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Binding to PCNA in Euryarchaeal DNA Replication Requires Two PIP Motifs for DNA Polymerase D and One PIP Motif for DNA Polymerase B

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Abstract:

Replicative DNA polymerases possess a canonical C-terminal proliferating cell nuclear antigen (PCNA)-binding motif termed the PCNA-interacting protein (PIP) box. We investigated the role of the PIP box on the functional interactions of the two DNA polymerases, *Pab*Pol B (family B) and *Pab*Pol D (family D), from the hyperthermophilic euryarchaeon *Pyrococcus abyssi*, with its cognate PCNA. The PIP box was essential for interactions of *Pab*Pol B with PCNA, as shown by surface plasmon resonance and primer extension studies. In contrast, binding of *Pab*Pol D to PCNA was affected only partially by removing the PIP motif. We identified a second palindromic PIP box motif at the N-terminus of the large subunit of *Pab*Pol D that was required for the interactions of *Pab*Pol D with PCNA. Thus, two PIP motifs were needed for *Pab*Pol D for binding to *Pab*PCNA. Moreover, the C-terminus of *Pab*PCNA was essential for stimulation of *Pab*Pol D activity but not for stimulation of *Pab*Pol B activity. Neither DNA polymerase interacted with the *Pab*PCNA interdomain connecting loop. Our data suggest that distinct processes are involved in *Pab*Pol D and *Pab*Pol B binding to PCNA, raising the possibility that Archaea require two mechanisms for recruiting replicative DNA polymerases at the replication fork.

Keywords: DNA replication; DNA polymerases; PCNA-binding motifs; PIP box; Archaea

Abbreviations: IDCL, interdomain connector loop of PCNA; *Pab*, *Pyrococcus abyssi*; PCNA, proliferating cell nuclear antigen; *Pab*PCNA, *Pyrococcus abyssi* PCNA; *Pab*Pol B, *Pyrococcus abyssi* family B DNA polymerase; *Pab*Pol D, *Pyrococcus abyssi* family D DNA polymerase; *Pab*Pol BΔpip and *Pab*Pol DΔpip, DNA polymerases lacking the C-terminal PIP box motif; *Pab*Pol DNcut, *Pab*Pol D lacking the N-terminus of the large subunit; PIP, PCNA-interacting protein; *Pfu*, *Pyrococcus furiosus*; RF-C, replication factor C; SPR, surface plasmon resonance.

INTRODUCTION

DNA replication is a functionally conserved mechanism among all organisms including Bacteria, Eukarya, Archaea and viruses[1,2] that ensures genome stability.[3] The process takes place through a variation of a theme with a protein triad composed of a DNA polymerase holoenzyme with two accessory factors, the sliding clamp and the clamp-loader. In *E. coli*, the chromosomal replicase is a DNA polymerase III complex encompassing the two protein factors in addition to the polymerizing core subunits.[4,5] Recently, O'Donnell and his co-workers suggested that three Pol III complexes could assemble to form a triple replisome.[6] In eukaryotes, two replicases, belonging to family B, ensure faithful copying of the entire genome. DNA polymerase δ is composed of three[7] to four[8,9] essential subunits, while DNA polymerase ε is active as a heterotetramer.[10] Recent genetic studies on DNA polymerases δ and ε strongly suggest that they are essential for DNA replication. DNA polymerase δ might be responsible for Okazaki fragments synthesis on the lagging strand while DNA polymerase ε is likely to perform leading strand synthesis.[11,12]

A fascinating relationship has been found between the eukaryal and archaeal DNA replication proteins. Indeed, informational proteins in Archaea are more closely related to their eukaryotic rather than their bacterial counterparts. Moreover, the functional interaction between the eukaryotic DNA polymerase δ and the archaeal accessory factors, PCNA and RF-C was conserved through evolution.[13,14]

Yet, the archaeal replication factors from thermococcales display unique functional properties. Indeed, while ATP hydrolysis is required for clamp-loading in Eukaryotes,[15] in Archaea, the RF-C complex could load the PCNA without the need for ATP hydrolysis.[16] Also, the PCNA stimulated the B and D family DNA polymerases on a circular DNA template in the absence of the RF-C.[17] Moreover, the spontaneous loading of the PCNA from

Pyrococcus abyssi, hereafter named *Pab*PCNA, can be enhanced not only by RF-C but also by Pol B.[18]

Within the archaeal domain, it has been established that significant divergence can be found within the main sub-domains, i.e. Euryarchaea and Crenarchaea. Indeed, up to three PCNA homologues were found in Crenarchaea[19,20] which were incriminated to form a heterotrimer *in vivo*,[21] while a single PCNA homologue that forms a homotrimer was found in Euryarchaea. These assemblies demonstrate how protein components have evolved distinctly in Archaea. Moreover, the spectrum of cellular DNA polymerases is highly divergent within these sub-domains. Crenarchaea possess mainly up to three family B monomeric DNA polymerases,[22,23] while Euryarchaea possess one monomeric family B DNA polymerase and one heterodimeric family D DNA polymerase supposed to be restricted to this sub-domain.[24-26] However, Pol D was also detected in the genome of *Candidatus Korarchaeum cryptofilum*,[27] *Nanoarchaeum equitans*[28] and *Cenarchaeum symbiosum*.[29]

Generally, replicative DNA polymerases need to be chaperoned by the sliding clamp to become rapid and processive.[30] Interactions of proteins with the sliding clamp are mediated through common motifs, like the PCNA Interacting Protein (PIP) box.[31] It can be defined as Qxx(h)xx(a)(a), where “x” is any amino acid, “h” is a hydrophobic residue (I, L or M), and “a” is a aromatic residues (F, Y or W). A large number of replication and repair proteins, including error prone DNA polymerases have the consensus PIP motif.[32,33] Recent studies have demonstrated that the PIP box is not always sufficient to account for interactions with PCNA. This seems to indicate that a region proximal to the core-conserved domain makes a significant contribution to PCNA interaction.[34] Moreover, a helix-hairpin-helix (HhH) motif, important for proper DNA primer binding of DNA polymerase λ, was shown to physically interact with PCNA.[33] In addition, a C-terminal region of DNA polymerase λ, which is not related to the PIP box, was described and suggested that specific PCNA-binding motifs could

