

## The Pol32 Subunit of DNA Polymerase $\delta$ Contains Separable Domains for Processive Replication and Proliferating Cell Nuclear Antigen (PCNA) Binding\*

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We have carried out a domain analysis of *POL32*, the third subunit of *Saccharomyces cerevisiae* DNA polymerase  $\delta$  (Pol  $\delta$ ). Interactions with *POL31*, the second subunit of Pol  $\delta$ , are specified by the amino-terminal 92 amino acids, whereas interactions with the replication clamp proliferating cell nuclear antigen (PCNA, *POL30*) reside at the extreme carboxyl-terminal region. Pol32 binding, *in vivo* and *in vitro*, to the large subunit of DNA polymerase  $\alpha$ , *POL1*, requires the carboxyl-proximal region of Pol32. The amino-terminal region of Pol32 is essential for damage-induced mutagenesis. However, the presence of its carboxyl-terminal PCNA-binding domain enhances the efficiency of mutagenesis, particularly at high loads of DNA damage. *In vitro*, in the absence of effector DNA, the PCNA-binding domain of Pol32 is essential for PCNA-Pol  $\delta$  interactions. However, this domain has minimal importance for processive DNA synthesis by the ternary DNA-PCNA-Pol  $\delta$  complex. Rather, processivity is determined by PCNA-binding domains located in the Pol3 and/or Pol31 subunits. Using diagnostic PCNA mutants, we show that during DNA synthesis the carboxyl-terminal domain of Pol32 interacts with the carboxyl-terminal region of PCNA, whereas interactions of the other subunit(s) of Pol  $\delta$  localize largely to a hydrophobic pocket at the interdomain connector loop region of PCNA.

Accurate and efficient DNA replication in eukaryotic cells requires the participation of at least three essential DNA polymerases, Pol  $\alpha$ ,<sup>1</sup> Pol  $\delta$ , and Pol  $\epsilon$ . Several lines of evidence indicate that Pol  $\alpha$  and Pol  $\delta$  function in the initiation and elongation of Okazaki fragment synthesis, respectively (reviewed in Refs. 1 and 2). Replication of the C-A-rich strand at telomeres, by nature a lagging strand process, is also dependent on these two DNA polymerases (3). Under special circumstances, *e.g.* in a yeast mutant carrying a deletion of the Pol  $\epsilon$  polymerization domain, Pol  $\delta$  probably also carries out leading strand synthesis (4–6). However, analyses of exonuclease-de-

ficient DNA polymerase  $\delta$  and  $\epsilon$  strains have revealed a strand specificity of mutation rates and spectra that is most easily reconciled with a dominant role for Pol  $\epsilon$  in leading strand synthesis (7–9).

Pol  $\delta$  from *Saccharomyces cerevisiae* consists of three subunits of 125, 55, and 40 kDa (10). An additional small fourth subunit is found in the enzymes from *Schizosaccharomyces pombe* and human, (11–13). Deletion of the *Cdm1* gene for the fourth subunit in *S. pombe* is genetically silent in the absence of additional mutations in *Cdc1* encoding the second subunit (14). However, efficient overproduction of soluble fission yeast Pol  $\delta$  in baculovirus required the presence of Cdm1 (15). The analogous p12 human subunit could be reconstituted with the human three-subunit Pol  $\delta$  into a stable four-subunit enzyme, and significantly stimulated its activity (13).

The catalytic subunit and the second subunit, encoded in *S. cerevisiae* by the *POL3* and *POL31* (*HYS2*) genes, respectively, are highly conserved in eukaryotes. The genes are also essential in the two yeasts. In contrast, the third subunit shows an extreme divergence at the primary amino acid level. For example, in very closely related yeasts such as *S. cerevisiae* and *Saccharomyces paradoxus*, which show a mean amino acid sequence identity of >90% for all proteins, only 82% sequence identity is observed for the Pol32 subunit. In comparison that for Pol3 and Pol31 subunits is 96 and 93%, respectively (16). A comparison between more distant eukaryotes, including *S. pombe* and human, identified only one motif that was highly conserved in this subunit, *i.e.* the consensus proliferating cell nuclear antigen (PCNA)-binding motif QXX(L/I)XXFF at the extreme carboxyl-terminus of the protein.

Hydrodynamic studies of *S. cerevisiae* Pol  $\delta$  have shown the three-subunit complex to be highly elongated, revealing an unusually high Stokes radius and an unusually small sedimentation constant (17). This deviation from globularity of the entire three-subunit complex could be attributed to the extremely elongated structure of Pol32. A recent study (18) of *S. pombe* Pol  $\delta$  confirmed and extended the *S. cerevisiae* study and showed that the elongated structure of Cdc27 was mainly caused by the central portion of this subunit; a deletion derivative lacking the central 110 amino acids was much more globular. This structure-function analysis of *S. pombe* Cdc27 also identified the N-terminal 160 aa domain as being required for interaction with the second subunit, and the C-terminal 20 aa domain for interaction with PCNA.

The *S. pombe* Cdc27 gene for the third subunit is essential for growth (19). Even point mutations in Cdc27 that eliminate *in vitro* binding to PCNA resulted in cell lethality in one study, although, in a second study, overexpression to high levels of C-terminal truncations lacking this domain rescued growth of the Cdc27 deletion (18, 20). Surprisingly, *S. cerevisiae* *POL32* is

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<sup>1</sup> The abbreviations used are: Pol  $\alpha$ ,  $\delta$ ,  $\epsilon$ , DNA polymerase  $\alpha$ ,  $\delta$ ,  $\epsilon$ ; PCNA, proliferating cell nuclear antigen; aa, amino acid; FEN1, flap endonuclease 1.

not essential, but *pol32* $\Delta$  strains show severe defects in DNA replication, repair, and mutagenesis (10). In addition, the combination of *pol32* $\Delta$  with conditional mutations in *POL3*, *POL31*, or *POL30* (PCNA) also resulted in cell lethality.

In this study we have addressed the functional role of the Pol32 subunit in DNA metabolism by carrying out a scanning deletion mutagenesis of the protein. The domain structure resulting from this study gives new insights in cell growth and DNA-damage induced mutagenesis in yeast, and of processive DNA synthesis *in vitro*. Surprisingly, we found that the essential functionalities are comprised in a small amino-terminal domain of Pol32, not much larger than that necessary to specify interaction with Pol31 subunit of Pol  $\delta$ .

#### MATERIALS AND METHODS

**Strains**—The yeast strains used were BJ2168 (*MATa*, *ura3-52*, *trp1-289*, *leu2-3,112*, *prb1-1122*, *prc1-407*, *pep4-3*) for overproduction, L40 (*Mata ade2 his3- $\Delta$ 200 leu2-3,112 trp1-901 LYS2::lexAop*), *HIS3 URA3::lexAop*, *gacZ*) for two-hybrid analysis, PY175 (*Mata ura3-52 trp1- $\Delta$  his3- $\Delta$ 200 leu2-3,112 lys2-801 pol32 $\Delta$ ::HIS3*) and PY188 (*Mata ura3-52 trp1- $\Delta$  his3- $\Delta$ 200 leu2-3,112 lys2-801 pol32 $\Delta$ ::HIS3*). PY188 was obtained by back-crossing PY175 with strain PY74 (10), which showed a high frequency of damage-induced mutagenesis, and screening the resulting spores for appropriate markers and an elevated frequency of mutagenesis.

**Plasmids**—The *Escherichia coli* overexpression plasmid pBL370-0 placed a His<sub>7</sub> tag, together with a linker of 8 amino acids, at the N terminus of the *POL32* gene, under control of the bacteriophage T7 promoter, in the expression vector pPY55. This plasmid was also used to create and express the *POL32* scanning deletion mutants by standard PCR mutagenesis. The plasmid carrying the full-length *POL32* gene is designated as pBL370-0 and a given derivative plasmid carrying truncation or internal deletion *pol32-x* as pBL370-x. The same truncations and deletions were also created, by standard subcloning, in the series of yeast complementation plasmid pBL387 (Bluescript *ARS CEN TRP1 POL32*), and in the yeast two-hybrid plasmid pBL390 (pGBT8 2  $\mu$ m ori *TRP1 lexA-POL32*), described previously (10). However, the pBL387 and pBL390 series of plasmids did not contain the His<sub>7</sub> tag nor the linker sequence. The series of yeast integration plasmids pBL388 (Bluescript *LEU2 POL32*) had selected *POL32* alleles swapped from the pBL387 member into integration vector pRS305. Vector pRS305 or the set of pBL388 plasmids were linearized by cutting with NarI inside the plasmid *LEU2* gene, prior to integrative transformation of PY175 into the *leu2-3,112* allele to leucine prototrophy. All plasmids and their sequences are available from the corresponding author upon request.

