Mutant DNA polymerase δ from thermosensitive *Schizosaccharomyces pombe* strains display reduced stimulation by proliferating cell nuclear antigen

Mylène PERDERISET*, Giovanni MAGA†, Karine PIARD*, Stefania FRANCESCONI*, Isabelle TRATNER*, Ulrich HÜBSCHER† and Giuseppe BALDACCI*¹

*CNRS-IFC1, Institut de Recherche sur le Cancer, UPR 9044, 7 Rue Guy Moquet BP8, 94801 Villejuif, France, and †Institut für Veterinärbiochemie, Universität Zürich-Irchel, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

We have isolated and characterized DNA polymerase δ (pol δ) from two thermosensitive *Schizosaccharomyces pombe* strains, pol δ ts1 and pol δ ts3, mutated in two different evolutionarily conserved domains of the catalytic subunit. At the restrictive temperature of 37 °C pol δ ts1 and pol δ ts3 mutant strains arrest growth in the S phase of the cell cycle. We show that at low levels of primer ends, *in vitro* stimulation by proliferating cell nuclear antigen (PCNA) of mutant enzymes is lower than stimulation of wild-type pol δ . Affinity for primer (3'-OH) ends and processivity of mutant enzymes do not appear different from wild-type pol δ .

In contrast, $V_{\max}$ values are lower than the wild-type value. The major *in vitro* defect appears to be decreased stimulation of mutant enzymes by PCNA, resulting in reduced velocity of DNA synthesis. In addition, ts1 pol δ is not stimulated by low PCNA concentration at 37 °C, although low concentrations stimulate activity at 25 °C, suggesting that this thermolability at low levels of primer ends could be its critical defect *in vivo*. Thus, both ts1 and ts3 pol δ mutations are located in regions of the catalytic subunit that seem necessary, directly or indirectly, for its efficient interaction with PCNA.

INTRODUCTION

DNA polymerase (pol) δ is a eukaryotic enzyme that is essential for *in vitro* replication of DNA containing the origin of replication of the simian virus 40 (SV40) [1]. Pol δ holoenzyme from *Schizosaccharomyces pombe* is believed to consist of five subunits (125, 55, 54, 42 and 22 kDa). The catalytic subunit (p125) is encoded by the *pol3*⁺ gene [2], p55 and p54 are encoded by *cdc1*⁺ and *cdc27*⁺ genes respectively [3], and p42 and p22 (encoded by the *cdm1*⁺ gene) have been isolated recently and their function is still unclear [4]. Genetic and biochemical studies have shown that p125 interacts with the product of the *cdc1*⁺ gene, and that the products of *cdc27*⁺ and *cdc1*⁺ genes interact together, suggesting that p125, p55 and p54 form a complex [3]. Recombinant p125 expressed in insect cells or in *Escherichia coli* is not activated by proliferating cell nuclear antigen (PCNA) [5,6]. Indeed, the p48 subunit of human pol δ (homologous to *S. pombe* p55) is essential for stimulation of polymerase activity by PCNA, indicating that functional p125–PCNA interaction requires additional proteins [7–9].

In budding and fission yeast the genes encoding the catalytic subunit of pol δ are essential for cell growth [10]. In addition, mutations affecting the 3' → 5' exonuclease activity of *Saccharomyces cerevisiae* pol δ result in an increased mutation rate *in vivo* [11]. It has also been proposed that the N-terminal region of *S. cerevisiae* p125 is necessary for a productive association with PCNA [12,13]. Accordingly, a mutant in the NT1 domain of human p125 did not show stimulation by PCNA [13]. Mutations in the gene encoding p125 that induce thermosensitive growth have been isolated in both budding and fission yeast

[10,14]. Pol δ holoenzyme from one of the *S. cerevisiae* mutant strains showed thermolabile activity *in vitro*, but the precise location of the mutation in p125 is unknown [15]. At restrictive temperatures *S. pombe* pol δ mutant strains arrest their growth in the S phase of the cell cycle with a DNA content comprised of between 1C (where 1C is the DNA content of haploid nuclei) and 2C [14,16]. Two of the strains, pol δ ts1 and pol δ ts3, are mutated in two different evolutionarily conserved domains of p125 [14,16]. The pol δ ts1 allele has two adjacent point mutations in the NT1 domain: Ala¹⁴³ → Val and Pro¹⁴⁴ → Ser. The pol δ ts3 allele contains a single amino acid change in the ZnF2 domain (a putative Zn finger) [17] at the C-terminus of p125: Arg¹⁰⁶⁴ → Gln. We characterized mutant p125 from these strains in order to obtain insights into the function of domains NT1 and ZnF2. In this paper, we report the partial purification and comparative analysis of pol δ from wild-type and from mutant *S. pombe* strains pol δ ts1 and pol δ ts3. We show that at low levels of primer ends, stimulation of both mutant enzymes by PCNA is lower than for wild-type pol δ , and that ts1 pol δ displays a temperature-dependent reduction of stimulation by PCNA.