be related in a negative regulation of the nucleotidyl transferase activity.[35] Looking over a random peptide library, a new polymorphic PCNA-binding motif (KA-box) was recently identified in human Pol δ and also in other proteins like members of MCM family, mismatch repair protein MSH6 or cyclin D3.[36] Finally, the binding of DNA polymerase δ to PCNA was shown to be mediated by a glycine-rich region (GX4GX8GX3YFY).[37] However, PCNA-interacting peptides play a role not only through their sequences but also at a structural level. Indeed, recent studies have shown that PCNA binding of PCNA-interacting peptides is independent of the specific sequence.[38] Moreover, the PIP-peptides are mediated through beta zipper formation at the interaction surface.[39] Usually, the PIP-box motifs interact preferentially with the hydrophobic pocket[40,41] of PCNA, defined by the C-terminus and the interdomain connecting loop (IDCL). Therefore, the PCNA interacting partners, and especially DNA polymerases, bind the clamp through a complex set of motifs that have no match with the PIP box. In accord with this idea, we searched for unraveled PCNA binding motifs within archaeal DNA polymerases in order to decipher the physical assembly of functional DNA Pols and PCNA in Archaea.

RESULTS

Functional and physical interactions between *Pab*PCNA and *Pab*Pol B but not *Pab*Pol D are mediated by the C-terminal PIP box motif.

The C-terminal PIP box of *Pab*Pol B and *Pab*Pol D was deleted (Fig. 1a). For *Pab*Pol B, physical interactions with the *Pab*PCNA were abolished, indicating the importance of this structural motif for PCNA binding (Fig. 1b). On the contrary, deletion of the putative PIP box of the *Pab*Pol D only moderately impaired the binding to *Pab*PCNA (Fig. 1b), suggesting that other critical residues may interact with *Pab*PCNA.

The functional interactions of *Pab*Pol B with *Pab*PCNA were also abolished when the PIP box was removed (Fig. 2, lanes 7-10). Indeed, the addition of *Pab*PCNA led to full-length M13 DNA synthesis by *Pab*Pol B (Fig. 2, lanes 7 and 8), whereas, the lengths of products synthesized by *Pab*Pol BΔpip were not modified by adding *Pab*PCNA (Fig. 2, lanes 9 and 10). On the contrary, removing the putative PIP box of the large subunit of *Pab*Pol D had no effect on the stimulation of the DNA polymerase activity by the *Pab*PCNA (Fig. 2, lanes 3-6). Quantitative analysis of the data in Fig. 2, performed on six independent experiments, also showed no significant difference in PCNA stimulation for wild-type and Δpip *Pab*Pol D.

Then, primer elongation activities of *Pab*Pol B and *Pab*Pol D have been tested in the presence of *Pab*PCNA and competitor peptides, PIPB and PIPD, the primary structures of which correspond to the respective PIP-boxes (Fig. 3). While adding the control peptide TEMD had no significant effect on primer elongation by *Pab*Pol B (Fig. 3a, lanes 4-5), the PIPB peptide clearly inhibited the stimulation of the sliding clamp for long fragments synthesis (Fig. 3a, lanes 6-7). This is a confirmation that the functional interaction of *Pab*Pol B is PIP box-dependent. Interestingly, the PIPD peptide suppressed the interaction between Pol B and PCNA signifying that the PIP box of *Pab*Pol D is functionally comparable to the PIP box of

*Pab*Pol B (Fig. 3a lanes 8-9). Furthermore, PIPD and PIPB peptides also entered in competition with *Pab*Pol D, as observed by the absence of full-length M13 fragments (Fig. 3b, lanes 3-4). This observation was reinforced by the lack of PCNA stimulation of *Pab*Pol DΔpip in presence of PIPD and PIPB peptides (Fig. 3c lanes 4 and 5), suggesting the need for an additional critical PCNA-binding motif within *Pab*Pol D.

A novel N-terminal motif in *Pab*Pol D is required for functional interactions with *Pab*PCNA to occur.

Since the C-terminal PIP box motif of *Pab*Pol D had no essential role in the interactions with *Pab*PCNA, we looked for other putative PCNA-binding motifs in DNA polymerase D. A novel motif localized at the N-terminus was identified. This putative PIP box (10-YEEMLQREIDKAY-22) could correspond to a palindromic motif schematized by the following sequence: (a)xx(h)x(Q)xx(h)xxx(a), where glutamine 15 is located in the middle of the sequence (Fig. 4a).

Functional interactions of *Pab*Pol D deleted at the N-terminus (*Pab*Pol DNcut) with *Pab*PCNA were strongly inhibited, although not abolished (Fig. 4b). Quantitative analysis of the data in Fig. 4b, performed on three independent experiments, showed that PCNA stimulation was significantly reduced in *Pab*Pol DNcut as compared to *Pab*Pol D.

Besides, primer elongation activities of *Pab*Pol D have been tested in the presence of *Pab*PCNA and the competitor peptides Nter 1 and Nter 2 (Fig. 5). These peptides correspond to the primary structures of the sequence upstream and downstream of glutamine 15, respectively. Stimulation of *Pab*Pol D was still observed in the presence of peptides Nter 1 and Nter 2, which were added separately with peptide TEMD (Fig. 5, lanes 8-15). However, stimulation of *Pab*Pol D processivity was highly reduced when both peptides were present

(Fig. 5, lanes 18 and 19). This result confirms clearly the role of the entire palindromic PIP box sequence at the N-terminus of *Pab*Pol D for interactions with the PCNA.

The C-terminus of *Pab*PCNA is essential for *Pab*Pol D but not for *Pab*Pol B stimulation

In order to highlight the critical residues of *Pab*PCNA involved in physical interactions with *Pab*Pols, different mutations have been introduced within conserved structural domains (Fig. 6). By using the crystal structure of *Pfu*PCNA[42] and different results on eukaryotic PCNA,[43-45] a *Pab*PCNA structure has been modelled and putative amino acids have been targeted for mutational analyses. Firstly, we generated three mutants in the IDCL region: EIE120, VDL123 and EL127. Then, proline and arginine located at the C-terminus were replaced by alanine. All these mutations are represented in Fig. 6.

Native polyacrylamide gel was used to estimate the homotrimeric organization of individual PCNA mutants which migrated as a single band at about 85 kDa like the wild type PCNA (Data not shown).

