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Subunit Interactions within the *Saccharomyces cerevisiae* DNA Polymerase ϵ (pol ϵ) Complex

DEMONSTRATION OF A DIMERIC pol ϵ*

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Saccharomyces cerevisiae **DNA polymerase epsilon (pol ϵ) is essential for chromosomal replication. A major form of pol ϵ purified from yeast consists of at least four subunits: Pol2p, Dpb2p, Dpb3p, and Dpb4p. We have investigated the protein/protein interactions between these polypeptides by using expression of individual subunits in baculovirus-infected Sf9 insect cells and by using the yeast two-hybrid assay. The essential subunits, Pol2p and Dpb2p, interact directly in the absence of the other two subunits, and the C-terminal half of *POL2*, the only essential portion of Pol2p, is sufficient for interaction with Dpb2p. Dpb3p and Dpb4p, non-essential subunits, also interact directly with each other in the absence of the other two subunits. We propose that Pol2p·Dpb2p and Dpb3p·Dpb4p complexes interact with each other and document several interactions between individual members of the two respective complexes. We present biochemical evidence to support the proposal that pol ϵ may be dimeric *in vivo*. Gel filtration of the Pol2p·Dpb2p complexes reveals a novel heterotetrameric form, consisting of two heterodimers of Pol2p·Dpb2p. Dpb2p, but not Pol2p, exists as a homodimer, and thus the Pol2p dimerization may be mediated by Dpb2p. The *pol2-E* and *pol2-F* mutations that cause replication defects *in vivo* weaken the interaction between Pol2p and Dpb2p and also reduce dimerization of Pol2p. This suggests, but does not prove, that dimerization may also occur *in vivo* and be essential for DNA replication.**

DNA polymerases (pol) [1] play essential roles in the duplication of genetic material and DNA repair in both prokaryotes and eukaryotes (1). In *Saccharomyces cerevisiae* there exist three essential nuclear DNA polymerases, pol α, δ, and ϵ (2–4). Despite years of study, several perhaps equally plausible models exist for the function of each polymerase during DNA replication. Pol α plays the role of a primase in the initiation of DNA replication on both leading and lagging strands in the simian virus 40 *in vitro* system (5). Pol δ and pol ϵ are required for the bulk of the replication on the leading and lagging strands (6–8). The precise location of pol δ and pol ϵ on leading and lagging strands, however, is not known. Pol α, pol δ, and pol ϵ have also been shown to participate in DNA repair (1).

Polymerase ϵ was the first proofreading polymerase purified from yeast (2). Since then it has been purified from a number of sources including *Schizosaccharomyces pombe*, the silkworm *Bombyx mori*, and HeLa cells (3–7). The *S. cerevisiae* pol ϵ consists of four subunits, Pol2p, Dpb2p, Dpb3p, and Dpb4p, with an estimated stoichiometry of 1:1:4:4 (4). These four proteins are encoded by the *POL2*, *DPB2*, *DPB3*, and *DPB4* genes (4, 8).[2] The *POL2* gene encodes the 256-kDa catalytic subunit of pol ϵ (8). Mammalian and *S. pombe* Pol2p show strong sequence similarity to yeast Pol2p (7, 9). The *DPB2*, *DPB3*, and *DPB4* genes encode the remaining 80-, 34-, and 29-kDa subunits of the yeast pol ϵ holoenzyme, respectively (4, 10, 11).[2] A functional and structural human homolog of *DPB2*, *DPE2*, has recently been described (12). Dpb2p has also recently been shown to share amino acid similarity with the B subunits of pol α (Pol12p), pol δ (Pol31p), and many other DNA polymerases (13). *DPB3* and *DPB4* from other sources have recently been identified (27). The *POL2* and *DPB2* genes are essential for the growth of yeast (8, 10), and the phenotype of temperature-sensitive *pol2* and *dpb2–1* mutants suggests a role for each in DNA replication (10). However, the precise function of *DPB2* is not known. The *DPB3* gene is non-essential, but *dpb3* mutants have increased frequency of spontaneous mutation as expected of a DNA replication defect (11). The role of the *DPB4* gene is currently unknown. Genetic analysis indicates that *DPB11*, which is essential for the initiation of DNA replication, may also be important for the function of the pol ϵ complex (14); however, Dpb11p does not seem to copurify with the holoenzyme. Another protein, encoded by *SLD2* (also known as *DRC1*), also appears to participate in DNA replication through interaction with Dpb11p and pol ϵ, but does not copurify with the complex (15, 16).

Early studies revealed two main classes of replication-defective *pol2* mutants. One class mapped to the N-terminal catalytic region, *pol2–9* and *pol2–1*8. The second class mapped in the very C-terminal amino acids, *pol2–11* and *pol2–12* (17–19). This suggested that the protein might have at least two essential functions. Supporting this idea, the C-terminal mutants were defective both in DNA replication and in the surveillance mechanisms that monitor DNA damage and replication blocks during S phase, the S/M, and the intra-S checkpoints (19, 20). The catalytic domain mutants, *pol2–9* and *pol2–1*8, in contrast, were defective only in DNA replication (19). We made serial 10 amino acid deletions over the C-terminal 120 amino acids to identify the amino acids specifically involved in the checkpoint function of the C terminus. A 20-amino acid stretch between

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[1] The abbreviations used are: pol, DNA polymerase; HA, hemagglutinin; PCR, polymerase chain reaction; aa, amino acid(s); NTA, nitrilotriacetic acid.