Two-hybrid plasmids pBL240 (*GAL4<sub>activation domain</sub>-POL30* in pACT2), pBL364 (*GAL4<sub>activation domain</sub>-POL31* in pACT2), and pACT2-FR034 (*GAL4<sub>activation domain</sub>-POL1* (aa 313-533)) have been previously described (10, 21, 22). Yeast overexpression plasmids pBL336 (2  $\mu$ m ori *TRP1 GAL1promoter-POL3*), pBL338 (2  $\mu$ m ori *LEU2 GAL1promoter-POL31*), and pBL340 (2  $\mu$ m ori *URA3 GAL10promoter-POL32*) have also been described previously (23). pBL340-10 (*URA3 GAL10promoter-pol32-10*) was made by subcloning the appropriate *pol32-10* fragment from pBL370-10 into pBL340.

**Enzymes**—Pol  $\delta$ , replication factor C, PCNA, and replication protein A were all purified as described previously (23). Mutant *pcna-79* (I126A/L128A) and *pcna-90* (P252A/K253A) were purified as described (24). Pol  $\delta$ -10 containing the mutant Pol32-10 subunit was overexpressed in strain BJ2168 and was purified analogous to Pol  $\delta$ . To remove traces of chromosomally expressed wild-type Pol32-containing Pol  $\delta$  from the purified preparation, 0.5 mg (1 ml) of the final fraction was adjusted to the same conductivity as that of buffer A<sub>100</sub> (30 mM Hepes-NaOH, pH 7.6, 1 mM EDTA, 10% glycerol, 3 mM dithiothreitol, 0.01% Nonidet P40, 100 mM NaCl) and passed over a 1 ml PCNA-agarose column in A<sub>100</sub>. The column was washed with 1 ml of A<sub>100</sub> and the combined eluates stored at -70 °C. Wild-type Pol  $\delta$ , but not Pol  $\delta$ -10 remained bound to this affinity column. This final preparation was free from wild-type Pol32 as shown by a FarWestern analysis (24).

**Expression and Purification of His-tagged Pol32 Proteins**—BL21(DE3) containing pBL370-x was grown in one liter Terrific Broth at 30 °C to A<sub>600</sub> = 0.6-0.8. The temperature was shifted to 15 °C and isopropyl-thiogalactoside was added to a final concentration of 2 mM when the cells reached an A<sub>600</sub> = 1.2-1.6. 12-16 h later, the cells were harvested and resuspended in 20 ml of 50 mM Tris-HCl, pH 8.1, 10% sucrose, and frozen on dry ice. After thawing, EDTA was added to 1 mM,

*p*-methylphenyl-sulfonyl fluoride was added to 0.5 mM, and lysozyme was added to 0.2 mg/ml. After 15 min on ice *p*-methylphenyl-sulfonyl fluoride was added to 0.5 mM. All further steps were carried out at 0-4 °C. After another 15 min on ice, Nonidet P-40 was added to 0.02%, and the cells were sonicated to complete lysis and reduce the viscosity of the lysate. Ammonium sulfate was added to 150 mM, followed by 40  $\mu$ l per ml of lysate of 10% polymin P to precipitate the nucleic acids. After stirring for 5 min, the lysate was cleared at 18,000 rpm for 30 min in an SS-34 rotor. To the supernatant was added 0.114 g of solid ammonium sulfate per ml. After stirring for 1 h, the precipitate was spun at 37,000 rpm in a Ti-45 rotor for 40 min. An additional 0.123 g/ml of solid ammonium sulfate was added to the supernatant and stirred for 1 h. The pellet obtained after spinning the precipitate for 40 min at 37,000 rpm was dissolved in 4 ml of buffer E<sub>100</sub> (40 mM triethanolamine-HCl, pH 7.9, 10% glycerol, 0.02% Nonidet P-40, 10 mM 2-mercaptoethanol, and 100 mM NaCl; subscript designates mM NaCl) and frozen on dry ice. After thawing and dialysis against buffer E<sub>200</sub>, the extract was loaded onto a 3-ml phosphocellulose column that was equilibrated in buffer E<sub>200</sub>. The column was washed with 4 column volumes of buffer E<sub>200</sub>, followed by a step elution with buffer E<sub>1000</sub>. The eluate (8 ml) was gently mixed by rotary inversion for 1 h with 0.7 ml of nickel-nitrilotriacetic acid agarose equilibrated in binding buffer Ni<sub>5</sub> (40 mM triethanolamine-HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole; subscript designates mM imidazole) for 1 h. The agarose was transferred to a column and washed with 4 column volumes of buffer Ni<sub>40</sub>, followed by a step elution with buffer Ni<sub>500</sub>. The peak fraction was frozen on dry ice. 400  $\mu$ l was injected onto a 20 ml Superose 12 column. The Superose 12 column was equilibrated in buffer E<sub>200</sub> and calibrated with bovine serum albumin and ovalbumin. The column was run at 0.4 ml/min at 25 °C, and 200  $\mu$ l fractions were collected and analyzed by 9% SDS-PAGE. The peak fractions from the gel filtration column had a purity of >90% and were devoid of DNA polymerase or nuclease activity. They were used in the reconstitution experiments, for the gel-mobility shift assays, and DNA replication assays.

**Gel-mobility Shift Assay**—Ph-PCNA (PCNA containing an amino-terminal phosphorylatable tag) was <sup>32</sup>P-labeled as previously described (24). A typical 10  $\mu$ l reaction mix contained about 18 fmol labeled PCNA, 250 fmol Pol  $\delta^*$ , and ~100 fmol Pol32 in binding buffer (40 mM triethanolamine-HCl, pH 7.9, 10% glycerol, 0.5  $\mu$ g bovine serum albumin, and 100 mM NaCl). The reaction mix without PCNA was preincubated on ice for 2 h. After addition of labeled PCNA, incubation was continued for 3 min at 30 °C. The mixture was chilled on ice before separation on a native low-ionic strength 4% polyacrylamide gel. The gel was transferred onto a Whatmann filter, dried, and subjected to phosphorimaging analysis.

**In Vitro DNA Replication Assay**—The standard 30  $\mu$ l reaction contained 40 mM Tris-HCl, pH 7.8, 8 mM magnesium-acetate, 0.2 mg/ml of bovine serum albumin, 1 mM dithiothreitol, 80  $\mu$ M each of dATP, dCTP, and dGTP, and 10  $\mu$ M of [<sup>32</sup>P]dTTP, 0.5 mM ATP, 50 fmol (100 ng) of singly primed single-stranded mp18 DNA (the 36-mer primer is complementary to nt 6330-6295), 1  $\mu$ g (9 pmol) of replication protein A, 1 pmol wild-type or mutant PCNA, 100 fmol of replication factor C, and NaCl, Pol  $\delta$ , or Pol  $\delta^*$ , and wild-type or mutant Pol32 as indicated in the legend to the figures. Incubations were at 30 °C for the times indicated. The reactions were stopped with 10  $\mu$ l of 60% glycerol/50 mM EDTA/1% SDS, and electrophoresed on a 1 or 1.5% alkaline agarose gel.