EXPERIMENTAL

Materials

Terminal deoxynucleotidyltransferase, *E. coli* pol I Klenow fragment, nucleotides and ribonucleotides were obtained from Boehringer Mannheim. Phosphocellulose (P11), glassmicrofibre filters (GF/C) and DEAE-81 cellulose filters were purchased from

Abbreviations used: pol, DNA polymerase; DTT, dithiothreitol; TCA, trichloroacetic acid; SV40, simian virus 40; RF-C, replication factor C; PCNA, proliferating cell nuclear antigen.

¹ To whom correspondence should be addressed (e-mail baldacci@infobiogen.fr).

Whatman BioSystems Inc. The TSK DEAE 5 PW HPLC column was from Beckman Instruments. [^3H]dATP ($17.5 \text{ Ci mmol}^{-1}$) was from Du Pont-New England Nuclear, [^3H]dTTP (58 Ci mmol^{-1}) was from ICN, [^3H]dCTP (25 Ci mmol^{-1}), [^{32}P]dATP ($3000 \text{ Ci mmol}^{-1}$) and ECL (enhanced chemiluminescence) Western blotting detection reagents were from Amersham Life Sciences, poly(dA)400 and oligo(dT)12–18 from Pharmacia Biotech, Nitrocellulose (Biotrace NT) was from Gelman Sciences S. A., and opti-fluor O scintillation cocktail was from the Packard Instrument Company.

Nucleic acid substrates

Poly(dA) was mixed with oligo(dT) at a molar ratio of 1:3, as described previously [18]. Single-stranded M13(mp11) DNA was prepared as described previously [19]. For the exonuclease assay, two substrates were used: as a first substrate the plasmid pUC19 DNA was digested with *EcoRI* and the 3'-end was labelled with [^3H]dATP (1500 c.p.m./pmol) using the Klenow fragment of *E. coli* pol I. The reaction was arrested by heating for 5 min at 65°C , slowly cooled to room temperature and loaded onto a Sephadex G-50 column pre-equilibrated with 10 mM Tris/HCl, pH 8/1 mM EDTA to eliminate unincorporated [^3H]dATP. As a second substrate, we used the partially mismatched template/primer poly(dA)/oligo(dT)-[^3H]dC-3'-end-labelled, prepared as described [6].

Yeast cultures and extract preparations

The *S. pombe* mutant strains pol δ ts1 and pol δ ts3 and the isogenic wild-type strain SP808 have been described previously [14]. Procedures used for the extraction and purification of pol activities from fission yeast cells were similar to those described previously for budding yeast [20]. Briefly, *S. pombe* cells were grown aerobically at 30°C (wild type) or 25°C (thermosensitive mutants) in 8 liters of 1% (w/v) yeast extract enriched with 3% (w/v) glucose and 12.5 g/l adenine. At a concentration of 5×10^7 /ml, cells were harvested by centrifugation at 2500 g at 4°C for 10 min and frozen in liquid nitrogen. Frozen cells were broken with glass beads (0.5 mm diameter; Sigma) in a Waring Blender. Lysing buffer [buffer A: 0.2 M Tris/HCl, pH 8.1/10% (v/v) glycerol/2 mM EDTA, 0.05% (v/v) brij 50/5 mM DTT (dithiothreitol)/10 mM $(\text{NH}_4)_2\text{SO}_4$ /1 mM PMSF/1 $\mu\text{g/ml}$ pepstatin A/1 $\mu\text{g/ml}$ leupeptin/5 kallikrein units/ml aprotinin/2 mM benzamidine] was added, and the mixture was centrifuged at 2500 g for 10 min at 4°C . Saturated ammonium sulphate solution (47 μl) was added to each 1 ml of supernatant, followed by 35 μl of 10% (v/v) polymin P. After stirring for 30 min at 0°C , the lysate was centrifuged at 40000 g for 30 min at 4°C . Solid ammonium sulphate was added to the supernatant at a final concentration of 0.30 g/ml and the suspension was stirred for 30 min at 0°C . The precipitate was collected by centrifugation at 40000 g for 30 min at 4°C and dissolved in buffer B [50 mM KH_2PO_4 , pH 7.0/10% (v/v) glycerol/2 mM EDTA/1 mM DTT/2 mM benzamidine/1 $\mu\text{g/ml}$ pepstatin A/1 $\mu\text{g/ml}$ leupeptin/5 kallikrein units/ml aprotinin] and dialysed against buffer B containing 25 mM KH_2PO_4 .

Phosphocellulose and analytical DEAE-HPLC columns

The dialysate was batch-absorbed to phosphocellulose pre-equilibrated with buffer B. After stirring for 2 h at 4°C , the phosphocellulose was packed into a column ($4 \times 2.5 \text{ cm}$) and washed with 5 column vols. of buffer B. Proteins were eluted in buffer B containing 500 mM KCl. Active fractions were analysed by Western blot and fractions containing pol δ were loaded onto

a ($7.5 \times 75 \text{ mm}$) TSK DEAE 5 PW HPLC column equilibrated with buffer B containing 25 mM KH_2PO_4 , pH 7.0/10% (v/v) glycerol/2 mM EDTA/1 mM DTT/2 mM benzamidine/1 $\mu\text{g/ml}$ pepstatin A/1 $\mu\text{g/ml}$ leupeptin/5 kallikrein units/ml aprotinin. Proteins were eluted with three linear KCl gradients (28 ml of 0–200 mM KCl, 12 ml of 200–350 mM KCl and 8 ml of 350–500 mM KCl). For quantification analyses, a preparative TSK DEAE 5PW column ($21.5 \times 150 \text{ mm}$) was used as the first chromatographic step, as described [20], and eluted pol δ was loaded on a phosphocellulose column as described above.

