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The PCNA from Thermococcus fumicolans Functionally Interacts with DNA Polymerase δ

Ghislaine Henneke[a], Jean-Paul Raffin[a*], Elena Ferrari[b], Zophonías O. Jónsson[b], Jacques Dietrich[a] and Ulrich Hübscher[b]

[a] Ifremer, Laboratoire de Biotechnologie des Microorganismes Hydrothermaux, DRV/VP, BP 70, 29280, Plouzané, France
b Institut fuer Veterinärbiochemie, Universität Zürich, Winterthurerstrasse 190, 8057, Zürich, Switzerland

* Jean-Paul Raffin is a Researcher at the Centre National de la Recherche Scientifique (CNRS).

Abstract: We have cloned the gene encoding proliferating cell nuclear antigen (PCNA) from the hyperthermophilic euryarchaeote Thermococcus fumicolans (Tfu). Tfu PCNA contains 250 amino acids with a calculated Mr of 28,000 and is 26% identical to human PCNA. Next, Tfu PCNA was overexpressed in Escherichia coli and it showed an apparent molecular mass of 33.5 kDa. The purified Tfu PCNA was tested first with recombinant Tfu DNA polymerase I (Tfu pol) and second with calf thymus DNA polymerase δ (pol δ). When tested with the homologous Tfu pol on bacteriophage λ DNA, large amounts of Tfu PCNA were required to obtain two- to threefold stimulation. Surprisingly, however, Tfu PCNA was much more efficient than human PCNA in stimulating calf thymus pol δ. Our data suggest that PCNA has been functionally conserved not only within eukaryotes but also from hyperthermophilic euryarchaeotes to mammals.

Keywords: PCNA; hyperthermophile; archaea; Thermococcus fumicolans; conservation; DNA polymerase δ

INTRODUCTION

DNA replication is a highly organized process involving a large set of proteins and enzymes to achieve faithful, fast and timely replication. A set of two protein classes confers speed and high processivity to the DNA polymerases (pols) involved. This high processivity is given by a clamp (e.g. the β-subunit in *E. coli*, the gp45 in bacteriophage T4 and PCNA in eukaryotes (reviewed in 1)) which forms a ring-like structure, and by a clamp-loader that can load the clamp in an ATP-dependent process (e.g. the γ-complex in *E. coli*, the gp44/66 complex in bacteriophage T4 and replication factor C, RF-C, in eukaryotes (reviewed in 2)).

PCNA was first characterized as a processivity factor for pol δ. At the same time PCNA has been shown to have roles in other cellular pathways than DNA replication, such as nucleotide excision repair, mismatch repair, base excision repair, cell cycle control, apoptosis and transcription. All these interactions led to the idea that PCNA plays a central role in connecting all these important cellular processes and can act as cellular communicator in cells.

PCNA forms a torus-like structure with a central cavity that accommodates double-stranded DNA (3). The eukaryotic clamp-loader RF-C, which is required to load PCNA onto the DNA duplex, is composed of five subunits with molecular masses of about 140, 40, 38, 37 and 36 kDa (reviewed in 2 and 4).

Recent genomic sequencing of archaea (5-10) has identified three genes encoding putative homologues of RF-C and PCNA. These genes encode proteins with sequences similar to those of the large RF-C subunit from eukaryotes (about 15 % identity), the p40 subunit of RF-C (about 37 % identity) and PCNA (about 26 % identity).

Assuming that the eukaryotic genome is a chimera of genes derived from archaea and Gram-Negative bacteria (11), the archaeal DNA replication system can be considered to be an ancestor of its eukaryotic counterpart. There should be substantial differences in the DNA replication

mechanisms of the two subdomains of the archaeota: crenarchaeota and euryarchaeota. The crenarchaeotes contain two or more homologues of the family B DNA polymerase (12-14), whereas the euryarchaeotes contain only one (15). A different pol (Pol II) has been identified in euryarchaeotes (16, 17). This novel pol has not up to now been detected in the genome of the crenarchaeote *Sulfolobus solfataricus* (F. Pisani, personal communication). If this result is corroborated, it raises an interesting question concerning the mechanisms of DNA replication in archaea.

The PCNA from the euryarchaeote *Pyrococcus furiosus* has been recently characterized (18). Also, two PCNA homologues have been described in the crenarchaeotes *Sulfolobus solfataricus* (19) and *Pyrobaculum aerophilum* (20). The PCNAs from *S. solfataricus* and *P. furiosus* increased the processivity of the respective family B DNA polymerases. However, high amounts of PCNA were required to obtain stimulation.

In this paper we have cloned, overexpressed and characterized PCNA from the hyperthermophilic euryarchaeote *Thermococcus fumicolans*. Surprisingly, we found that the *Tfu* PCNA itself is more efficient in stimulating mammalian pol δ than the homologous, recombinant *Tfu* pol.

MATERIALS AND METHODS

Thermococcus fumicolans

T. fumicolans ST557 (21) is an extremely thermophilic archaea that was isolated from a deep-sea hydrothermal vent in the North Fiji Basin. It is strictly anaerobic and grows at an optimal temperature of 85°C, preferentially in the presence of elemental sulfur.