**Co-immunoprecipitation Assay**—Pol  $\delta^*$  (360 ng, 2 pmol) was incubated in buffer H<sub>200</sub> (40 mM Hepes-NaOH, pH 7.4, 10% glycerol, 1 mM EDTA, 0.5 mM EGTA, 0.01% Nonidet P40, 5 mM dithiothreitol, 50  $\mu$ g/ml bovine serum albumin, 200 mM NaCl; subscript designates mM NaCl) in a final volume of 20  $\mu$ l together with 100 ng (2.5 pmol) of Pol32 or Pol32-8 for 1 h at 0 °C. Controls consisted of Pol  $\delta$  and of Pol  $\delta^*$ . Rabbit antiserum (1.5  $\mu$ l) raised against the Pol31 subunit was added to each of the reactions and incubation continued for another hour on ice. Magnetic beads (15  $\mu$ l) containing sheep anti-rabbit IgG, equilibrated in E<sub>200</sub>, were added to the reactions and the reactions were left on ice with occasional mixing for 1 h. The beads were washed with 4  $\times$  30  $\mu$ l of H<sub>400</sub>, followed by 30  $\mu$ l of H<sub>70</sub>. Pol  $\alpha$  (2 pmol, 600 ng) was added to the beads in a final volume of 20  $\mu$ l of H<sub>70</sub>. After 15 min, the beads were washed with 3  $\times$  30  $\mu$ l of H<sub>70</sub>, followed by elution with 2  $\times$  15  $\mu$ l of H<sub>500</sub>. The supernatants from the H<sub>500</sub> elution were combined and subjected to 8% SDS-PAGE, followed by a Western analysis with monoclonal antibodies against the catalytic subunit of Pol  $\alpha$ .

#### RESULTS

The Pol32 subunit is an excellent target for a domain analysis by genetic and biochemical means for several reasons.

	1	100	200	300	350	Cell growth		Interaction strength			UV-Mutagenesis	
						12 °C	75mM HU	POL31	POL1	POL30	%Survival #1, #2	CAN <sup>R</sup> #1, #2
						None		None		None		None
wt=0								++++	++	++	9, 18	0.4, 1
1								-	+	+	6, 12	1.3, 0
2								-	+++	++	7, 21	0.9, 0.7
3								++++	++	++	47, 44	55, 175
4								++++	++	++	45, nd	60, nd
5								++++	++	++	60, nd	53, nd
6								++++	++	++	57, nd	72, nd
7								++++	+	++	48, nd	53, nd
8								++++	-	++	72, nd	49, nd
9								++++	+	-	22, 28	12, 65
10								++++	++	-	28, 35	22, 133
11								++++	-	++	62, 55	47, 155
26								++++	nd	++	nd, 27	nd, 16
27								-	nd	++	nd, 18	nd, 3

FIG. 1. **Protein-protein interactions, cell growth, and UV-induced mutagenesis in *POL32* mutants.** Strains PY175 and PY188 (both *pol32-Δ trp1 CAN1*) were transformed with either empty vector pRS314 (*None*) or the series of pBL387-x plasmids (*CEN TRP1 pol32-x*). The polypeptide endpoints of the truncations and deletions are indicated. 10-fold serial dilutions (from ~50,000–50 cells) were plated on YPD and grown at 12 °C, or on YPD + 75 mM hydroxyurea and grown at 30 °C. At 30 °C on YPD media, no great difference in growth was observed for any of the transformed PY175 or PY188 strains (not shown). For each strain, survival on selective media after irradiation by UV light (30 J/m<sup>2</sup>) was measured, as well as the frequency of canavanine resistant colonies (CAN<sup>R</sup>) per 10<sup>6</sup> survivors after irradiation with 30 J/m<sup>2</sup>. Spontaneous mutation frequencies to canavanine-resistance (no UV irradiation) under these conditions (0.3–1 10<sup>-6</sup>) were subtracted. The two sets of data for % survival and canavanine resistant colonies (CAN<sup>R</sup>) colonies (×10<sup>6</sup>) are those for strain PY175 (#1) and PY188 (#2), respectively. Each data point is the median of at least three independent experiments. The two-hybrid analysis was carried out in yeast strain L40 carrying a plasmid with the (mutant) *POL32* gene fused to the bacterial LexA DNA-binding domain and either *POL31*, *POL1*, or *POL30* to the *GAL4* activation domain.  $\beta$ -Galactosidase assays were carried out in triplicate and tabulated as follows: -, <2 units; +, 2–5 units; ++, 5–15 units; +++, 15–50 units; +++++, >50 units of  $\beta$ -galactosidase. Allele *POL32-0*, wild-type (*wt*); *None*, empty vector; *nd*, not determined.

First, the *S. cerevisiae* gene is not essential but shows at least three distinct phenotypes, *i.e.* cold-sensitivity for growth, sensitivity to the replication inhibitor hydroxyurea, and a defect in damage-induced mutagenesis. Secondly, interactions of Pol32 with at least four proteins have putatively been identified. These include Pol31, the second subunit of Pol  $\delta$ , Pol1, the catalytic subunit of Pol  $\alpha$ , PCNA, and Srs2 (10, 22, 24, 25). Some of these protein-protein interactions have only been identified by a two-hybrid analysis and have awaited analysis by more direct methods. For none of these interactions has its relevance for cellular DNA metabolism and chromosome maintenance been established. Finally, the functionality of Pol32 and its (deletion) mutations can be easily assessed biochemically in replication studies. The catalytic and the second subunit form an isolatable heterodimeric complex, Pol3-Pol31, which we have designated as Pol  $\delta^*$  (23). Fully active Pol  $\delta$  can be easily reconstituted by incubation of purified Pol  $\delta^*$  with Pol32, and the particular phenotype, *e.g.* complex stability, processivity, PCNA interaction, etc., of a particular Pol32 mutant determined biochemically.

**Domain Analysis of *Pol32***—To ascertain which domain of Pol32 is required for each of the proposed protein-protein interactions and which domains contribute to one or more of the observed phenotypes of the *pol32* deletion, we carried out a scanning deletion mutagenesis study of *POL32* (Fig. 1). A series of truncations and internal deletions of the *POL32* gene was fused to the DNA-binding domain of the bacterial *LexA* gene. The deletion constructs were then assayed in the two-hybrid system in strains containing either the *POL1*, *POL30*, or *POL31* gene fused to the *GAL4* activation domain (26). Interaction of *POL32* with *POL31* requires the N-terminal 92 aa, as alleles -1, -2, and -27 were defective, but -3 and -26 showed full interaction strength by the two-hybrid method.

The extreme carboxyl-terminus of the 350-amino acid Pol32 protein contains the PCNA-consensus motif 338-**QGTLES-FFKRKAK**-350 (conserved amino acids in bold). Not surprisingly, both allele -10, which deletes the last seven amino acids including the two conserved phenylalanines, and allele -9, which has an internal deletion from Lys-311 through Ser-343 are defective for interaction with PCNA (*POL30*) by two-hybrid analysis. Finally, the interaction with the catalytic subunit of Pol  $\alpha$ -primase, *POL1*, resides within a discrete domain just upstream of the domain that interacts with PCNA. Alleles -7 and -9 showed partial defects, whereas allele -8 was completely defective. It is important to note that observed interaction defects in the deletion alleles are not caused by failure of a particular allele to express or be imported into the yeast nucleus. For each allele, at least one interaction could still be observed; *e.g.* *pol32-1*, defective for interaction with *POL31*, still showed comparable to wild-type interactions with *POL1* and *POL30*. The only exception was *pol32-27*, for which mutant a several-fold lower signal with *POL30* was observed, suggesting that decreased expression and/or stability could contribute to the observed phenotype. From this analysis we conclude that Pol31, Pol1, and PCNA interact with discrete and separate domains on the Pol32 protein.

Previous interaction studies by the yeast two-hybrid method have also identified putative interactions of Pol32 with itself and with Srs2. The two-hybrid interaction of Pol32 with itself, suggesting the existence of a Pol32-Pol32 dimer, has been shown to be indirect and mediated by PCNA, *i.e.* Pol32-PCNA-Pol32 (17). An interaction between Pol32 and Srs2 was also suggested from a weak two-hybrid signal between *POL32* and a small carboxyl-terminal region of *SRS2* (25). The observed two-hybrid signal was too small to permit a systematic analysis similar to that carried out with the other three

genes. Furthermore, no two-hybrid signal was observed when we attempted to measure interactions of *POL32* with full-length *SRS2* in either combination, *i.e.* LexA<sup>binding domain</sup>-*POL32* with GAL4<sup>interaction domain</sup>-*SRS2*, or LexA<sup>binding domain</sup>-*SRS2* with GAL4<sup>interaction domain</sup>-*POL32*.