Then, primer elongation activities of *Pab*Pol B, *Pab*Pol D and its mutants *Pab*Pol DΔpip and *Pab*Pol DNcut were tested in the presence of the mutated *Pab*PCNA in optimal stimulation conditions for each DNA polymerase (Fig. 7). All of PCNA mutants stimulated *Pab*Pol B (Fig. 7). *Pab*Pol D was stimulated by EIE120, VDL123 and EL127 PCNA mutants while PR245 was not able to enhance primer extension by *Pab*Pol D (Fig. 7). This result indicates that the C-terminus of PCNA is a critical factor for physical interaction with Pol D. Also, *Pab*Pol DΔpip and *Pab*Pol DNcut were stimulated by PCNA mutants with the exception of PR245 (Fig. 7). Overall, our results demonstrated that PCNA binding of *Pab*Pol D is probably carried out *via* the PIP motifs location at the N- and C-terminus respectively.

DISCUSSION

PCNA-binding motifs appear to be divergent and even the PIP box has a polymorphous nature. In the human DNA polymerase δ p66 subunit, a N-terminal region of the canonical PIP motif contributed significantly to the interactions with PCNA.[34] In the Pol X family, a non-canonical PIP box belongs to a longer PCNA-binding motif with a conserved helix-hairpin-helix (HhH) domain.[33] Also, PIP box-like motifs can be found in the core of most DNA polymerases. As an example, PIP box-like motifs were found in the large subunit of *Pfu*Pol D at amino acids positions 1096-1103 and 1253-1261.[17] In *Pab*Pol B, the LxxFG motif is found at positions 745-750 and two LxxFY motifs are found in the large subunit of *Pab*Pol D in the amino acids sequence 894-904. Therefore, it can be assumed that functional PIP box motifs are located at the terminal parts of the interacting proteins. In this study, we gave further insight in to the physical and functional interactions of *Pab*Pols with its cognate PCNA. While *Pab*Pol B was shown to carry only one PIP box motif at the C-terminus, *Pab*Pol D was demonstrated to contain two PIP-type motifs that interact with PCNA. Therefore, these results suggest that at the replication fork *Pab*Pol D might be loaded and stimulated differently by PCNA compared with *Pab*Pol B. Contrary to *Pab*Pol B, removing the canonical C-terminal PIP motif from *Pab*Pol D did not disrupt the physical interactions with the PCNA, although we demonstrated, by the use of competitor peptides, that this shortened motif was functionally a PIP box. Interestingly, the N-terminal PCNA-interacting motif in *Pab*Pol D is a palindromic putative PIP box defined by the sequence (10-YEEMLQREIDKAY-22). We show here that this sequence is part of the surfaces interacting with the sliding clamp. Besides, both peptides corresponding to the N-terminus inhibited interactions between *Pab*Pol D and *Pab*PCNA at higher amounts than PIPD peptide (500 µM versus 30 µM). Finally, while the situation is clear for *Pab*Pol B, which interacts with PCNA

via one motif, PCNA-binding of *Pab*Pol D is carried out at least by two motifs at the N- and C-terminus respectively. This result does not fit in with those from recent *in vitro* experiments[46] which show clearly that *Pfu*Pol D interacts functionally with *Pfu*PCNA at its C-terminal PIP box. This is surprising since Pol D from *Pyrococcus abyssi* and *Pyrococcus furiosus* share strong similarities within their primary structure and particularly through their PIP box motif. Moreover, these authors demonstrated the binding of *Pfu*Pols to *Pfu*PCNA in the absence of DNA, contrary to our previous results[47] where the presence of DNA was fully required. Besides, the absence or the presence of DNA, the amount of PCNA, relative to the DNA polymerases, is also an important factor. Indeed, as shown in Fig. 4b, for the mutant Pol D, an excess of PCNA would lead to the erroneous conclusion that the N-terminal part is the only PCNA-interacting motif.

In the eukaryotic PCNA, three domains are known to be involved in protein-protein interactions:[48,49] the interdomain connecting loop is a major interaction site and is recognized by several proteins, such as Pol δ, p21, Fen1 and DNA ligase 1; the N-terminal region comprising the inner α-helices forms part of the binding site for cyclin D; the C-terminal tail important for interactions with Pol ε and RF-C. Since *Pab*Pol D as well as *Pab*Pol DΔpip did not interact with the C-terminus mutant of the PCNA (see Fig. 7), and given that the interactions between *Pab*Pol D and *Pab*PCNA are inhibited by competitor peptides mimicking the PIP boxes, we can assume that the two PIP-motifs of *Pab*Pol D interact in the same zone of the PCNA (i.e. the C-terminus). In addition, since no PCNA mutant affected the binding of *Pab*Pol B, we cannot propose any hypothesis about the region of binding of this DNA polymerase on the PCNA. More studies are required to answer these questions. This hypothesis raises the question of steric accessibility of the PCNA sites, especially when high molecular weight complexes are involved. Besides, the PIP box motif may be a flexible connector.[50] In addition, PCNA interactions with more than one partner have to be considered

as proposed for the crenarchaeal heterotrimeric clamp.[21] Besides, it has been shown in the bacteriophage RB69 that the DNA polymerase, owing to a PIP box-like peptide, binds only to one site on the sliding clamp, the other two preserving the ability to bind additional proteins.[50] This property would allow, through a structural reorganisation of the bound protein, accessibility to the free PCNA binding sites for another protein. Additionally, it has been shown that p21 interferes strongly with PCNA stimulation by Pol δ but does not effectively inhibit the loading of the PCNA by RF-C,[51] which argues for different binding sites for Pol δ and RF-C. Furthermore, it has been proposed that RF-C travels with Pol δ and PCNA.[52] Nevertheless, we cannot answer if both motifs interact in the same monomer or two different monomers. Besides, we cannot say whether two motifs of *Pab*Pol D interact on PCNA at the same time, alternatively or in a preferential order depending on replication and/or on repair partners steps. Finally, it is clear that PCNA binding occurs differently both DNA polymerases of *Pyrococcus abyssi*, which may imply that PCNA acts as a pivotal replication factor to coordinate and enhance DNA synthesis of the two *Pab*Pols at the leading and lagging strands, as hypothesized by previous studies.[47]

MATERIALS AND METHODS

Recombinant proteins

The *Pyrococcus abyssi* family B DNA polymerase (*Pab*Pol B) used was the Isis™ DNA polymerase commercialized by Qbiogene (Illkirch, France). *Pab*Pol D and *Pab*PCNA were prepared as described earlier.[16,26] Mutant versions of both DNA polymerases with the deleted PIP box were obtained using the QuickChange™ site-directed Mutagenesis kit (Stratagene Europe, Amsterdam, The Netherlands). The codons for lysine 768 and lysine 1440 were replaced with a stop codon just upstream of the PIP box, respectively for *Pab*Pol B and *Pab*Pol D (Fig. 1a).

