

## Characterization of the Two Small Subunits of *Saccharomyces cerevisiae* DNA Polymerase $\delta^*$

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Yeast DNA polymerase  $\delta$  (Pol $\delta$ ) has three subunits of 125, 58, and 55 kDa. The gene for the 125-kDa catalytic subunit (*POL3*) has been known for several years. Here we describe the cloning of the genes for the 58- and 55-kDa subunits using peptide sequence analysis and searching of the yeast genome data base. The 58-kDa subunit, encoded by the *POL31* gene, shows 23–28% sequence similarity to the 48-kDa subunit of human Pol $\delta$  and to *S. pombe* Cdc1. *POL31* is allelic to *HYS2* and *SDP5*. The 55-kDa subunit is encoded by the *POL32* gene (ORF YJR043c in the yeast data base). Very limited sequence similarity was observed between Pol32p and *Schizosaccharomyces pombe* Cdc27, the functionally analogous subunit in *S. pombe* Pol $\delta$ . The *POL32* gene is not essential, but a deletion mutant shows cold sensitivity for growth and is sensitive to hydroxyurea and DNA damaging agents. In addition, lethality was observed when the *POL32* deletion mutation was combined with conditional mutations in either the *POL3* or *POL31* gene. *Pol32 $\Delta$*  strains are weak antimutators and are defective for damage-induced mutagenesis. The *POL32* gene product binds proliferating cell nuclear antigen. A gel filtration analysis showed that Pol32p is a dimer in solution. When *POL31* and *POL32* were co-expressed in *Escherichia coli*, a tetrameric (Pol31p-Pol32p)<sub>2</sub> species was detected by gel filtration, indicating that the two subunits form a complex.

DNA polymerase  $\delta$  (Pol $\delta$ )<sup>1</sup> is the major replicative DNA polymerase in the eukaryotic cell. This insight is based on extensive *in vitro* studies using the simian 40 virus as a model system, and on genetic and biochemical studies in the yeast *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (reviewed in Refs. 1 and 2). These genetic studies have not only shown a role for Pol $\delta$  in bulk DNA replication but also for maintaining genome fidelity via the proofreading exonuclease activity of this enzyme (3, 4). A similar, but perhaps less defined role has also been identified for DNA polymerase  $\epsilon$ ,

whereas the synthetic function of DNA polymerase  $\alpha$ -primase appears to be limited to that of initiator RNA-DNA synthesis for priming Okazaki fragments on the lagging strand of the DNA replication fork (5–7).

The best characterized Pol $\delta$  from mammalian cells is the enzyme purified from fetal calf thymus tissue, a heterodimer with a catalytic subunit of 125 kDa and a second subunit of 48 kDa (8). The small subunit is required for efficient stimulation of the polymerase processivity by the proliferating cell nuclear antigen (PCNA) (9, 10).

The forms of Pol $\delta$  isolated from bakers' and fission yeast are more complex. Our previous studies of *S. cerevisiae* Pol $\delta$  indicated that the enzyme might consist of three or more subunits (11). Very recently, Pol $\delta$  has been isolated from *S. pombe* as an enzyme with five distinct subunits, four of which also appear to be subunits based on genetic arguments (12).

In this paper we describe an improved purification of *S. cerevisiae* Pol $\delta$  as a three-subunit enzyme, the cloning of the two small subunits of Pol $\delta$ , and a genetic and biochemical analysis of those subunits. Surprisingly, deletion of the gene for the smallest subunit, *Pol32*, was not a lethal mutation. Nevertheless, it resulted in a phenotype consistent with its involvement in DNA replication. We also show that this subunit interacts with PCNA. In the accompanying article (55), the properties of a two-subunit form of Pol $\delta$ , which is analogous to mammalian Pol $\delta$ , is compared with the three-subunit form.

### MATERIALS AND METHODS

**Strains**—The *Escherichia coli* strains used were DH5 $\alpha$ , BL21 (DE3), and ABLE-C, lac(lacZ $\omega$ -)[KanR McrA- McrCB- McrF- Mrr- HsdR(rK-mK-)] [F' proAB lacIqZDM15 Tn10 (TetR)]. Some of the plasmids containing the *POL32* gene were toxic to *E. coli*. They were successfully propagated in ABLE-C cells (Stratagene, La Jolla, CA) which maintained these plasmids at a reduced copy number. The yeast strains are listed in Table I. Most strains were constructed using standard genetic methods. To obtain a "START to STOP" *POL32* deletion strain, the *HIS3* gene was PCR amplified with two-hybrid *POL32-HIS3* primers as described (13). The PCR product was used to transform diploid strain YM4590 to His<sup>+</sup>. Correct integration of *HIS3* in the *POL32* locus was confirmed by PCR with appropriate primers and by Southern analysis. The resulting diploid was sporulated to give PY74a. To obtain additional *POL32* deletion mutants, genomic DNA from PY74 was PCR amplified with two primers which were located about 330 nucleotides upstream and 250 nucleotides downstream of the *HIS3* integration site, respectively, and the appropriate *his3* strains, e.g. PY116, transformed with the PCR product to His<sup>+</sup>, PY117 (Table I). Proper integration and loss of the *POL32* gene were confirmed by PCR and by Southern analysis.

To obtain the strains used for the synthetic-lethal analysis, PY42 was crossed with PY84 and the diploid sporulated. Desired isogenic haploid strains were obtained that were either *pol32 $\Delta$* , *cdc2-1*, or both, and were in addition *ura3-52* and contained pBL384. These strains were transformed with plasmid pBL389. Subsequently, isolates which had lost plasmid pBL384 were obtained by growth on tryptophan-containing media, resulting in strains PY131, PY132, and PY130, respectively (Table I). A derivative of PY130 that contained both plasmids pBL384 and pBL389 was also used in the analysis (Table III). *Pol31<sup>ts</sup>* (*hys2-1*)

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<sup>1</sup> The abbreviations used are: Pol $\delta$ , DNA polymerase  $\delta$ ; Pol $\delta^*$ , Pol $\delta$  lacking Pol32p; PCNA, proliferating cell nuclear antigen; RF-C, DNA replication factor C; PCR, polymerase chain reaction; MMS, methylmethane sulfonate; BuPhdGTP, *N*<sup>2</sup>-(*p*-*n*-butylphenyl)-2'-deoxyguanosine-5'-triphosphate; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; Tricine; *N*-[2-hydroxy-1,1-bis(hydroxy-methyl)ethyl]glycine; HPLC, high performance liquid chromatography.

TABLE I  
Yeast strains

Strain	Genotype	Source
BJ405	<i>MAT<math>\alpha</math> trp1 prb1-1122 prc1-407 pep4-3.</i>	E. Jones
KSH542	<i>MAT<math>\alpha</math> ade1 his2 his3-<math>\Delta</math>200 trp1 ura3 leu2 hys2-1</i>	Ref. 24
KSH542-1	<i>MAT<math>\alpha</math> ade1 his2 his3-<math>\Delta</math>200 trp1 ura3 leu2 hys2-1 (pBL389 [POL32 URA3])</i>	This study
L40	<i>MAT<math>\alpha</math> ade2 his3-<math>\Delta</math>200 leu2-3,112 trp1-901 LYS2:::(lexAop)<sub>4</sub>HIS3 URA3:::(lexAop)<sub>3</sub>lacZ</i>	S. Elledge
PY42	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 trp1<math>\Delta</math> his3-<math>\Delta</math>200 can1</i>	This study
PY74a	<i>MAT<math>\alpha</math> ura3-52 his3-<math>\Delta</math>200 lys2-801 leu2-3,112 trp1 tyr1 can1 pol32<math>\Delta</math>::HIS3</i>	This study
PY80	<i>MAT<math>\alpha</math> trp1-<math>\Delta</math> his3-<math>\Delta</math>200 cdc2-1</i>	This study
PY82	<i>MAT<math>\alpha</math> ade2-101 leu2-3,112 lys2-801 ura3-52 his3-<math>\Delta</math>200 pol32<math>\Delta</math>::HIS3</i>	This study
PY83	<i>MAT<math>\alpha</math> ade2-101 leu2-3,112 lys2-801 ura3-52 his3-<math>\Delta</math>200</i>	This study
PY84	<i>MAT<math>\alpha</math> trp1-<math>\Delta</math> his3-<math>\Delta</math>200 cdc2-1 pol32<math>\Delta</math>::HIS3(pBL384 [POL32 TRP1])</i>	This study
PY116	<i>MAT<math>\alpha</math> ura3-52 trp1-<math>\Delta</math> his3-11,15 leu2-3,112 pep4-3 prb1-1122 nucl::LEU2</i>	This study
PY117	<i>MAT<math>\alpha</math> ura3-52 trp1-<math>\Delta</math> his3-11,15 leu2-3,112 pep4-3 prb1-1122 nucl::LEU2 pol32<math>\Delta</math>::HIS3</i>	This study
PY130	<i>MAT<math>\alpha</math> cdc2-1 his3-<math>\Delta</math>200 leu2-3,112 ura3-52 trp1<math>\Delta</math> can1 pol32<math>\Delta</math>::HIS3 (pBL389[POL32 URA3])</i>	This study
PY131	<i>MAT<math>\alpha</math> his3-<math>\Delta</math>200 leu2-3,112 ura3-52 trp1<math>\Delta</math> can1 pol32<math>\Delta</math>::HIS3 (pBL389 [POL32 URA3])</i>	This study
PY132	<i>MAT<math>\alpha</math> cdc2-1 his3-<math>\Delta</math>200 leu2-3,112 ura3-52 trp1<math>\Delta</math> (pBL389 [POL32 URA3])</i>	This study
PY133	<i>MAT<math>\alpha</math> ade1 his3-<math>\Delta</math>200 trp1 ura3 leu2 hys2-1 pol32<math>\Delta</math>::HIS3 (pBL389 [POL32 URA3])</i>	This study
YM4590	<i>MAT<math>\alpha</math>/MAT<math>\alpha</math> ura3-52/ura3-52 his3-<math>\Delta</math>200/his3-<math>\Delta</math>200 lys2-801/lys2-801 leu2-3,112/leu2-3,112 trp1/trp1 tyr1/tyr1 can1/+</i>	M. Johnston

