The Hyperthermophilic Euryarchaeota *Pyrococcus abyssi* Likely Requires the Two DNA Polymerases D and B for DNA Replication

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Abstract:

DNA polymerases carry out DNA synthesis during DNA replication, DNA recombination and DNA repair. During the past five years, the number of DNA polymerases in both eukarya and bacteria has increased to at least 19 and multiple biological roles have been assigned to many DNA polymerases. Archaea, the third domain of life, on the other hand, have only a subset of the eukaryotic-like DNA polymerases. The diversity among the archaeal DNA polymerases poses the intriguing question of their functional tasks. Here, we focus on the two identified DNA polymerases, the family B DNA polymerase B (*Pab*polB) and the family D DNA polymerase D (*Pab*polD) from the hyperthermophilic euryarchaeota *Pyrococcus abyssi*. Our data can be summarized as follows: (i) both *Pab*polS are DNA polymerizing enzymes exclusively; (ii) their DNA binding properties as tested in gel shift competition assays indicated that *Pab*polD has a preference for a primed template; (iii) *Pab*polD is a primer-directed DNA polymerase independently of the primer composition whereas *Pab*polB behaves as an exclusively DNA primer-directed DNA polymerase; (iv) *Pab*PCNA is required for *Pab*polD to perform efficient DNA synthesis but not *Pab*polB; (v) *Pab*polD, but not *Pab*polB, contains strand displacement activity; (vi) in the presence of *Pab*PCNA, however, both *Pab*polS D and B show strand displacement activity, and (vii) we show that the direct interaction between *Pab*polD and *Pab*PCNA is DNA-dependent. Our data imply that *Pab*polD might play an important role in DNA replication likely together with *Pab*polB, suggesting that archaea require two DNA polymerases at the replication fork.

**Keywords:** DNA replication; euryarchaea; gap filling; strand displacement; DNA polymerase
Summary

DNA polymerases carry out DNA synthesis during DNA replication, DNA recombination and DNA repair. During the past five years, the number of DNA polymerases in both eukarya and bacteria has increased to at least 19 and multiple biological roles have been assigned to many DNA polymerases. Archaea, the third domain of life, on the other hand, have only a subset of the eukaryotic-like DNA polymerases. The diversity among the archaeal DNA polymerases poses the intriguing question of their functional tasks. Here we focus on the two identified DNA polymerases, the family B DNA polymerase B (PabpolB) and the family D DNA polymerase D (PabpolD) from the hyperthermophilic euryarchaeota Pyrococcus abyssi. Our data can be summarised as follows: (i) both Pabpols are DNA polymerizing enzymes exclusively; (ii) their DNA binding properties as tested in gel shift competition assays indicated that PabpolD has a preference for a primed-template; (iii) PabPolD is a primer-directed DNA polymerase independently of the primer composition whereas PabpolB behaves as an exclusively DNA primer-directed DNA polymerase; (iv) PabPCNA is required for PabpolD to perform efficient DNA synthesis but not PabpolB; (v) PabpolD, but not PabpolB, contains strand displacement activity (vii) in the presence of PabPCNA, however, both Pabpols D and B show strand displacement activity and (viii) we show that the direct interaction between PabpolD and PabPCNA is DNA-dependent. Our data imply that PabPolD might play an important role in DNA replication likely together with PabpolB, suggesting that archaea require two DNA polymerases at the replication fork.

Keywords: DNA replication, euryarchaea, gap filling, strand displacement, DNA polymerase, Okazaki fragments, PCNA.
Introduction

Living organisms are faced with the tremendous task of maintaining the integrity of the genome. Enzymes called DNA polymerases (pols) are involved in highly sophisticated mechanisms for maintenance of the genome such as DNA replication, DNA recombination and DNA repair. Our knowledge of DNA transactions in hyperthermophilic archaea was enlarged when the recent availability of the genomic sequences \(^1\) demonstrated the striking similarity between these extremophilic organisms and eukarya. Archaea, as the third kingdom of life, might serve as a useful model to understand DNA transactions in the more complex eukaryotic systems \(^2\). Biological studies are already yielding valuable data for studying the simpler archaeal DNA replication system. Indeed, a single replication origin in the euryarchaeal *Pyrococcus abyssi* (*P. abyssi*) was identified (ori C) \(^3\) and bi-directional DNA replication was shown to be initiated \(^4\). In addition, short eukaryotic-like Okazaki fragments were detected in the two main archaeal phyla, *P. abyssi* and *Sulfolobus acidocaldarius* (*S. acidocaldarius*) \(^5\). Thus, archaea replicate their circular genome, as do bacteria i.e., much more rapidly than eukarya \(^6\), and they do this despite containing eukaryotic-like DNA replication proteins \(^3\). However, the situation became controversial within the crenarchaeota phyla. One study identified two origins of replication in the archaeon *Sulfolobus solfataricus*, (*S. solfataricus*) \(^7, 8\), while another used a different technique to show that both *S. solfataricus* and *S. acidocaldarius* have three functional origins \(^7, 8\). In eukarya, on the other hand, initiation of DNA replication takes place at multiple replication origins along the genome and this may correlate to the larger size of the eukaryal genome and the coordination of differential gene expression during development \(^9\).