The construction of an expression system for the *Pab*Pol DNcut deletion mutant consisted to in deleting the N-terminal first's 22 amino acids by introduction of an NdeI recognition sequence. The gene encoding *Pab*Pol DNcut, cloned into the plasmid pET25b+ (Promega), was amplified by PCR using the oligonucleotides 5'-CAGAGGGAGATAGATAAGGCCCATATGATAGCTAAAAAGGCGAGG-3' and 3'-GTCTCCCTCTATCTATTCCGGGTATACTATCGATTTTTCCGCTCC-5'. The NdeI recognition sequence is underlined. The gene was digested by NdeI and reintroduced in the pET25b+ plasmid. The entire nucleotide sequence was confirmed by sequencing. The gene was co transformed into *E. coli* HMS174 (DE3) pLysS with the plasmid pET28b+ containing the gene encoding the small subunit of *Pab*Pol D.

In the PCNA mutants, proline 245 and arginine 246 (PR245), glutamic acid 120, isoleucine 121 and glutamic acid 122 (EIE120), valine 123, aspartic acid 124 and leucine 125 (VDL123), glutamic acid 127 and leucine 128 (EL127) were replaced for by alanine (Fig. 6). The mutants were obtained using the following oligonucleotides: 5'-TGAT ATTCCTCCTGGCTGCCGCGGTTGAGGAG-3' and 3'-

GACTATAAGGAGGACCGACGGCGCCAACTCCTC-5’ (PR245) ; 5’-ATAGATGTTGAGGCGGCCGCAGTTGACTTGCCAGAG-3’ and 3’-ATCTACAACTCCGCCGGCGTCAACTGAACGGTCTC-5’ (EIE120) ; 5’-GAGGAGATCGAAGCTGCCGCGCCAGAGTTACCC-3’ and 3’-CTC CTCTAGCTTCGACGGCGCGGTCTCAATGGG-5’ (VDL123) ; 5’-ATCGAAGTTGACTTGCCAGCGGCACCCTTCACG-3’ and 3’-TAGCTTCAACTGAACGGTCGCCGT GGGAAGTGC-5’ (EL127). The PCR products were cloned into the plasmid pET25b+ (Promega) and the entire nucleotide sequence was confirmed by sequencing. All mutants have been expressed with Rapid Translation System 500 *E. coli* HY Kit by Roche Diagnostics (Mannheim, Germany).

The peptides used as competitor for PCNA were synthesised by Eurogentec. PIPB corresponded to the C-terminal extremity of *Pab*Pol B (765-QKTKQVFLGAWLKF-778), PIPD corresponded to the C-terminal extremity of *Pab*Pol D large subunit (1438-PKKKRVISLEEFFSRKSK-1455), Nter 1 corresponded to the N-terminus of *Pab*Pol D large subunit (1- MELPKEMEEYEEMLQ-15), Nter 2 corresponded to the N-terminal domain of *Pab*Pol D large subunit (15-QREIDKAYEIAKKAR-29) and TEMD corresponded to an internal sequence of *Pab*Pol D (83-EIIEGKFGDLGSREKYAEQAV-103) and was used as control. The theoretical pI of the competitor peptides PIPB, PIPD, Nter1, Nter2 and TEMD were 10.3, 10.57, 3.98, 9.52 and 4.59, respectively.

Primer extension assay

The 32-nt oligonucleotide used to prepare the primed DNA substrate for primer extension (5’-TGCCAAGCTTGCATGCCTGCAGGTCGACTCTA-3') was synthesized, labelled at the 5’ terminus with 6-carboxyfluorescein, and purified by Eurogentec (Belgium). M13ssc was

annealed to the 32-nt oligonucleotide at a 1:3 molar ratio, in 10 mM Tris-HCl pH 8.0, 50 mM NaCl, by heating at 75°C for 10 min followed by cooling to room temperature.

Primer extension was performed in a final volume of 11 µl containing the following components: 0.09 pmol of template/primer, 2 pmol of each dNTP, 0.02 to 1.5 pmol of *Pab*PCNA and *Pab*Pols to be tested in the buffer (50 mM Tris-HCl pH 8.8, 1 mM dithiothreitol (DTT), 10 mM KCl, 2 mM $MgCl_2$). Reactions were incubated at 60°C for 30 min and stopped on ice by adding 10 µl of stop buffer (98 % (v/v) formamide, 0.2 mM EDTA, pH 8.0).

Peptides (30 µM to 1000 µM as indicated) and *Pab*Pols were added simultaneously to start the reaction and incubations were performed at 60°C for 15 min and then stopped on ice. The products were heated at 95°C for 5 min and resolved for 16 h at 30 V at 4°C on a 1 % (w/v) alkaline agarose gel (50 mM NaOH, 1 mM EDTA). Finally, the products were visualized on a Typhoon 9400 imager (Amersham Biosciences).

Surface plasmon resonance (SPR) experiments

SPR analyses were performed on a BIAcore X apparatus (BIAcore, Uppsala, Sweden. DNA surfaces consisted in approximately 200 resonance units (RU) of biotinylated template immobilized onto a streptavidin surface (SA sensor chip, BIAcore) as already described.[47]

The interaction between *Pab*Pols and the complex DNA/PCNA were monitored as follows: *Pab*PCNA (200 nM) was injected over the DNA chip and *Pab*Pols (200 nM) were supplemented 30 seconds after injection. 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) surfactant P20).

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FIGURES

Figure 1: PIP box motifs in *Pab*Pol B and D.

(a) Consensus motifs in *Pab*Pol B and D. The PIP box motif localized at level of C-terminal sequence of *Pab*Pol B and large subunit of *Pab*Pol D. The amino acid residues of PIP box are framed. Arrows show lysine mutated to a stop codon to truncate each protein.