Chemicals

Deoxyribonucleoside 5'-triphosphates were purchased from Appligene-Oncor, Strasbourg, France. T4 DNA ligase, and modification and restriction enzymes were obtained from Roche-Diagnostics Mannheim, Germany. Activated calf-thymus DNA was purchased from Sigma-Aldrich, St Louis, USA.

Lambda DNA and Oligo-F (5'-AGGTCGCCGCCCCGTAACCTGTCGGATCACCGGAAA-3') were kindly supplied by Appligene-Oncor, Strasbourg, France. [*methyl*,1',2'-^{3}H]Thymidine 5'-triphosphate, ammonium salt (119 Ci/mmole) and [α-^{32}P]dCTP (3000 Ci/mmole) were obtained from Amersham-France, Les Ulis. Polyacrylamide gel electrophoresis reagents were purchased from Bio-Rad S.A., France. Whatman supplied the GF/C filters. Molecular weight markers, Source Q, Superdex-200, poly(dA)$_{1000\text{-}1500}$ and oligo(dT)$_{12\text{-}18}$ were all obtained from Pharmacia-Biotech, Uppsala, Sweden.

All other chemicals were molecular biology grade reagents from Sigma-Aldrich, St Louis, USA.

Bacterial strains

The transformation-competent *E. coli* strains, DH5α and BL21(De3)[pLysS], were obtained from Clontech Laboratories, Basingstoke, UK and Stratagene, Cambridge, UK respectively.

Buffers

The following buffers were used: buffer A (100 mM sodium citrate pH 5.5); buffer B (50 mM Tris-HCl pH 7.5, 1 mM DTT); buffer C (50 mM Tris-HCl pH 7.5, 1mM DTT, 1 M NaCl); buffer D (50 mM Tris-HCl pH 7.5, 1mM DTT, 50 % (v/v) glycerol); buffer E (50 mM Bis-Tris-HCl pH 6.5, 1 mM DTT, 0.25 mg/ml BSA); buffer F (50 mM Tris-HCl pH 8.8, 1 mM DTT, 10 mM KCl, 5 mM $MgCl_2$, 0.4 mg/ml BSA).

Nucleic acid substrates and DNA polymerases

For preparation of the primed lambda DNA, 400 fmol were mixed with the 36-mer oligonucleotide F (1200 fmol) in buffer F in a final volume of 300 µl. The mixture was heated at 90°C for 10 min and then allowed to cool slowly to 30°C over 1 h. Calf thymus Pol δ and poly(dA)/oligo(dT) (base ratio 10:1) were obtained as previously described (22). The recombinant form of the *T. fumicolans* DNA polymerase (*Tfu* pol) was obtained from Appligene-Oncor, Strasbourg, France.

Cloning of the PCNA gene from *Thermococcus fumicolans* (*Tfu*)

The *Pyrococcus abyssi* PCNA gene (sequence obtained as part of a collaborative sequencing project) was amplified by PCR using genomic DNA and was used as a probe for screening a *Tfu* genomic ZAPII library, constructed according to the manufacturer's protocol (Stratagene,

Cambridge, UK). To subclone the *Tfu* PCNA gene, PCR primers were designed such that the amplified DNA contained the recognition sites for the restriction endonucleases *Nde*I and *Bam*HI at its 5'- and 3'-ends respectively. The PCR products obtained with these primers were digested with the *Nde*I and *Bam*HI and ligated into an expression vector under the control of the T7 promoter. The resulting plasmids were used to transform *E. coli* DH5α cells. The plasmid was prepared from the *E. coli* cells and the sequence of the *Tfu* PCNA gene was tested by standard sequencing method.

Overproduction and purification of the *Tfu* PCNA

The *E. coli* strain BL21(De3)[pLysS] was transformed with the *Tfu* PCNA expression construct and cultured at 37°C in 200 ml 2xYT broth containing 100 μg/ml ampicillin. Overproduction of the protein was induced, at an $O.D._{600}$ of 0.6, by adding Isopropyl -D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The cells were cultured for a further 5h, pelleted by centrifugation, resuspended in 8 ml of buffer A and disrupted by sonication (3 x 15 s). The insoluble debris was removed by centrifugation at 15,000 x g for 45 min at 4°C. The cleared lysate was heated for 40 min at 60°C, cooled rapidly on ice, and finally centrifuged for 45 min at 15,000 x g and 4°C. The resulting supernatant (about 7.5 ml) was loaded onto a Source Q column (1.6 x 7.5 cm) previously equilibrated with buffer B. The column was washed with 40 ml of the buffer B at a flow rate of 1 ml/min and proteins were eluted with a 90-ml linear gradient from buffer B to buffer C. The eluted fractions were pooled, concentrated to a final volume of 500 μl and loaded onto a Superdex-200 column (1.6 x 50 cm) previously equilibrated with buffer B (1 ml/min). The *Tfu* PCNA fractions were pooled, concentrated and finally dialyzed against buffer D.

DNA polymerase assays

(i) DNA polymerase assay with primed bacteriophage λ DNA.