mutant strain KSH542 was transformed with centromere plasmid pBL331 (*POL31 TRP1*). In this plasmid-containing strain, the *POL32* gene was deleted by integrative transformation with the *HIS3* cassette as described above. This strain was then transformed with plasmid pBL389 and subsequently, isolates that had lost plasmid pBL331 were obtained by growth on tryptophan-containing media, resulting in strain PY133 (Table I). A derivative of PY133 which contained both plasmids pBL331 and pBL389 was also used in the analysis (Table III).

**Media**—Standard media were used as described (14). YPDA medium is yeast extract/peptone/dextrose with 20  $\mu$ g/ml adenine. Hydroxyurea (75 mM final concentration) was added to YPDA after autoclaving.

**Plasmids**—With the exception of pBL385, all plasmids in this study are derived from standard ColE1 based Amp<sup>R</sup> vectors. Gene coordinates are based on those given in the *Saccharomyces* genome data base (<http://genome-www.stanford.edu/Saccharomyces/>) with the first nucleotide of the translation initiation codon defined as +1. In the genome data base the *POL31* (*HYS2,SDP5*) gene is YJR006w and the *POL32* gene is YJR043c. Plasmid pBL331 contains a genomic *XbaI-SalI POL31* fragment (coordinates -139 to +2044) cloned into the *SpeI-SalI* sites of pRS314 (Bluescript *ARS CEN TRP1*) (15). The *POL31* gene was PCR amplified with an amino-terminal primer which introduces an *NcoI* site at the initiation codon position and a carboxyl-terminal primer which extends beyond the genomic *PvuII* site (coordinate +1559). The *NcoI-PvuII* fragment was cloned into the *NcoI-HindIII* (filled) sites of pPY55, thereby putting the *POL31* gene under control of the phage T7 promoter (16). This is plasmid pBL361. The accuracy of the amplified DNA was confirmed by DNA sequence analysis. pBL384 contains a 2.2-kilobase yeast genomic *SpeI-PstI* fragment (coordinates -542 to +1656) cloned into the *SpeI-PstI* sites of pRS314 (Bluescript *ARS CEN TRP1*). pBL389 was derived from pBL384 by recloning *POL32* into pRS316 (Bluescript *ARS CEN URA3*) using appropriate polylinker restriction sites. The *POL32* gene in pBL384 was PCR amplified with a primer which introduces a *BclI* site at nucleotides 3-8 and a carboxyl-terminal primer which extends beyond the *HincII* site (nucleotides +1113). The *BclI* (filled)-*HincII* fragment was cloned into the *NcoI* (filled)-*SalI* (filled) sites of pMON5839 (pACYCori Kan<sup>R</sup>) putting the gene under control of the Ptac-G10L promoter-leader. This is plasmid pBL385.

The plasmids used in the two-hybrid analysis were made in vector pCH435 (derived from pGBT8 by insertion of a more extensive polylinker; a gift of Dr. Chris Hardy, Washington University) for fusions to the bacterial *lexA* DNA-binding domain, and in pACT2 for fusions to the yeast *GAL4* activation domain. In the *POL30*, *POL31*, and *POL32* fusion constructs, the translational start sites of these genes were placed at appropriate polylinker sites, whereas in the *POL3* construct the *NcoI* site at amino acid 9 was used for fusions. The resulting fusion plasmids were pBL237 (*lexA-POL30*), pBL240 (*GAL4AD-POL30*), pBL323 (*GAL4AD-POL3*), pBL363 (*lexA-POL31*), pBL364 (*GAL4AD-POL31*), pBL390 (*lexA-POL32*), and pBL391 (*GAL4AD-POL32*).

**Purification of Pol $\delta$** —The purification is largely based on the one published previously (11). All purification steps were carried out at 0-4  $^{\circ}$ C. Unless otherwise indicated, all buffers contained 10% glycerol, 1 mM EDTA, 0.2 mM EGTA, 3 mM dithiothreitol, 0.02% Nonidet P-40, 10 mM NaHSO<sub>3</sub>, 2  $\mu$ M pepstatin A, and 4  $\mu$ M leupeptin. The NaCl concentration in millimolar in the buffer is indicated with a subscript. Strain

BJ405 was grown, broken open, and fractionated with ammonium sulfate as described before (11).

The ammonium sulfate precipitate from 2 kg of yeast cells was resuspended in 500 ml of GF buffer (25 mM potassium phosphate, pH 7.2) and passed through a 4000-ml Bio-Gel P-4 desalting column in the same buffer. The desalted material contained residual ammonium sulfate and was diluted with buffer GF to obtain a conductivity equivalent to GF<sub>80</sub>. It was batch-absorbed to 500 ml of S-Sepharose equilibrated in GF<sub>50</sub>. The beads were washed with 1000 ml of GF<sub>100</sub> and eluted with 1200 ml of GF<sub>600</sub>. Protein-containing fractions (500 ml) were dialyzed against 2  $\times$  3 liters of buffer QS (40 mM triethanolamine-HCl, pH 7.3) for 4 and 16 h. The dialysate which had a conductivity equivalent to QS<sub>40</sub> was cleared by centrifugation and loaded onto a 300-ml Q-Sepharose HR column (Pharmacia). After washing with 2 column volumes of QS<sub>40</sub> the column was eluted with a 3-liter linear gradient of Q<sub>40</sub> to Q<sub>500</sub>. This step separated Pol $\delta$  from the other DNA polymerases. The Pol $\delta$ -containing fractions eluted early in the gradient at  $\sim$ QS<sub>150-200</sub>. They were loaded on a 100-ml heparin-agarose column equilibrated in GF buffer containing 50 mM ammonium sulfate. The column was washed with 200 ml of the same buffer and eluted with a 1200-ml linear gradient from 50 to 700 mM ammonium sulfate in buffer GF. The Pol $\delta$ -containing fractions eluted late in the gradient at  $\sim$ 400-500 mM ammonium sulfate. They were dialyzed against 2 volumes of saturated ammonium sulfate. The precipitate was collected by centrifugation, dissolved in 15 ml of buffer PR (30 mM potassium phosphate, pH 7.2, 10% ethylene glycol, 0.2% ampholytes 3.5-9, and no glycerol, no Nonidet P-40) containing 1 M ammonium sulfate. Just prior to injection onto the HPLC column, 3 ml of saturated ammonium sulfate was added to bring the final ammonium sulfate concentration to 1.8 M in buffer PR. The suspension was injected onto a 20-ml propyl-silica gel HPLC column in PR containing 1.8 M ammonium sulfate and the column eluted with a 300-ml linear gradient from 1.8 to 0 M ammonium sulfate in buffer PR. Pol $\delta$  eluted prior to bulk protein early in the gradient at  $\sim$ 1.2-1.3 M ammonium sulfate. Fractions were dialyzed against buffer MS (30 mM Hepes-NaOH, pH 7.4, 0.1% ampholytes 3.5-9) until the conductivity was equivalent to that of MS<sub>100</sub> and fractionated on a 1-ml MonoS column with a 15-ml gradient from 100 to 500 mM NaCl in buffer MS. The active fractions which eluted at 300-400 mM NaCl were diluted with 2 volumes of buffer MQ (30 mM triethanolamine-HCl, pH 7.3, no NaHSO<sub>3</sub>, 0.1% ampholytes 3.5-9) and fractionated on a 0.3-ml MonoQ column with a 5-ml linear gradient from 50 to 500 mM NaCl in MQ buffer. Pol $\delta$  eluted as a single peak at MQ<sub>200</sub>.