(b) SPR measurements of *Pab*Pols and *Pab*PCNA interactions. At time 0, *Pab*PCNA was injected over the immobilized primed ssDNA[47] as indicated by the black arrow. Then, either *Pab*Pol B or *Pab*Pol D and their PIP box deleted mutants, respectively *Pab*Pol BΔpip and *Pab*Pol DΔpip, were injected as indicated by the grey arrow. The background resulting from the injection buffer alone was subtracted from the data in each case before plotting.

Figure 2: The PIP box of *Pab*Pol B, but not of *Pab*Pol D, is required for functional interaction with *Pab*PCNA.

Primer extension studies were performed with M13mp18 template (90 fmol), hybridized to a 5'-fluorescein-labeled primer and contained 0.3 pmol of *Pab*Pol D, 0.6 pmol of *Pab*Pol DΔpip, 0.02 pmol of *Pab*Pol BΔpip or 0.04 pmol of *Pab*Pol B. PCNA was added to a ratio 1:1 relative to the DNA polymerase. Primer extension studies were performed as outlined in the materials section. Reactions were incubated at 60°C for 30 min, and products were resolved on denaturing alkaline 1% agarose gels and visualized by Typhoon 9400 imager. Lanes 1 and 2 correspond to controls without the DNA polymerase.

A quantitative analysis was performed by determining the relative amounts of the full-length product by densitometry analysis of the gel. Data are expressed as means ± SD of the percent full-length product as related to total elongation products. Differences between means were tested by Student's *t* test for equal variances. Different from control without PCNA: * P <

0.01 ; ** P < 0.005 (n = 6). Data with *Pab*Pol D and *Pab*Pol DΔpip were not significantly different, in presence or absence of the PCNA, respectively.

Figure 3: Functional interaction of *Pab*Pols with *Pab*PCNA in the presence of competitor peptides corresponding to the PIP box motifs.

Primer extension studies were performed with M13mp18 template (90 fmol) hybridized to a 5'-fluorescein-labeled primer. PIPB corresponded to the C-terminal part of *Pab*Pol B, PIPD corresponded to the C-terminal part of *Pab*Pol D and TEMD was a control peptide corresponding to the amino acids 83-103 of *Pab*Pol D. Primer extension studies were performed as outlined in the materials section. Reactions were incubated at 60°C for 15 min, and products were resolved on denaturing alkaline 1% agarose gels and visualized by Typhoon 9400 imager.

(a) PIP box peptides inhibit functional interactions of *Pab*Pol B with *Pab*PCNA. The reactions contained 0.04 pmol of *Pab*Pol B. *Pab*PCNA was added to a ratio 1:1. Lane 1 corresponds to control without the polymerase. Peptides were added (10 and 30 µM) at the same time than *Pab*Pol B.

(b) PIP box peptides affect functional interactions of *Pab*Pol D with *Pab*PCNA. The reactions contained 0.3 pmol of *Pab*Pol D and *Pab*PCNA was added at an equimolar ratio. Peptides were added (30 µM) at the same time than *Pab*Pol D.

(c) PIP box peptides affect functional interactions of *Pab*Pol DΔpip with *Pab*PCNA. The reactions contained 0.6 pmol of *Pab*Pol DΔpip and *Pab*PCNA was added at an equimolar ratio. Peptides were added (30 µM) at the same time than *Pab*Pol DΔpip.

Figure 4: Putative PIP box motif localized at N-terminus of large subunit of *Pab*Pol D.

(a) The palindromic PIP box motif localized at level of N-terminal sequence of large subunit of *Pab*Pol D. The amino acid residues forming the motif are framed. Arrow show the glutamic acid mutated to a start codon.

(b) The N-terminus of large subunit of *Pab*Pol D is not essential for functional interaction with *Pab*PCNA. Primer extension studies were performed with M13mp18 template (90 fmol), hybridized to a 5'-fluorescein-labeled primer and contained 0.3 pmol of *Pab*Pol D and 0.7 pmol of *Pab*Pol DNcut. PCNA was added to ratios 1:3, 1:1 and 3:1 relative to DNA polymerase (symbolized by a triangle). Primer extension studies were made as outlined in the materials section. Reactions were incubated at 60°C for 30 min, and products were resolved on denaturing alkaline 1% agarose gels and visualized by Typhoon 9400 imager. Lanes 1 corresponds to control without the DNA polymerase.

A quantitative analysis was performed by determining the relative amounts of the full-length product by densitometry analysis of the gel. Data are expressed as means ± SD of the percent full-length product as related to total elongation products. Differences between means were tested by Student's *t* test for equal variances. Different from control without PCNA: ** $P < 0.005$ ($n = 3$). Data with *Pab*Pol D and *Pab*Pol DΔpip were significantly different when the PCNA was present ($P < 0.005$).

Figure 5: Functional interaction of *Pab*Pol D with *Pab*PCNA in the presence of competitor peptides mimicking to the N-terminus of *Pab*Pol D.

Primer extension studies were performed with M13mp18 template (90 fmol) hybridized to a 5'-fluorescein-labeled primer. Nter 1 and Nter 2 corresponded to the N-terminal part of large subunit of PabPol D, respectively the amino acids 1-15 and 15-29 (see Materials and Methods). TEMD was a control peptide corresponding to the amino acids 83-103 of *Pab*Pol D. The reactions contained 0.3 pmol of *Pab*Pol D. *Pab*PCNA was added to an equimolar

ratio. Increasing amounts of peptides were added (50 µM, 100 µM, 500 µM and 1000 µM) at the same time than *Pab*Pol D, excepted for lanes 4 to 7 where the amount of TEMD were doubled, i.e. 100 µM, 200 µM, 1000 µM and 2000 µM. Primer extension studies were made as outlined in the materials section. Reactions were incubated at 60°C for 15 min, and products were resolved on denaturing alkaline 1% agarose gels and visualized by Typhoon 9400 imager. Lane 1 corresponds to control without the DNA polymerase.

Figure 6: Mutations effected on IDCL and C-terminus of *Pab*PCNA corresponding to the hydrophobic pocket.
On the right, the 3D structure of a PCNA monomer with the mutated amino acids.