pol assays

Replication factor C (RF-C)-independent pol assay

pol activity was assayed with poly(dA)/oligo(dT) as template/primer at different molar ratios. A final volume (50 μl) in buffer C (50 mM Bistris, pH 6.5/1 mM DTT/250 $\mu\text{g/ml}$ BSA/6 mM MgCl_2) contained the following components: 10 μM [^3H]dTTP (1.5 Ci/mmole), 200 ng of *S. pombe* recombinant PCNA, 0.5 μg poly(dA)/oligo(dT) and 10 μl of chromatographic fraction. DNA synthesis was carried out at 37°C for 60 min and the reaction was stopped by the addition of cold 10% (w/v) trichloroacetic acid (TCA). Insoluble radioactive material was collected, washed and counted as described [21].

RF-C-dependent pol assay.

The final reaction volume of 25 μl contained: 50 mM Tris/HCl (pH 7.5), 1 mM DTT, 0.2 mg/ml BSA, 10 mM MgCl_2 , 200 ng of *S. pombe* recombinant PCNA, 450 ng of *E. coli* single-stranded DNA-binding protein (SSB), 1 mM ATP, 100 ng of singly primed M13 DNA, [^3H]dTTP (5 Ci/mmole), 6 μM dCTP, 6 μM dATP, 6 μM dGTP, 0.02 units of pol δ and 0.03–0.05 units of calf thymus RF-C. Reactions were incubated at 37°C , stopped by the addition of 1 ml of ice-cold 10% TCA and acid precipitable radioactivity was determined. One unit of RF-C-dependent activity corresponds to the incorporation of 1 nmol of dNMPs into acid-precipitable material during 1 h at 37°C .

To measure processivity, the sizes of products synthesized with an excess of poly(dA)/oligo(dT) template/primer were determined. The reaction mixture (70 μl) was as described above, except that [^3H]dTTP was replaced by [$\alpha^{32}\text{P}$]dTTP (52000 c.p.m./pmol). After incubation at 37°C for 20 min, the reaction was stopped by the addition of SDS to 2%. Aliquots of 10 μl were processed as above to determine total incorporation and the rest of the sample was precipitated with ethanol, dried and dissolved in 10 μl of sample buffer (60% deionized formamide in Tris/borate/EDTA containing 0.01% xylene cyanol). After heating at 95°C for 5 min, products were separated on 6% PAGE in Tris/borate/EDTA with 7 M urea.

Exonuclease assays

For the exonuclease assays, the final volume (50 μl) in buffer C contained: 3 $\mu\text{g/ml}$ [^3H]pUC19 DNA (10000 c.p.m.) and 10 μl of chromatographic fraction. After 30 min at 37°C , the reaction was arrested with 100 μl of stop solution (25 mM sodium pyrophosphate/20 mM EDTA/30 μg of salmon sperm DNA as carrier) and 0.5 ml of 10% TCA. Residual DNA-bound radioactivity was adsorbed on glass fibre and determined as described above. The second deoxyribonuclease test was performed in a final volume of 50 μl in buffer C containing: 2 $\mu\text{g/ml}$ poly(dA)/oligo(dT)12–18-[^3H]dC (10000 c.p.m.) and 10 μl of each chromatographic fraction. Incubation was carried out at 37°C for 30 min. Samples were then adsorbed onto 2 cm^2 DEAE-81 paper

disks, washed in 0.3 M ammonium formate, pH 7.8, washed in H_2O , dehydrated twice in 95% ethanol and counted in a scintillation counter. One unit of exonuclease activity is defined as the amount required to remove 1 pmol [3H]dAMP or [3H]dCMP during 30 min at 37 °C.

Primase assays

The primase assay was as previously described [22]. Reaction volumes (50 μ l) contained: 20 mM Tris/HCl (pH 7.5), 15 mM $MgCl_2$, 10 mM DTT, 200 μ g/ml BSA, 50 μ M dATP, 50 μ M dCTP, 50 μ M dGTP, 20 μ M [3H]dTTP (1.5 Ci/mmol), 400 ng of single-stranded phage M13 DNA as template, 1 mM CTP, 1 mM GTP, 1 mM UTP, 2 mM ATP, 0.5 unit of DNA polymerase I Klenow fragment and 10 μ l of each chromatographic fraction. Samples were incubated for 1 h at 37 °C and incorporated [3H]dNMP was counted on a glass filter as described [21].

Steady-state kinetic measurements

For steady-state kinetic measurements, the final volume (25 μ l) in buffer C contained 20 μ M [3H]dTTP (1.5 Ci/mmol), 0.02 units of pol δ , 200 ng of PCNA and different concentrations of poly(dA)/oligo(dT) measured as 3'-OH ends (2, 4, 8, 20, 40, 200, 400 nM). PCNA titration was performed in a final volume of 50 μ l containing 10 μ M [3H]dTTP, 0.15 units pol δ , 0.5 μ g poly(dA)/oligo(dT) and different concentrations (2–200 nM) of PCNA in buffer C. Reactions mixtures were incubated at 37 °C for 45 min, precipitated with TCA and the insoluble radioactive material was determined as described [21]. K_m and V_{max} values were calculated according to the Michaelis–Menten equation.