**Phenotypic Defects in *POL32* Deletion Alleles**—Strains deleted for *POL32* show close to normal growth at 30 °C although late S phase cells do accumulate (10, 22). However, growth at low temperatures is severely inhibited and so is growth at 30 °C in the presence of hydroxyurea. In addition, deletion mutants are defective for damage-induced mutagenesis, indicating an essential role for Pol  $\delta$  in error-prone DNA synthesis past sites of damage (10, 25). Similar conclusions have been reached from the study of mutations in the catalytic subunit of Pol  $\delta$  (27, 28). Eliminating the interaction between Pol32 and PCNA in alleles -9 and -10 did not deleteriously affect growth at 12 °C or on hydroxyurea-containing media (Fig. 1). Similarly, no defects were detected when the interaction with *POL1* was eliminated in alleles -8 or -11. In fact, defects were only detected when deletions approached the N-terminal region of *POL32* required for interaction with *POL31*. Whereas allele *pol32-11*, which deleted the central half of *POL32* showed wild-type growth, slight growth defects were observed in *pol32-26* even though the interaction with *POL31* was apparently still intact. A further deletion of 10 amino-terminal amino acids in *pol32-27* completely eliminated the interaction with *POL31*.

One possible problem with the interpretation of these results is that the fusion of the *POL32* alleles with the LexA DNA-binding domain used for the interaction analysis in the two-hybrid analysis might effect any of the factors related to protein folding, protein stability, nuclear import, etc., differently from the analogous alleles with only native *POL32* sequences. Therefore, *pol32- $\Delta$*  strains were transformed with the pBL390-x series of plasmids containing the LexA-*POL32* fusion constructs and the growth studies were repeated. The identical results were obtained, *i.e.* strains containing LexA-*pol32-11* showed no defects, those containing LexA-*pol32-26* showed partial growth defects, and those containing LexA-*pol32-27* were as defective as the deletion strains (data not shown).

**The PCNA-binding Motif in *POL32* Enhances the Efficiency of Mutagenesis**—UV-induced mutagenesis of the series of mutants was investigated at a single dose of 30 J/m<sup>2</sup>. The analysis was carried out in two distinct *pol32- $\Delta$*  strains. PY188 containing *POL32* shows a higher frequency of UV-induced mutations in the *CAN1* gene than strain PY175 with *POL32* (see "Materials and Methods" and Fig. 1). The reason for this difference is unknown.

Mutants *pol32-9* and *pol32-10* that inactivate the PCNA-binding motif showed a slight but significant reduction in survival after UV irradiation. This was accompanied by a similar minor significant reduction in the frequency of mutagenesis, particularly for allele *pol32-9* (Fig. 1). No defects in mutagenesis were detected in the alleles *pol32-8* and *pol32-11* defective for interaction with *POL1*. On the other hand, all alleles defective for interaction with *POL31*, *e.g.* *pol32-1*, *pol32-2*, and *pol32-27*, were also completely defective for mutagenesis, suggesting that the Pol32 subunit mediates its role in mutagenesis through the Pol  $\delta$  complex.

Interestingly, mutant *pol32-26* that interacted efficiently with *POL31* was still defective for mutagenesis (Fig. 1). The fusion gene LexA-*pol32-26*, which showed the strong interaction with *POL31* by two-hybrid analysis, was similarly defective for mutagenesis when transformed into a *pol32- $\Delta$*  strain (data not shown). This result suggests that determinants for mutagenesis are located within the first 102 aa because

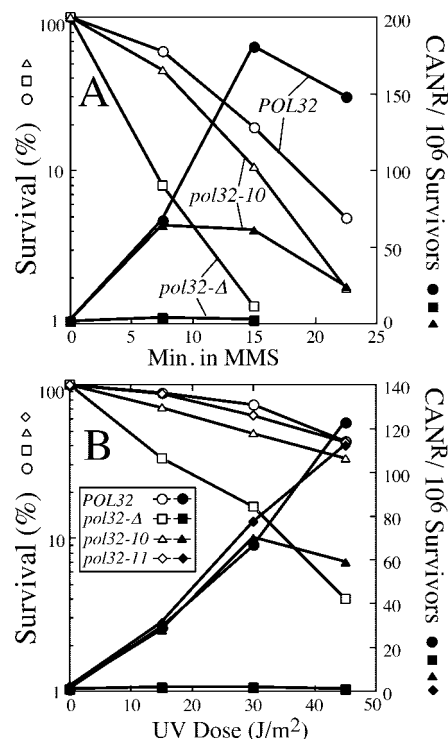


FIG. 2. The PCNA-binding domain of *POL32* enhances damage-induced mutagenesis at high loads of damage. Survival curves and survival-corrected mutation frequencies to canavanine resistance are shown. A, *POL32* deletion strain PY175 into which either empty vector (pRS305), or pBL388 (*POL32* in pRS305), or pBL388-10 (*pol32-10* in pRS305) had been integrated into the chromosome. B, *POL32* deletion strain PY188 into which either empty vector pRS314 (*pol32- $\Delta$* ), or plasmids pBL387 (*POL32* in pRS314), pBL387-10 (*pol32-10* in pRS314), or pBL387-11 (*pol32-10* in pRS314) had been transformed. The strains were grown to saturation and treated with 0.75% methylmethane sulfonate (MMS) (A) or irradiated with the indicated dose at 254 nm (B). Open symbols, survival in %; closed symbols, mutation frequencies. For symbol usage in A, see inset in B. The experiments in A and B were carried out four independent times in duplicate. Standard errors per experimental set were 20%. Results of an average experiment are shown.

*pol32-3*, which deletes aa 103-142, showed no defect. However, when we fused the N-terminal 102 aa domain to the C-terminal 40 aa domain, the resulting clone showed a strong isolate-dependent variability in mutagenesis, even though all isolates interacted with *POL31*. The same variability was observed when we extended the N-terminal domain to 127 aa. Therefore, although it appears that determinants for DNA damage-induced mutagenesis are more demanding than those for *POL31* interaction, we have been unable to exactly differentiate between these two determinants by this simple deletion analysis.

To investigate more closely the role of the PCNA-binding motif of *POL32* in mutagenesis, we measured the induced mutation frequency as a function of the damage load, either UV or methylmethane sulfonate, for *pol32-10* defective for PCNA binding and the large internal deletion mutant *pol32-11* (Fig. 1). The dose-response studies show that at very low levels of damage, *pol32-10* is as efficient for mutagenesis as wild-type (Fig. 2). However, at higher levels of damage defects in mutagenesis become apparent in the *pol32-10* mutant. Strain *pol32-11* was as effective as wild-type at all damage loads. These data suggest that the PCNA-binding domain of *POL32* enhances mutagenic bypass of chromosomal DNA regions that have received multiple hits. Induced mutagenesis in the *pol32-9* mutant strains was compromised to a much greater extent than for *pol32-10* (Fig. 1). However, interaction with *POL1* was also weakened in this mutant, possibly adding to the

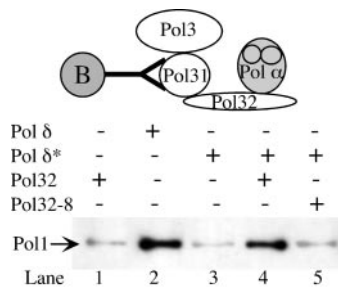


FIG. 3. **Pol  $\delta$  and Pol  $\alpha$  interact via the Pol32 subunit.** Native Pol  $\delta$  (lane 2) or Pol  $\delta$  reconstituted from Pol  $\delta^*$  and Pol32 (lane 4) or Pol32-8 (lane 5, see Fig. 1), was attached to magnetic beads via antibodies to Pol31. The beads were incubated with Pol  $\alpha$ , and protein-bound was detected by a Western analysis with antibodies to Pol1. See "Materials and Methods" for details. A schematic of the complex bound to the beads is shown.

observed defect. Although a role for Pol  $\alpha$  in mutagenesis has not been identified, *e.g.* see the data for *pol32-8* in Fig. 1, our more extensive analysis focused on *pol32-10* to avoid that possible complication.