The standard reaction mixture contained in final volume of 20 µl: 0.2 mM of each of the four dNTPs, 0.35 µM [^{3}H]-dTTP (119 Ci/mmol) and 15 fmol of primed bacteriophage λ DNA (Oligo-F, a 36-mer, is complementary to nucleotides 48502 to 48466), in buffer E (Pol δ buffer) or buffer F (*Tfu* pol buffer). *Tfu* PCNA was incubated with various amounts (in units) of *Tfu* pol, for 20 to 30 min at 45 to 70°C, as indicated in the legends of the Figures. The reaction was stopped by chilling on ice and 15 µl of the reaction mixture was precipitated with trichloroacetic acid and adsorbed onto a Whatman GF/C filter and counted in a Beckman scintillation counter. For analysis of the reaction products, the assay mixture contained in a final volume of 50 µl: 0.1 nM primed bacteriophage λ DNA, 1 U of *Tfu* pol and 30 µCi/ml of [α-^{32}P]dCTP. The assay mixture was incubated at 70°C and aliquots (20 µl) were taken and counted as acid-insoluble material. Further 20 µl aliquots were subjected to denaturing electrophoresis in a 1% agarose gel and the products were detected by autoradiography. One unit of DNA polymerase is defined as the amount of enzyme catalyzing the incorporation of 1 nmole/min of dNTP into acid-insoluble product.

(ii) DNA polymerase assays with poly(dA)/oligo(dT).

The reaction mixture contained in a final volume of 25 µl the following components: 25 µM [^{3}H]dTTP (1.5 Ci/mmol), 0.5 µg of poly(dA)/oligo(dT)10:1 in buffer E or F, 0.1 U of pol δ or 0.01-1 U of *Tfu* pol and various amounts of PCNA. The reactions were incubated for 30 min at

the appropriate temperature and acid-insoluble radioactivity was precipitated with 10% trichloroacetic acid and scintillation counted as described elsewhere (23).

Protein determination

Protein concentration was determined by the method of Bradford (24) using the Bio-Rad reagent and bovine serum albumin as the standard.

Nucleotide sequence accession number

The PCNA nucleotide sequence has been submitted to the EMBL/GenBank/DDBJ databases (accession number AJ130939).

RESULTS

Amino acid sequence and predicted structure of *Tfu* PCNA

We have screened a *Tfu* ZAPII library and identified a 750 bp open reading frame encoding a 249 amino-acid peptide with a sequence similar to those of eukaryotic and archaeal PCNAs. The protein had a predicted molecular mass of 28 kDa and isoelectric point of 4.3. We retrieved similar primary sequences from the BLASTP search program and aligned them using ClustalW (1.73). Several conserved regions were identified (Fig. 1), particularly in the domain-connecting loop (aa 118-135) and the SHV43 loop at the front of the torus (25). The back-side loop (aa 180-190) was greatly reduced in all archaea. Human and *Tfu* PCNA were found to be 26 % identical. Structure-based alignment showed that each of the strands and helices were preserved. The β-α-β-β-β motif, which forms a pattern involving two-fold symmetry within each domain, was also observed.

Functional overexpression of *Tfu* PCNA in *E. coli*

Tfu PCNA was overproduced in *E. coli* as a soluble and active protein. For purification, the cell lysate was heated at 60°C for 40 min. This heat treatment resulted in the denaturation of most *E. coli* proteins. After this stage, *Tfu* PCNA was purified to near homogeneity (Fig. 2) from the clarified solution by the two chromatographic steps. *Tfu* PCNA migrated as 33.5 kDa in SDS-PAGE, which is higher than that of the 28 kDa derived from the amino acid sequence (Fig.1).

Tfu PCNA can only moderately stimulate the homologous DNA polymerase (_Tfu_ pol) in an assay using primed linear DNA

Tfu pol activity can be detected on bacteriophage λ DNA at temperatures as low as 45°C (data not shown). Processivity was unaffected at lower temperatures, reflecting thermal tolerance over a large range of temperatures. First we investigated the effect of *Tfu* PCNA on *in vitro* DNA synthesis catalyzed by *Tfu* pol, using a singly primed linear template. *Tfu* PCNA stimulated the *Tfu* pol activity only 3-4 times and this only at high *Tfu* PCNA (500 ng) tested (data not shown). A product analysis of this stimulation is shown in Fig. 3a. 400 ng of PCNA gave half maximal stimulation and lower amounts had no effect (data not shown). Our results suggest a distributive mode of DNA synthesis by *Tfu* pol and size of DNA products was not PCNA-dependent (Fig. 3a). From Fig. 3b it is evident that the stimulation was more pronounced at lower *Tfu* pol amounts used (0.01-0.02 Units).

The hyperthermophilic euryarchaeote _Tfu_ PCNA can stimulate mammalian pol δ

Finally we tested the effect of *Tfu* PCNA on calf thymus pol δ (Fig. 4). In the control reaction with human PCNA and calf thymus pol δ the expected stimulation pattern was evident at 37° C and also at 45°C with a maximum of 150 ng PCNA. When *Tfu* PCNA was tested under the same assay conditions and the same amount of calf thymus pol δ a sharp maximum of stimulation was seen between 10 and 30 ng *Tfu* PCNA. Surprisingly the stimulation by the hyperthermophilic euryarchaeote PCNA was much stronger than with human PCNA. Finally we noticed that the stimulation had a very sharp optimum, reaching almost background level at an amount of PCNA (150 ng) where maximum stimulation with human PCNA was evident.