**Peptide Sequence Analysis**—Pol $\delta$  MonoQ fraction (30  $\mu$ g) was concentrated by acetone precipitation and separated by 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue, appropriate gel slices were excised and treated with lysyl endopeptidase as described (17). The peptides were recovered from the gel slices by centrifugation through a nylon mesh (18). They were separated by reverse phase HPLC and sequenced by the protein chemistry facility at Washington University.

**Overexpression of POL31 and POL32 in E. coli**—Strain BL21 (DE3) contained pBL361 (*POL31*), pBL385 (*POL32*), or both. A single colony was grown overnight in 10 ml of LB medium with 100  $\mu$ g/ml ampicillin, 50  $\mu$ g/ml kanamycin, or both, and inoculated into 1 liter of the same medium at 37  $^{\circ}$ C. When the OD<sub>595</sub> reached 0.6, isopropyl  $\beta$ -D-thiogalac-

topyranoside was added to the culture to a final concentration of 1 mM, and the culture shaken for another 3 h at 37 °C. The culture was then harvested and the cells suspended in 5 ml of 50 mM Tris-HCl, pH 8.1, 10% sucrose, and an equal volume of 2  $\times$  lysis buffer was added (lysis buffer is 50 mM Tris-Cl, pH 8.1, 2 mM EDTA, 0.2 mM EGTA, 2  $\mu$ M leupeptin, 2  $\mu$ M pepstatin A, 5 mM sodium bisulfite, and 3 mM dithiothreitol). All further steps were carried out at 0–4 °C. Lysozyme was added to 0.6 mg/ml and the mixture was stored on ice for 30 min with occasional mixing. Nonidet P-40 and phenylmethylsulfonyl fluoride were then added to 0.05% and 1 mM, respectively, and, after another 10 min on ice, the mixture was sonicated to reduce the viscosity. After a spin at 27,000  $\times$  g for 20 min, the supernatant was discarded and the precipitate was washed with 10 ml of wash buffer (lysis buffer, except that the pH of the Tris-HCl was reduced to 7.5 and NaCl was added to 2 M final). After repeating the wash procedure, the precipitate was homogenized with 10 ml of denaturation buffer (50 mM Tris-HCl, pH 7.5, 6 M urea, 2 mM EDTA, 0.2 mM EGTA, 10 mM sodium bisulfite, 2  $\mu$ M leupeptin, 2  $\mu$ M pepstatin A, 0.5 mM phenylmethylsulfonyl fluoride). The suspension was shaken gently for 1 h and spun for 30 min at 27,000  $\times$  g. The supernatant was diluted to a protein concentration of 1 mg/ml with denaturation buffer and dialyzed for 2  $\times$  3 h against 2  $\times$  200 ml of dialysis buffer (40 mM Hepes pH 7.4, 20% glycerol, 1 mM EDTA, 0.1 mM EGTA, 3 mM dithiothreitol, 2  $\mu$ M pepstatin A, 10 mM NaHSO<sub>3</sub>, 2  $\mu$ M leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 200 mM ammonium sulfate). The solution was cleared by centrifugation and stored at –70 °C. Protein concentrations were determined according to Bradford (19).

**Gel Filtration Analysis**—200- $\mu$ l samples were injected onto a 20-ml Superose 12 column in 40 mM Hepes-NaOH, pH 7.5, 10% ethylene glycol, 1 mM EDTA, 0.02% Nonidet P-40, 0.2 M NaCl, 1 mM dithiothreitol, 5 mM NaHSO<sub>3</sub>, and 2  $\mu$ M each of leupeptin and pepstatin A. The column was run at 25 °C at 0.4 ml/min. Fractions of 300  $\mu$ l were collected and analyzed by 10% SDS-PAGE.

**Protein Interaction Blots**—Protein-protein interaction blots were carried out with a PCNA derivative containing an amino-terminal phosphorylatable tag, Ph-PCNA, MRRASVGS-PCNA. Ph-PCNA was overproduced in *E. coli* from plasmid pMM83 (a gift of Michael McAlear, Wesleyan University) and purified as wild-type PCNA (20). The replication properties of Ph-PCNA were indistinguishable from wild-type (data not shown). Phosphorylation of 1  $\mu$ g of Ph-PCNA was carried out in a 50- $\mu$ l reaction containing 20 mM Hepes-NaOH, pH 7.0, 12 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 100 mM NaCl, 50  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP, and 3 units of bovine heart cAMP-dependent protein kinase (catalytic subunit, Sigma) at 30 °C for 30 min. The labeled protein was passed through a Sephadex G-50 column, equilibrated in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, to remove unincorporated radioactivity.

All blotting steps were carried out at room temperature. Yeast Pol $\delta$  or urea extracts from *E. coli* cell lysate pellets were electrophoresed in a 10% SDS-polyacrylamide gel and the proteins transferred electrophoretically to GeneScreen. The membrane was equilibrated in Buffer A (40 mM Tris-HCl pH 7.6, 150 mM NaCl, 3 mM MgCl<sub>2</sub>, 1 mM dithiothreitol), then incubated for 10 min in 7 guanidinium hydrochloride in buffer A, followed by sequential 2-fold dilutions with buffer A for 10 min each until a concentration of 0.4 M guanidinium was reached. The membrane was then immersed for 10 min in Buffer A, followed by incubation in 5% nonfat milk in buffer A for 10 min and 25% fetal calf serum in Buffer A for 20 min to block the membrane. The membrane was probed with 100 ng/ml of <sup>32</sup>P-labeled Ph-PCNA (1–2  $\times$  10<sup>6</sup> cpm/ml) in Buffer A containing 25% fetal calf serum for 2 h at room temperature, then washed in 2  $\times$  50 ml of buffer A for 1–2 min, and processed for autoradiography (21).

**DNA Polymerase Assay**—The 50- $\mu$ l reaction contained 20 mM Tris-HCl, pH 7.8, 8 mM MgAc<sub>2</sub>, 0.2 mg/ml bovine serum albumin, 4% glycerol, 1 mM dithiothreitol, 80  $\mu$ M each dATP, dGTP, and dCTP, 20  $\mu$ M [<sup>3</sup>H]dTTP (400 cpm/pmol), 200  $\mu$ g/ml activated salmon sperm DNA, 1 mM spermidine, and enzyme. Assays were assembled on ice and incubated at 37 °C for 30 min. They were stopped by addition of 100  $\mu$ l of 25 mM EDTA, 25 mM sodium pyrophosphate, and 50  $\mu$ g/ml salmon sperm DNA, followed by 1 ml of 10% trichloroacetic acid. After 10 min on ice, the mixture was filtered over a GF/C filter. The filter was washed with 2  $\times$  2 ml of 1 M HCl, 0.05 M sodium pyrophosphate, rinsed with ethanol, dried, and counted in a counting fluid in a liquid scintillation counter. One unit of enzyme incorporates 1 pmol/min of nucleotide into acid-insoluble radioactivity. When inhibition by BuPhdGTP was measured, the concentration of dGTP was lowered to 10  $\mu$ M.

**Measurements of Spontaneous Mutation Rates and Damage-induced Mutagenesis**—To measure forward mutation rates to canavanine resistance, PY82 or PY83 cells were grown to saturation in YPDA broth,

TABLE II  
Purification of Pol $\delta$

One unit of polymerase incorporates 1 pmol/min of nucleotide into acid-insoluble radioactivity using activated DNA as a template-primer. See "Materials and Methods" for details.

