
**Glycerol Gradient Centrifugation**—To demonstrate that the activity observed with preparations of PolB was not due to contamination with *E. coli* pols, a portion of the HiTrap-Q purified protein fractions (140 μg in 200 μl buffer A) was applied to a 5-ml 15–35% glycerol gradient in buffer A containing 500 mM NaCl. After centrifugation at 45,000 rpm (190,000 × *g*) for 19 h in an SW50.1 rotor at 4 °C, fractions (200 μl) were collected from the bottom of the tube. The distribution of PolB was detected following 10% SDS-PAGE and staining with Coomassie Brilliant Blue (R-250) and by the assay of each fraction for PolB activity (using 1 μl of each fraction diluted 50-fold in buffer A) using the standard replication conditions at (70 °C) as described above.

The interaction between *mthRPA* and PolB was examined by incubating 140 μg of each protein (in 200 μl) alone or together for 10 min at 70 °C and then subjecting the mixtures to a glycerol gradient centrifugation step as described above. After centrifugation, fractions were analyzed by 10% SDS-PAGE followed by staining with Coomassie Brilliant Blue (R-250).

## 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 characterized individually and as the complex. The genes encoding PolB1 and PolB2 (open reading frames *MTH1208* and *MTH208*, respectively (7)) were inserted individually and together into *E. coli* expression vectors and expressed as fusion proteins containing N-terminal His<sub>6</sub> tags (see "Experimental Procedures"). The PolB complex, containing both subunits, was soluble and was purified to near homogeneity by affinity chromatography onto Ni<sup>2+</sup> chelate and a HiTrap-Q column (Amersham Pharmacia Biotech) (Fig. 2A).



**FIG. 1. Comparison of the mthPolB proteins with other type B DNA polymerases.** A, sequence domains of the mthPolB proteins. The positions of the 3'-5' exonuclease and the polymerase (*palm*, *fingers*, and *thumb*) domains (based on the structure of homologous pol from *Thermococcus gorgonarius* (40)) are shown above a scheme of the protein sequences. Conserved sequence regions found in all group I archaeal DNA polymerases (see B and Ref. 11) are boxed and stippled. Regions also conserved in all type B DNA polymerases are shown in black. Arrowheads indicate intein integration points in other group I DNA pols (see B). Sequence lengths are in amino acids. B, sequence relation between different type B archaeal group I DNA pols. Conserved regions (see A) from all sequences were used to compute the dendrogram. All branch points are significant, having bootstrap values above 880/1000. mthPolB is highlighted, and DNA pols with inteins are marked with bullets. Species and sequence accession numbers (from the NCBI Entrez protein sequences data base) are as follows: *Mth*, *M. thermoautotrophicum*  $\Delta H$  (accession numbers 3913522 and 2621253); *Mja*, *M. jannaschii* (accession number 3915679); *Mvo*, *Methanococcus voltae* (accession number 1706513); *PocB3*, *Pyrodictium occultum* (accession number 807830); *Afu*, *Archaeoglobus fulgidus* (accession number 3122019); *Tli*, *Thermococcus litoralis* (accession number 154686); *TspTY*, *Thermococcus* sp. strain TY (accession number 3913524); *Pfu*, *P. furiosus* (woesei) (accession number 399403); *PspGB-D*, *Pyrococcus* sp. strain GB-D (accession number 2494186); *Pho*, *Pyrococcus horikoshii* (accession number 3913526); *PspGE23*, *Pyrococcus* sp. strain GE23 (accession number 3913530); *Pab*, *Pyrococcus abyssi* (accession number 3913529); *Tfu*, *Thermococcus fumicolans* (accession number 3913528); *Tsp9oN-7*, *Thermococcus* sp. strain 9oN-7 (accession number 1197452); *PspKOD1*, *Pyrococcus* sp. strain KOD1 (accession number 2129415).

PolB1 alone was marginally soluble (few percent) and was purified by affinity chromatography on  $\text{Ni}^{2+}$  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  $\text{Ni}^{2+}$  chelate in the presence of 6 M urea (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.