DISCUSSION

Recent analysis of the complete genome sequences of euryarchaeotes has shown that most of the genes involved in DNA replication are more similar to those found in eukaryotes than to those in bacteria (5-10). However, our knowledge of the mechanism of DNA replication in archaea is still limited. In this communication we report the cloning and overproduction of the *Tfu* PCNA as a soluble active protein. As shown by SDS-PAGE, *Tfu* PCNA was purified to near-homogeneity and, as already observed with eukaryotic PCNA (26), it ran more slowly than predicted in SDS-PAGE with an apparent molecular mass of 33.5 kDa. The recombinant *Tfu* PCNA stimulated the recombinant form of *Tfu* DNA polymerase I (B-type) only moderately on a primed linear DNA. Surprisingly *Tfu* PCNA strongly stimulated mammalian pol δ at even lower concentrations than the homologous counterpart.

The sequence of *Tfu* PCNA is 26% identical to the human PCNA sequence and 84% identical to the *Pyrococcus abyssi* PCNA sequence. The overall conservation of PCNA between the two kingdoms is reflected in the tertiary structure of the molecule. In yeast PCNA (3), an outer layer of six β sheets forms a circular collar that supports twelve α-helices at the inner surface of the ring. The PCNA trimer has quasi-six-fold symmetry, with each monomer composed of two domains (27). The domain-connecting loop, the most prominent feature on the surface of eukaryotic PCNA (25), is conserved in *Tfu* PCNA. The back-side loop, which has been implicated in the interaction with pol ε (28, 29) is much smaller in all archaea than in eukaryotes. Dynamic light scattering suggests that *Tfu* PCNA, like eukaryotic PCNA (30), behaves as a trimer in solution (results not shown). Moreover, increasing temperature shifts the equilibrium towards the trimeric form. *Tfu* PCNA should be structurally homologous to its counterpart in

eukaryotes since the sizes and isoelectric points of *Tfu* and eukaryotes PCNA are also similar. It is therefore not surprising that *Tfu* PCNA and human PCNA are similar in terms of shape, size and internal architecture.

The homologous *Tfu* pol was stimulated by a factor of 3-4 only but large amounts of PCNA (500 ng) were required. By using a singly-primed linear replication assay, PCNA switched the *Tfu* pol to a more distributive mode (Fig. 3). It is known that the archaeal pol I, which belongs to the Family B pols, can elongate long fragments of primed DNA *in vitro* under PCR conditions (Fig. 3). In contrast, however, calf thymus DNA pol δ, which is known to synthesize full-length products in the presence of the sliding-clamp PCNA, was found to be efficiently stimulated by *Tfu* PCNA. The sensitivity of pol δ was similar at 37°C and 45°C. Surprisingly the stimulation was higher that with human PCNA and a lower amount (10 ng *Tfu* PCNA versus 150 ng human PCNA) was required for maximal stimulation. Human PCNA has been shown to stimulate the activity of archaeal FEN-1 (31). Therefore, despite the low level of sequence similarity between human and archaeal PCNAs (see 19), their functional three-dimensional structures are probably very similar. Sequence alignment showed that key amino acids in the domain-connecting loop are conserved in *Tfu* PCNA (Fig. 1). The Leu126 and Ile128 residues of human PCNA were found to be part of a hydrophobic cleft in which a hydrophobic side chain of an interacting protein, such as human pol, may be inserted (28). This region contains more hydrophobic residues in *Tfu* PCNA. This may result in a heteroenzyme complex between pol δ and *Tfu* PCNA being more productive.

Surprisingly, the newly identified archaeal dimeric DNA polymerase form (pol II) (16, 32) showed low sensitivity to PCNA. Indeed, the *Pyrococcus furiosus* Pol II required 2 μg of PCNA for a two fold stimulation (18). More experiments are nevertheless required to explain the low

sensitivity of archaeal pol to stimulation by homologous PCNA. As it becomes increasingly clear, the eukaryotic pol δ have three or four subunits and some of them can bind to PCNA (33). The pol δ used in this study was purified from cells, as a PCNA-dependent DNA pol on poly(dA)/oligo(dT) template-primer (22), while the *Tfu* pol was a recombinant form that could be devoid of auxiliary proteins necessary for PCNA stimulation. Also, from the results in Fig. 3 one should conclude that *Tfu* PCNA, despite stimulating calf thymus pol δ *in vitro*, could have a different function *in vivo*.

Whatever, the pol δ stimulation data showed that PCNA has been functionally conserved not only within eukaryotes but also from hyperthermophilic euryarchaeotes to mammals.

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Fig. 1: Amino-acid alignment for seven PCNAs of seven species.

Black boxes indicate highly conserved regions and grey boxes indicate moderately well conserved regions. Abbreviations are: PCNAYEAST, Yeast *S. cerevisiae* PCNA; PCNAHUMA, human PCNA, PCNAMETH, PCNA from *Methanococcus jannaschii*; PCNAHORI, PCNA from *Pyrococcus Horikoshii*; PCNAPFUR, PCNA from *Pyrococcus furiosus*; PCNAPAB, PCNA from *Pyrococcus abyssi*; PCNATFU, PCNA from *Thermococcus fumicolans*.