**Pol  $\delta$  Forms a Complex with Pol  $\alpha$** —The observed two-hybrid signal between Pol32 and Pol1 suggests an interaction between these two DNA polymerases that may be important for properly regulated lagging strand DNA synthesis (Fig. 1 and Ref. 22). To assess whether the two-hybrid signal reflects a physical interaction between Pol  $\alpha$  and Pol  $\delta$ , we carried out a co-immunoprecipitation experiment with the purified polymerases. The presence of the large subunit of Pol  $\alpha$  in the complex was then detected in a Western analysis with antibodies to this subunit. Indeed, this analysis showed that Pol  $\delta$  interacts with Pol  $\alpha$ , whereas Pol  $\delta^*$  does not (Fig. 3, compare lanes 1 and 3 with lanes 2 and 4). Moreover, reconstitution of Pol  $\delta^*$  with Pol32-8, rather than wild-type Pol32 produced a background signal, in agreement with the 2-hybrid data (Fig. 1). Control experiments described below show that Pol  $\delta^*$  and Pol32-8 efficiently reconstitute into a functional Pol  $\delta$  complex (Fig. 7).

A two-hybrid interaction was also reported between Pol32 and a small domain of Srs2 (25). As stated above, this interaction could not be verified in a two-hybrid analysis with the full-length genes. Using the same methodology that allowed us to verify the existence of a Pol  $\alpha$ /Pol  $\delta$  complex, we were unable to detect a complex between Pol  $\delta$  and Srs2. Possibly, if such a complex exists, it may be an indirect one mediated by a third protein.

**Reconstitution of Pol  $\delta$  from Pol  $\delta^*$  and Pol32 Requires the N-terminal Domain**—Previously, we have shown that incubation of Pol  $\delta^*$  with Pol32 reconstitutes fully functional Pol  $\delta$  (23). This reconstitution can be directly verified by gel filtration analysis. Because of the highly elongated shape of Pol32, a large shift in Stokes radius, from 51 Å for Pol  $\delta^*$  to 71 Å for Pol  $\delta$ , is observed upon successful reconstitution (17). We have extended this reconstitution analysis to the Pol32 alleles, -1, -2, -3, -8, and -10. With the exception of Pol32-1 and Pol32-2, all mutant forms of Pol32 reconstituted efficiently with Pol  $\delta^*$  into the respective mutant forms of Pol  $\delta$  as judged by a shift in elution pattern (results not shown). Therefore, in agreement with the two-hybrid data, an amino-terminal domain < 102 amino acids is required for proper reconstitution of Pol  $\delta$ .

**DNA-independent Interaction between PCNA and Pol  $\delta$  Is Mediated by Pol32**—We have previously shown by overlay blots that PCNA binds Pol32 *in vitro* (24). This same assay showed that Pol32-10 failed to bind PCNA (Fig. 4). This result is consistent with several studies (18, 20, 29), including those

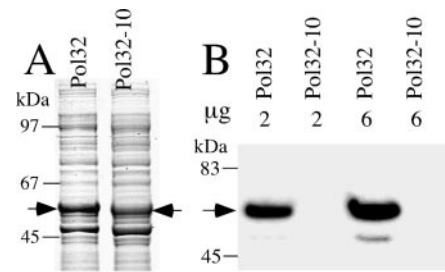


FIG. 4. **PCNA binding to POL32 detected by overlay blots.** For details see "Materials and Methods." A, lysates (10  $\mu$ g each) of *E. coli* cells overexpressing Pol32 or Pol32-10. B, the indicated amounts of each lysate were separated on a 10% SDS/polyacrylamide gel, the proteins electrophoretically transferred to nitrocellulose, and probed with  $^{32}$ P-labeled PCNA. Bound PCNA was visualized by phosphorimaging analysis. Migration positions of the Pol32 subunit and its truncation derivative is indicated. See "Materials and Methods" for details.

with Pol32 orthologs in *S. pombe* and human, which have shown that the phenylalanines in the PCNA-binding motif are essential for interaction.

However, direct binding of PCNA to the large subunit as well as the second subunit of Pol  $\delta$  has also been demonstrated (30-33), although other studies failed to detect such interactions, suggesting that they are likely quite weak (10, 24, 31, 33-35). To determine to what extent the PCNA-binding domain of Pol32 is responsible for the interaction between the three-subunit Pol  $\delta$  and PCNA, we carried out native gel electrophoresis of several reconstituted Pol  $\delta$  complexes as well as surface plasmon resonance analysis of one particular mutant Pol  $\delta$ -10.

In the native gel assay, reconstituted forms of (mutant) Pol  $\delta$  were incubated with labeled PCNA, and complexes formed with PCNA analyzed on a native 4% polyacrylamide gel. In addition to unbound PCNA, three additional complexes could be detected by phosphorimaging analysis (Fig. 5). A streak of radioactivity migrating up from the position of free PCNA was observed when Pol  $\delta^*$  was mixed with PCNA, indicative of an unstable complex (lane 2). A poorly defined complex more retarded in migration resulted from interaction of PCNA with Pol32, *e.g.* in lanes 3-7. A well defined slowly migrating complex between Pol  $\delta$  and PCNA was only observed if Pol32 reconstituted with Pol  $\delta^*$  into a stable Pol  $\delta$  complex and if Pol32 retained a functional PCNA-binding domain. Therefore, this complex was not observed with Pol32-1 and Pol32-2 because they are defective for interaction with Pol31 (lanes 4 and 5) nor with Pol32-9 because this allele was defective for PCNA binding (lane 8, see also Fig. 1).

We purified mutant Pol  $\delta$ -10 from a yeast overproduction system and measured its interactions with PCNA by surface plasmon resonance. Interestingly, binding of Pol  $\delta$  to PCNA attached to a chip was virtually eliminated when the PCNA-binding motif in Pol32 was mutated in Pol  $\delta$ -10 (Fig. 6). Therefore, in agreement with the native gel analysis in Fig. 5, binding between Pol  $\delta$  and PCNA, in the absence of effector DNA, is overwhelmingly determined by this one interaction to Pol32.

**Functional Interaction between Pol  $\delta$  and PCNA**—The conclusion that the PCNA-binding domain of Pol32 is essential for efficient interaction of Pol  $\delta$  with PCNA in the absence of effector DNA does not address the functional importance of this domain during replication of the ternary DNA-PCNA-Pol  $\delta$  complex. Nor does it follow from the lack of an interaction phenotype in the central region of Pol32, *i.e.* in alleles -3 through -7, that any of these domains is not required for appropriate and processive replication by this ternary complex. To address the functional importance of the different regions of Pol32, the purified mutant proteins were mixed with Pol  $\delta^*$  to reconstitute

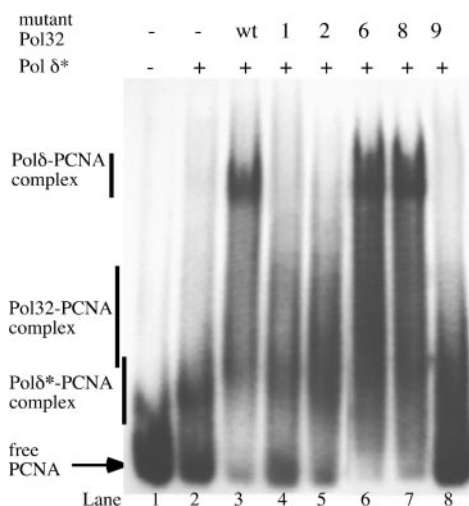


FIG. 5. **Native gel electrophoresis of PCNA-Pol  $\delta$  complexes.** Pol  $\delta^*$  was preincubated with the Pol32 mutants as indicated (see Fig. 1 for allele identification), followed by incubation with [ $^{32}$ P]PCNA prior to analysis on a 4% polyacrylamide gel. The migration positions of the different complexes are indicated; that of PCNA-Pol32 was determined in separate experiments (not shown). See "Materials and Methods" for details.