Figure 7: Functional interaction of *Pab*Pol B and *Pab*Pol D, *Pab*Pol DΔpip and *Pab*Pol DNcut with *Pab*PCNA mutated in the hydrophobic pocket.
Primer extension studies were performed with M13mp18 template (90 fmol) hybridized to a 5'-fluorescein-labeled primer. Primer extension studies were made as outlined in the materials section. Products were resolved on denaturing alkaline 1% agarose gels and visualized by Typhoon 9400 imager. The reactions contained (a) 0.04 pmol of *Pab*Pol B. Wild type and mutants PCNA were added at an equimolar ratio, as related to the Pol; (b) 0.3 pmol of *Pab*Pol D; (c) 0.6 pmol of *Pab*Pol DΔpip; (d) 0.7 pmol of *Pab*Pol DNcut.
PCNA : Pol ratio was 3:1.

Fig 1

a)

***Pab*Pol B : PAVERILRAFGYRKEDLKYQKTKQVGLGAWLKF**
746 **778**

***Pab*Pol D : TEKAEKAEKPRKKSDEKPKKKRVISLEEFFSRKSK**
1421 **1455**

b)

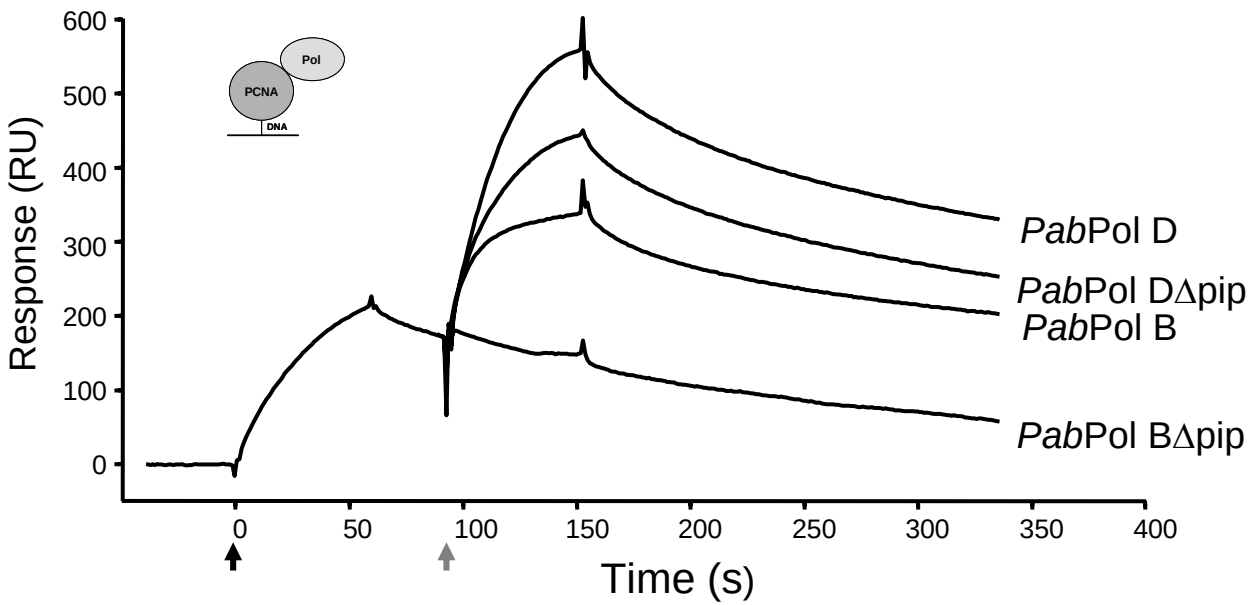
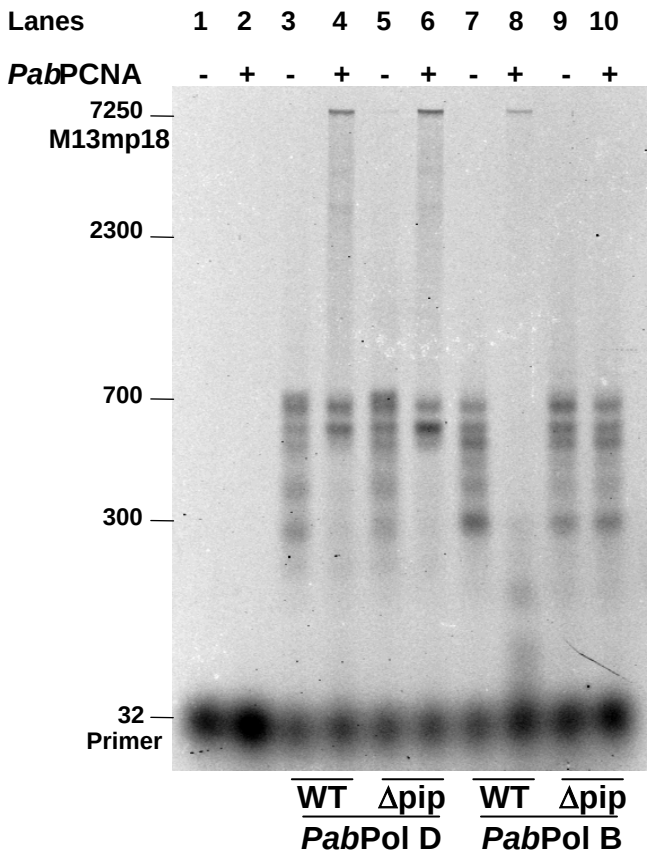
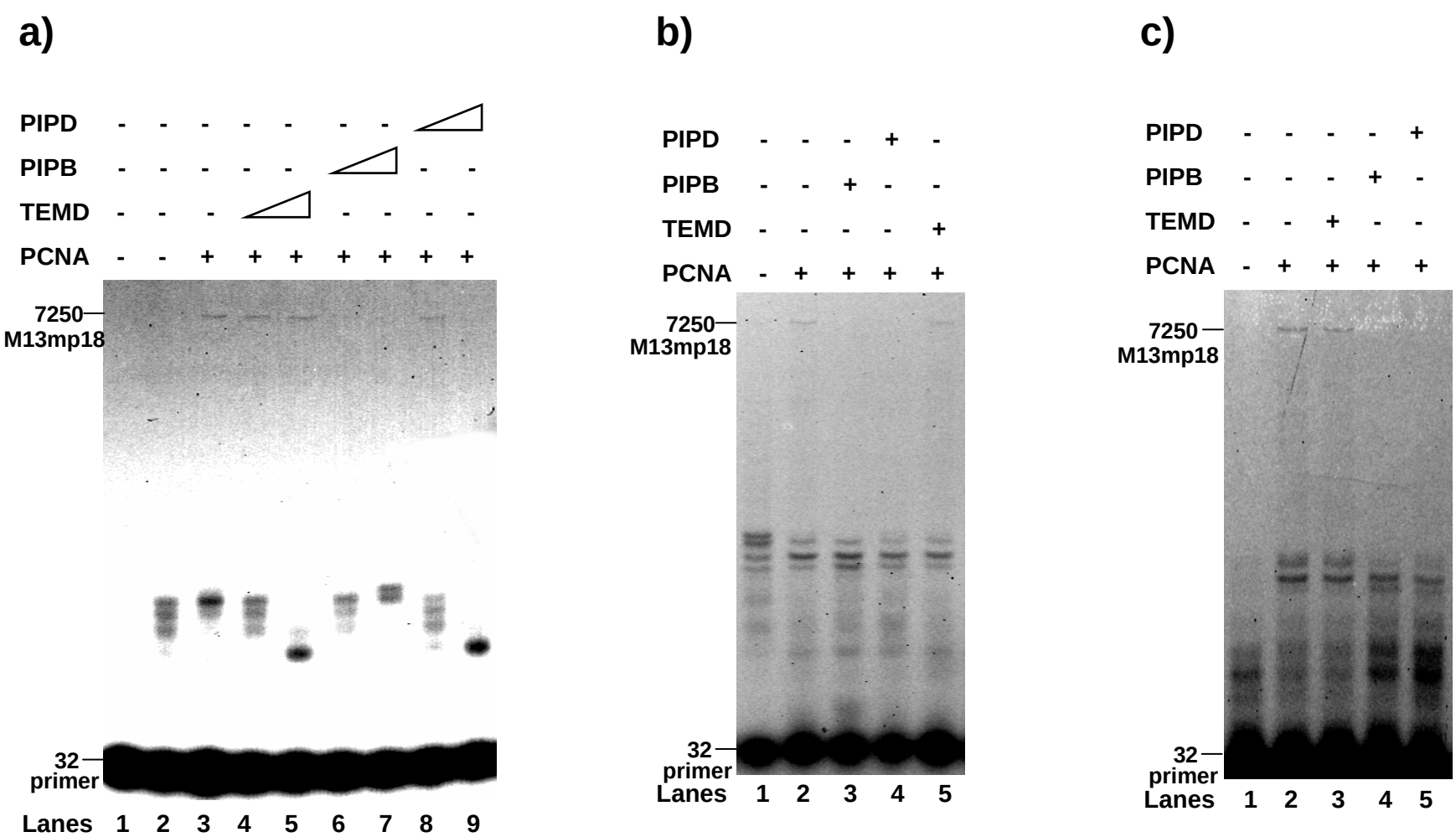