Antibodies production

Rabbit polyclonal anti-*S. pombe* pol δ antibodies were obtained according to the following procedure: Sf9 insect cells were infected with a recombinant baculovirus containing the *S. pombe* pol3⁺ gene encoding p125 devoid of its intron [23]. Cells were harvested, sonicated and heated at 100 °C for 5 min. Proteins were separated by preparative SDS/PAGE. *S. pombe* pol δ was identified according to its predicted 125 kDa size, excised from the gels, destained and electroeluted. Antisera were obtained from two rabbits. Anti-pol α antibodies were prepared as previously described [24]. Anti-*S. cerevisiae* pol ϵ antibodies were a gift from Dr. Akio Sugino (Research Institute for Microbial Diseases, Osaka University, Japan).

Protein assays, SDS/PAGE and immunoblotting analyses

Protein concentration was measured by the Bradford procedure [25] using reagents and protocol supplied by Bio-Rad. SDS/PAGE was performed according to the method of Laemmli [26] using a 3% stacking gel and a 10% or 7.5% running gel. Proteins were transferred to nitrocellulose according to previously described procedures [24].

RESULTS

Purification of *S. pombe* wild-type and mutant pols

From 40 g of wild-type *S. pombe* cells, 1 g of crude extract was obtained containing about 860 units of total pol activity (Table 1). After DEAE-HPLC the activity of wild-type and mutant enzymes increased about 25–40-fold in the presence of PCNA. Wild-type and mutant DNA pol δ showed similar specific activities in the presence of PCNA (Table 1). Wild-type DEAE-

HPLC fractions were tested for activity on poly(dA)/oligo(dT) (1:3) template/primer, in the presence or absence of 200 ng of PCNA (Figure 1A) and analysed with polyclonal antibodies raised against pol α , pol ϵ or pol δ (Figures 1B, 1C and 1D). In the absence of PCNA, three activity peaks were found: fractions 33–38, 41–45 and 45–47. Immunoblot analyses with anti-pol α antibodies revealed a minor band of about 140 kDa in fractions 33–38 and two bands of about 160 and 180 kDa eluted in fractions 41–45 (Figure 1B). In fractions 45–47 anti-*S. cerevisiae* pol ϵ antibodies cross-reacted with two bands of about 180 and 200 kDa (Figure 1C). When PCNA was added, an activity peak appeared in fractions 34–39 where anti-pol δ antibodies revealed a major band of 125 kDa (Figure 1D). To analyse the different pol activities further, we performed primase and exonuclease assays (Figure 1E). A primase activity peak was found in fractions 38–45 containing the pol α 180 kDa major band. Exonuclease activity was tested on 3'-end-labelled pUC19 DNA. A first peak of exonuclease activity was found in fractions 16–30 that are devoid of detectable pol activity (Figure 1A). A second smaller peak from fractions 32–36 contained pol δ (Figure 1D). No exonuclease activity was detectable in fractions 38–48, where pol α was present (Figure 1B). Finally, an exonuclease activity peak is present in late eluting fractions that are devoid of pol activity (Figure 1A). The different *S. pombe* DNA polymerase activities eluted at KCl concentrations similar to those reported for the corresponding enzymes from *S. cerevisiae*: pol δ at about 150 mM; pol α at about 170 mM; and pol ϵ at about 180 mM [20].

DNA polymerase activity of the pol δ ts1 strain eluting in DEAE-HPLC fractions 36–40 was stimulated by PCNA (Figure 2A), where a 125 kDa band was revealed by anti-pol δ antibodies (Figure 2C). As in the case of wild-type profile, the ts1 pol δ activity was followed by pol α (Figure 2B). Since fraction 54 cross-reacted with anti-*S. cerevisiae* pol ϵ antibodies (results not shown), this activity peak seems to be related to pol ϵ . In Figure 2(D) the exonuclease activity peak specific for a 3' $\rightarrow$ 5' proof-reading activity co-purified with the PCNA-dependent pol δ activity.

DNA polymerase activity of the pol δ ts3 strain eluting in DEAE-HPLC fractions 40–48 was stimulated by PCNA (Figure 3A). Immunoblot analysis of these fractions with anti-pol δ antibodies revealed a 125 kDa band (Figure 3C). As in the case of the wild-type profile, the ts3 pol δ activity was followed by pol α in fractions 46–54, the major 180 kDa band eluting in fractions 48–52 (Figure 3B). The activity peak eluting in fractions 60–68 seems to be related to pol ϵ , as revealed by anti-*S. cerevisiae* pol ϵ antibodies (results not shown). In Figure 3(D) the exonuclease activity peak specific for a 3' $\rightarrow$ 5' proof-reading activity co-purified with the PCNA-dependent pol δ activity.

Taken together, these results show that ts1 pol δ and ts3 are stimulated by PCNA on poly(dA)/oligo(dT) (1:3) as template/primer and are associated with 3' $\rightarrow$ 5' exonuclease activity *in vitro* at 37 °C, a temperature that is restrictive for growth *in vivo*.