Chromosomal DNA replication in eukaryotes require the three distinct Pol \(\alpha\), Pol \(\delta\) and Pol \(\varepsilon\) (reviewed in refs \(^{10, 11}\)). Pol \(\alpha\), with its associated DNA primase activity, is involved in the initiation step at the leading strand and in the repeated priming events required for
replication of the lagging strand (reviewed in ref \(^{12}\)). However, Pol α/primase is only able to synthesize a short RNA-DNA primer of about 10 ribonucleotides and 20 deoxyribonucleotides, that is subsequently elongated by other pols. Pols $\delta$ and $\varepsilon$ are believed to be the two major replicative pols in metazoans, with Pol $\delta/\varepsilon$ acting on the leading strand and Pol $\delta$ at the lagging strand \(^{13}\). Given that eukaryotic DNA replication requires the coordinated action of at least three distinct pols at the replication fork, there must be a tight regulation to fulfill the efficient duplication of the genetic material. This mechanism involves polymerase switching from Pol α to Pol $\delta$ during the synthesis of each Okazaki fragment. The clamp PCNA and the clamp loader RF-C, are required for polymerase switching \(^{14}\). Since the closed PCNA ring cannot assemble onto a primed-template junction by itself, it requires the ATP-dependent loading by RF-C. Through physical interactions with the Pol $\delta$ and Pol $\varepsilon$, PCNA promotes rapid and processive DNA replication (reviewed in \(^{10}\)). Maturation of Okazaki fragments, finally, requires the concerted action of several enzymes consisting of the removal of the RNA primer by Fen1, filling of the gap by Pol $\delta$ and sealing the resulting nick by DNA ligase I \(^{15}\).

Chromosomal DNA replication in bacteria appears to be simpler than in eukarya. In \(E.\ coli\), DNA elongation involves the replicase, Pol III. Pol III is a complex molecular assemblies called holoenzyme \(^{16}\) composed of (i) the core enzyme ($\alpha$, $\varepsilon$ and $\theta$) containing the polymerase and proofreading activities; (ii) a $\tau$-subunit that causes the core enzyme to dimerize; (iii) a $\beta$ processivity clamp that tethers the core enzymes to the chromosome and (iv) the five subunit clamp loader ($\gamma$, $\delta$, $\delta'$, $\psi$ and $\chi$) called $\gamma$-complex that allows to open and lock the ring clamp on the DNA \(^{16}\). Thus, in \(E.\ coli\), there is one complex with two pols that replicates the leading as well as the lagging strand. Moreover, the two core enzymes are not pre-dedicated to one strand and can be interchanged \(^{17}\). The cycle of Okazaki fragments synthesis starts with the primase DnaG \(^{18}\), to which it remains stable bound through its
association with the single-strand binding protein (SSB). Then, the γ-complex of Pol III displaces the primase from the primer-template junction upon binding SSB. Subsequently, the lagging strand Pol III transits from the 3’-OH terminus of the newly completed Okazaki fragments to the new primer terminus, thus leading to DNA elongation. The cycle ends by removal of the previous RNA primer by RNase H, gap filling with Pol I and ligation by DNA ligase to create a duplex DNA. However, little is known so far about the concerted action of Pol III and Pol I during Okazaki fragments maturation.

Archaea, the third domain of life, have an universally conserved replication machinery, including the sliding clamp PCNA, the clamp loader RF-C and (a) replicative pol (s). Even though several pols belonging to the B family (α, δ, ε) have been implicated in chromosomal DNA replication in eukarya, it is not clear whether members of B-type DNA polymerases are the replicative pols in archaea. According to the distribution of genes into the archaeal genomes, there is evidence for a family B pol to function solely during DNA replication in crenarchaeota and either a B- or D-type pol, or both in euryarchaeota. Data obtained so far indicated that the family D pol (Pol D) might be a key-enzyme of DNA replication in euryarchaeota. Pol D is composed of a small subunit (DP1) which shows significant homologies with the small subunit of eukaryotic pols α, δ and ε and a large subunit (DP2) containing the polymerizing activity. The pol D heterodimer possesses efficient DNA polymerizing and a 3’->5’ exonuclease (proofreading) activities. Pol D from P. abyssi appears to be a heterodimer whereas Pol D from Pyrococcus horikoshii (PhopID) is like Pol δ a heterotetramer. Moreover, its small subunit, DP1, physically interacts with PCNA. Genetic analysis indicated that the two subunits of Pol D are clustered in an operon located adjacent to the replication origin in Pyrococcus containing essential genes for maintenance of the genome. Moreover, its ability to interact with multiple proteins involved in DNA replication, recombination and repair together with its newly
characterised Mre11-like nuclease activity\textsuperscript{29} suggest that Pol D may participate, in addition of its proposed role in chromosomal replication, in DNA repair and recombination. However, due to the lack of genetic tools in archaea, it is not known whether both Pols B and D are essential for viability and what their exact functions are at the replication fork.