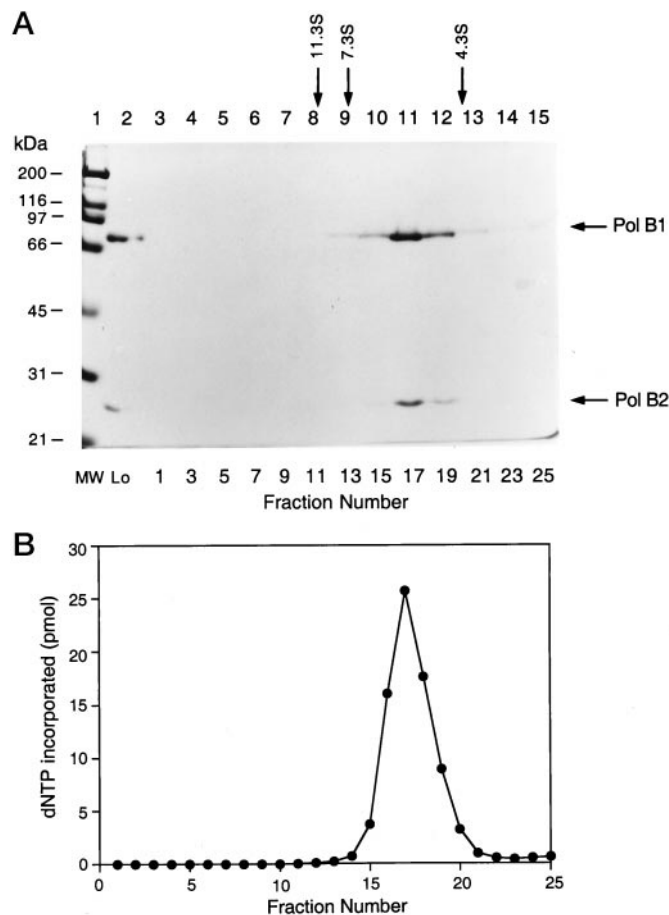
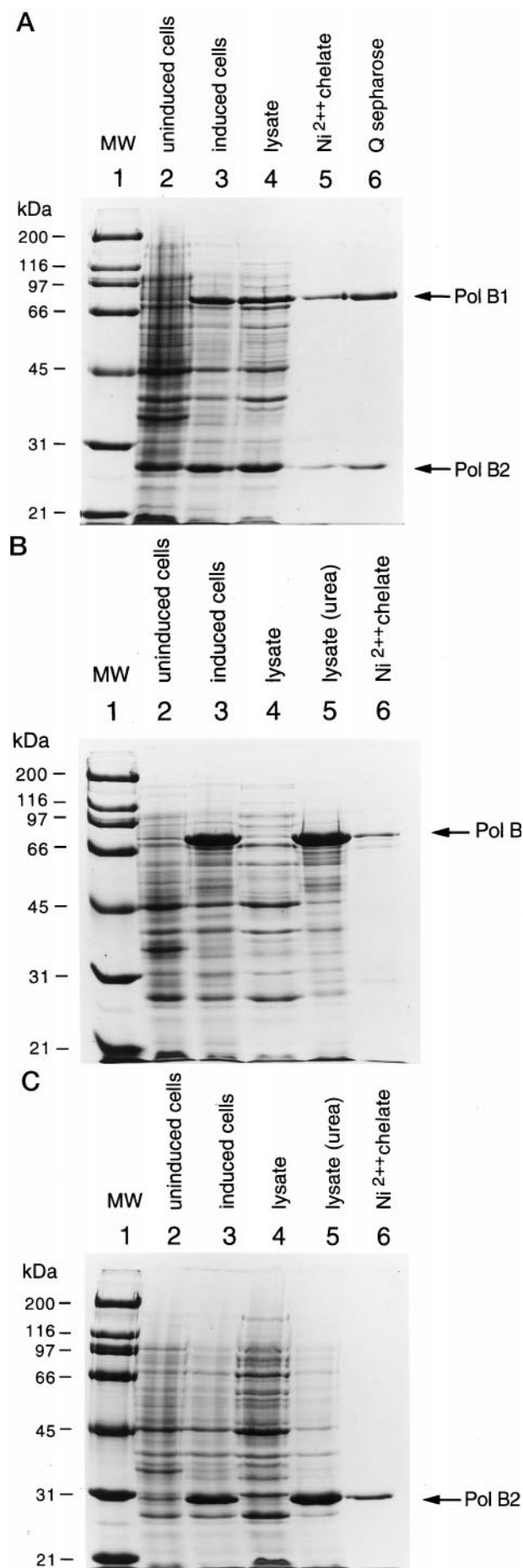
Glycerol gradient centrifugation of the pooled HiTrap-Q fractions of PolB yielded a single peak of DNA synthetic activity that sedimented between aldolase and BSA (Fig. 3). SDS-PAGE analysis of the gradient fractions revealed that the peak of pol activity sedimented coincidentally with both PolB proteins (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  $\text{Mg}^{2+}$  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  $\text{Mg}^{2+}$ , and at pH 7.5 (data not presented). The effects of several pol inhibitors were also examined. *N*-Ethylmaleimide and aphidicolin, which inhibit eukaryotic  $\text{pol}\alpha$ ,  $\text{pol}\epsilon$ , and  $\text{pol}\delta$  (1), and *N*(2)-(Butylphenyl)dGTP (kindly provided by George Wright, University of Massachusetts), which specifically inhibits  $\text{pol}\alpha$ , did not affect 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* polII (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.  $\text{pol}\alpha$ ). The amino acid sequence of



**FIG. 3. Glycerol gradient sedimentation of PolB.** This step was carried out as described under "Experimental Procedures." **A**, aliquots (20  $\mu$ l) 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."

PolB includes a putative exonuclease domain located between amino acids 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  $^{\circ}$ C, efficient removal of the  $^{32}$ P-labeled nucleotide from the 3'-end of the primed DNA was observed at 70  $^{\circ}$ C (Fig. 5A). At 50  $^{\circ}$ C, the efficiency of exonuclease activity was lower than that observed at 70  $^{\circ}$ C but greater than that detected at 30  $^{\circ}$ C. These results are similar to

**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  $\mu$ g); *lane 5*,  $\text{Ni}^{2+}$  chelate column (2  $\mu$ g); *lane 6*, Q-Sepharose eluate (2  $\mu$ g). **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  $\mu$ g); *lane 5*, cell lysate solubilized in 6 M urea (10  $\mu$ g); *lanes 6*,  $\text{Ni}^{2+}$  chelate column eluate (2  $\mu$ g). **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  $\mu$ g); *lane 5*, cell lysate solubilized in 6 M urea (10  $\mu$ g); *lane 6*,  $\text{Ni}^{2+}$  chelate column (2  $\mu$ g).