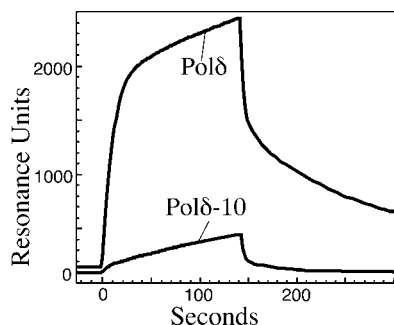


FIG. 6. **DNA-independent interactions between PCNA and Pol  $\delta$ .** PCNA was attached to a CM5 chip as described previously (37). The running buffer was 30 mM Hepes-NaOH 7.4, 5 mM MgAc<sub>2</sub>, 5% glycerol, 0.01% Nonidet P-40, 100 mM NaCl, 1 mM dithiothreitol, 100  $\mu$ g/ml bovine serum albumin. Pol  $\delta$  or Pol  $\delta$ -10 (Fig. 1) at 25 nM were flowed across the chip at a rate of 45  $\mu$ l/min.

the respective mutant forms of Pol  $\delta$ . These were then tested in a DNA replication assay at high salt and low PCNA concentrations where Pol  $\delta^*$  alone showed low processivity (Fig. 7, lane 2) (23). Three distinct replication patterns were observed. Replication with Pol32-1 and Pol32-2 gave the same activity as that of Pol  $\delta^*$  alone, confirming the two-hybrid data (Fig. 1), the gel filtration analysis, and the native acrylamide analysis (Fig. 5) that the N-terminal region of Pol32 is required for interaction with Pol31. Internal deletions -3 through -8 and even the larger internal deletion -11 yielded the same type processive replication products as native Pol  $\delta$  (lane 1) or Pol  $\delta$  reconstituted from Pol  $\delta^*$  together with wild-type Pol32 (lane 3). Finally, replication with Pol32-9 and Pol32-10, which were defective for interaction with PCNA yielded slightly less processive replication products than wild-type Pol32. These studies indicate that the PCNA-binding domain of Pol32 contributes to processive synthesis by Pol  $\delta$ , but much less so than expected. Surprisingly, it appears that the ability of Pol32 to interact with Pol31 is the major determinant for processive DNA synthesis.

*In Vitro Epistasis Analysis of Interaction Domains between Pol  $\delta$  and PCNA*—Of all known PCNA-binding proteins, Pol  $\delta$  is unique because this complex contains at least two, and possibly three PCNA-binding domains. These binding domains likely

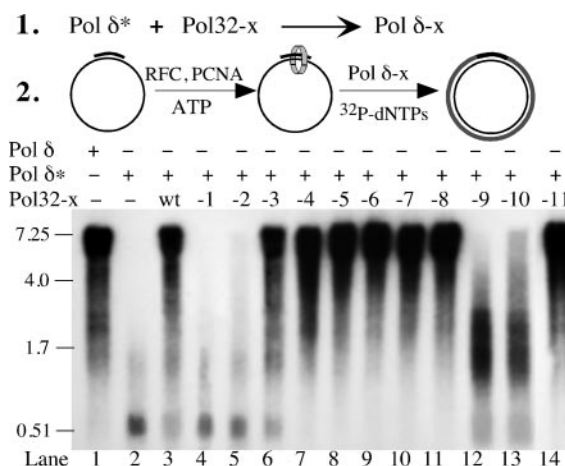
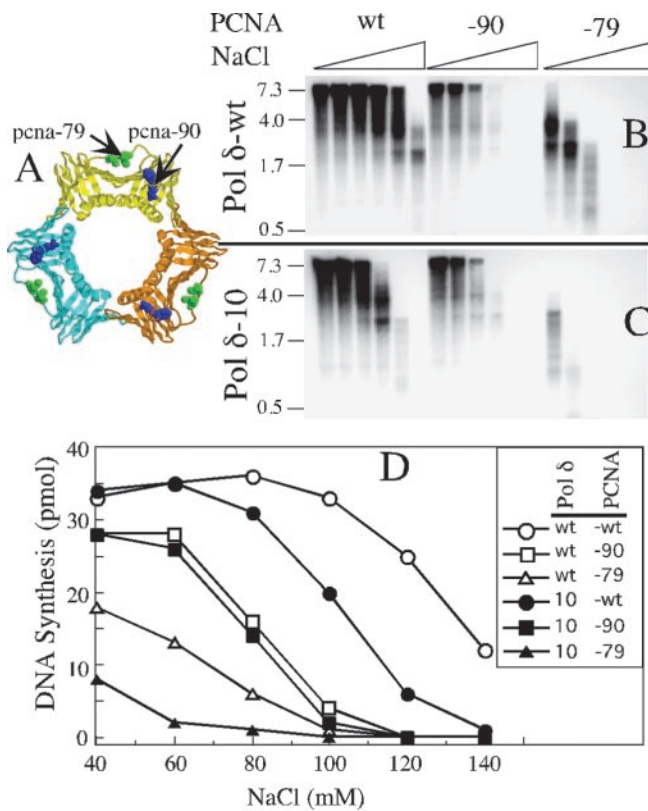


FIG. 7. **Functional interaction between mutant Pol  $\delta$  and PCNA.** Pol  $\delta^*$  (100 ng, 500 fmol) was mixed with (mutant) Pol32 (50 ng, 1.2 pmol) in a total volume of 5  $\mu$ l of buffer H<sub>200</sub>. After 2 h at 0  $^{\circ}$ C, 1  $\mu$ l was added to a standard DNA replication reaction containing 50 fmol singly primed mp18 DNA, 10 pmol of replication protein A, 200 fmol of PCNA trimers, 100 fmol of replication factor C, and 125 mM NaCl. After a 4 min incubation at 30  $^{\circ}$ C, the reaction products were separated by electrophoresis on an 1% alkaline agarose gel, the gel was dried and subjected to phosphorimaging analysis. The numbering of the mutants is as in Fig. 1. Lane 1, 100 fmol of 3-subunit Pol  $\delta$ ; lane 2, no Pol32 added; lane 3, wild-type Pol32.

are all distinct; only Pol32 has the well known consensus motif QXX(L/D)XXFF. Therefore, it is reasonable to assume that these distinct motifs interact with different regions on PCNA. And the order of importance of these different interactions may depend on the particular metabolic pathway in which Pol  $\delta$  functions. Here, we have tested this model for processive DNA synthesis by Pol  $\delta$ .

Previously, we have carried out both a genetic and biochemical analysis of a large number of PCNA mutants (24, 36). Two mutants, pcna-79 and pcna-90, have been extremely useful in understanding protein-PCNA interaction on or off the effector DNA. These mutant PCNAs are efficiently loaded onto template-primer DNA by replication factor C and, therefore, any defect encountered should be the result of their interaction with other proteins. Pcn-79 (I126A/L128A) eliminates a hydrophobic pocket in the interdomain connector loop of PCNA (Fig. 8A). Consequently, proteins with the consensus PCNA-binding motif QXX(L/I)XXFF fail to form a binary complex with pcna-79 in solution, *i.e.* in the absence of DNA. For instance, like Pol32, the flap endonuclease 1 (FEN1) has a single PCNA consensus motif near its carboxyl-terminus. Its binding to PCNA was decreased from 7 nM to 10  $\mu$ M when the hydrophobic pocket was mutated in pcna-79 (37). Yet, this mutant PCNA formed an efficient complex with FEN1 on the DNA, indicating that determinants other than the hydrophobic pocket are important for stability of the ternary DNA-FEN1-pcna-79 complex. The opposite result was obtained with mutant pcna-90 (P252A/K253A) with mutations in the carboxyl-terminal tail of PCNA. Whereas the stability of the binary FEN1-pcna-90 complex was like that of wild-type, no ternary complex could be detected when pcna-90 encircled the DNA, and therefore this mutant did not stimulate FEN1 activity (37). Analogous results were obtained with the yeast AP-endonuclease Apn2 (38).