Step	Mg	Units ( $\times 10^{-3}$ )	Units/mg ( $\times 10^{-3}$ )
Ammonium sulfate	16,000	9,200	0.6
S-Sepharose	2,400	7,800	3.2
Q-Sepharose HR	355	1,800	5.0
Heparin-agarose	75	1,500	15
Propyl silica gel	0.9	650	720
MonoS	0.38	420	1,100
MonoQ	0.23	300	1,300

diluted to approximately 100 cells/ml in 20 separate cultures for each strain, and again grown to saturation in YPD. Cells were then plated on complete synthetic media without arginine and with 80  $\mu$ g/ml canavanine (14). Colonies appearing after 4 days of growth at 30 °C were counted. Reversion or suppression of the *lys2-1* ochre mutation was measured similarly in strains PY71 and PY125. Plating was on synthetic complete media lacking lysine.

20-ml cultures of PY82 and PY83 each were grown to saturation at 30 °C. The cells were collected by centrifugation, washed with water, resuspended to 5  $\times$  10<sup>7</sup> cells/ml in 50 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2, briefly sonicated to disperse clumped cells, and treated with 0.75% MMS at room temperature. Aliquots were quenched in an equal volume of 10% cold Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. The cultures were either further diluted and plated on complete synthetic medium lacking arginine to determine survival, or concentrated 4-fold and plated on complete synthetic medium without arginine and with 80  $\mu$ g/ml canavanine to determine mutation frequencies. Several canavanine plates were used to obtain mutation frequency data points when survival was low. To obtain UV-survival curves and UV-induced mutagenesis frequencies, the sonicated cells in phosphate buffer were plated on the two media and immediately irradiated with the indicated doses of UV light. Colonies appearing after 4 days of growth at 30 °C were counted.

## RESULTS

**Purification of Pol $\delta$** —Our attempts to obtain acceptable quantities of Pol $\delta$  based on the original purification scheme were hampered by several problems relating to enzyme instability, enzyme loss, and proteolysis (11). In particular, use of a mildly hydrophobic HPLC column (propyl silica gel) resulted in large losses in yield and activity. Yet this matrix appeared very desirable as it greatly separated Pol $\delta$  from bulk protein. We found that inclusion of broad-range ampholytes (3.5–9) in the buffers substantially increased both yield and activity of Pol $\delta$ , in particular when silica gel columns were used. Therefore, ampholytes were included in all columns starting at the propyl silica gel step. The purification is summarized in Table II.

Analysis of the final preparation by SDS-PAGE showed three polypeptides of 125, 58, and 55 kDa which copurified (Fig. 1). The presence of the 55-kDa polypeptide had varied considerably between preparations of Pol $\delta$ . It was present at apparent stoichiometrical levels in some preparations (11), but virtually absent from others (22). This variation was found to be due to highly variable staining intensity of this subunit with silver. The silver staining method by Morrissey (23) gave the most consistent results and confirmed that the 55-kDa polypeptide was present in all preparations of Pol $\delta$  analyzed (data not shown). When 30  $\mu$ g of Pol $\delta$  was separated by SDS-PAGE in preparation for peptide sequencing and the gel stained by Coomassie, the same three polypeptides and no significant levels of other polypeptides were observed (data not shown).

**Isolation of the POL31 Gene**—Amino acid sequence analysis resulted in only one short peptide sequence for the 58-kDa subunit (Fig. 2). A BLAST search of the complete yeast data base revealed only one perfect match to this sequence, but there were several other genes with only one mismatch. The perfect match was with *HYS2*. This gene was initially isolated

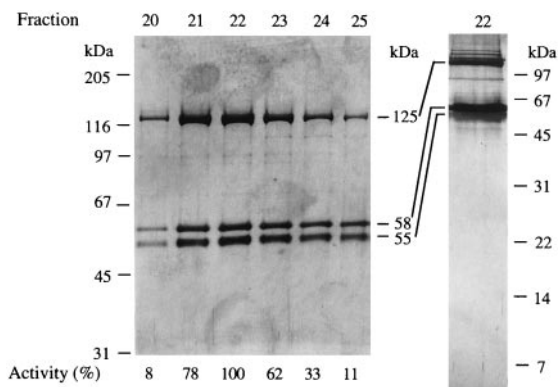


FIG. 1. SDS-PAGE of the MonoQ step of Polδ. MonoQ fractions were analyzed on a 10% SDS-PAGE gel and the peak fraction also on a 10–20% SDS-PAGE gradient gel in Tricine buffer (46). Bands were visualized by silver staining (23).

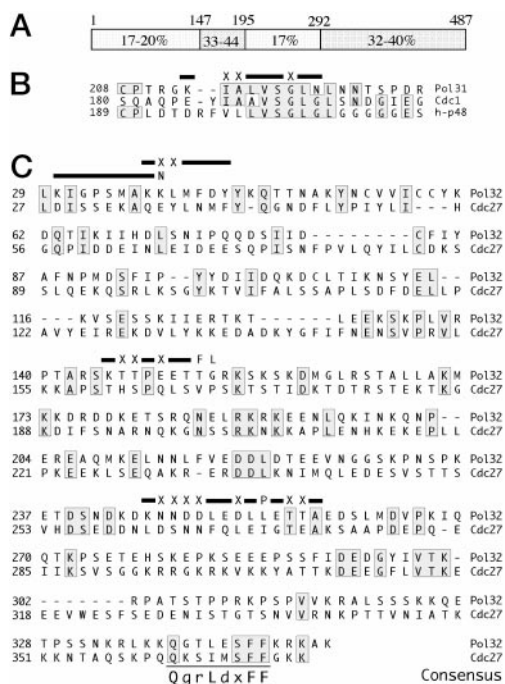


FIG. 2. Analysis of the *POL31* and *POL32* genes. A, diagram of sequence similarities between Pol31p, *S. pombe* Cdc1, and the human 48-kDa subunit. B, sequence comparison for a small region of Pol31p and location of a peptide sequence obtained from the 58-kDa subunit. C, sequence comparison between Pol32p and *S. pombe* Cdc27. Locations of peptide sequences obtained for the 55-kDa subunit are also indicated. A bar designates identity, an X indicates an uninformative position in the peptide sequence, and when the actual amino acid is shown, a disagreement exists with the amino acid sequence deduced from the *POL32* gene sequence. The underlined octapeptide sequence (338–345 for *POL32*) shows strong similarity to the consensus PCNA-binding domain identified by Warbrick *et al.* (29).

in a screen for yeast mutants which confer hydroxyurea-sensitive cell growth (24). Hydroxyurea sensitivity can result from defects in nucleotide metabolism, DNA replication, DNA repair, or cell-cycle checkpoints. *HYS2* is an essential gene and the *hys2-1* mutant isolated by Sugimoto *et al.* (24) showed a temperature-sensitive growth defect with a terminal phenotype consistent with a role for the *HYS2* gene product in DNA replication. Recently, the same gene has been identified as an extragenic suppressor, *sdp5-1*, of the temperature-sensitive growth of a *POL3* mutant (25). The *HYS2* (*SDP5*) gene shows strong sequence similarity with the small subunit gene of human Polδ and the second largest subunit of *S. pombe* Polδ (Fig. 2). This strongly indicates that the *HYS2* (*SDP5*) gene encodes

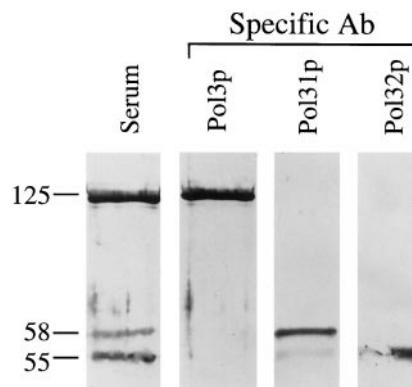


FIG. 3. Immunoblot analysis of Polδ subunits. Partially purified Polδ was separated by 10% SDS-PAGE, and transferred to GeneScreen. The membrane was cut in 4 strips and the strips were separately probed with an antiserum raised against the Polδ complex (lane 1), with antibodies raised against the catalytic subunit (lane 2), with immunoaffinity purified antibodies using *E. coli* expressed Pol31p (lane 3), with immunoaffinity purified antibodies using *E. coli* expressed Pol32p (lane 4). Immunoaffinity purification of the antiserum raised against the Polδ complex was carried out as described (47).

one of the small subunits of Polδ. We have designated this gene as *POL31* to indicate that it is the second subunit of Polδ. Positive confirmation that *POL31* encodes the 58-kDa subunit of Polδ came from two additional experiments: (i) expression of *POL31* in *E. coli* yielded a polypeptide with the exact same electrophoretic mobility as the 58-kDa subunit (data not shown). (ii) Pol31p was overproduced in *E. coli* and the protein bound to nitrocellulose. The filter was incubated with a rabbit serum raised against the entire Polδ complex. The bound antibodies were eluted from the filter with an alkaline buffer and used in a Western analysis of yeast Polδ; they recognized the 58-kDa subunit specifically (Fig. 3).