In this work we focus on the two known family B and D Pols of \textit{P. abyssi}. We compared the recombinant Pols B and D by a variety of biochemical properties and our data indicated that \textit{Pab}PolD might play an important role in DNA replication likely together with \textit{Pab}polB, suggesting that archaea might have two Pols at the replication fork.
Results

*Pab* DNA polymerases B and D preferentially replicate synthetic DNA but not RNA. Our previous studies indicated that the newly identified family D pol from *P. abyssi* contains both polymerase and 3’->5’ exonuclease activities as does the family B 25. Both enzymes are primer-directed DNA polymerases 25. To gain further insight into the precise roles of the two pols, in a first approach, synthetic primed-templates different in composition were tested as outlined in Materials and Methods. When the homopolymeric poly(dA)/Oligo(dT)_{10:1} or poly(dA)-oligo(dT)_{40:1} were tested, both *Pab*pols were with a moderate reduced activity in presence of poly(dA)-oligo(dT)_{40:1}. On the other hand both *Pab*pols were inactive with the poly(rA)/oligo(dT) at a both molar ratios of 40:1 and 10:1, suggesting lack of reverse transcriptase activity (Figure 1). Furthermore, both pols neither perform rNTPs incorporation nor *de novo* synthesis (data not shown).

*PabpolD* binds preferentially primed-DNA templates and *PabpolB* binds single-strand and primed-DNA template. The recognition of DNA structures by B- and D-type pols from archaea was never compared before. We therefore compared the two *Pab*pols for their DNA substrate binding abilities. Since *Pab*pols are potent pols and exonucleases in presence of divalent metal cations (24), EDTA was included in the binding reaction. To measure the difference of DNA binding abilities among the two pols, a titration analysis was performed in presence of several templates that can occur during DNA elongation. *PabpolD* was found to bind strongly to primed DNA (A2/B2) independently of Mg$^{2+}$. The dose response was linear between 1-10 pmol and a single shifted species was observed (data not shown). In order to determine the specificity of *PabpolD* for the primed DNA (A2/B2, represented in Figure 2A) in the mobility shift assay, various nucleic acid competitors were
added to the binding reaction (Figure 2B). Single-strand DNA and single-strand RNA competed inefficiently. An approximately 125-fold molar excess of single-strand DNA competitor was required to compete the binding of PabpolD away from the labeled primed DNA (Figure 2B, lanes 3-6). The single-strand RNA failed to compete even at a 125-fold excess (Figure 2B, lanes 17-20). RNA and DNA primed-templates, double-strand DNA competed efficiently with the labeled DNA primed-template. Double-strand DNA competed less efficiently (Figure 2B, lanes 7-10) than the DNA (Figure 2B, lanes 21-24) and RNA primed-templates (Figure 2B, lanes 11-14). These results suggest the requirement of a primed-template for strong binding of PabpolD. When, on the other hand, PabpolB was tested onto a primed-template, two different shifted species were observed (data not shown). Similarly to PabpolD, we found that PabpolB could bind the single-strand DNA independently of Mg$^{2+}$ in a dose dependent manner. This dose response was linear between 5 and 15 pmol but only one single shift was observed (data not shown). Thus, we next measured the binding specificity of PabpolB to single-strand DNA by titrating derivatives of DNA structures (Figure 3A). Single-strand RNA and double-strand DNA could not compete efficiently the binding of PabpolB away from the labeled single-strand DNA (Figure 3B, lanes 17-24). Upon addition of unlabeled primed DNA templates, the shifted template was no longer detectable in the presence of a 25-fold molar excess of competitor. The reduced binding of PabpolB was observed with DNA- and RNA primed-template competitors (Figure 3B, lanes 3-10). In summary, PabpolD binds preferentially primed-DNA templates and PabpolB binds single-strand as well as primed-DNA templates.

PabPCNA can stimulate PabPolD and PabPolD can elongate an DNA and RNA primers. Next the abilities of both pols were tested on primed circular M13mp18 templates (Figure 4A) The results illustrated in Figure. 4B showed that PabpolB could incorporate
dNTPs into M13mp18 to the same efficiency in presence of primers containing 3’-OH DNA ends (short DNA, DNA and hybrid RNA-DNA). Interestingly, PabpolB was inactive with an RNA primer (Figure 4B) and this even when higher amount of PabpolB were tested (data not shown). When these template-primer combinations (Figure 4A) were tested with PabpolD incorporation of dNTPs was observed independently with all four primers, including RNA. The amounts of nucleotides incorporated was moderately reduced in presence of a short DNA primer (Figure 4B). The DNA primer utilization by PabpolB was 3-fold higher than for PabpolD. Next, primer extension assays were carried out with 5’-end labeled primed M13mp18 DNA template (Figure 4A and 4C). Both Pabpols could elongate primers containing DNA 3’-OH ends. PabpolB accumulated full-length products (Figure 4C, lanes 2, 5 and 8) whereas PabpolD did stop after 3600 nucleotides (Figure 4C, lanes 3, 6 and 9) and longer incubation time did not increase the size of the products (data not shown). Moreover, the yield of full-size products was higher with PabpolB than with PabpolD, suggesting that the enzyme does not utilize the same amount of primers (Figure 4C, compare the lanes 3 and 6, 9 with the lanes 2, 5 and 8). These data correlate with the results of nucleotides incorporation observed in Figure 4B. Next the effect of PabPCNA (0.7 pmol) was investigated. Figure 4C shows that addition of PabPCNA stimulated the extension ability of PabpolD, since full-DNA synthesis could be observed (Figure 4C, compare lanes 15, 18 and 21 to lanes 3, 6 and 9) but, still, with lower efficiency compared to PabpolB alone (Figure 4C, lanes 14, 17 and 20). Increasing amounts of PabPCNA added into a PabpolD reaction did not affect the amount of the utilised primers (data not shown). As mentioned in Figure 4B, nucleotide incorporation in the presence of an RNA primer could only be observed with PabpolD. In order to detect significant RNA primer elongation, the length of single-strand template was shortened and the reaction products were visualised on a denaturing polyacrylamide gel. Replication was allowed to proceed with or without PabPCNA for both
*Pab*pols as indicated (Figure 4D). Interestingly, *Pab*polD could elongate the RNA primer to the full-length even without *Pab*PCNA. Moreover, the inability of *Pab*polB for RNA primer extension was confirmed and that could not be overcome by the addition of *Pab*PCNA. These data demonstrate that the family D pol is an DNA and an RNA primer-directed pol whereas the family B pol can only use DNA primers.