As shown in Fig. 6, stability of the binary, DNA-independent, PCNA-Pol  $\delta$  complex is predominantly accomplished through Pol32. Previously, we have shown that mutant pcna-79 no longer forms a binary complex with Pol32, nor with Pol  $\delta$ , whereas pcna-90 is still functional (24). Therefore, as with FEN1, the hydrophobic pocket of PCNA is a crucial determi-



**FIG. 8. Epistasis analysis of PCNA-Pol  $\delta$  interaction domains.** A, localization of amino acids mutated in *pcna-79* (I126A/L128A) and *pcna-90* (P252A/K253A). B and C, replication of singly primed single-stranded mp18 DNA (50 fmol) was carried out under standard replication conditions as described under "Materials and Methods," except that NaCl was varied as indicated in D. The entire assay mixture except for the polymerase was preincubated at 30 °C for 30 s to allow loading of PCNA, Pol  $\delta$  or Pol  $\delta$ -10 was added and reaction continued for another 120 s at 30 °C. This is the time required for the average complexes to completely replicate the viral DNA. PCNA, *pcna-79*, or *pcna-90* (200 fmol each) were added where indicated. B, wild-type (*wt*) Pol  $\delta$  (100 fmol). C, Pol  $\delta$ -10 (100 fmol). D, quantitation of the total amount of DNA replicated (pmol of dNTP incorporated) in B and C. No incorporation (<1 pmol) was observed in the absence of PCNA.

nant for binary complex formation with Pol32 and consequently also with Pol  $\delta$ .

Applying this methodology to PCNA-Pol  $\delta$  interactions on the DNA is more complicated because of the presence of multiple functional PCNA-binding motifs during DNA replication. Our working hypothesis for establishing the functional PCNA-interacting domains is: If Pol32 interacts uniquely at a specific site X on PCNA when it encircles the DNA, then the *in vitro* replication defect of the *pcna-x* mutant should not be exacerbated when in addition the PCNA-binding motif in Pol32 is mutated. We measured the affinity of the ternary DNA-PCNA-Pol  $\delta$  complex indirectly by assessing the influence of NaCl on processivity of DNA synthesis.

Replication assays were carried out at increasing concentrations of NaCl for wild-type Pol  $\delta$  and Pol  $\delta$ -10, and for PCNA, *pcna-79*, and *pcna-90*, in all six combinations (Fig. 8, B-D). With wild-type PCNA, both forms of Pol  $\delta$  were fully functional at low to moderate NaCl levels, and a processivity defect with Pol  $\delta$ -10 could only be detected at >80 mM NaCl. However, with *pcna-90*, both forms of Pol  $\delta$  showed defects in processive replication at >60 mM NaCl. Strikingly, no significant difference was observed between Pol  $\delta$  and Pol  $\delta$ -10, exactly a result one would expect if the PCNA-binding domain of Pol32 failed to interact functionally with *pcna-90* on the DNA. Finally, with *pcna-79*, severe replication defects by Pol  $\delta$  had previously been

documented, particularly in its ability to traverse regions of secondary structure (24). The additional inactivation of PCNA binding in Pol32 crippled processive replication even further. As the previous data have shown that on the DNA, Pol32 interacts with the carboxyl-terminus rather than the interdomain connector loop region of PCNA, it follows that the functional interaction of the other subunit(s) of Pol  $\delta$  with the hydrophobic pocket is impaired in the *pcna-79* mutant.

In conclusion, the epistasis analysis in Fig. 8 indicates that interaction between Pol32 and PCNA, both on and off the DNA, closely resembles that of FEN1. Conversely, the PCNA-binding domain(s) on Pol3 and/or Pol31 do not interact, or poorly interact with PCNA in solution, but do localize at least in part to the interdomain connector loop when PCNA encircles the DNA.

#### DISCUSSION

From comprehensive biochemical studies in the *S. cerevisiae*, *S. pombe*, and human systems a remarkably consistent picture of Pol  $\delta$  has emerged. The three largest subunits of this complex, Pol3, Pol31 (designated Cdc1 in *S. pombe*, p50 in human), and Pol32 (Cdc27, p68) form a heterotrimeric complex in which Pol31 forms the bridge between Pol3 and Pol32. A small fourth subunit is found in *S. pombe* (Cdm1) and human (p12), but is lacking from purified preparations of *S. cerevisiae* Pol  $\delta$ , and various search algorithms failed to identify a putative *S. cerevisiae* ortholog.<sup>2</sup> The third subunit of all organisms is an extremely elongated protein with an unusually high content of charged amino acids (30–35%) and an unusually slow migration in a SDS/polyacrylamide gel. In fact, the extremely elongated structure and consequentially the very large Stokes radius of this subunit initially gave the appearance by gel filtration chromatography that this subunit, as well as Pol  $\delta$ , might be dimeric in structure (10, 15, 23, 39). Subsequently, more extensive studies that took the unusual shape of the third subunit in account or used techniques that revealed molecular weights independent of shape showed Pol  $\delta$  to be monomeric (17, 18).

*In Vivo Analysis of POL32 Domains*—The domain analysis of the Pol32 subunit of *S. cerevisiae* Pol  $\delta$  reported here allows us to draw strong analogies with the *S. pombe* *Cdc27* gene, even though *Cdc27* is an essential gene whereas *POL32* is not. Recent domain studies (18) of *Cdc27* showed that rescue of the lethality of *cdc27* $\Delta$  required both the N-terminal Cdc1-interacting region as well as the C-terminal PCNA-interacting region of *Cdc27*. However, the presence of just the N-terminal region sufficed for rescue if it was over-produced ~30-fold in the cell. *Cdc27* mutants that have the internal region deleted, leaving just 160 amino acids at the N terminus and 30 amino acids at the C terminus, grow like wild-type. The role of *Cdc27* and its domains in mutagenesis has not been studied.

Certainly, in *S. cerevisiae* the phenotypes of *POL32* mutants are much more mild than for *Cdc27* in *S. pombe*. Yet, like in *S. pombe*, the N-terminal region of Pol32 is most important for rescue. Thus, the N-terminal amino acid region of Pol32 was essential for rescuing all phenotypes of *pol32* $\Delta$ , *i.e.* cold-sensitivity, hydroxyurea sensitivity, and damage-induced mutagenesis; the only measurable effect of the presence of the C-terminal PCNA-interacting region was the increased efficiency in mutagenesis. Damage-induced mutagenesis (UV or methylmethane sulfonate) of *pol32-10*, which is defective for PCNA binding, was comparable to wild-type at low doses of damage. However, at higher doses of damage the efficiency of mutagenesis dropped significantly, and this was accompanied by an increase in lethality. Possibly, adjacent sites of damage which are normally replicated processively by the same mutagenic

<sup>2</sup> E. Johansson and P. M. J. Burgers, unpublished results.

replication complex containing Pol  $\delta$ , cannot be replicated similarly if the Pol  $\delta$ -10 complex is less processive at regions of damaged DNA.

Our studies strongly suggest that the mere binding of Pol32 to Pol31 suffices to suppress the cold-sensitivity and hydroxyurea-sensitivity of the deletion mutant. However, although it is apparent that additional determinants are required for mutagenesis, it is not certain whether these determinants identify additional interactions with Pol31 or interactions with another as yet unidentified actor. There is also an uncertainty in the delineation of the required sequences. Mutants *pol32-1* (deleting aa 1–39) and *pol32-2* (deleting aa 37–102) are defective for interaction with *POL31* and defective for mutagenesis, whereas mutant *pol32-3* (deleting aa 103–142) and *pol32-11* (deleting aa 142–309) are completely proficient (Fig. 1). On the other hand, *pol32-26* (deleting aa 93–309) interacts with *POL31* but is deficient for mutagenesis. We tried to delineate more precisely the domain required for mutagenesis by studying mutants that were intermediate between *pol32-26* and *pol32-11*, maintaining either 102 or 127 N-terminal amino acids together with the C-terminal 40 aa domain. Unfortunately, these mutants yielded widely varying results between different isolates, and therefore did not allow us to draw any reliable and reproducible conclusions (data not shown). Therefore, a more intensive analysis besides this simple domain analysis is required to gain definite insights in the required function of *POL32* for mutagenesis.

Previously, a two-hybrid analysis identified a putative interaction between *POL32* and *POL1* (22). Here, we have shown biochemically that Pol  $\alpha$  interacts with Pol  $\delta$ , and, moreover, that this interaction is defective in the scanning deletion mutant Pol32–8 (Figs. 1 and 3). An interaction between these two enzymes may serve an important role in the regulation of the initiation and elongation of Okazaki fragments. However, this particular loss of interaction is not associated with a discernable phenotype, perhaps suggesting a redundancy in interactions of the lagging strand complex.