**Isolation of the *POL32* Gene**—The amino acid sequence analysis of the 55-kDa subunit yielded four short sequences (Fig. 2). A BLAST search of the yeast genome data base yielded the YJR043c open reading frame as the only possible candidate gene. Three of the four peptide sequences did not exactly correspond to the amino acid sequence predicted by the YJR043c gene in the genome data base. Therefore, the cloned gene was sequenced again. The sequence was identical to that present in the data base. We conclude that the amino acid changes, 4 out of a total of 27 informative positions, originate from peptide sequence misassignments (Fig. 2). We have designated this gene as *POL32* to indicate that it is the third subunit of Polδ. Like many other genes involved in DNA replication, the *POL32* gene has a putative *MluI* cell cycle box located 141 nucleotides upstream of the translational start site. The *MluI* cell cycle box confers periodic expression in the cell cycle with an increased expression at the G<sub>1</sub>/S phase (26).

The following biochemical experiments positively identify the *POL32* gene as the one for the 55-kDa subunit: (i) expression of *POL32* in *E. coli* yielded a polypeptide with the same electrophoretic mobility as the 55-kDa subunit (data not shown, but see Fig. 7). (ii) When Pol32p overproduced in *E. coli* was used for the immunoaffinity purification of serum raised against the entire Polδ complex, the purified antibodies recognized the 55-kDa subunit specifically (Fig. 3). (iii) Biochemical fractionation of extracts from a *POL32* mutant strain yielded a defective form of Polδ (see below). Genetic experiments described below are consistent with the biochemical results.

A BLAST search of the protein data base did not reveal any proteins with strong sequence similarities. Nor were any detected by various types of FASTA searches of the data base. Interestingly, the *S. pombe cdc27*<sup>+</sup> gene was identified among

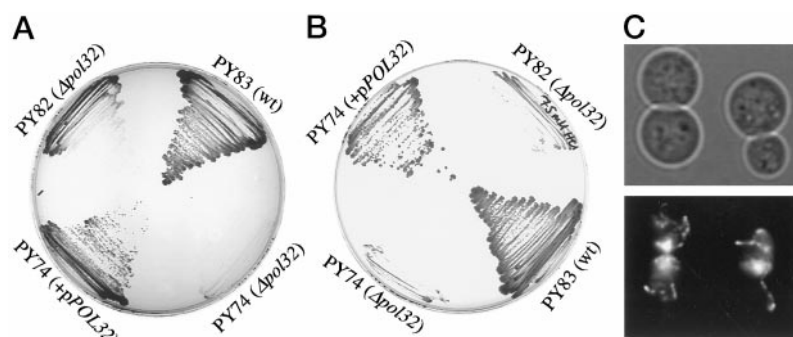


FIG. 4. **Growth defects in *POL32* deletion mutants.** Strain PY74 (*pol32 $\Delta$ ) with vector or complementing plasmid pBL384, and strains PY82 (*pol32 $\Delta$ ) and PY83 (*POL32*) were grown on YPDA medium at 13 °C for 10 days (A) or on YPDA containing 75 mM hydroxyurea at 30 °C for 3 days (B). C, strain PY74 (*pol32 $\Delta$ ) was grown in liquid YPDA medium at 30 °C in early log phase followed by growth at 13 °C for 16 h. The cells were fixed and stained with 4,6-diamidino-2-phenylindole as described (48). Shown in the upper and lower panel, respectively, are a Nomarski view and 4,6-diamidino-2-phenylindole fluorescence of two cells arrested in G<sub>2</sub>/M.***

the high scoring genes in a pattern-based FASTA search (27). The optimal alignment between Pol32p and Cdc27 contained several gaps with an overall sequence identity of 20% (Fig. 2). Many of the amino acid identities are in charged amino acids which both proteins have in abundance, diminishing the strength of the alignment. However, other considerations rather than the weak sequence similarity indicate that these two genes are functional homologues. Both Pol32p and Cdc27 are highly charged, each having 35% of strongly charged amino acids in comparison to 20–25% for most proteins. Both proteins migrate anomalously in SDS-polyacrylamide gels. Pol32p with a calculated molecular mass of 40.3 kDa migrates as a 55-kDa protein by SDS-PAGE (Fig. 1), and Cdc27 with a calculated molecular mass of 42.2 kDa migrates as a 54-kDa protein (12). Furthermore, both proteins have a box of sequence similarity at the carboxyl terminus which has previously been identified as a PCNA-binding domain (Fig. 2) (28, 29).

***POL32* Deletion Mutants Are Viable and Cold-sensitive for Growth**—The entire *POL32* gene was deleted in diploid strain YM4590 while tagging the deletion with the *HIS3* auxotrophic marker as described under “Materials and Methods.” Sporulation of the heterozygous diploid, *i.e.* *POL32/pol32 $\Delta$ ::*HIS3*, and tetrad analysis showed a 2:2 segregation for His<sup>+</sup>, indicating that the *POL32* gene is not essential for yeast cell growth. Southern analysis confirmed the deletion of the *POL32* gene in the His<sup>+</sup> spores (data not shown).*

A further investigation of the deletion strain revealed a slow-growth phenotype at 30 °C and a failure to grow at 13 °C (Fig. 4A). An analysis by fluorescence microscopy of the mutant cells incubated at the restrictive temperature showed an accumulation of large budded cells with the nucleus positioned at the neck of the bud, consistent with an arrest in the G<sub>2</sub>/M phase of the cell cycle due to replication defects (Fig. 4C).

Like the *hys2-1* mutant allele of *POL31*, *pol32 $\Delta$ , mutants are hypersensitive to the replication inhibitor hydroxyurea (24). This was expressed as a failure to grow on YPDA plates containing 75 mM hydroxyurea, which allow growth of wild-type cells (Fig. 4B). When cells were grown for 17 h in liquid YPD medium containing 200 mM hydroxyurea, the plating efficiency of the *pol32 $\Delta$  strain was 9% in comparison to 210% for the isogenic wild-type strain, *i.e.* the wild-type cells had doubled (data not shown).**

**The *pol32 $\Delta$  Mutation Exhibits Synthetic Lethality with Mutations in Other Pol $\delta$  Genes***—Our studies strongly suggest that Pol32p is part of the Pol $\delta$  complex. In order to determine further genetic interactions between *POL32* and other components of Pol $\delta$  holoenzyme, we attempted to isolate double mutants by strain crossing. In *S. cerevisiae*, the catalytic subunit of Pol $\delta$  is encoded by the *CDC2* gene, which is now commonly

referred to as *POL3* (30, 31). The *cdc2-1* mutation confers temperature-sensitive growth upon yeast (32). When crosses were carried out between a *cdc2-1* strain and a *pol32 $\Delta$  strain, no progeny were obtained which were *cdc2-1 pol32 $\Delta$ , suggesting synthetic lethality between the two mutations (data not shown). To confirm synthetic lethality, the *cdc2-1 pol32 $\Delta$  double mutant was made in the presence of a complementing centromere plasmid carrying the wild-type *POL32* gene and the *URA3* gene as a selectable marker. The strain was grown on non-selective media for 10 generations, and plasmid loss was determined in the double mutant by plating on 5-fluoroorotic acid-containing media which allows growth of those cells which have lost the plasmid (Table III). No growth was observed indicating that loss of the *POL32* complementing plasmid was not tolerated and that, therefore, a *cdc2-1 pol32 $\Delta$  double mutation is lethal. In comparison, the single mutant control strains showed 55–80% loss of the *POL32* plasmid. Loss of the complementing plasmid with the *URA3* marker in the double mutant was also tolerated if the strain contained an additional *POL32* plasmid, *e.g.* pBL384 (*POL32 TRP1*) (Table III, entry 2). Similarly, a double mutant carrying the temperature-sensitive *hys2-1* allele of *POL31* together with *pol32 $\Delta$  failed to lose a complementing centromere plasmid carrying the wild-type *POL32* gene and the *URA3* maker, indicating synthetic lethality between *pol31* and *pol32* mutations. Again, the single mutant control strain KSH542-1 readily lost the *POL32* plasmid (Table III).*****