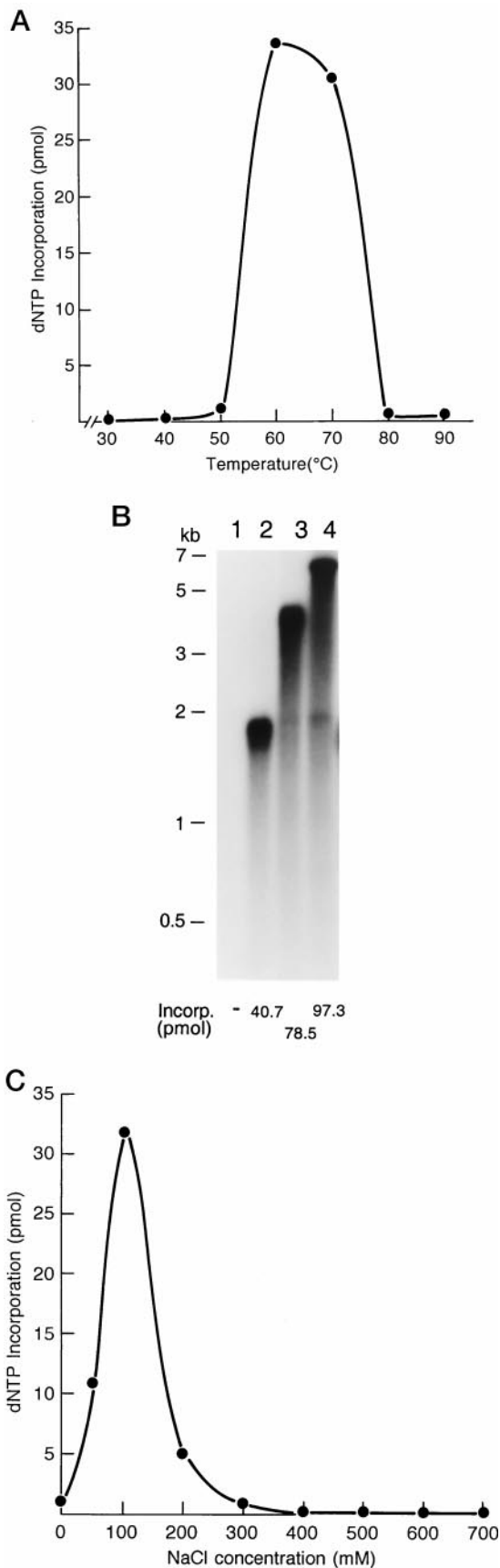


FIG. 4. **DNA synthesis by PolB.** A, effect of temperature on the replication activity of PolB was performed as described under "Experimental Procedures." Reaction mixtures (20  $\mu$ l) containing 8.3 fmol, singly primed M13 single-stranded DNA, 50 fmol of PolB and 0.1 M NaCl were incubated for 30 min at the indicated temperatures and analyzed as described under "Experimental Procedures." B, Pol B-

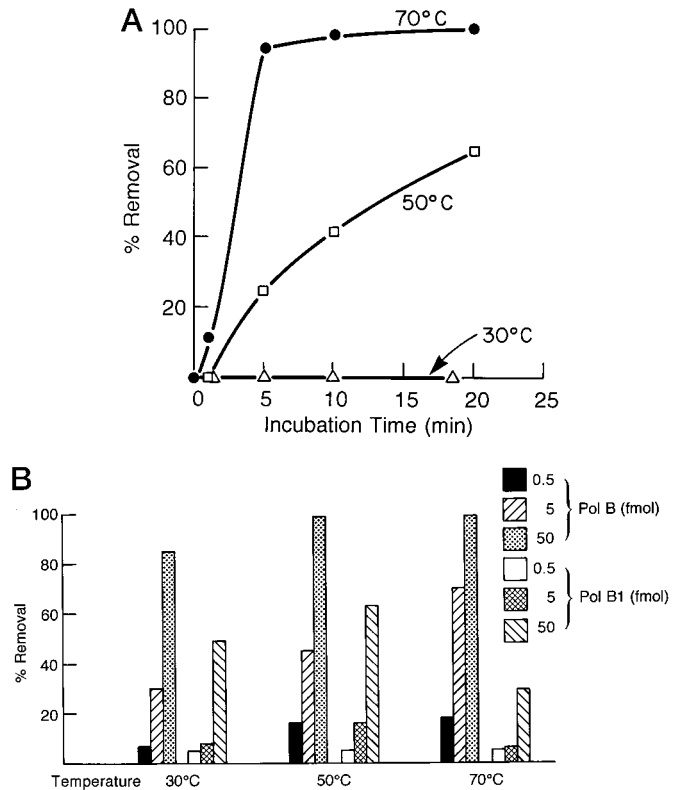


FIG. 5. **Exonuclease activity of PolB.** A, reaction mixtures (20  $\mu$ l) containing 50 fmol of PolB and 20 fmol of 3'-end-labeled singly primed M13 ssDNA as substrate were incubated at 30, 50, or 70 °C for the time indicated and analyzed as described under "Experimental Procedures." B, reaction mixtures (20  $\mu$ l) containing 20 fmol of 3'-end-labeled oligonucleotide and PolB or PolB1 at the indicated concentrations were incubated for 10 min at 30, 50, or 70 °C and analyzed as described under "Experimental Procedures."

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. 5 (50 fmol of PolB), 3'-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  $\mu$ l) described under "Experimental Procedures" in the presence of 12.8 fmol of DNA and 0.1 M 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 1. 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.

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* (Taq) (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 jannaschii* 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 SSB 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 $\alpha$  interacts with RPA (21), *E. coli* polIII 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 glycerol gradient 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.

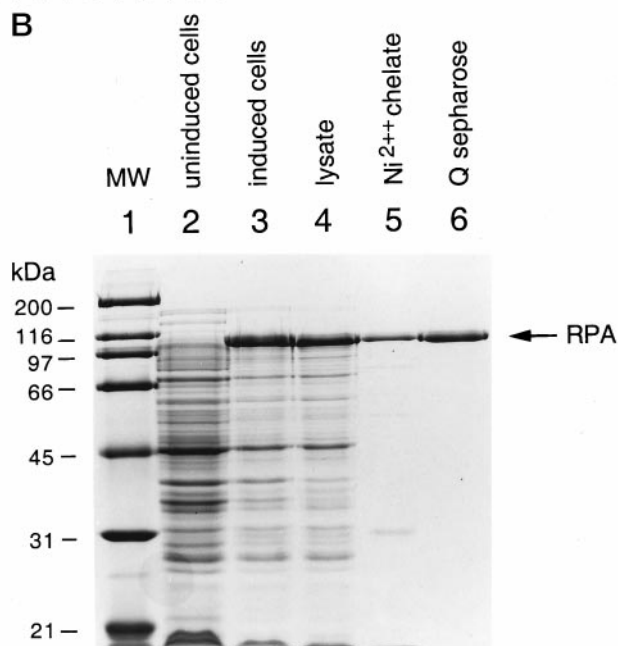
**RFC and PCNA Relieve the Inhibitory Effect of mthRPA**—In bacteria and eukaryotes, replicative pols have low processivity unless they are associated with a ring-shaped accessory protein, a DNA sliding clamp (reviewed in Refs. 25 and 26). The sliding clamp is assembled around DNA by a protein complex 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