Template utilization of *S. pombe* pol δ

Two different templates [circular M13 DNA or poly(dA)/oligo(dT)] were tested with wild-type and mutant enzymes. Pol δ is able to elongate a 40-mer hybridized to single-stranded circular M13 DNA in the presence of RF-C [27]. The reaction requires PCNA and ATP, which act as substrates for RF-C, as well as a single-stranded DNA-binding protein which covers the DNA to prevent unspecific RF-C binding. Under these conditions, wild-type *S. pombe* pol δ was able to synthesize DNA at 37 °C in the presence of *S. pombe* PCNA and RF-C from calf thymus (Figure

Table 1 Purification of pol from wild-type and mutant *S. pombe* strains

One unit of enzyme catalyses the incorporation of 1 nmol dTTP during 60 min at 37 °C using poly(dA)/oligo(dT) (1:3) as template in the presence or absence of *S. pombe* PCNA (150 ng). nd, not determined.

Strain	Fraction	Protein (mg)	DNA polymerase activity			
			(total units)		(units/mg)	
			— PCNA	+ PCNA	— PCNA	+ PCNA
Wild-type pol δ	Crude lysate	1000	860	nd	0.86	nd
	DEAE-HPLC	10	27	330	2.7	33
	Phosphocellulose	0.5	1.2	50	2.4	100
ts1 pol δ	Crude lysate	700	500	nd	0.71	nd
	DEAE-HPLC	8	17	180	2.1	22
	Phosphocellulose	0.3	0.8	20	2.6	66
ts3 pol δ	Crude lysate	650	600	nd	0.92	nd
	DEAE-HPLC	12	12	210	1	17.5
	Phosphocellulose	0.4	0.7	30	1.75	75

Figure 1 Analytical DEAE-HPLC and immunoblot profiles of pol, primase and exonuclease activities of *S. pombe* wild-type pol δ

(A) Aliquots (10 μ l) were assayed for pol activity at 37 °C on poly(dA)/oligo(dT) (1:3) in the presence (●) or absence (○) of 200 ng of PCNA. (B, C and D) Immunoblots of pol α , pol ϵ and pol δ respectively. Aliquots (80 μ l) of DEAE-HPLC fractions were loaded on a 7.5% SDS polyacrylamide gel. Positions of protein standards are shown on the right. (E) Aliquots (10 μ l) were assayed for DNA primase (●) and for exonuclease (○) activity at 37 °C. The substrate for the exonuclease assay was 3 H-3'-end-labelled pUC19 DNA.

Figure 2 Analytical DEAE-HPLC and immunoblot profiles of pol activities from pol δ ts1 strain

(A) Aliquots (10 μ l) were assayed for pol activity at 37 °C on poly(dA)/oligo(dT) (1:3) in the presence (●) or absence (○) of 200 ng of PCNA. (B and C) Immunoblots of pol α and pol δ respectively. Freeze-dried aliquots (200 μ l) of DEAE-HPLC fractions were loaded on a 7.5% SDS polyacrylamide gel. Positions of protein standards are shown on the right. (D) Aliquots (10 μ l/assay) were tested at 37 °C for PCNA-dependent pol activity (●) and for exonuclease activity on poly(dA)/oligo(dT)-3'-end labelled with 3 H]dC (○).

4A). The low amount of DNA synthesized at 37 °C by both wild-type and mutant enzymes was probably due to the hetero-specific origin of RF-C [4]. The template/primer poly(dA)/oligo(dT)

Figure 3 Analytical DEAE-HPLC and immunoblot profiles of pol activities from $\text{pol}\Delta\text{ts3}$ strain

(A) Aliquots (10 μl) were assayed at 37 $^{\circ}\text{C}$ for pol activity on poly(dA)/oligo(dT) (1:3) in the presence (●) or absence (○) of 200 ng of PCNA. (B and C) Immunoblots of pol α and pol δ respectively. Aliquots of DEAE-HPLC fractions were loaded on a 7.5% SDS polyacrylamide gel. Positions of protein standards are shown on the right. (D) Aliquots (10 μl /assay) were tested at 37 $^{\circ}\text{C}$ for PCNA-dependent pol activity (●) and for exonuclease activity on poly(dA)/oligo(dT)-3'-end-labelled with [^3H]dC (○).

Figure 4 Template utilization of wild-type and mutant pol δ at 37 $^{\circ}\text{C}$

(A) M13 singly primed DNA was elongated at 37 $^{\circ}\text{C}$ for different times by 0.02 units of wild-type (○), ts1 (●) and ts3 (□) pol δ in the presence of 0.03–0.05 units of RF-C from calf thymus, 200 ng of *S. pombe* PCNA and 450 ng of *E. coli* SSB protein. (B) The template poly(dA)/oligo(dT) at different molar ratios (1:3, 1:10, 10:1 and 30:1) was elongated at 37 $^{\circ}\text{C}$ for 30 min by 0.03 units of wild-type (white), ts1 (black) or ts3 (grey) pol δ , in the presence (full bars) or in absence (hatched bars) of 150 ng of *S. pombe* PCNA.

was tested at four different molar ratios (Figure 4B). The concentration of oligo(dT) 3'-OH ends is 100-fold higher at a poly(dA)/oligo(dT) ratio of 1:10 compared with a ratio of 10:1.