***Pol32 $\Delta$  Mutants Are Damage-sensitive and Deficient for Induced Mutagenesis***—Pol $\delta$  participates in various forms of DNA repair including repair of methylation damage and UV damage (25, 34–36). The *pol32 $\Delta$  strain was more sensitive to UV irradiation than wild-type. Also, exposure to MMS had a profound effect on cell viability (Fig. 5).*

Spontaneous mutation rates were measured in two loci. Suppression of a *lys2* ochre mutation was decreased 2-fold in the *pol32 $\Delta$  strain in comparison to wild-type. Similarly, forward mutation rates in the *CAN1* gene to canavanine resistance were decreased 1.6-fold in the *pol32 $\Delta$  strain in comparison to wild-type. This indicates that *pol32 $\Delta$  is a weak antimutator.***

Because an antimutator phenotype may result from a defect in damage-induced mutagenesis, we determined mutation frequencies to canavanine resistance in the *pol32 $\Delta$  strain PY82 in response to UV irradiation or treatment with MMS. Maximal mutation induction in the *pol32 $\Delta$  strain in response to MMS treatment and to UV irradiation was only 2- and 5-fold, respectively, over the uninduced mutation frequencies. In comparison, induced mutation frequencies in the wild-type strain PY83 were 100- and 300-fold, respectively, over the uninduced mutation frequencies (Fig. 5). Therefore, lack of the small subunit**

TABLE III  
Plasmid loss in double mutants

All strains contained centromere plasmid pBL389 (*POL32 URA3*). The strains were obtained as described under "Materials and Methods." Their genotypes are given in Table I. An additional plasmid pBL384 (*POL32 TRP1*) or pBL331 (*POL31 TRP1*) was present as indicated. Cells were grown at 23 °C in YPDA medium for 10 generations, and approximately  $10^3$  cells plated on YPDA plates to determine cell number and  $10^3$  and  $10^5$  cells on 5-FOA plates to identify cells that had lost plasmid pBL389 (33).

Strain	Chromosomal genotype	Additional plasmid	Plasmid loss
			%
PY130	<i>pol32<math>\Delta</math> pol3<sup>ts</sup></i>		0
PY130	<i>pol32<math>\Delta</math> pol3<sup>ts</sup></i>	pBL384	30
PY131	<i>pol32<math>\Delta</math> POL3</i>		55
PY132	<i>POL32 pol3<sup>ts</sup></i>		80
PY133	<i>pol32<math>\Delta</math> pol31<sup>ts</sup></i>		0
PY133	<i>pol32<math>\Delta</math> pol31<sup>ts</sup></i>	pBL331	30
KSH542-1	<i>POL32 pol31<sup>ts</sup></i>		80

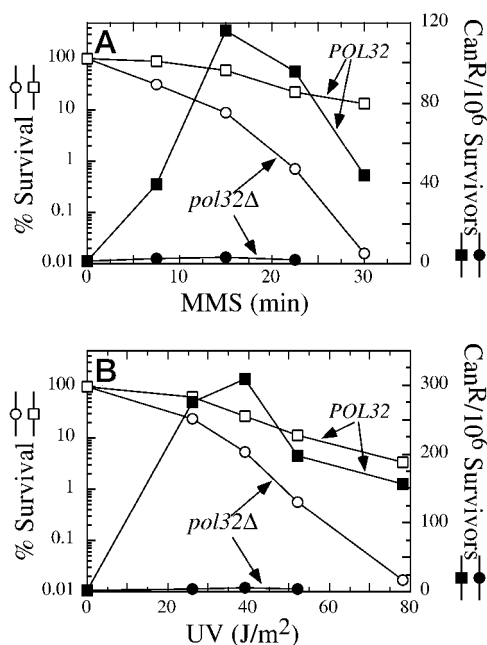


FIG. 5. *POL32* deletion strains are deficient for damage-induced mutagenesis. See "Materials and Methods" for details. Survival curves and survival-corrected mutation frequencies to canavanine resistance are shown for the *pol32 $\Delta$*  strain PY82 and the isogenic wild-type strain PY83 in response to treatment with MMS (A) or UV irradiation (B). The entire experiment was performed four times. Between experiments some variation (2–5-fold) was noted in the absolute, but not the relative, rates of killing by MMS. Variations in the UV killing curves and induced mutagenesis rates were less than 2-fold between experiments. The representative experiment is given in this figure.

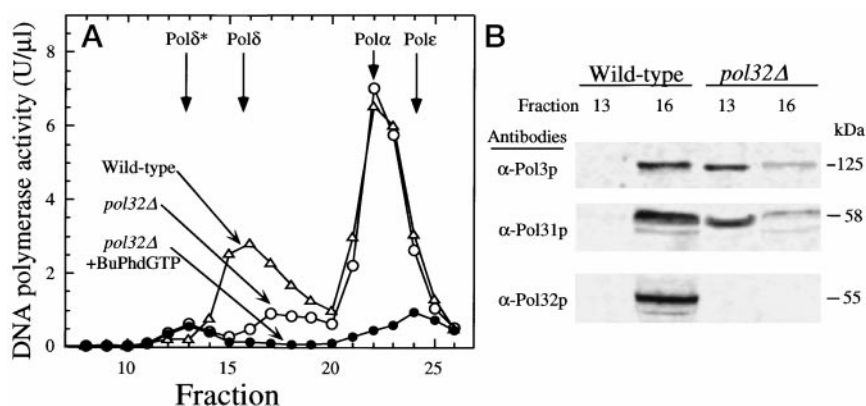
of Pol $\delta$  results in an almost complete defect in damage-induced mutagenesis.

**Defective Pol $\delta$  from a *pol32 $\Delta$*  Strain**—Lack of the 55-kDa subunit of Pol $\delta$  has serious consequences *in vivo*. To assess its effect *in vitro*, the *POL32* deletion was introduced in a protease-deficient strain used in our laboratory for biochemical studies. The protease-deficient strain and its *pol32 $\Delta$*  derivative were grown up in gram quantities, extracts were made and fractionated by phosphocellulose chromatography and MonoQ HPLC. Fractionation of extracts from wild-type cells separated, in order of elution, Pol $\delta$  from Pol $\alpha$  and Pol $\epsilon$  (Fig. 6A). In contrast, fractionation of extracts from the isogenic *pol32 $\Delta$*  strain lacked Pol $\delta$ , but showed a novel peak of polymerase activity eluting prior to that Pol $\delta$ . We have designated this new activity Pol $\delta^*$ . A Western analysis showed that the Pol $\delta^*$  peak from

*pol32 $\Delta$*  extracts contained both the catalytic subunit and the 58-kDa subunit of Pol $\delta$ , but not the 55-kDa subunit, whereas the Pol $\delta$  peak from wild-type extracts contained all three subunits (Fig. 6B).

**Interactions between Pol $\delta$  Subunits and PCNA**—A comprehensive two-hybrid analysis was carried out to determine protein-protein interactions between the three subunits of Pol $\delta$  and of PCNA with each of these three subunits (51). The *POL3* gene was fused to the acidic activation domain of *GAL4*, and the *POL31*, *POL32*, and *POL30* (PCNA) genes to both the *GAL4* activation domain and the bacterial *lexA* DNA-binding domain. Pairwise combinations of binding domain and activation domain plasmids were tested for interaction by measuring  $\beta$ -galactosidase activity in strain L40 which places this marker gene under transcriptional control of the *lex* operator (52). The results are given in Table IV. Strong interaction signals were obtained for Pol3p-Pol31p, Pol3p-Pol32p, and Pol31p-Pol32p combinations. A weak, but statistically significant interaction signal was obtained for Pol32p-Pol32p, indicating that this subunit may form a homodimer, whereas the Pol31p-Pol31p combination yielded background levels of  $\beta$ -galactosidase activity. When interactions with PCNA were measured, all interaction signals were low, like observed previously with other two-hybrid experiments involving PCNA (53, 54). For instance, even though PCNA is known to form a stable trimer, only 1.9 units of  $\beta$ -galactosidase activity was measured for the PCNA-PCNA pair (Table IV). In comparison, the interaction signal between PCNA and Pol32p was high, especially in the orientation with *POL32* fused to the *LexA* DNA-binding domain and *POL30* to the *GAL4* activation domain. The analogous orientation for the Pol31p-PCNA pair gave a very low but statistically significant signal, whereas background levels of  $\beta$ -galactosidase activity were measured for the pair in the opposite orientation. In conclusion, this analysis supports a model in which Pol3p interacts with Pol31p and with Pol32p, Pol31p interacts with Pol32p, Pol32p interacts with itself, *i.e.* forms a dimer, and PCNA interacts with Pol32p, and perhaps also with Pol31p. However, because the two-hybrid method may detect both direct and indirect interactions, we turned to biochemical methods to investigate a possible interaction between these polypeptides.