Fig. 2: *Tfu* PCNA during the various purification steps.

A 12% SDS-PAGE of the products of various *Tfu* PCNA purification steps was performed. Lane 1: Low molecular weight standards (Pharmacia-Biotech); lane 2: crude extract, uninduced; lane 3: crude extract, induced; lane 4: crude extract, induced and heated at 60°C for 45 min; lane 5: Source Q column, pooled active fractions; lane 6: Superdex-200 column, pooled active fractions.

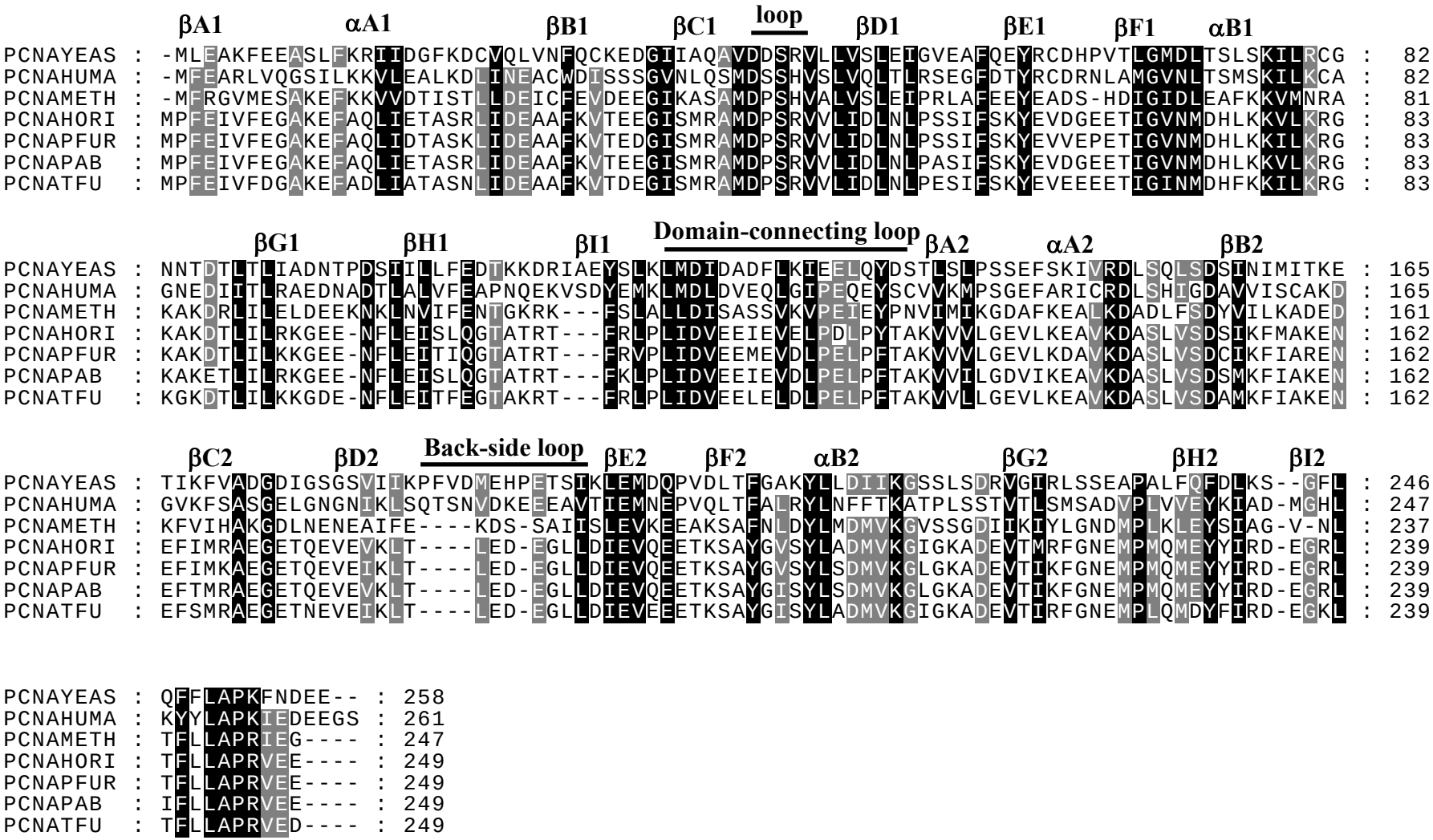
Fig. 3: *Tfu* PCNA can only moderately stimulate the homologous DNA polymerase (*Tfu* pol) in an assay using primed linear DNA.

a: Analysis of elongation products. 0.25 U of *Tfu* pol was incubated in the absence (lanes 1) or presence (lanes 2) of 500 ng of *Tfu* PCNA, at 70°C for 30 min, as described in "Materials and Methods". The reaction products were analyzed by denaturing agarose gel electrophoresis and autoradiography. **b**: Relative activity of *Tfu* pol in the presence of 500 ng *Tfu* PCNA. The relative *Tfu* pol activity in the absence of PCNA is chosen as 1. Reaction conditions were as described in "Materials and Methods" by using buffer F and measuring [^{3}H]dTTP incorporation. Incubation was 70°C for 30 min.