Figure 5 Stability of wild-type and mutant pol δ at 37 $^{\circ}\text{C}$

(A) pol δ (0.03 units) from analytical DEAE chromatography fractions was pre-incubated at 37 $^{\circ}\text{C}$ for increasing times in 50 mM Bistris, pH 6.5/1 mM DTT/250 $\mu\text{g}/\text{ml}$ BSA/6 mM MgCl_2 . Reactions were then triggered by addition of 4 pmol poly(dA)/oligo(dT) (1:3), 150 nM PCNA and NTPs. Wild-type pol δ (○), ts1 pol δ (●) and ts3 pol δ (□). (B) pol δ (0.03 units) was pre-incubated at 37 $^{\circ}\text{C}$ for 10 min in 50 mM Bistris, pH 6.5/1 mM DTT/250 $\mu\text{g}/\text{ml}$ BSA/6 mM MgCl_2 at the indicated poly(dC) concentrations. Reactions were then triggered by the addition of 150 nM PCNA, 4 pmol poly(dA)/oligo(dT) (1:3) and NTPs. Wild-type pol δ (○), ts1 pol δ (●) and ts3 pol δ (□). 100% activity corresponds to 30 pmol [^3H]dTTP incorporation.

Similar dTTP incorporations were observed with wild-type or mutant enzymes on poly(dA)/oligo(dT) at a ratio of 1:10. In contrast, when the poly(dA)/oligo(dT) ratio was 10:1, decreased activity was observed with mutant enzymes (Figure 4B). The low activity of mutant pol δ under these conditions is not due to a temperature effect, since it is also observed at 25 $^{\circ}\text{C}$ (results not shown), but could be explained by different processivity, different affinity for primer 3'-OH ends and/or different stimulation by PCNA.

Thermal stability of mutant pol δ

Since $\text{pol}\Delta\text{ts1}$ and $\text{pol}\Delta\text{ts3}$ strains arrest their growth in the S phase of the cell cycle [14,16] we tested mutant pol δ stability at 37 $^{\circ}\text{C}$. Figure 5(A) summarizes four independent experiments that produced fairly similar results. After up to 20 min pre-incubation at 37 $^{\circ}\text{C}$, wild-type and mutant enzymes show similar stability. When pre-incubation is extended to 60 min, ts1 pol δ still retains about 70% activity, ts3 pol δ about 50% and wild-type pol δ 40%. At present we do not know the reason for the increased thermal stability of mutant pol δ , which appears only after a long incubation at 37 $^{\circ}\text{C}$. According to the mechanism proposed by Ng et al. [28], on poly(dA)/oligo(dT) template/primer pol δ binds template first, followed by the primer and then by the template-directed dNTP. Thus, the possibility exists that mutant pol δ enzymes were specifically inactivated by excess template at a low primer/template molar ratio. We tested this hypothesis using poly(dC) as template competitor in a pre-incubation step (Figure 5B). No specific inactivation of mutant enzymes by single-stranded DNA is evident. Actually ts1 pol δ is even less sensitive than the wild-type to poly(dC).

Processivity of wild-type and mutant pol δ

Figure 6 shows the effect of PCNA on the processivity of wild-type or mutant pol δ on poly(dA)/oligo(dT) at 37 $^{\circ}\text{C}$. In the absence of PCNA, the enzymes synthesized very short products (lanes 3, 5, 7, 10, 12, 14). In the presence of PCNA, wild-type pol δ synthesized products of multiple sizes on poly(dA)/oligo(dT)

Figure 6 Processivity of wild-type and mutant pol δ at 37 °C

Aliquots of phosphocellulose peak fractions (Table 1) corresponding to 0.03 units of pol δ from wild-type, ts1 or ts3 strains were assayed for processivity of pol δ . Lane 1, size markers in bp; lanes 2–8, poly(dA)/oligo(dT) (1:10); lanes 9–15, poly(dA)/oligo(dT) (10:1). Lanes 2 and 9, substrate alone. Lanes 3 and 10, wild-type pol δ ; lanes 4 and 11, wild-type pol δ with 150 ng of PCNA. Lanes 5 and 12, ts1 pol δ ; lane 6 and 13, ts1 pol δ with PCNA. Lanes 7 and 14, ts3 pol δ ; lanes 8 and 15, ts3 pol δ with PCNA.

Table 2 Apparent kinetic constants for wild-type (wt) and mutant pol δ

$V_{\max}$ values are in pmol/min; K_m values are in nM.