**A**

```

ATG AAG GAA GAA TTG AAG AGG GAA TAT GAA AGA ATT AAG GAC AGG ATC TCC CCT GAG GAA TTT GAG GAA CTC ATA GAA AAA AAG AAA GAG 90
1 M K E E L K R E Y E R I K D R I S P R E F E E L I E K K E
GAA TTA GGG GAC ATA GGA TTC ATG GAT GAC CTT ACC ATT GCA TCG ACA GTC GTT GAT GAT ATC CTG AAG GAA AAA AAC ACC ATG CTA TCC 180
E L G L G D I G F M D D L T I A S T V V D C D I L K E K N T M L S
31 GAG AAA CCC GAA CAC AGG ATG GAC ACA ATA TCC AAG CTT GAG GAG GGA GCC GAA ACC CCT GTG ACA GGG AGG GTC ATG AAG ATA TCA ACC 270
E K P E H R M D T I S K L E E G A E T P V T G M K I S S
61 CCC AGG ACC TTC ACC ACC AAG AAG GGA AGG GAG GGG AAA CTC GCC AAC GTG ATA ATC GCA GAT GAT ACA GGT GAA CTC AGG GCA GTG TTC 360
E G K L A N V I I A D D T G E L R A V F P R T F T T R K G R
91 TGC ACA GAG AAC ATC AAG CTC CTC AAG AAA TTC AGG GAG GGC GAT GTG ATA AGG ATA AAG GAT GTT AAC GAA GGA GTC GGC GGC 450
W T E N I K L L K K F R E E G D V I R I K D V N I R G G F G G
121 AAG AAG GAG GCC CAC CTC ATG CCA AGA TCC ACC GTG GAG GTC CTT GAC CCC GAG GAC TAC CCT GAA TTC CCT GAG TAC CGT GAG GAG ATA 540
R K E A H L M P R S T V E V L D P E D Y F F E Y R E E I
151 ACC CCG ATA GGA GAT CTG GTG GAG GAT GAT GAG GTC AAT GTC ATA ACA AGG ATC ACA GGA ATA TCC CCT GTA AGG ACC TTT GAG AGG GAC 630
T P I G D L V E D D E V N V I A R I T G V S R V R T F E R D
181 GGA AGG GAG GGC AGG TTC ATC TCC CTG GAC ATC ATG GAT GCA ACC GGT TCA CGC ACC TAC ACC CTC TGG AAC AAT GAG ATA AAC CTC GTT 720
G R E G R F I S L D I M D A T G S T T Y T L W N N D V N L V
212 GAG GAA CTG GGA CTG AAG GAG GGT GAT GCA GTG AAG ATA CTC TGG CCG GAC CCA AGG AGA AGG GAT AAG GTT ACC CTC ACC CAC ACC 810
E E L G L K E G D A V K I L W A Q P R R R D D A G T P L T H T
241 AGC CTC ACA AGG GTG GTG CCG GGT GAA TAC GAT GTA CCT GAA TTC AGG GAG GAG CTC GTG AAG ATA GGG GAC CTC GAT GAT AGA AAC 900
S L T R V V F G E Y D V F E F R E E L V I G D L H E M R N
271 GTC ACT GTG ATG GGC CTT GTG ACA AAG GTC AAT GAC CCG GTA GAA TTT GAG AGA AAC GAC GCC ACA ACA GGT TCT GTG AAG TCC ATT GAG 990
V T V M G L V T K V N D P V E F E R N D G T T G S V G S I E
301 ATA GCC GAT GAC ACG GGA TCT GCA AGG GTA ACC CTC TGG GAT GAG GAT ACA CGC ATC AAA ATC AAC AAG GGG GAT ATC ATC AGA ATA TCA 1080
I A D D T G S A R V T L M D E D T R I K I N C K G D I I R I S
331 GGT GCC AAT GTC GAG TTC GAT GAC TTC AAC CAG TCC TAC AGG ATA AAC ACG AAC TTC AAC ACC CTC ACC CTC AAC CCT GAA TCC GAC 1170
G A N V E F D D F N Q S Y R I N T N F N T R I T L N P E S D
361 GGT GCA CTG CTG AAG GTC CTT GAG GAG TAC AGG GAA CAG ATG AGG CCA ATG AAG ATA TCT GAG ATA CTT GAA ATG GAG GAT GAG GGA GAG 1260
G A L L K V L E E Y R E Q M R P M K I S E I L E M E D E G E
391 GAA GTC GAT GTC GTT GGA AGG ATC TTC ACC CTC AGT GAC CCC CCG GAA TTT GAG AGG GAA GAC GGG ACC GGA ATC GTG AGG TCA ATG GAA 1350
E V D V V G R I F S L S D P R E F E R E D G T G I V R S M E
421 CTT GCC GAT GAG ACA GGC AAG ATA AGG ATC AGT CTC TGG GAT GAA AAG GCC GAG AAA CCC ATG AAT ATC GGC GAC ACA GTC AGA ATA GAA 1440
L A D E T G K I R I S L W D E K A E R L E I G D A V R I E
451 AAC GCA CCG ATA AAG CTT GGA CTC TAC AGC GTG GAA CTC AGC GCC GGA CGA ACA ACA AGG ATA GTG AAC CCC CTG CCA GAG GAC ATG GAG 1530
N A R I R L G L Y S V E L S A G R T T R I V N H P L F E D M E
481 GAC CTT CCA TCC TTT GAG GAA CTT GAG GAG ATG CTC TAT CAG ACA AAG AAA ATA GCC GAC CTC GAG GAG GAC GAC AAG AAC ATA AGG ATA 1620
D L P S F E E L E E M L Y Q T K K I A D L E E D D R N I R I
511 ATT GCA AGG GTC GTT GAC CTC TTT GAA CCC AGG GAG TTC CAG AAG GGT GAC GGC ACC CCC GGA CTT GTG AAG ACA GCT GAA TTC GCT GAT 1710
I A R V V D L F E P R E F Q R G D G T Y T L W N N D V N L V
541 GAC ACG GGA TCA ATA AGG GCC ACC CTC TGG GAT GAT GCT GCT GAG AAA CCC CTG AGC ATA GGG GAC CCC CTG AAG ATA GAA AAT CCC CGG 1800
D T G S I R A S L W D D A A E K P L S I G D F V K I E N P R
571 GTG GPT TTC AGG GAT GAT ATG GGT GGT GGA AGA CTT GAA CTC AGC ATA GGA AAC AGT TCA AGG ATT GAA CCG GCC AGC GAG AGG GAC CTT 1890
V V F R D D M G C G G R L E L S I G N S S R I E P A S E R D L
601 GAG GGT CTC CCA TCC TTC GAT GAA CTC GAG GAG ATC CTC TAT CCC CAC COT GAT ATT GOG GAT CTG GAT GAT TCA AAG AAC GTC CTC 1980
E G L P S F D E L Q C A G E M L Y F H R D I A D L D E D S R N V L
631 ATA GAG GGT GAG CTG ATC GAG ATG TCA GGA AGA CCG ATT CTC TCC ATA AAG TCC CCA TCC TGC AAG GAG GAT GAT GTC ACC GAT GAG 2070
I E G E L I E M S G R R I L S I K C P S C N E R L D L S D E
661 AAC ATA TCC AAC TTC TCC GGG GAA CTG GTG GAT GAA CCA CCG TAC CTC ATG ATC CCC GGG AGG ATC ATC GAG GAC ACC GGT GAG GTC 2160
N I C N C G E L V D E P R Y L L M I P G R I H D D T G E V
691 ATG ATA ACC TTC TTT GGA AGG GAG GCA GAG AGT ATC CTT GAA ATG ACA ACA GAT GAG GPT GTG AAC ATT ATC AAC CAG TCT GCA GAT GAA 2250
M I T F F G R E A E S I L E M T T D E V V N I I N Q S A D E
721 TCT GCA CTT GAA GAA CCF GTG GAG GAT CTG AAT GGA GTA ACC GTG AGG GTT ATT GGC AAT GCA GAT ATG GAT GTT TAC ACC GAG GAG CTA 2340
S A L E E R V E D L N G V T V R V I E G N A D M D V Y S E E L
751 AAG TTC ATA CCC AGA AAG GTC GTA AAG AAG GAA CTT TAA 2379
R F I P R K V V K K E L
    
```