Fig. 4: Titration of pol δ with *Tfu* or human PCNA.

0.1 U of pol δ was incubated for 30 min in buffer E in the presence of [^{3}H]-TTP, $MgCl_2$, oligo(dA)/poly(dT) and various amounts of PCNA as described in "Materials and Methods", and acid-insoluble material was determined. The incubation temperature was either 37°C (solid line) or 45°C (dotted line): *Tfu* PCNA (●) or human PCNA (■).

Footnote: Jean-Paul Raffin is a Researcher at the Centre National de la Recherche Scientifique (CNRS)


βA1 αA1 βB1 βC1 loop βD1 βE1 βF1 αB1
PCNAYEAS : -MLEAKFEEASLFKRIIDGFKDCVQLVNFQCKEDGIIAQAVDDSRVLLVSLEIGVEAFQEYRCDHPVTLGMDLTSLSKILRCG : 82
PCNAHUMA : -MFEARLVQGSILKKVLEALKDLINEACWDISSSGVNLQSMDSSHVSLVQLTLRSEGFDTYRCDRNLAMGVNLTSMSKILKCA : 82
PCNAMETH : -MFRGVMESAKEFKKVVDTISTLLDEICFEVDEEGIKASAMDPSHVALVSLEIPRLAFEEYEADS-HDIGIDLEAFKKVMNRA : 81
PCNAHORI : MPFEIVFEGAKEFAQLIETASRLIDEAAFKVTEEGISMRAMDPSRVVLIDLNLPSSIFSKYEVDGEETIGVNMDHLKKVLKRG : 83
PCNAPFUR : MPFEIVFEGAKEFAQLIDTASKLIDEAAFKVTEDGISMRAMDPSRVVLIDLNLPSSIFSKYEVVEPETIGVNMDHLKKILKRG : 83
PCNAPAB : MPFEIVFEGAKEFAQLIETASRLIDEAAFKVTEEGISMRAMDPSRVVLIDLNLPASIFSKYEVDGEETIGVNMDHLKKVLKRG : 83
PCNATFU : MPFEIVFDGAKEFADLIATASNLIDEAAFKVTDEGISMRAMDPSRVVLIDLNLPESIFSKYEVEEEETIGINMDHFKKILKRG : 83
βG1 βH1 βI1 Domain-connecting loop βA2 αA2 βB2
PCNAYEAS : NNTDTLTLIADNTPDSIILLFEDTKKDRIAEYSLKLMDIDADFLKIEELQYDSTLSLPSSEFSKIVRDLSQLSDSINIMITKE : 165
PCNAHUMA : GNEDIITLRAEDNADTLALVFEAPNQEKVSDYEMKLMDLDVEQLGIPEQEYSCVVKMPSGEFARICRDLSHIGDAVVISCAKD : 165