Poly(dA)/oligo(dT) ratio	wt pol δ		ts1 pol δ		ts3 pol δ	
	$V_{\max}$	K_m	$V_{\max}$	K_m	$V_{\max}$	K_m
10:1	2.90 $\pm$ 0.5	57 $\pm$ 11	1.04 $\pm$ 0.24	13 $\pm$ 2	0.55 $\pm$ 0.2	63 $\pm$ 12
1:10	5.5 $\pm$ 0.23	50 $\pm$ 15	7 $\pm$ 0.4	4.6 $\pm$ 1.5	3 $\pm$ 1.5	60 $\pm$ 22

at a ratio of 1:10, as expected on the basis of the geometry of the template/primer (lane 4). In contrast, mutant pol δ synthesized lower amounts of long molecules, suggesting lower velocity (lanes 6 and 8). This is confirmed by results obtained on poly(dA)/oligo(dT) at a ratio of 10:1 (lanes 9–15), where long products were detectable in much lower amounts for mutant pol δ , as also indicated in Figure 4(B). However, since the size of the longest products synthesized by wild-type or mutant pol δ were similar, their processivity is not significantly different. Indeed, when 250 μ g/ml heparin was added to reactions after 3 min, in order to trap pol δ not involved in synthesis [29], no differences in size were observed in DNA synthesized by mutant or wild-type pol δ , again confirming similar processivity (results not shown).

Kinetic analysis

Relative affinities of wild-type and mutant pol δ for poly(dA)/oligo(dT) template/primer were studied by steady-state kinetic approaches using increasing concentrations of poly(dA)/oligo(dT) in the presence of 150 nM PCNA at 37 °C (Table 2). $V_{\max}$ and K_m were determined from Lineweaver–Burk double-reciprocal plots. Wild-type and ts3 pol δ show K_m (3'-OH) values

of about 55 nM and ts1 pol δ shows a more than 10-fold lower apparent K_m (3'-OH) at a template/primer ratio 1:10. Apparent $V_{\max}$ values of wild-type pol δ are similar at both template/primer ratios. In contrast, with poly(dA)/oligo(dT) at a ratio of 10:1, the apparent $V_{\max}$ decreases about 7-fold for ts1 pol δ and more than 5-fold for ts3 pol δ (Table 2). Decreased $V_{\max}$ values of mutant enzymes at low primer concentration indicate inefficient formation of the pol δ –DNA complex. Since productive pol δ –DNA interaction is dependent on PCNA, these observations suggest poor stimulation of both mutant enzymes by PCNA. This is in agreement with the reduced amount of full-length molecules synthesized by mutant enzymes (Figure 6). In addition, low K_m values of ts1 pol δ , particularly evident at high primer concentration, suggest frequent association/dissociation of the enzyme from the template. Thus, the reduced amount of full-length molecules synthesized by ts1 pol δ could be explained by a nearly distributive mode of action of this enzyme due to its poor interaction with PCNA.

PCNA stimulation of wild-type and mutant pol δ

Since major differences in precursor incorporation between wild-type and mutant pol δ were evident at a poly(dA)/oligo(dT) ratio of 10:1, we analysed the stimulation of DNA synthesis at this ratio by different concentrations of PCNA (Figure 7). At 37 °C, 11-fold and 5-fold maximum stimulation of wild-type and ts3 pol δ were obtained at 26 nM PCNA respectively. No stimulation of ts1 pol δ was detected at PCNA concentrations lower than 60 nM (Figure 7, 37 °C). At 25 °C, 12-fold and 5-fold maximum stimulation of wild-type and ts1 pol δ respectively were reached at 6 nM PCNA (Figure 7, 25 °C). In contrast, ts3 pol δ showed 5-fold maximum stimulation at 80 nM of PCNA. Thus, at low primer/template ratio, mutant pol δ never reached the same level of PCNA stimulation as the wild-type enzyme, indicating lowered velocity at both temperatures. In addition, ts1 pol δ displayed a temperature-dependent defect at low PCNA concentrations.

Figure 7 Effect of PCNA on the activity of wild-type and mutant pol δ

Aliquots of phosphocellulose peak fractions (Table 1) corresponding to 0.03 units of pol δ from wild-type, ts1 or ts3 strain were assayed for pol activity at 37 °C and 25 °C on poly(dA)/oligo(dT) (10:1) in the presence of different concentrations of PCNA. Fold indicates the ratio between pmol [3 H]dTTP incorporated in the presence or absence of PCNA by wild-type pol δ (○), ts1 pol δ (●) and ts3 pol δ (□).

DISCUSSION

Analytical DEAE-HPLC chromatography was crucial for the complete separation of wild-type *S. pombe* pol α , δ and ϵ reported in this paper. The elution profiles of DNA polymerase activities from wild-type or mutant strains are similar to that described for *S. cerevisiae* [20].