**FIG. 6. mthRPA sequence and isolation.** A, nucleotide and deduced amino acid sequences of mthRPA. Nucleotides are numbered on the right-hand side and amino acids on the left-hand side. The position in which adenine was inserted in the published sequences (7) is indicated by an arrow. B, purification of mthRPA; 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  $\mu$ g); lane 5, Ni<sup>2+</sup> chelate column (2  $\mu$ g); lane 6, Q-Sepharose eluate (2  $\mu$ g).

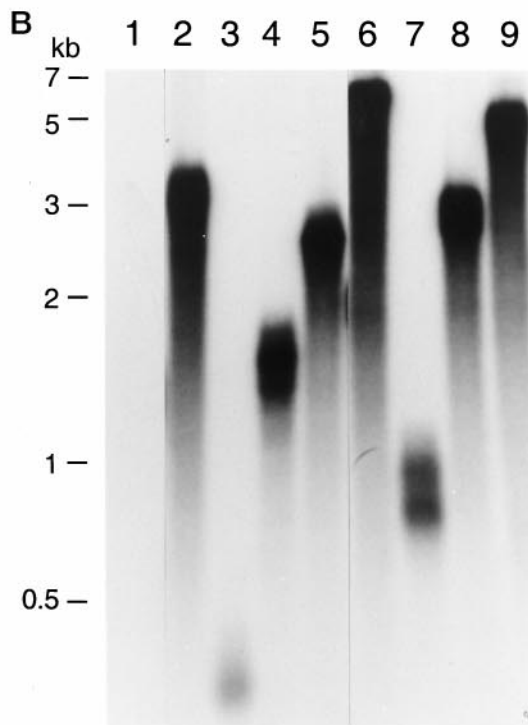
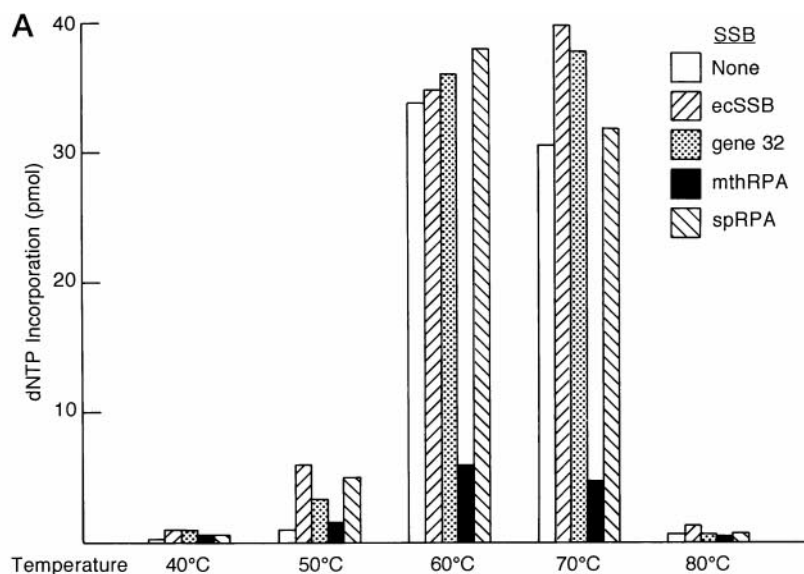


mthRPA. As shown in Fig. 10, the presence of RFC and PCNA not only relieved the inhibitory effects of mthRPA on PolB activity but also stimulated DNA synthesis compared with the activity observed in reactions containing PolB alone.