**Pab*polD, but not Pab*polB, can perform strand displacement DNA synthesis.** Since eukaryotic pols β and δ can perform strand displacement synthesis\(^3\), we next tested *Pab*pols for these properties. A single-strand circular M13mp18 with a defined gap of 25-nt containing either an RNA or an DNA downstream primer (Figure 5A) was used. Elongation was monitored by incubating *Pab*pols at a 3-fold excess over the template. Gap filling synthesis results in a 57-nt fragment and strand displacement activity gives longer products up to 87-nt. *Pab*polD could displace the DNA downstream primer after completion of the gap (Figure 5B, lane 3). However, an accumulation of 57-base pair products occurred in presence of an RNA downstream primer (Figure 5B, lane 6). When, on the other hand, *Pab*polB was tested DNA synthesis proceeded until the 5’-end of the DNA downstream primer was reached corresponding to the 57-nt elongated product (Figure 5B, lane 9). No displacement of the DNA downstream fragment by *Pab*polB even at longer incubation times could be detected and an increase of the dNTPs concentration had no effect (data not shown). Finally, an RNA downstream primer prevented the precise gap filling by *Pab*polB (Figure 5B, lane 12).

**The gap size does neither influence the gap filling nor strand displacement DNA synthesis by the Pab*pols B and D.** We next addressed whether the gap size affects the *Pab*pols. Gap sizes of 1, 10, 25, 50 and 100 nucleotides were prepared as outlined in Table 1 and Figure 6A. *Pab*polD was able to fill gaps of all sizes tested and was able to displace
efficiently the DNA downstream primer and this independently of the gap size (Figure 6B, lanes 3-8). Again, when the downstream DNA primer was replaced by an RNA primer, strand displacement activity of PabpolD was prevented (Figure 6B, lanes 9-14). PabpolB, on the other hand, was able to fill the gap, but no strand displacement activity was observed even by decreasing the gap size (Figure 6C, lanes 3-8). DNA primer elongation was severely reduced in presence of gapped template containing an RNA downstream primer (Figure 6C, lanes 9-14). These data indicated that, irrespectively of the gap size, only PabpolD can perform strand displacement synthesis only in the presence of DNA.

The strand displacement synthesis of Pabpol D and B are stimulated by PabPCNA in presence of a defined downstream primer-template. PabPCNA was next tested for its effect on gap filling reaction and on strand displacement synthesis by Pabpol D and B. To address this question, the dNTP concentration was lowered 2-fold and M13mp18 with a defined gap of 25-nucleotides containing either an RNA or an DNA downstream primer were used (Figure 7A). Under these reaction conditions, strand displacement activity by PabpolD on an DNA oligonucleotide was stimulated by PabPCNA (Figure 7B, lanes 4-5). Interestingly, PabpolD could displace the RNA primer in the presence of PabPCNA (Figure 7B, lanes 6-7). Moreover, when PabPCNA was added to PabpolB, gap filling was now seen until the 5’-end of the downstream DNA primer was reached and the strand displacement activity was enhanced (Figure 7C, lanes 4-5). No effect of PabPCNA onto PabpolB activity in presence of the RNA downstream primer was observed (Figure 7C, lanes 6-7). In summary, PabPCNA can enhance the strand displacement synthesis of both PabpolD and B.