Fig 2



	*Pab*Pol D	*Pab*Pol DΔpip
- PCNA	0.4 ± 0.8	1.1 ± 0.8
+ PCNA	6.7 ± 3.8**	5.0 ± 2.6*

Fig 3



*Pab*Pol B

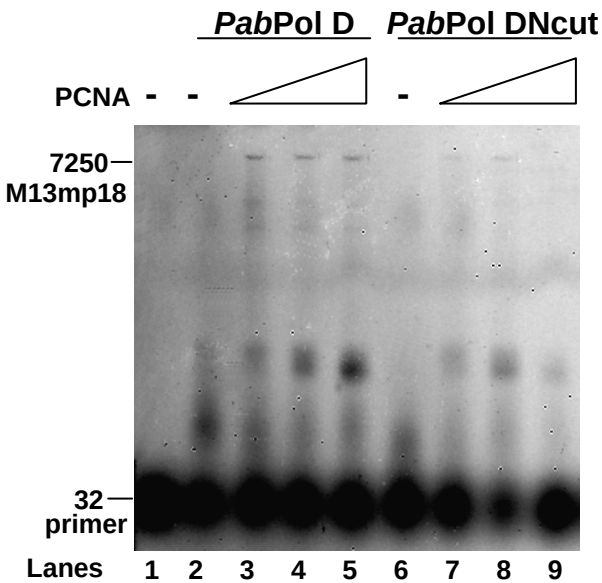
*Pab*Pol D

*Pab*Pol DΔpip

Fig 4

a)

b)



	*Pab*Pol D	*Pab*Pol DNcut
- PCNA	0.1 ± 0.1	0.3 ± 0.6
+ PCNA	5.2 ± 1.4**	0.4 ± 0.4

Fig 5

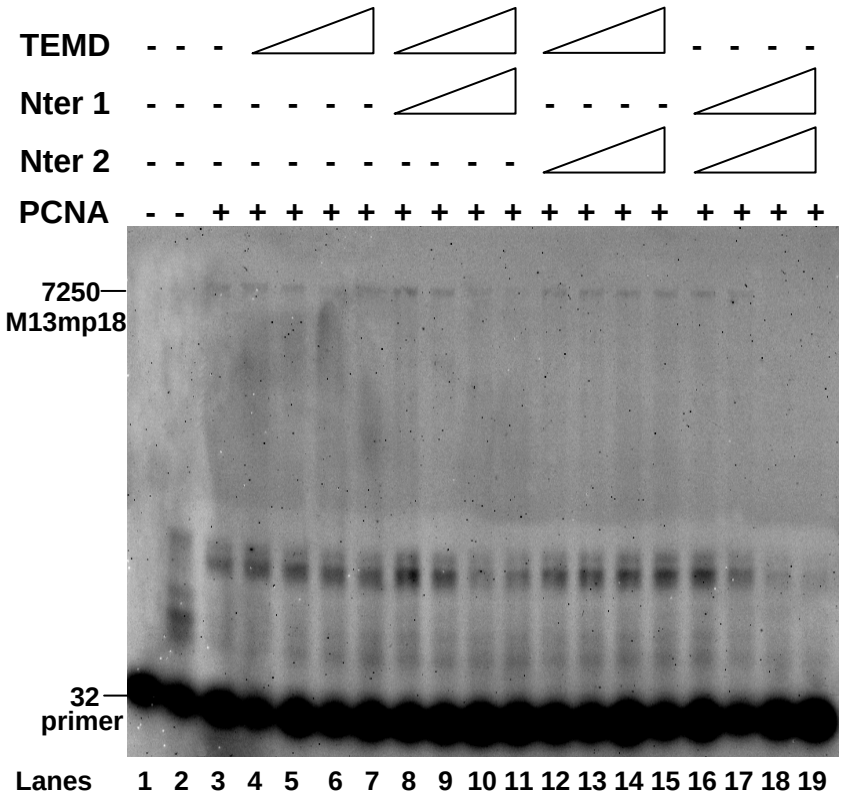

TEMD - - -
Nter 1 - - - - - - - - - - - - -
Nter 2 - - - - - - - - - - - - - - -
PCNA - - + + + + + + + + + + + + + + + +
7250
M13mp18
32
primer
Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Fig 6