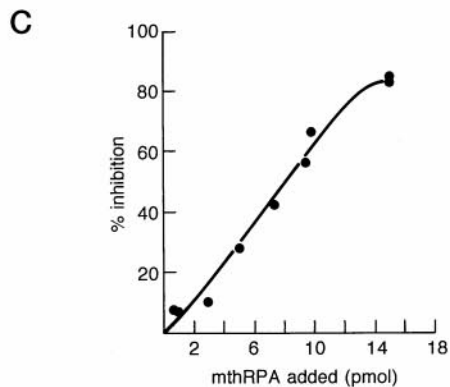
In these reactions, the rate of elongation of singly primed M13 DNA by PolB alone was decreased by reducing the temperature of the reaction to 50 °C and by the presence of 0.25 M NaCl (see Fig. 4). The effects of mthRPA, RFC, and PCNA on

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  $\mu$ l) 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  $\mu$ l) 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  $^{\circ}$ C for 20 min, and an aliquot (2  $\mu$ l) 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.



Incorp. (pmol) - 74.3 42.6 111 76.5  
 12.8 60.8 30 88.5



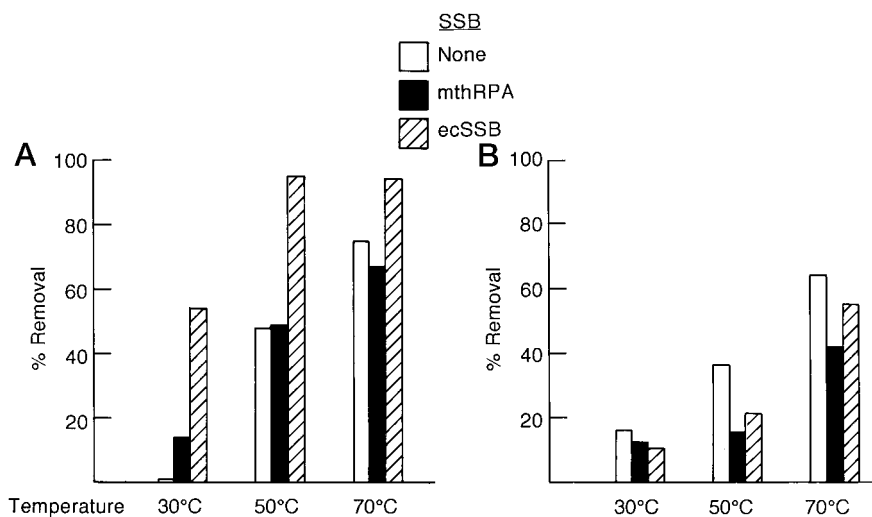


FIG. 8. **Effect of SSBs on the 3'-5' exonuclease activity of PolB.** A, exonuclease assays were performed as described under "Experimental Procedures" in reaction mixture (20  $\mu$ l) containing 20 fmol of 3'-labeled singly primed M13 ssDNA, 50 fmol of PolB, and either no SSB, 7.25 pmol of mthRPA, 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  $\mu$ l) 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.

#### 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 factors, each archaeal organism contains a slightly different set of proteins. This study describes the isolation and characterization of a pol from the archaeon *M. thermoautotrophicum*. The PolB of *M. thermoautotrophicum* is unique in being split into two proteins that interact to form a dimeric active enzyme. All other pols of the B-type are coded by a single gene and are active as a single protein. Several euryarchaeote B-type pols contains inteins (Fig. 1B) that are removed post-translationally leading to a single contiguous polypeptide chain (28-30). Interestingly, a C-type cyanobacterial replicative pol is also split into two separate gene products that are joined to form a single polypeptide chain by an intermolecular intein-directed splicing event (31, 32). In contrast, the two polypeptides constituting mthPolB are split at a region that is different from the characterized intein integration sites found in type B pols (these sites in intein-containing pols are noted by arrowheads in Fig. 1A). In mthPolB, no amino acid remnants of inteins are found. Although some euryarchaeotes can each contain 10-19 inteins (5, 8, 33),<sup>2</sup> *M. thermoautotrophicum* possesses only a single intein that is localized to a ribonucleotide reductase subunit (7). These findings suggest that the mthPolB is not protein-

spliced and its gene organization most likely resulted from a genomic rearrangement that divided the original PolB gene in two.

This study demonstrates that the complex of the two subunits, PolB1 and PolB2, possesses both pol and 3' to 5' exonuclease activities. Thus, the properties of PolB are similar to the majority of pols in the B family. As expected from a thermophile that grows optimally between 65 and 70 °C, both pol and exonuclease activities are temperature-dependent, exhibiting maximal activity at temperatures similar to the optimal growth conditions of *M. thermoautotrophicum*.

Although this study did not address whether the subunit PolB2 alone has pol activity, due to technical difficulties (PolB2 is not soluble), several lines of evidence suggest that only the complex is active. Whereas each PolB subunit is insoluble (PolB2) or marginally soluble (PolB1) when overexpressed in *E. coli* alone, the two subunits form a soluble 1:1 dimer after coexpression (as judged by scanning of a Coomassie-stained SDS-PAGE), suggesting that a complex of the proteins exists within the *M. thermoautotrophicum* cell. The three-dimensional structures of all pols studied to date have revealed a conserved overall structural organization, referred to as fingers, palm, and thumb (reviewed in Refs. 34 and 35). In the mthPolB, a portion of the palm and the entire thumb domains are encoded by PolB2, and the remaining conserved structures are encoded by PolB1 (Fig. 1A). These domains are all needed to form the active enzyme, suggesting that each subunit of PolB alone would not be sufficient for pol activity as was shown for PolB1 (data not shown). Furthermore, antibodies generated against PolB2 inhibited the activity of PolB suggesting that the PolB2 subunit is required for polymerase activity.