The physical interaction between Pabpol and PabPCNA is DNA-dependent. Since we demonstrated that PabPCNA is required to stimulate primer elongation and strand
displacement synthesis by *Pab*pols D and B (Figures 4C and 7B-7C), we therefore investigated whether *Pab*PCNA can physically interact with the two *Pab*pols using the SPR assay. No direct interaction between *Pab*PCNA and either *Pab*polD (Figure 8A, curve 1) or *Pab*polB (Figure 8B, curve 1) was seen. This result was similarly found in a negative pull-down assay (data not shown). However, when an DNA primed-template was immobilised onto the sensor chip, both *Pab*pols were able to bind the immobilised primed-template (Figure 8A and B, curves 2). When *Pab*PCNA and *Pab*pols were added sequentially over the primed-template sensor chip, a stronger level of binding could be observed (Figure 8A and B, curves 3), suggesting that *Pab*PCNA promotes the association of *Pab*pols with the PCNA/DNA complex. Taken together, we assume that the physical interactions between *Pab*pols D and B and *Pab*PCNA are DNA-dependent.
Discussion

Archaea, the third domain of life, are fascinating microorganisms since they show a bacterial-like DNA replication with its circular chromosome, a high DNA replication speed and a bi-directional replication. On the other hand they use eukaryal-like DNA replication proteins. Compared to eukaryotes, archaeal replication requires structurally simpler DNA replication complexes. They have evolved differently to initiate duplication of their genome. Here we focused on the two pols D and B from the hyperthermophilic euryarchaeota *Pyrococcus abyssi*. The family D and B pols are bona fide DNA polymerases and require a primer to initiate DNA synthesis (Figure 1). *Pab*polD preferentially binds primed-templates (Figure 2B) consistent with its ability to extend primers independently of their composition. *Pab*polD initiated DNA synthesis in presence of various primed-templates but to a lower efficiency than *Pab*polB. *Pab*PCNA could stimulate DNA synthesis of full-length products (Figure 4C). Therefore, *Pab*PCNA is likely required for *Pab*polD to perform efficient DNA synthesis. Moreover, the length of single-strand regions seems to be a pre-requisite in order to detect the RNA primer elongation by *Pab*polD, thus restricting the size of the newly extended product (Figure 4D). In contrast, *Pab*polB could discriminate between an RNA or DNA primer and could only extend an DNA primed-template (Figure 4B and C). When *Pab*polB was tested in a gel shift competition assay, binding was observed to single-strand DNA with primed-templates as good competitors (Figure 3B). This is not surprising because it involves the already known properties of archaeal family-B. However, the observation that an RNA primed-template could compete the single-strand binding of *Pab*polB with only a 25-fold molar excess was unexpected, taking into account that *Pab*polB could not elongate an RNA primer and this even in the presence of *Pab*PCNA (Figure 4C) and/or a short single-strand region (Figure 4D). Therefore, *Pab*polB can interact with an RNA-primed template. Hence,
this explanation would corroborate with the identification of an RNA-binding domain at the N-terminus of the family B pols \(^3^4\).

\textit{Pabh} polD can efficiently perform strand displacement DNA synthesis when the downstream nucleic acid was DNA but not RNA (Figure 5B) and these properties were unchanged by varying the size of the gap (Figure 6B). On the other hand, \textit{Pabh} polB did not show displacement of DNA or RNA (Figure 5B). In addition, an RNA downstream primer prevented the precise gap filling by \textit{Pabh} polB under all conditions tested (Figure 6C). \textit{Pabh}PCNA could stimulate strand displacement DNA activity of \textit{Pabh} polD (Figure 7B). Stimulation of the strand displacement activity of \textit{Pabh} polB was only detected for DNA when \textit{Pabh}PCNA was added (Figure 7C).

The results presented in this paper suggest that \textit{Pabh} pols D and B might both be involved in DNA replication. Indeed, a replicase like human Pol \(\delta\) is known to perform strand displacement DNA synthesis of the pre-existing downstream Okazaki fragment thus resulting in an intermediate flap structure that is the target of the subsequent concerted action of Fen1, PCNA and DNA ligase I \(^3^0\). By analogy with the eukaryotic mechanisms, \textit{Pabh} polD would appear to be a candidate to carry out the completion of Okazaki fragments and to create PCNA-dependent displacement of the encountering RNA-DNA primer. Our data together with those published so far let us to propose a model for archaeal replication: A primase is recruited to the replication origin to initiate synthesis of a short RNA primer \(^3^5, 3^6\). This is followed by the incorporation of a short stretch of DNA by Pol D at both the lagging and leading strands. Subsequently, the RNA-DNA primer is further elongated by the replicases, in which we propose that one enzyme is active on the leading strand and the other on the lagging strand. Pol B might be the candidate to replicate the leading strand while Pol D could synthesize the lagging strand. Completion of the Okazaki fragments by Pol D would lead to
the strand displacement of the RNA-DNA primer, thus targeting maturation enzymes such as RNaseH2, Fen1 and DNA ligase to generate an intact DNA strand.\textsuperscript{32}

**Abbreviations used:** *Pab, Pyrococcus abyssi*; dNTP, deoxyriboNucleosideTriPhosphate; nt, nucleotide; pol, polymerase; RF-C, Replication Factor C; PCNA, Proliferating Cell Nuclear Antigen; Fen1, Flap endonuclease 1.