**Pol31p and Pol32p Form a Heterotetramer**—In order to test whether the interactions between the small subunits suggested from the two-hybrid analysis could be reproduced *in vitro*, the small subunits were overproduced in *E. coli*. Both Pol31p and Pol32p when overproduced in *E. coli* were found in inclusion bodies. This was also observed when Pol31p and Pol32p were overproduced simultaneously (data not shown). The three different precipitates were dissolved in a buffer containing 6 M urea and the urea was removed by stepwise dialysis. The preparations were analyzed by gel filtration. Analysis of the Pol31p preparation was uninformative. The subunit was largely aggregated and the bulk of the protein eluted in the void volume, while the rest streaked across the entire elution profile (Fig. 7B). In contrast, very little Pol32p was aggregated and the majority eluted as a single peak with an apparent  $M_r$  of 105,000, consistent with a dimeric form for this subunit (Fig. 7A). When the preparation resulting from the simultaneous overexpression of *POL31* and *POL32* was analyzed, most of Pol31p was still aggregated and most of Pol32p eluted as a dimer. However, a peak of Pol31p and a shoulder of Pol32p was observed at fractions 31 and 32, corresponding to a molecular weight of 230,000 (Fig. 7C). Quantitation of the bands in the Coomassie-stained gels showed an approximate 1:1 ratio of Pol31p and Pol32p in these fractions (Fig. 7E). To confirm that



**FIG. 6. Analysis of DNA polymerase in a *POL32* deletion strain.** *A*, protease-deficient strain PY116 and the *pol32* $\Delta$  derivative PY117 were grown, lysed, and fractionated by phosphocellulose chromatography and MonoQ HPLC as described (49). Polymerase activity in the fractions was determined. See "Materials and Methods" for details. The activity in fractions 12–16 of PY117 was due to unidentified forms of Pol $\alpha$ , as the activity was inhibited by *N*<sup>2</sup>-(*p*-n-butylphenyl)-2'-deoxyguanosine-5'-triphosphate (*BuPhdGTP*) (50). *B*, immunoblot analysis of fraction 13, corresponding to the Pol $\delta^*$  peak, and fraction 16, corresponding to Pol $\delta$  peak. Aliquots (100  $\mu$ l) of fractions 13 and 16 from the wild-type and *pol32* $\Delta$  strains were concentrated by precipitation with acetone and separated by 10% SDS-PAGE. This procedure was carried out for three separate gels. Protein was then transferred to GeneScreen. The three membranes were separately probed with an antiserum raised against the catalytic subunit of Pol $\delta$  (*top*), with immunoaffinity purified antibodies against Pol31p (*middle*), and with immunoaffinity purified antibodies against Pol32p (*bottom*). Only the regions of interest are shown. See "Materials and Methods" and the legend to Fig. 3 for details.

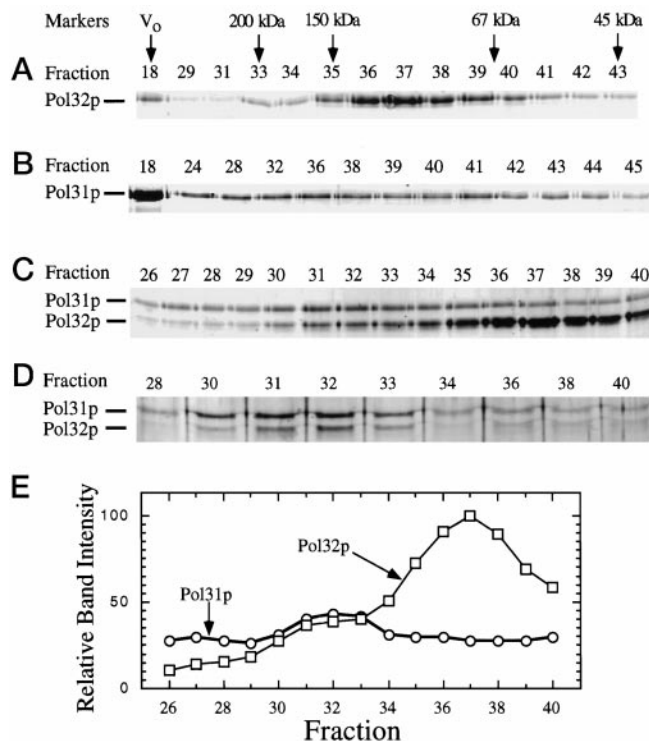
TABLE IV  
*In vivo* protein-protein interactions

Strain L40 (Table I) was transformed with a *lexA*-binding domain plasmid and a *GAL4* activation domain plasmid. Three independent transformants were grown up and  $\beta$ -galactosidase activity determined as described (14). The experiment was carried out twice for strains with >10 units and three times for strains with <10 units of  $\beta$ -galactosidase activity. Control strains contained the binding domain vector plus each of the activation domain plasmids, or the activation domain vector plus each of the binding domain plasmids. Activities in all control strains were 0.5–0.8 ( $\pm$ 0.2) units of  $\beta$ -galactosidase activity. The data in the table are given in standard units of  $\beta$ -galactosidase activity ( $\pm$ S.D.) and are corrected for background activity.

GAL4-activation domain fusion	LexA-binding domain fusion		
	POL31	POL32	POL30
POL3	140 (10)	21 (5)	0
POL31	0	84 (27)	0
POL32	121 (30)	1.3 (0.4)	2.0 (0.5)
POL30	1.1 (0.6)	8 (3.1)	1.9 (0.6)

fractions 31 and 32 contained a distinct complex, these fractions were combined and reinjected onto the column. Coelution of the bulk of both subunits at fraction 31 and 32 confirmed the existence of a complex with an apparent molecular weight of 230,000 (Fig. 7D). However, because the subunits were visualized by silver staining, quantitation of the data could not be carried out. This analysis shows that Pol31p and Pol32p form a complex in the absence of the catalytic subunit of Pol $\delta$  and indicate that this complex may be that of a dimer of a heterodimer:(Pol31p·Pol32p)<sub>2</sub>.

**Pol32p Interacts with PCNA**—The subunits of Pol $\delta$  were separated by SDS-PAGE, transferred to nitrocellulose, and probed with a <sup>32</sup>P-labeled form of PCNA. Under the experimental conditions used, binding of PCNA was only observed to the Pol32p subunit, but not to Pol3p nor to Pol31p (Fig. 8). These data largely agree with the two-hybrid results (Table IV). Extracts from *E. coli* cells overexpressing *POL32*, but not extracts from cells overexpressing *POL31*, showed binding of <sup>32</sup>P-labeled PCNA confirming that the binding was specific for this subunit. The signal was abolished by inclusion of an excess of cold PCNA (Fig. 8) or Pol32p (data not shown) in the blotting solution. Regardless of the different experimental conditions used in this assay (renaturation and probing were carried out



**FIG. 7. A heterotetrameric complex of Pol31p and Pol32p.** Pol32p (*A*), Pol31p (*B*), or Pol31p/Pol32p (*C*), derived from overexpression in *E. coli* were analyzed by gel filtration on a Superose 12 column. *D*, re-injection of fractions 31 and 32 from the analysis in *C*. *E*, scan of the analysis shown in *C*. Proteins were separated by 10% SDS-PAGE and visualized by Coomassie staining (*A-C*) and silver staining (*D*). In all four separations, the same fraction numbers correspond to the same elution positions. Size markers are apoferritin (445 kDa),  $\beta$ -amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), and ovalbumin (45 kDa).

at pH 6.8; 0.05% Triton X-100 was included during renaturation and probing; the guanidinium hydrochloride renaturation was omitted; probing was carried out in different buffers, e.g. with EDTA instead of Mg or in 30 mM NaCl instead of 150 mM NaCl) no interaction signal between PCNA and Pol31p could be detected (data not shown).