*Processive DNA Synthesis by Pol  $\delta$  Requires Two Separate Domains of Pol32*—Previously, we have studied the processivity of the two-subunit Pol  $\delta^*$  and the three-subunit Pol  $\delta$  in the absence and presence of PCNA. Without PCNA present, Pol  $\delta^*$  showed a very low processivity of 2–3 (nucleotides incorporated per binding event) on poly(dA)-oligo(dT) under the reaction conditions used (23). The processivity of Pol  $\delta$  was much higher at 5–10 nucleotides on this template-primer system. Not surprisingly, this increase in processivity did not require the PCNA-binding domain of Pol32, and the processivity of Pol  $\delta$  and Pol  $\delta$ -10 on poly(dA)-oligo(dT) were identical (data not shown). In the presence of PCNA, both Pol  $\delta$  and Pol  $\delta^*$  were fully processive on this template, although a much higher level of PCNA was necessary to stimulate processive DNA synthesis by Pol  $\delta^*$  than by Pol  $\delta$  (23).

Replication of natural DNA by the PCNA-Pol  $\delta^*$  complex was also much less processive than that of the PCNA-Pol  $\delta$  complex (Fig. 7, lanes 1 and 2). Replication pause sites with Pol  $\delta^*$  are much more pronounced than with Pol  $\delta$ , indicating that sites of secondary structure form major replication barriers for this two-subunit enzyme. The poor replication efficiency of Pol  $\delta^*$  could be ascribed to the frequent disassembly of replication complexes, perhaps at sites of secondary structure, because a large molar excess of PCNA stimulated Pol  $\delta^*$ -mediated replication (23). To a large extent, the defects observed with Pol  $\delta^*$  were eliminated by providing that domain of Pol32 which suffices for interaction with Pol31 (Fig. 7). A further stimulation was then observed when in addition Pol32 contained its PCNA-binding domain. However, the distinction between Pol  $\delta$  and

Pol  $\delta$ -9 or Pol  $\delta$ -10 was only apparent under conditions, e.g. high salt levels, that challenged the stability of the complex. At below 80 mM NaCl, both Pol  $\delta$  and Pol  $\delta$ -10 replicated single-stranded mp18 DNA with virtually identical kinetics (Fig. 8).

These *in vitro* replication results agree qualitatively with a recent study of the *S. pombe* enzyme, where major importance for processive DNA synthesis could similarly be ascribed to the Cdc1-interacting domain of Cdc27, and full processivity was only observed if in addition the PCNA-binding domain of Cdc27 was provided (18). However, although in *S. cerevisiae* under normal growth condition in the cell, the PCNA-binding domain of Pol32 is dispensable, it is not in *S. pombe*, indicating that quantitatively, this domain serves a more important function in this organism and perhaps also in mammalian cells (18, 20).

In *S. cerevisiae*, the only observable defect of a lack of the PCNA-binding domain is that of less efficient mutagenesis, particularly at high loads of DNA damage (Figs. 1 and 2). The observed defects in mutagenesis were less severe with *pol32-10*, a C-terminal 7 aa truncation (–F<sub>344</sub>FKRKAK), than with the internal deletion mutant *pol32-9* deleting aa 310–343 which include QGTLES<sub>343</sub> of the motif, suggesting the existence of residual interactions between Pol32–10 and PCNA *in vivo*. Because interaction with *POL1* was also weakened in mutant *pol32-9*, the interpretation of these results is more complex. In support of the conclusion that the primary mutagenesis defect in *pol32-9* is due to defective interaction with PCNA are the results with *pol32-8*, which show a complete defect in interaction with Pol  $\alpha$  and, yet, no defect in mutagenesis (Fig. 1). Therefore, our domain analysis of *POL32* is consistent with a model that processive mutagenic replication of closely juxtaposed sites of damage requires this PCNA-binding domain.

*The Subunits of Pol  $\delta$  Interact with Distinct Domains on PCNA*—Determining the PCNA-interacting domains on Pol  $\delta$  has remained an elusive undertaking, because interactions with PCNA on or off the DNA vary widely in strength and functional importance. In a mutational study of FEN1-PCNA interactions we proposed that loading of PCNA onto the DNA causes conformational transitions of PCNA which exposes a carboxyl-terminal domain for binding FEN1 (37). At the same time, the hydrophobic pocket at the interdomain connector loop of PCNA, which is crucial for binding FEN1 in solution, appears no longer to participate in binding FEN1 on the DNA. The PCNA-binding domain on Pol32, which is analogous to that of FEN1, also appears to follow this mode of interaction, i.e. binding to PCNA off the DNA is abolished in the hydrophobic pocket mutant *pcna-79* (Fig. 8A and Ref. 24), whereas functional interaction on the DNA appears to require the carboxyl-terminal domain of PCNA mutated in *pcna-90*. This conclusion was drawn from the observation that the processivity of replication of the double mutant complex *pcna-90*-Pol  $\delta$ -10 was no more sensitive to high salt than that of the single mutant *pcna-90*-Pol  $\delta$  (Fig. 8D). We note, however, that the *pcna-90* mutant alone is more defective than Pol  $\delta$ -10 alone, indicating that mutations PK252,253AA in the carboxyl-terminal tail of PCNA may affect additional interactions with Pol  $\delta$ , or PCNA function, or both.

On the other hand, the PCNA-binding domains on Pol3 and/or Pol31 poorly function in binding PCNA off the DNA (Fig. 6), although weak binding to either subunit of human Pol  $\delta$  has been demonstrated (30–33). However, these domains are the most important determinants for binding and for processivity of Pol  $\delta$  when PCNA encircles the DNA, as *pcna-79* mutants are severely defective for DNA replication (Fig. 8 Ref. 24). Thus, at least in part the interactions of these subunits to PCNA on the DNA are mediated through the hydrophobic pocket at the

interdomain connector loop of PCNA. Obviously, as these mutants were originally generated to study genetic defects in DNA replication and repair, none of these mutants can be expected to have completely disrupted essential interactions with Pol  $\delta$ . Therefore, even the double mutant complex pcna-79-Pol $\delta$ -10 still shows significant DNA replication activity.

These studies delineate at least two separate domains on Pol  $\delta$  with interactions to at least two separate domains on PCNA. The interactions between the three subunits and their arrangement with regard to N and C termini is: Pol3<sup>N</sup>Pol31<sup>C</sup>Pol32<sup>C</sup> (Fig. 1) (18, 19). Pol32 is an extremely elongated protein in solution with its PCNA-binding domain localized at the very carboxyl-terminus. This would separate the PCNA-binding domain on Pol32 by at least 50 Å from any putative PCNA-binding domain on Pol3 or Pol31, if Pol32 extended linearly from Pol  $\delta^*$  as is suggested by the extreme Stokes radius of 71 Å for Pol  $\delta$ . However, a deletion that removes almost half of the protein, and thereby most of the rod-like region of the third subunit (Pol32-11 in *S. cerevisiae* and Cdc27-6 M in *S. pombe*), and therefore should bring these PCNA-binding domains much closer together, still functions like wild-type in processive replication and shows no phenotypic defects in the cell (Fig. 7 and Ref. 18). The adaptability of PCNA-Pol  $\delta$  interactions to deletions in Pol32 suggests that on the DNA this third subunit may adopt a more flexible and compact orientation, and perhaps also that some degree of freedom exists in domain binding. The hydrophobic pocket and the C-terminal region marked by mutations *pol30-79* and *pol30-90* are separated by only 15–20 Å if present on the same monomer (40). However, these regions on adjacent monomers would be separated by 45–65 Å, depending on the direction of orientation of these regions. Given the large size of Pol  $\delta$  on one hand and on the other hand the very close juxtaposition of the hydrophobic pocket and the C-terminal tail on the same PCNA monomer, it is likely that Pol  $\delta$  binds at least two PCNA monomers when replicating DNA. An important step in the understanding of these functional complexes would be that of the atomic resolution structure of a ternary complex of a consensus PCNA-binding protein, *e.g.* FEN1, with PCNA when it encircles the effector DNA.

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