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Materials and Methods

Chemicals and Enzymes

$[^3]H$dTTP (1.5 Ci/mmol), $[^\gamma]^{32}P$ATP (3000 Ci/mmol), and $[^\alpha]^{32}P$dATP (3000 Ci/mmol) were from Amersham Biosciences. Unlabeled dNTPs, poly(dA), poly(rA) and Oligo(dT)$_{12-18}$ were from Roche Molecular Biochemicals. Single-strand circular (ssc) M13mp18 was purchased from Amersham Biosciences. T4 polynucleotide kinase was from New England Biolabs. $P_{ab}$polB and $P_{ab}$polD were cloned, expressed and purified as described earlier 25. Recombinant $P_{ab}$PCNA was produced in $E$. coli and purified to homogeneity as described 37. 1 unit of $P_{ab}$pols activity corresponds to the incorporation of 1 nmol of total dTMP into acid precipitable material per min at 50°C in a standard assay containing 0.5 µg (as nucleotides) of poly(dA)/oligo(dT)$_{10:1}$. Whatman was the supplier of the GF/C filters. All other reagents were of analytical grade and purchased from Sigma-Aldrich and Fluka.

Nucleic acid substrates

Oligonucleotides used to prepare the substrates for primer extension and gap filling assays were synthesised and purified by Eurogentec (Belgium). When appropriate, labeling at the 5’-end was performed using $[^\gamma]^{32}P$ATP and T4 polynucleotide kinase. Free $[^\gamma]^{32}P$ATP was removed on Microspin™ G-25 columns. The gapped and primed DNA templates were prepared by annealing oligonucleotides and M13mp18 template in a 3:1 molar ratio in 30 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA. The mixture was heated to 75°C and slowly cooled down to room temperature. The DNA templates for the short single-strand RNA primer extension and the mobility shift competition assays were prepared according to the
same protocol with the complementary oligonucleotides mixed in mol:mol ratio. The sequences of the different oligonucleotides used in this work are listed in Table 1.

**DNA polymerase assays**

Pol activities on poly(dA)/oligo(dT)$_{40:1}$, poly(dA)/oligo(dT)$_{10:1}$, poly(rA)/oligo(dT)$_{40:1}$ and poly(rA)/oligo(dT)$_{10:1}$, were determined in a final volume of 20 µl containing the following components: 0.5 µg of template/primer, 5 µM [$^3$H]dTTP (5 Ci/mmol), 50 µM of unlabeled dNTP and 0.2 pmol of Pabpol to be tested in their respective buffer: (i) for PabpolD: 20 mM Bis-Tris (pH 6.5), 1 mM dithiothreitol (DTT), 0.4 mg/ml of bovine serum albumin (BSA), 10 mM MgCl$_2$; (ii) for PabpolB: 50 mM Tris-HCl (pH 8.8), 1 mM DTT, 0.4 mg/ml BSA, 2 mM MgCl$_2$, 10 mM KCl. Reactions were incubated at 50°C for 30 minutes and stopped on ice for 5 minutes. The DNA products were precipitated with 10% (w/v) TCA and insoluble radioactive material was determined by scintillation counting.
Primer extension on primed sscM13mp18 DNA

**Acid precipitable assay.** Primer extension was performed in a final volume of 20 µl containing the following components: (i) for *PabpolD*: 20 mM Bis-Tris (pH 6.5), 1 mM dithiothreitol (DTT), 0.4 mg/ml of bovine serum albumin (BSA), 10 mM MgCl₂, 100 ng of different primed DNA templates, 200 µM of unlabeled dNTP, 20 µM [³H]dTTP and enzyme to be tested; (ii) for *PabpolB*: 50 mM Tris-HCl (pH 8.8), 1 mM DTT, 0.4 mg/ml BSA, 2 mM MgCl₂, 10 mM KCl, 100 ng of different primed DNA templates, 200 µM of unlabeled dNTP, 20 µM [³H]dTTP and enzymes to be tested. All reactions were incubated for 30 minutes at 55°C and the DNA was precipitated with 10% trichloroacetic acid (TCA). Insoluble radioactive material was determined by scintillation counting as described.

**Product analysis.** The composition of buffers, DTT, BSA, and MgCl₂ for *PabpolD* and *PabpolB* were identical to the acid precipitable assay. The final volume of 15 µl contained unlabeled 200 µM dNTP, 100 ng of different primed DNA templates and 0.2 pmol of *Pabpols* with or without 0.7 pmol *PabPCNA*, as indicated in the respective Figure legends. The reactions were incubated for 30 minutes at 55°C, samples were quenched on ice by adding 30 mM NaOH, 1 mM EDTA and the products were separated on a 0.8% (w/v) denaturing alkaline agarose gel and analysed by autoradiography.

RNA primer extension on a single strand linear DNA template

This assay was performed in a final volume of 15 µl containing: 0.25 pmol of labeled template, 200 µM dNTP and 0.2 pmol of *Pabpols* in their respective buffer, DTT, BSA, and MgCl₂ conditions (see above) either in the absence or in the presence of *PabPCNA* (0.7 pmol). The reaction was carried out at 55°C for 30 minutes and quenched on ice for 5 minutes and stopped by addition of the 2.5x stop buffer (95% (v/v) formamid, 20 mM EDTA, 0.05%
(w/v) bromophenol blue and 0.05% (w/v) xylene cyanol). Products were separated on 15% denaturing polyacrylamide gels and visualised by autoradiography.