On phage M13 singly primed DNA, wild-type or mutant enzymes are able to synthesize similar amounts of DNA in the presence of *S. pombe* PCNA and calf thymus RF-C. Thus, RF-C interaction with the PCNA–pol δ –template/primer complex is probably not implicated in DNA synthesis arrest of mutant strains at 37 °C. Using poly(dA)/oligo(dT) we could test PCNA-stimulated activity of mutant pol δ at different template/primer ratios. The results show a loss of about 80 % of ts1 and 60 % of ts3 activity on poly(dA)/oligo(dT) at a 10:1 molar ratio compared with values obtained at a ratio of 1:10 (Figure 4B). Since apparent K_m values of wild-type and ts3 pol δ are similar at both ratios, the differences in activity are not due to different affinities for the primer. The decreased apparent K_m values of ts1 pol δ , suggesting increased affinity for the substrate, could result from poor PCNA stimulation. Both mutant pol δ are able to synthesize long products, albeit in amounts lower than wild-type pol δ . Thus, loss of activity of mutant pol δ is not due to reduced processivity. In contrast, mutant pol δ V_{max} values significantly decrease at low primer/template ratios (Table 2). Since it has been shown that PCNA increases the rate of single-nucleotide incorporation, resulting in higher V_{max} [28], we tested whether mutant pol δ showed low stimulation by PCNA. Results shown in Figure 7 indicate a reduced extent of DNA synthesis by both mutant enzymes at different PCNA concentrations. In contrast to ts3 pol δ , ts1 pol δ does not show detectable activity at low PCNA concentration at 37 °C and shows partial recovery at 25 °C. These results raise the possibility that inefficient PCNA–ts1 pol δ –template/primer interaction could account for DNA synthesis arrest of the pol δ ts1 strain at 37 °C. Amino acid changes in the catalytic subunit of ts1 pol δ (Ala¹⁴³ → Val and Pro¹⁴⁴ → Ser) are located in an evolutionarily conserved domain, called NT-1, which is outside the catalytic site [17]. Decreased PCNA stimulation of ts1 pol δ is in agreement with a similar observation obtained with recombinant human pol δ mutated in this region (His¹⁴² → Arg and Phe¹⁴⁴ → Ser). Lowered stimulation by PCNA of the mutant form suggested that the N-terminus of the human pol δ catalytic subunit could be involved in its

interaction with PCNA [13]. In addition, relatively low apparent K_m values (Table 2), increased thermal stability (Figure 5A) and reduced inactivation by poly(dC) (Figure 5B) of ts1 pol δ suggest that this enzyme could be stabilized through increased affinity for the substrate.

We also observed reduced stimulation of ts3 pol δ by PCNA, but we have not detected differences of activity between 25 °C and 37 °C. The ts3 mutation (Arg¹⁰⁶⁴ → Gln) is located in a region of the catalytic subunit corresponding to a putative zinc finger that is conserved in different species. This portion of the protein is of great functional importance, because although *S. pombe* pol δ cannot replace *S. cerevisiae* pol δ *in vivo* (and *vice versa*), chimaeras possessing homo-specific C-terminal regions are active in both yeasts [23]. In *S. cerevisiae*, mutations in this region can be lethal for the cell or can confer a thermosensitive growth defect suppressed by a second site mutation in the 55 kDa pol δ subunit [30]. In addition, two mutations of the HYS2 gene encoding the p55 subunit of pol δ can be complemented by over-expression of the catalytic subunit [31].

Taken together, these results show that mutations located in the N- or C-terminus of the catalytic subunit affect stimulation of *S. pombe* pol δ by PCNA. The molecular details of PCNA–pol δ holoenzyme interactions are not yet known and the results presented in this paper do not allow us to discriminate between direct or indirect p125–PCNA contact. However, in addition to p125, other subunits of *S. pombe* pol δ are necessary for its functional interaction with PCNA [5]. Since the ts3 mutation is located in the C-terminus of the catalytic subunit that appears to be implicated in interaction with others subunits of pol δ , reduced PCNA stimulation could be the consequence of inefficient interaction of ts3 p125 with another subunit. The segment of *S. pombe* p125 N-terminus where the mutation in ts1 pol δ is located is conserved in human pol δ [6], and a 20 amino acid peptide corresponding to this region has been shown to interact directly with human PCNA [13]. Thus, it is possible that ts1 mutation affects a region of direct contact between the catalytic subunit and PCNA that is unmasked when p125 interacts with other subunits of pol δ *in vivo*. It is interesting to note that over-expression of the genes *cdc1+* or *cdc27+*, encoding two subunits of *S. pombe* pol δ holoenzyme [3], does not rescue growth at 37 °C of pol δ ts1 and pol δ ts3 strains (S. MacNeill, personal communication). We also over-expressed PCNA under the control of three promoters of different strength without complementing the growth arrest of the mutant strains at 37 °C (results not shown). These results suggest that the arrest of DNA synthesis at 37 °C *in vivo* is accompanied by a conformational change impairing replisome activity, which cannot be rescued by over-expressing PCNA or individual components of pol δ holoenzyme.

In conclusion, our results indicate that lower stimulation of ts1 and ts3 pol δ by PCNA results in lower velocity of DNA synthesis, which is particularly evident on long templates at both 25 °C and 37 °C. If the local concentration of PCNA at the replication fork is lower than 60 nM, the defect of ts1 pol δ at 37 °C is accounted for by our *in vitro* results. ts1 and ts3 pol δ show a defect at 25 °C also *in vivo*. In fact, the products of genes *hus1+* and *rad1+*, dispensable for the growth of otherwise wild-type *S. pombe* cells, are necessary for the growth of pol δ ts1 and pol δ ts3 strains [32]. Since the functions of *hus1+* and *rad1+* gene products are not yet established, the molecular aspects of their interaction with the catalytic subunit of pol δ remain uncertain. However, these results show that important protein–protein interactions of the catalytic subunit, that are necessary *in vitro* for normal PCNA stimulation, are also impaired *in vivo* at 25 °C, and become completely defective at 37 °C, in pol δ ts1 and pol δ ts3 strains.

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