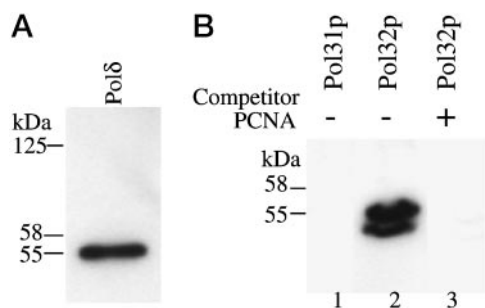


FIG. 8. **Interaction of Pol32p with PCNA.** 200 ng of Pol $\delta$  (A) or 2  $\mu$ g of total protein of a 6 M urea extract from an *E. coli* strain overproducing Pol31p (lane 1) or Pol32p (lanes 2 and 3) (B) was separated by 10% SDS-PAGE and transferred to GeneScreen. The filters were probed with 100 ng of  $^{32}$ P-labeled PCNA. In the experiment in lane 3, 20  $\mu$ g of cold PCNA were added to the probing solution. See "Materials and Methods" for details. The 50-kDa band in panel B, lane 2 is due to proteolysis of Pol32p in *E. coli* extracts.

#### DISCUSSION

**Subunit Structure of Pol $\delta$** —The enormous advantage of the availability of the *Saccharomyces* genome data base is exemplified in the project described in this paper. Peptide sequences derived from the two small subunits of Pol $\delta$  were obtained in 1992–1993. However, these were too small for any attempts at gene isolation. Periodic BLAST searches of the growing yeast sequence data base gave positive hits in 1995. Further biochemical studies allowed an unambiguous assignment of the genes for the 58-kDa as YRJ006w and the 55-kDa subunit as YJR043c, respectively. These genes have been renamed *POL31* and *POL32*, respectively, to indicate that they are subunits of Pol $\delta$ .

Whereas the catalytic subunit of Pol $\delta$  is highly conserved between species with about 45% sequence identity between the *S. cerevisiae* and *S. pombe* subunit, there is much less sequence conservation among the small subunits. It is about 25–30% for the second subunit and only about 15–20% for the third subunit (Fig. 2). Both *S. cerevisiae* *POL31* and its *S. pombe* homologue *cdc1*<sup>+</sup> are essential for cell growth (24, 37). Surprisingly, the *POL32* gene is not essential whereas the *S. pombe* *cdc27*<sup>+</sup> gene is. However, growth of *pol32 $\Delta$  strains is impaired, particularly at 13 °C, and lethality ensues when the replication apparatus is further crippled by mutations in either the catalytic or second subunit of Pol $\delta$  (Table III). Preliminary results also show synthetic lethality of *pol32 $\Delta$  with cold-sensitive mutants in the *POL30* gene, encoding PCNA.<sup>2</sup>**

No other subunits were detected in our most purified preparations of Pol $\delta$ . The *S. pombe* form of Pol $\delta$  has two additional subunits, one of 42 kDa encoded by an unknown gene on cosmid c9G1, and one of 22 kDa encoded by the *cdm1*<sup>+</sup> gene. Disruption of the *cdm1*<sup>+</sup> gene showed no phenotype, but the gene is a likely subunit of Pol $\delta$  as overproduction of Cdm1 suppresses the temperature sensitivity of a *cdc1* mutant (38). A search of the *S. cerevisiae* data base did not identify a gene with sequence similarity to Cdm1. The 42-kDa gene shows 43% amino acid identity with the *S. cerevisiae* *GCD14* gene. *GCD14* was originally isolated in a genetic screen for mutants which show constitutive expression of *Gcn4p*, which is required for basal expression of some amino acid synthetic genes. Recent studies have shown that *Gcd14p* is required for maturation of the initiator methionine tRNA (39). Although a possible link between translation and the elongation of DNA replication can-

not be excluded, based on our current understanding of cell cycle control, *Gcd14p* should not be considered a likely subunit of Pol $\delta$ .

**Repair Defects in Pol $\delta$** —A large number of mutations have been isolated in the *POL3* gene, three mutations in the *POL31* gene and the single deletion mutation of the *POL32* gene described here (24, 25, 36, 40–42). All conditional mutants tested are sensitive to hydroxyurea, indicating defects in DNA replication. Similarly, sensitivity to alkylating agents is greatly enhanced in mutants containing mutations in one of the three Pol $\delta$  genes (e.g. see Fig. 6). However, Pol $\delta$  mutations appear to fall in two classes with regard to the effect of UV irradiation. Most *POL3* mutants are not sensitive to UV. These include the *cdc2-1*, *cdc2-2*, *hpr6-1*, *mut7-1*, *pol3-01*, and *pol3-14* alleles of *POL3* (25, 36, 40–42). When mapped, the mutations fall in the exonuclease or polymerase domains of this subunit. A *POL31* mutant (*hys2-1*) is also insensitive to UV irradiation (24). Only two *POL3* mutants, *pol3-11* and *pol3-13*, with mutations in the carboxyl-terminal cysteine-rich domain of this subunit are sensitive to UV irradiation (25). The *pol32* deletion mutant is also UV-sensitive. These UV-sensitive mutants are also deficient for damage-induced mutagenesis (Fig. 6) (25). In addition, *pol32 $\Delta$  strains are weak antimutators, which may indicate that their primary repair defect is in error-prone repair. The Rev3p DNA polymerase is required for error-prone DNA repair. A *rev3* deletion mutant is deficient for error-prone repair and is a weak antimutator (43). The experiments described here and by Giot *et al.* (25) show that Pol $\delta$  also functions in the mutagenic repair of UV lesions, presumably in cooperation with Rev3p. Recent genetic studies have also implicated Rev3p and Pol $\delta$  in the repair of MMS-induced lesions, perhaps via a mutagenic pathway as well (35).*

**Biochemical Interactions between Pol32p and PCNA**—Both Pol32p and Cdc27 show a box of homology (<sup>338</sup>QGTLESFF<sup>345</sup> for Pol32p) which has previously been proposed to bind PCNA (Fig. 2). This sequence is also present in FEN-1 and in mammalian p21 (28, 29). X-ray structure analysis of the complex between human PCNA and a p21 peptide shows that the amino acids in the PCNA-binding box make contacts with the interdomain connector loop region of PCNA (44). Particularly, Met<sup>147</sup>, Phe<sup>150</sup>, and Tyr<sup>151</sup> in p21 make contacts within and near a hydrophobic pocket in PCNA formed mostly by the interdomain connector loop of PCNA. The amino acids in Pol32p analogous to p21 which are predicted to be essential to PCNA binding are Leu<sup>341</sup>, Phe<sup>344</sup>, and Phe<sup>345</sup> (Fig. 2). The hydrophobic pocket on PCNA includes Leu<sup>126</sup> and Ile<sup>128</sup>, which are universally conserved amino acids in this otherwise poorly conserved loop (21). In a recent mutational study of PCNA we showed that a mutant PCNA, *pca-79*, in which Leu<sup>126</sup> and Ile<sup>128</sup> were changed to alanine, fails to interact with Pol32p (21). These data support the proposal that proteins containing this consensus PCNA-binding domain target their interaction to the interdomain connector loop. Despite these clear similarities in protein interaction domains between yeast and human cells, considerable divergence of these interaction domains has still taken place as exemplified by the very weak interaction between human p21 and *S. cerevisiae* PCNA (45).

**Subunit Structure of Pol $\delta$** —Subunit interaction studies of *S. pombe* Pol $\delta$  have revealed interactions between the catalytic subunit and the second subunit, Pol3-Cdc1, and between the second and third subunit, Cdc1-Cdc27 (38). Our two-hybrid analysis confirms these interactions in *S. cerevisiae* Pol $\delta$  and extends it to indicate possible interactions between the catalytic and the third subunit, Pol3p-Pol32p (Table IV). Interestingly, the two-hybrid analysis indicates that Pol32p, but not Pol31p, may form a homodimer. Size fractionation studies with

<sup>2</sup> Synthetic lethality was observed between *pol32 $\Delta$  and *pol30-52* in one strain (20). In another strain, however, the double mutant is viable albeit extremely slow-growing (A. Pautz and P. Burgers, unpublished observations).*

subunits overproduced in *E. coli* confirmed the suggestion from the two-hybrid data that Pol32p is a homodimer and, moreover, that Pol31p and Pol32p interact to form a tetramer (Fig. 7). Because Pol31p overproduced in *E. coli* did not renature efficiently, we could not determine whether this subunit is a monomer or a dimer. However, the human p48 subunit expressed in *E. coli* as a soluble protein is a monomer (10). If the dimeric state of Pol32p persists in larger complexes containing the catalytic subunit of Pol $\delta$ , the interesting possibility is presented that Pol $\delta$  may consist of two catalytic cores. These studies will be reported in the second paper.

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