**Gap filling assays**

The standard reaction mixture (15 µl) and the respective buffer, DTT, BSA, and MgCl₂ conditions (see above) for *Pab*polB and *Pab*polD were identical to the primer extension assay. For varying the size of the gap, the appropriate DNA upstream primer was mixed with the downstream RNA or DNA primer and the sscM13mp18 DNA template. The reaction included unlabeled dNTPs (200 µM), 0.2 pmol of *Pab*pol and 0.07 pmol of gapped templates as indicated in the respective Figure legends. For *Pab*PCNA stimulation of *Pab*pol, the dNTP concentration was lowered to 100 µM. All reactions were carried out at 60°C for 30 min and stopped by adding denaturing gel loading buffer (95% (v/v) formamid, 10 mM EDTA, 0.05% bromophenol blue, 0.05 % xylene cyanol). The products were resolved by electrophoresis on a denaturing resolution gel and by autoradiography.

**Electrophoresis mobility shift assay (EMSA)**

Binding reactions were carried out in a total volume of 20 µl containing: 50 mM Tris-HCl (pH 8.0), 20 mM NaCl, 5 mM EDTA, 50 µg/ml BSA, 4% Ficoll, 0.5 pmol of labeled oligonucleotide probe, and the indicated amount of *Pab*pol. Following a 20-min incubation at 25°C, the reactions were loaded onto a 5% polyacrylamide gel containing 0.5x TBE and run first at 50 V for 45 minutes and then at 100 V for 90 minutes. The gels were exposed to a screen and the bands were visualised by phosphoimager (Biorad). When more than one oligonucleotide was part of a competitor oligonucleotide complex (e.g. A1/B2 in the double-strand structure), the number of pmol in the figure corresponds only to the oligonucleotide
B2, and in order to constitute the complete competitor structure the other oligonucleotides listed in Table 1 was added at an equimolar ratio to B2.

**Surface Plasmon Resonance (SPR) experiments**

SPR analysis were performed in a BIAcore X apparatus (BIAcore, Uppsala Sweden) using either a DNA or a PCNA sensor chip. When a DNA chip was used, a biotinylated template B2 hybridised to the primer A2 (Table 1) was attached to the chip (SA sensor chip, BIAcore) via the streptavidin-biotin linkage. Approximately 200 RU (Resonance Unit) of template were immobilised. The interaction between Pabpols and the complex DNA/PCNA were monitored as followed: PabPCNA (200 nM) was injected over the DNA chip and 1.4 pmol of Pabpols was supplemented 30 seconds after injection. When a PCNA chip was used, approximately 150 RU of PabPCNA were covalently fixed on the surface of a dextran chip (CM5) by an amine coupling method according to the manufacturer's instructions. In all experiments, the interactions were monitored at 25°C at a flow rate of 30 µl/min in HBS-P buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% (v/v) P20).
Figure Legends

Figure 1. *Pab* DNA polymerases preferentially replicate synthetic DNA but not RNA. The assays were performed as outlined in Materials and Methods and included 0.5 µg of poly(dA)/oligo(dT) or poly(rA)/oligo(dT) in a ratio of template : primer of either (10 : 1) or (40 : 1). The activities of *PabhpolD* and *PabhpolB* were each compared to those of poly(dA)/oligo(dT) (10:1).

Figure 2. *PabhpolD* specifically binds primed DNA. A, Schematic template bound. B, Gel shift competition reactions were carried out as described under Materials and Methods. Each reaction contained 10 pmol of purified *PabhpolD* and the indicated amount of the following nucleic acid competitor: lanes 1 and 2, no competitor; lanes 3-6, B2; lanes 7-10, A1/B2; lanes 11-14, C1/B2; lanes 15-16, no competitor; lanes 17-20, C1; lanes 21-24, A2/B2. The positions of free and complexes labeled templates are indicated on the left. The templates are represented in Table 1.

Figure 3. *PabhpolB* binds single-strand and primed-DNA templates. A, Template used in this study. B, Binding reactions were carried out as described under Materials and Methods. Each reaction contained 15 pmol of purified *PabhpolB* and the indicated amounts of the following nucleic acid competitors (see Table 1 for details): lanes 1 and 2, no competitor; lanes 3-6, A2/B2; lanes 7-10, C1/B2; lanes 11-12, no competitor; lanes 13-16, B2; lanes 17-20, C1; lanes 21-24, A1/B2. The positions of free and complexes labeled templates are indicated on the left.
Figure 4. *Pab*PCNA can stimulate *Pab*PolD and *Pab*PolD can elongate DNA and RNA primers. A, the different primers hybridised to the M13mp18 template were: RNAp, DNAp, short DNA and RNA-DNA hybrid primer. 100 ng of these four primed-M13mp18 DNA templates (base ratio 3:1) were tested in the respective polymerase reactions for *Pab*polD and *Pab*polB at 55°C for 30 minutes with 0.2 pmol of each *Pab*pols. B, dNTP incorporation into the four different primed-templates were tested by acid precipitation. C, primer extension assays were performed with the 5’-labeled primers hybridised to the M13mp18 template (100 ng) in the presence or absence of *Pab*PCNA (0.7 pmol) and 0.2 pmol of *Pab*pols as indicated. The extended products were separated on a 0.8% (w/v) denaturing alkaline agarose gel. D, RNA primer elongation was measured on a short single-strand linear DNA template in the respective polymerase reactions for *Pab*polD and *Pab*polB with or without *Pab*PCNA and analysed by a 15% denaturing polyacrylamide gel as outlined in Materials and Methods.