In the DP2 family of pols, the active pol is also a dimer of two distinct polypeptide chains, DP1 and DP2. Although both subunits are essential for pol activity, all of the conserved domains essential for catalytic activity reside in the large subunit (DP2) (36). This differs from mthPolB in which the domains essential for pol activity are distributed between two subunits. Interestingly, the small subunit of the *P. furiosus*, DP1, has homology to the small (non-catalytic) subunits of other heteropolymeric pols (e.g. pol $\delta$  and pol $\epsilon$ ) (37).

Three pols have been identified in *M. thermoautotrophicum*: PolB, described here, a DP2-like pol, and a member of family X.

<sup>2</sup> S. Pietrokovski, unpublished results.

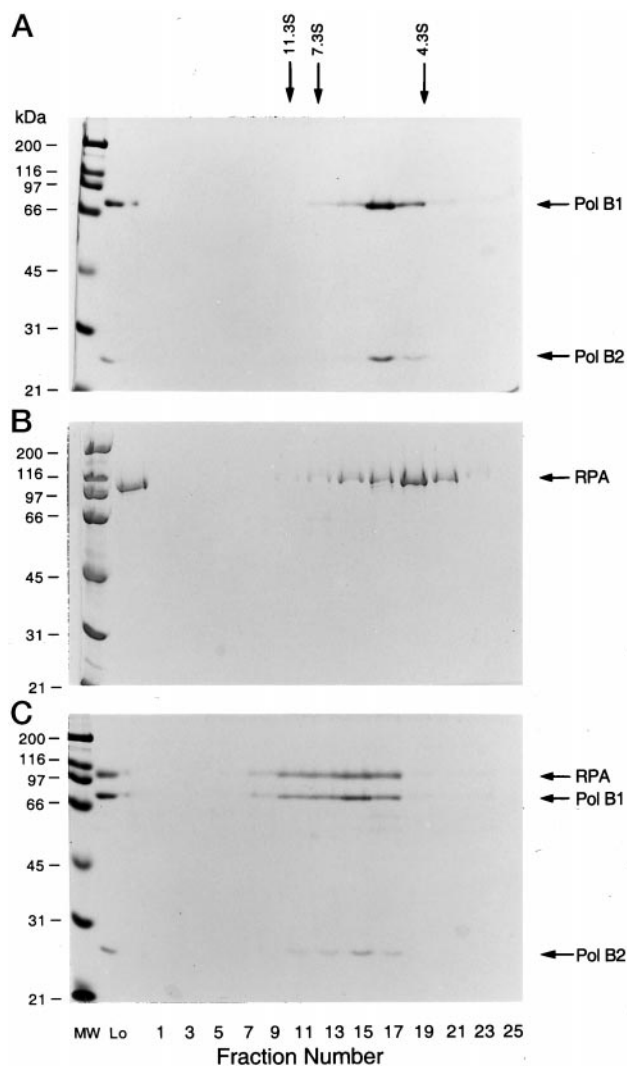


FIG. 9. **PolB interacts with mthRPA.** 140  $\mu$ g (2 nmol) of PolB was applied to a 5-ml 15–35% glycerol gradient as described under “Experimental Procedures.” After centrifugation, fractions (20  $\mu$ l) were collected from the bottom of the tube. The distribution of proteins was detected following 10% SDS-PAGE of 20  $\mu$ l from indicated fractions and staining with Coomassie Brilliant Blue. *B*, conditions were as described in *A* using 140  $\mu$ g (2 nmol) of mthRPA. *C*, mixtures were as described with *A* using 140  $\mu$ g of PolB together with 140  $\mu$ g of mthRPA.

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 the *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, 3' to 5' 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 Pole, a eukaryotic member of the B-type pols. Pole was suggested to play a role in Okazaki fragment maturation

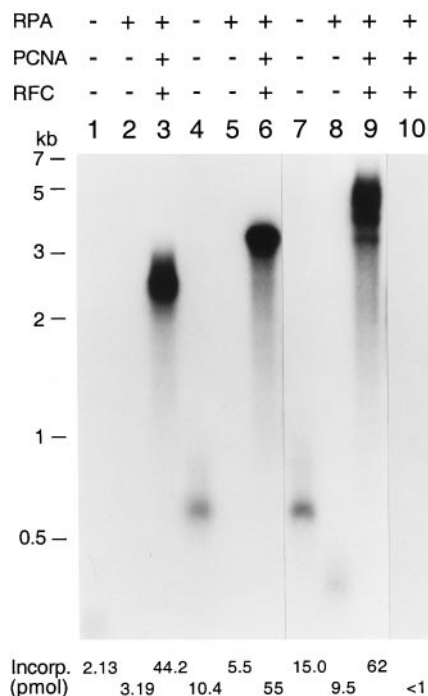


FIG. 10. **mthRFC and mthPCNA relieve the inhibition of PolB by mthRPA.** Reaction mixtures (20  $\mu$ l) were as described under “Experimental Procedures” for the elongation of singly primed M13 DNA but included NaCl (final concentration of 0.25 M) and where indicated 15 pmol of mthRPA, 3 pmol of mthRFC, 3 pmol of mthPCNA, and either 0.14 (lanes 1–3), 0.42 (lanes 4–6), or 1.4 (lanes 7–9) pmol of PolB. Lane 10 contained 15 pmol of mthRPA, 3 pmol of mthRFC, and 3 pmol of mthPCNA but no PolB. Reactions were incubated for 30 min at 50 °C. An aliquot (2  $\mu$ l) was used to measure DNA synthesis, and the remaining mixture was subjected to alkaline-agarose gel electrophoresis. After drying, gels were autoradiographed for 15 min at –80 °C and then developed. Marker lengths (in kb) are indicated on the left of the autoradiogram.

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 pol $\alpha$  (21) and pol $\delta$ ,<sup>3</sup> *E. coli* polIII (22), and bacteriophages T4 and T7 pols (23, 24). The interactions between these enzymes and their cognitive SSBs play different roles. For example, Pol $\alpha$  does not bind stably to the DNA template unless supported by its interaction with RPA,<sup>3</sup> 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

<sup>3</sup> 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.