PCNAMETH : KAKDRLILELDEEKNKLNVIFENTGKRK---FSLALLDISASSVKVPEIEYPNVIMIKGDAFKEALKDADLFSDYVILKADED : 161
PCNAHORI : KAKDTLILRKGEE-NFLEISLQGTATRT---FRLPLIDVEEIEVELPDLPYTAKVVVLGEVLKEAVKDASLVSDSIKFMAKEN : 162
PCNAPFUR : KAKDTLILKKGEE-NFLEITIQGTATRT---FRVPLIDVEEMEVDLPELPFTAKVVVLGEVLKDAVKDASLVSDCIKFIAREN : 162
PCNAPAB : KAKETLILRKGEE-NFLEISLQGTATRT---FKLPLIDVEEIEVDLPELPFTAKVVILGDVIKEAVKDASLVSDSMKFIAKEN : 162
PCNATFU : KGKDTLILKKGDE-NFLEITFEGTAKRT---FRLPLIDVEELELDLPELPFTAKVVLLGEVLKEAVKDASLVSDAMKFIAKEN : 162
βC2 βD2 Back-side loop βE2 βF2 αB2 βG2 βH2 βI2
PCNAYEAS : TIKFVADGDIGSGSVIIKPFVDMEHPETSIKLEMDQPVDLTFGAKYLLDIIKGSSLSDRVGIRLSSEAPALFQFDLKS--GFL : 246
PCNAHUMA : GVKFSASGELGNGNIKLSQTSNVDKEEEAVTIEMNEPVQLTFALRYLNFFTKATPLSSTVTLSMSADVPLVVEYKIAD-MGHL : 247
PCNAMETH : KFVIHAKGDLNENEAIFE----KDS-SAIISLEVKEEAKSAFNLDYLMDMVKGVSSGDIIKIYLGNDMPLKLEYSIAG-V-NL : 237
PCNAHORI : EFIMRAEGETQEVEVKLT----LED-EGLLDIEVQEETKSAYGVSYLADMVKGIGKADEVTMRFGNEMPMQMEYYIRD-EGRL : 239
PCNAPFUR : EFIMKAEGETQEVEIKLT----LED-EGLLDIEVQEETKSAYGVSYLSDMVKGLGKADEVTIKFGNEMPMQMEYYIRD-EGRL : 239
PCNAPAB : EFTMRAEGETQEVEVKLT----LED-EGLLDIEVQEETKSAYGISYLSDMVKGLGKADEVTIKFGNEMPMQMEYYIRD-EGRL : 239
PCNATFU : EFSMRAEGETNEVEIKLT----LED-EGLLDIEVEEETKSAYGISYLADMVKGIGKADEVTIRFGNEMPLQMDYFIRD-EGKL : 239
PCNAYEAS : QFFLAPKFNDEE-- : 258
PCNAHUMA : KYYLAPKIEDEEGS : 261
PCNAMETH : TFLLAPRIEG---- : 247
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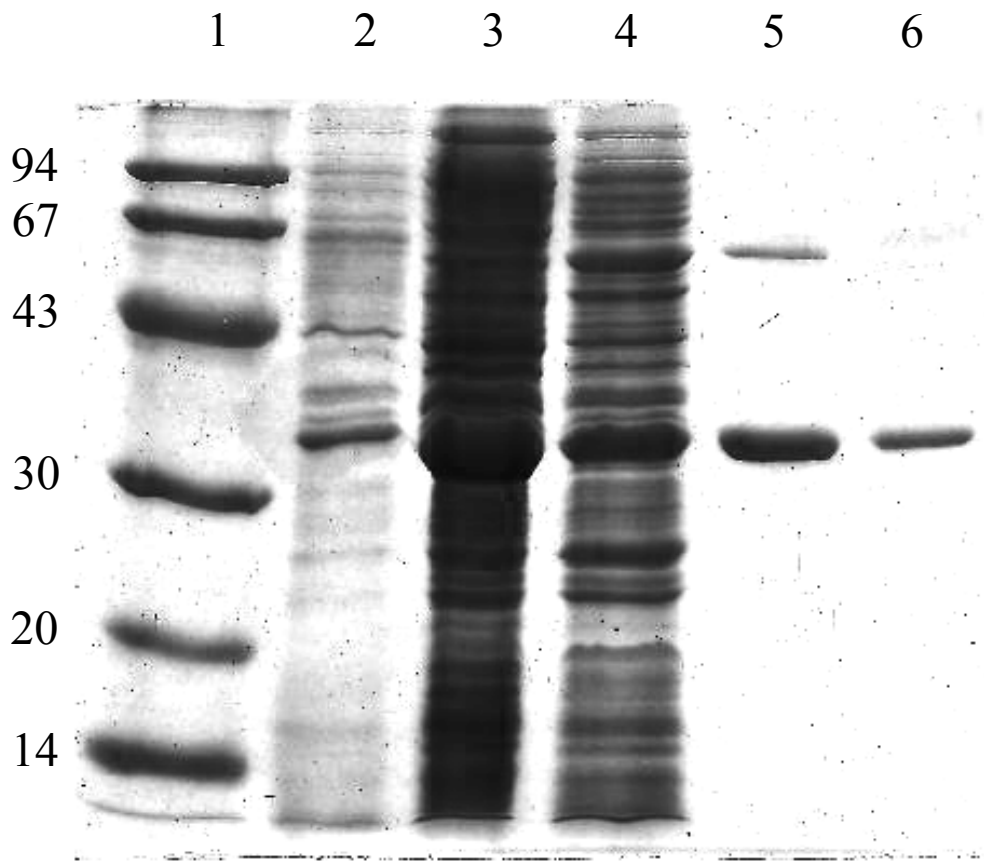
PCNATFU : TFLLAPRVED---- : 249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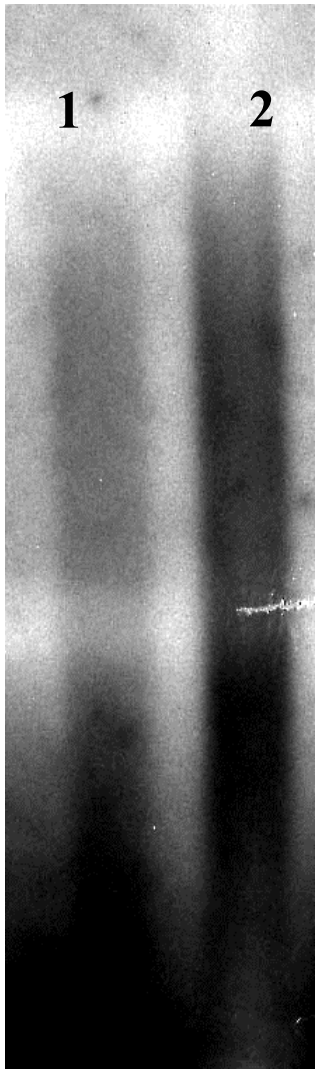
a