Figure 5. *Pab*polD, but not *Pab*polB, can perform strand displacement DNA synthesis. A, the DNA template used was single-strand M13mp18 with a 5’-labeled upstream DNA and a downstream DNA or RNA oligonucleotide, respectively. The gap was 25-nt. The reaction was performed at 60°C for 30 minutes as described in Materials and Methods with 0.2 pmol *Pab*pols, 200 µM dNTP and 0.07 pmol of 25-nt gapped M13mp18 DNA templates. Samples were analysed on a 15% denaturing polyacrylamide gel and by autoradiography. B, Gap filling and strand displacement activities by *Pab*polD (lanes 1-6) and *Pab*polB (lanes 7-12). Lanes 1, 4, 7 and 10 were the control without enzyme. Lanes 2, 5, 8 and 11 were time zero. Lanes 3, 6, 9 and 12 were 30-min incubation time.

Figure 6. The gap size does neither influence the gap filling nor strand displacement DNA synthesis by the *Pab*pols B and D. A, the DNA template used in the study was a
M13mp18 with a downstream DNA or RNA primer and different 5’-labeled upstream DNA oligonucleotides in order to create the appropriate gap size. Reactions were performed as described in Figure 5. B, gap filling and strand displacement synthesis by PabpolD (0.2 pmol) were tested at various gap sizes in the presence of DNA (lanes 3-8) or RNA (9-14). C, gap filling and strand displacement synthesis by PabpolB (0.2 pmol) were tested at various gap sizes in the presence of DNA (lanes 3-8) or RNA (9-14).

Figure 7. The strand displacement synthesis of Pabpol D and B are both stimulated by PabPCNA in presence of a defined downstream primer-template. A, the template used is the 25-nt gapped M13mp18 DNA indicated in the Figure 5. B, 0.2 pmol of PabpolD were incubated alone or in the presence of PabPCNA (0.7 pmol). C, 0.2 pmol of PabpolB were incubated alone or in the presence of PabPCNA (0.7 pmol). Lanes 1 and 2 are the DNA markers corresponding to the 57-mer (filled gap) and 87-mer (displaced strand). Lanes 3-7 contained the primers, the pols and PCNA as indicated above the gel. Reactions were performed at 60°C for 30 minutes with 0.07 pmol of template and 100 µM of dNTP as outlined in Materials and Methods. The products were resolved by a 15% denaturing polyacrylamide gel and revealed by autoradiography

Figure 8. The physical interaction between Pabpol D and PabPCNA is DNA-dependent. A, Curve 1: PabpolD (1.4 pmol) was injected over the immobilised PabPCNA. Curve 2: control injection of PabpolD binding to the primed-template (A2/B2). Curve 3: PabPCNA and PabpolD were sequentially added over a primed-template (A2/B2) sensor chip. B The PabpolB experiment were performed as described for PabpolD. , Curve 1: PabpolB injection over the immobilised PabPCNA. Curve 2: Control injection of PabpolB binding to the primed-template. Curve 3: PabPCNA and PabpolB were sequentially added over the primed-
template sensor chip. The background resulting from the injection buffer alone was subtracted in each case from the data before plotting.
References


Figure 1

Bar graph showing relative activity of $PabpolB$ and $PabpolD$ enzymes with different nucleotide ratios.

- Relative activity (%) along the y-axis.
- Nucleotide ratios (dA-dT) 10:1, (dA-dT) 40:1, (rA-dT) 10:1, (rA-dT) 40:1 along the x-axis.
Figure 2

2A

2B

Lanes
Complex
Free probe

competitor (pmol) 0 0 2.5 5 25 125 2.5 5 25 125 2.5 5 25 125

Single strand DNA

Double strand DNA

3’RNA

Lanes
Complex
Free probe

competitor (pmol) 0 0 2.5 5 25 125 2.5 5 25 125

Single strand RNA

3’DNA
**Figure 3**

3A

![Diagram showing 3'-P-5' and B2 competitor (pmol)]

3B

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4A

RNAp  
DNAp  
Short DNAp  
RNA-DNAp

\( \text{M13mp18} \)

(7250-nt)

\( 5' \)

4B

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pmol/30 min
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Figure 4C_D
Figure 5

5A

RNA or DNA downstream primer (30-nt)

Gap (25-nt)

DNA upstream primer (32-nt)

M13mp18
7250-nt

5'

5A

Pol D

5B

Pol B

87 nt
57 nt
32 nt

Lanes 1 2 3 4 5 6

DNAd
RNAd

87 nt
57 nt
32 nt

Lanes 7 8 9 10 11 12

DNAd
RNAd
Figure 6

6A

DNA or RNA downstream primer (30-nt)

DNA upstream primer (32-nt)

M13mp18 5'

32 nt

57 nt

134 nt

238 nt

markers

gap size (1, 10, 25, 50, 100-nt)

6B

6C
7A

RNA or DNA downstream primer (30-nt)

Gap (25-nt)

DNA upstream primer (32-nt)

M13mp18
7250-nt

7B

7C

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