**Acknowledgments**—We thank Dr. John Reeve for providing us with *M. thermoautotrophicum* genomic DNA and Dr. George Wright for N(2)-(Butylphenyl)dGTP.

## REFERENCES

- Kornberg, A., and Baker, T. (1992) *DNA Replication*, W. H. Freeman & Co., New York
- Braithwaite, D. K., and Ito, J. (1993) *Nucleic Acids Res.* **21**, 787–802
- Uemori, T., Sato, Y., Kato, I., Doi, H., and Ishino, Y. (1997) *Genes Cells* **2**, 499–512
- Woese, C. R., and Fox, G. E. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5088–5090
- Bult, C. J., White, O., Olsen, G. J., Zhou, L., Fleischmann, R. D., Sutton, G. G., Blake, J. A., Fitzgerald, L. M., Clayton, R. A., Gocayne, J. D. *et al.* (1996) *Science* **273**, 1058–1073
- Klenk, H.-P., Clayton, R. A., Tomb, J.-F., White, O., Nelson, K. E., Ketchum, K. A., Dodson, R. J., Gwinn, M., Hickey, E. K., Peterson, J. D. *et al.* (1997) *Nature* **390**, 364–370
- Smith, D. R., Doucette-Stamm, L. A., Deloughery, C., Lee, H., Dubois, J., Aldredge, T., Bashirzadeh, R., Blakely, D., Cook, R., Gilbert, K. *et al.* (1997) *J. Bacteriol.* **179**, 7135–7155
- Kawarabayasi, Y., Sawada, M., Horikawa, H., Kaikawa, Y., Hino, Y., Yamamoto, S., Sekine, M., Baba, S., Kosugi, H., Hosoyama, A. *et al.* (1998) *DNA Res.* **5**, 55–76
- Edgell, D. R., and Doolittle, W. F. (1997) *Cell* **89**, 995–998
- Koonin, E. V., Mushegian, A. R., Galperin, M. Y., and Walker, D. R. (1997) *Mol. Microbiol.* **25**, 619–637
- Edgell, D. R., Klenk, H.-P., and Doolittle, W. F. (1997) *J. Bacteriol.* **179**, 2632–2640
- Zeikus, J. G., and Wolfe, R. S. (1972) *J. Bacteriol.* **109**, 707–713
- Perler, F. B. (1998) *Cell* **92**, 1–4
- Ishiai, M., Sanchez, J. P., Amin, A. A., Murakami, Y., and Hurwitz, J. (1996) *J. Biol. Chem.* **271**, 20868–20878
- Henikoff, S., Henikoff, J. G., Alford, W. J., and Pietrokovski, S. (1995) *Gene (Amst.)* **163**, 17–26
- Schuler, G. D., Altschul, S. F., and Lipman, D. J. (1991) *Proteins* **9**, 180–190
- Pietrokovski, S. (1994) *Protein Sci.* **3**, 2340–2350
- Pietrokovski, S. (1998) *Protein Sci.* **7**, 64–71
- Pietrokovski, S. (1998) *Curr. Biol.* **8**, 634–635
- Kelly, T. J., Simancek, P., and Brush, G. S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 14634–14639
- Dornreiter, I., Erdile, L. F., Gilbert, I. U., vonWinkler, D., Kelly, T. J., and Fanning, E. (1992) *EMBO J.* **11**, 769–776
- Molineux, I. J., and Gefter, M. L. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71**, 3858–3862
- Cha, T. A., and Alberts, B. M. (1988) *Cancer Cells* **6**, 1–10
- Kim, Y. T., Tabor, S., Churchich, J. E., and Richardson, C. C. (1992) *J. Biol. Chem.* **267**, 15032–15040
- Kelman Z., and O'Donnell, M. (1994) *Curr. Opin. Genet. & Dev.* **4**, 185–195
- Kuriyan, J., and O'Donnell, M. (1993) *J. Mol. Biol.* **234**, 915–925
- Brown, J. R., and Doolittle, W. F. (1997) *Microbiol. Mol. Biol. Rev.* **61**, 456–502
- Perler, F. B., Comb, D. G., Jack, W. E., Moran, L. S., Qiang, B., Kucera, R. B., Benner, J., Slatko, B. E., Nwankwo, D. O., Hempstead, S. K., Carlow, C. K. S., and Jannasch, H. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 5577–5581
- Takagi, M., Nishioka, M., Kakihara, H., Kitabayashi, M., Inoue, H., Kawakami B., Oka, M., and Imanaka, T. (1997) *Appl. Environ. Microbiol.* **63**, 4504–4510
- Niehaus, F., Frey, B., and Antanikian, G. (1997) *Gene (Amst.)* **3**–158
- Gorbalenya, A. E. (1998) *Nucleic Acids Res.* **26**, 1741–1748
- Hong, W., Hu, Z., and Liu X.-Q. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 9226–9231
- Perler, F. B. (1999) *Nucleic Acids Res.* **27**, 346–347
- Steitz, T. A. (1993) *Curr. Opin. Struct. Biol.* **3**, 31–38
- Jager, J., and Pata, J. D. (1999) *Curr. Opin. Struct. Biol.* **9**, 21–28
- Cann, I. K. O., Komori, K., Toh, H., Kanai, S., and Ishino, Y. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 14250–14255
- Makiniemi, M., Pospiech, H., Kilpelainen, S., Jokela, M., Vihinen, M., and Syvaoja, J. E. (1999) *Trends Biochem. Sci.* **24** 14–16
- Ishino, Y., Komori, K., Cann, I. K. O., and Koga, Y. (1998) *J. Bacteriol.* **180**, 2232–2236
- Burgers, P. M. J. (1998) *Chromosoma* **107**, 218–227
- Hopfner, K.-P., Eichinger, A., Engh, R. A., Laue, F., Ankenbauer, W., Huber, R., and Angerer, B. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 3600–3605