← λ

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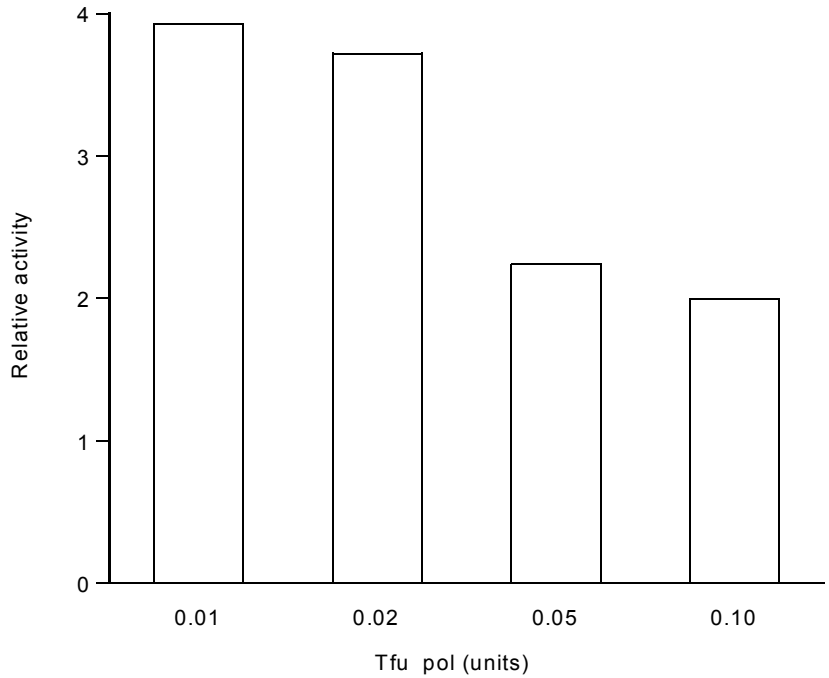
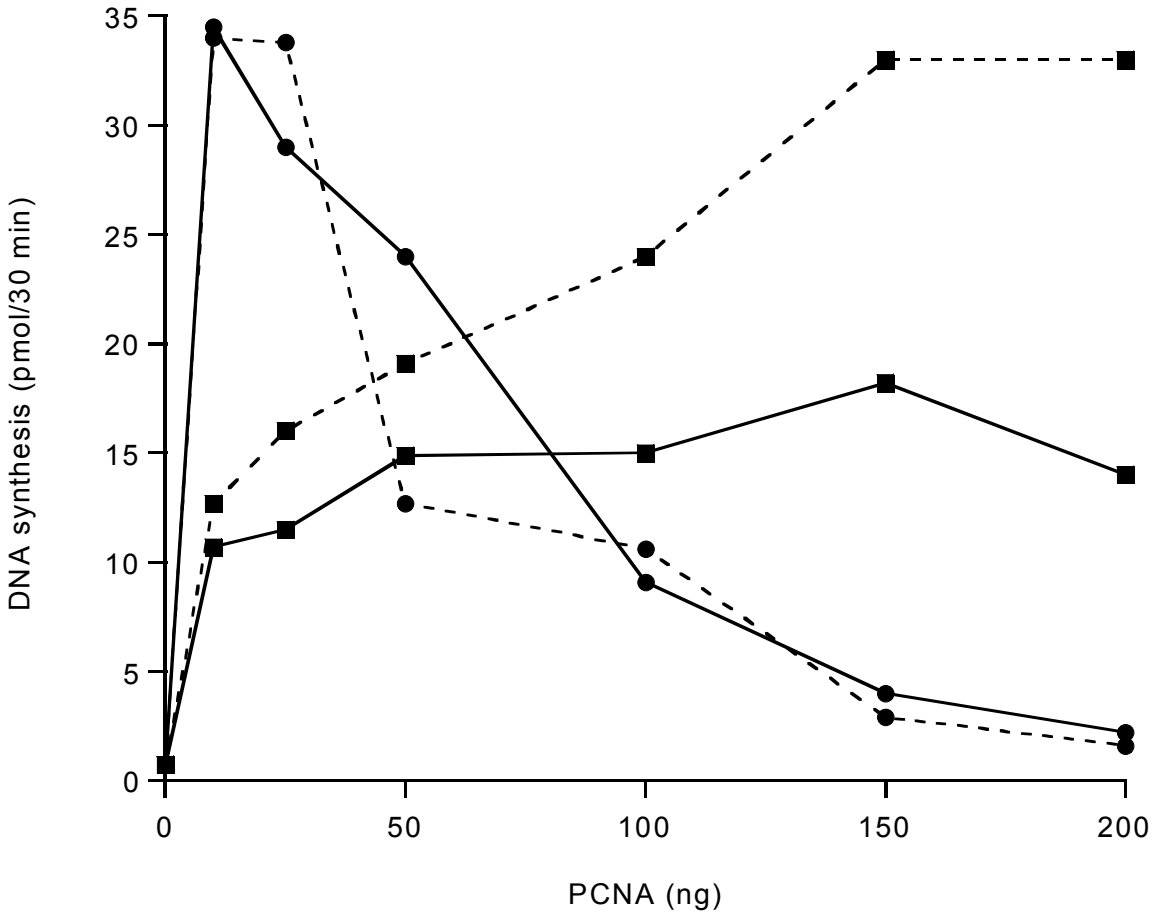
b




DNA synthesis (pmol/30 min)
35
30
25
20
15
10
5
0
0
50
100
150
200
PCNA (ng)