Proteins	Mutations on IDCL
PCNA	$_{115}$LIDVEEIEVDLPELPFT$_{131}$
EIE120	$_{115}$LIDVEAAAVDLPELPFT$_{131}$
VDL123	$_{115}$LIDVEEIEAAAPELPFT$_{131}$
EL127	$_{115}$LIDVEEIEVDLPAAPFT$_{131}$

Proteins	Mutations on C-terminus
PCNA	$_{243}$LAPRVEE$_{249}$
PR245	$_{243}$LAAAVEE$_{249}$

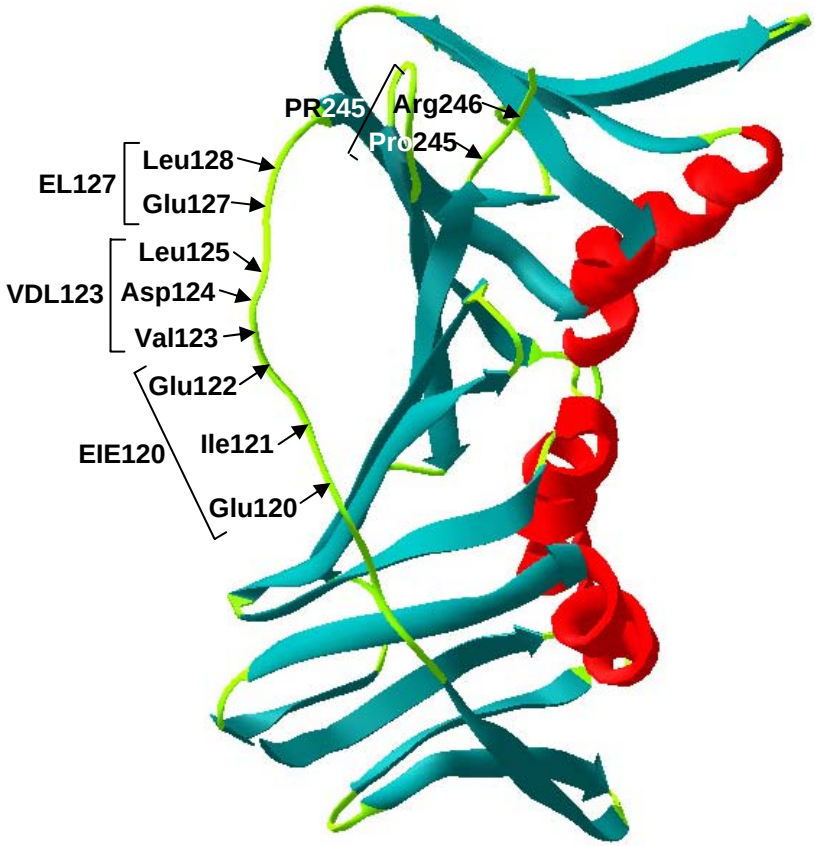
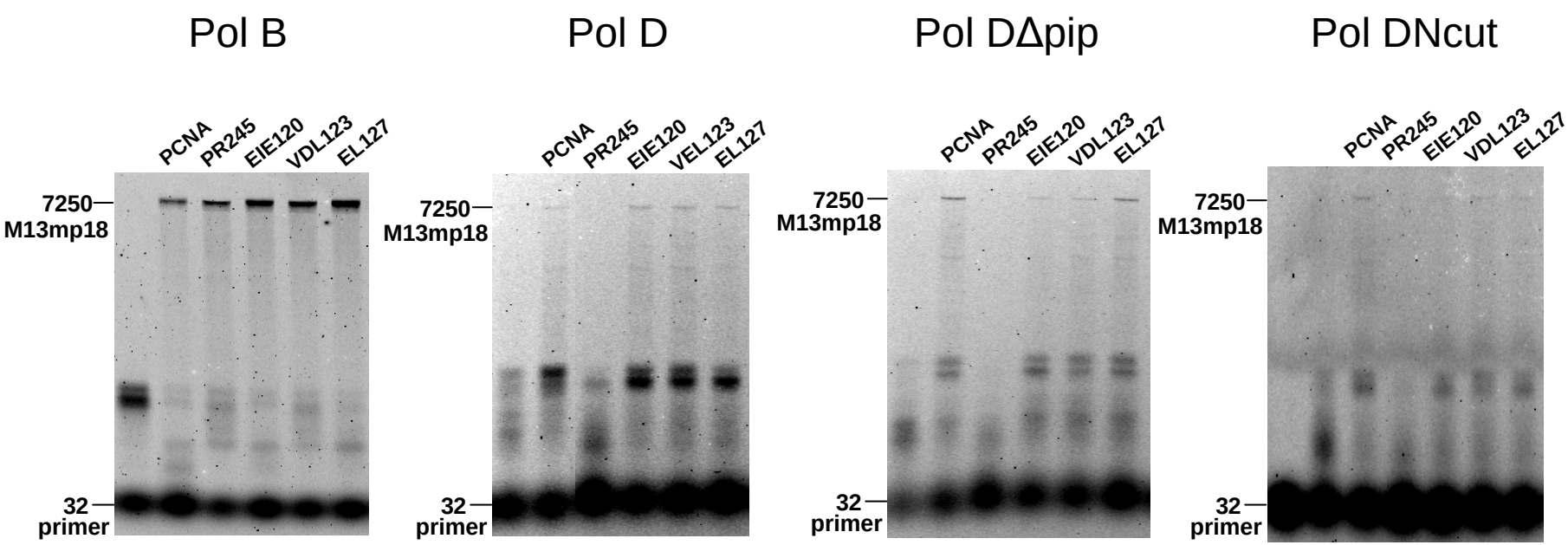


Fig 7


Pol B
Pol D
Pol DΔpip
Pol DNcut
PCNA
PR245
EIE120
VDL123
VEL123
EL127
7250
M13mp18
32
primer