[2] A. Sugino, unpublished data.

two putative zinc fingers (see Fig. 8) was critical for both DNA replication and the S/M checkpoint. Given the phenotypes of the C-terminal mutants, we reasoned that the mutations might affect either protein/DNA or protein/protein interactions. In fact, the mutations affected at least two important protein/protein interactions. We discovered that the Pol2 protein dimerized using two-hybrid assays, and that the inter-zinc finger mutants failed to do so, suggesting that the dimerization was not an artifact of the two-hybrid assay (21). Second, we found that the C terminus of Pol2p interacted strongly with the Dpb2p subunit by two-hybrid analysis, and that the inter-zinc finger mutations also destabilized this interaction, although they did not abolish it (21). These results led to the model that Pol2p C-terminal amino acids were involved in assembling an active holoenzyme, as also suggested by Sugino, Araki, and collaborators (22) based on studies of the holoenzyme purified from yeast.

The importance of the Pol2p C terminus at the replication fork was made clear when it was found that the entire catalytic domain of Pol2p can be deleted and cells remain viable, as long as the C-terminal 110-kDa fragment of the protein is intact (23, 24). Thus, it is the C-terminal half of the protein that provides the only non-redundant essential role in DNA replication, rather than, as would have been expected, the catalytic polymerase activity.

The large size of the pol ϵ complex currently precludes direct structural studies. However, a first order picture of the arrangement of subunits that assemble into pol ϵ can be obtained by methods similar to those used to analyze the *Escherichia coli* replicase, *i.e.* purification of individual subunits or mixtures of subunits and analysis of their interactions. We have analyzed the protein/protein interactions in pol ϵ by both biochemical analysis and two-hybrid assay of pairwise combinations of subunits. We find that Pol2p and Dpb2p form a stable complex and that Dpb3p and Dpb4p form a tight complex. It seems logical to propose that these two subcomplexes may interact to form the holoenzyme observed in yeast extracts, since Pol2p and Dpb3p interact strongly. We also find that the Pol2p·Dpb2p complex is dimeric, with dimerization of the catalytic subunit, Pol2p, being mediated by dimerization of Dpb2p. Mutations that reduce Pol2p/Dpb2 interaction and dimerization have severe growth defects. This suggests, but does not prove, that pol ϵ is active as a dimeric protein.

EXPERIMENTAL PROCEDURES

Materials—Plasmid PTZ18 was from Bio-Rad. Plitmus39, M13KO7, restriction enzymes, T4 DNA ligase, and Klenow large fragment were from New England Biolabs. *E. coli* CJ236 and mutagenesis kit were obtained from Bio-Rad. Grace's medium and supplements for culturing Sf9 insect cells were from Invitrogen. PfastbacI and pfastbacHTb baculovirus vectors, *E. coli* DH10bac, Cellfectin reagent, SFM medium for insect cells, and heat-inactivated fetal bovine serum were obtained from Life Technologies, Inc. Agarose Ni^{2+}/NTA beads were from Qiagen. Oligonucleotides were synthesized by the oligonucleotide facility at California Institute of Technology. Plasmid miniprep kits were from Qiagen. Western blotting reagents and nitrocellulose membrane were obtained from Amersham Pharmacia Biotech. The His_6 monoclonal antibody was from Sigma. Polyclonal antibody for the pol ϵ complex was provided by Dr. Akio Sugino (Osaka University, Osaka, Japan).

Construction of POL2, DPB2, DPB3, and DPB4 Recombinant Baculoviruses—The pSEY18-POL2 vector (17) was digested with *Sac*I, and the purified *POL2* fragment was cloned at the *Sac*I site in the PTZ18 vector to create PTZ18-POL2. To excise the C terminus of *POL2*, PTZ18-POL2 was digested with *Bsr*GI and the vector was ligated with itself. The ligated vector containing the N terminus of *POL2* was transformed into *E. coli* CJ236, and cells were infected with M13KO7 phage to make a *POL2* phagemid. Uracil containing single-stranded DNA phagemid template was prepared as described earlier (21). The single-stranded DNA template was annealed to an oligonucleotide, which was designed to introduce a *Sac*I restriction enzyme site near the ATG start of *POL2*. Mutagenesis was carried out as described (21). The mutagenic oligonucleotide used is as follows: P1, 5′-C AAA CAT CAT A G A GC T C TC CCC TGA G-3′.

The mutation was confirmed by DNA sequencing. This resulted in PTZ181-POL2. The C-terminal BsrGI fragment of *POL2* (21) was subcloned into the PTZ181-POL2 to reconstitute the full-length *POL2* gene resulting in PTZ182-POL2. For *pol2-E* and *pol2-F* mutants, plitmus39 containing the mutagenized C terminus (21) was used to reconstitute the full-length *pol2-E* and *pol2-F*, which resulted in PTZ183-POL2 and PTZ184-POL2, respectively. The PTZ182-POL2, PTZ183-POL2, and PTZ184-POL2 were digested with *Sac*I, and the purified fragment was subcloned in the pfastbacI baculovirus vector at *Sac*I site. Colonies were screened by colony hybridization, and positive clones were confirmed by restriction digestion and DNA sequencing.

The *DPB2* gene was excised from the pGal4BD-DPB2 vector (21) by *Nco*I/*Sal*I digestion and cloned in the pfastbacHTb vector. The *DPB3* gene (21) was cloned at *Bam*HI/*Xho*I sites in pfastbac1 and pfastbacHTb vectors. The open reading frame of clone YDR121W, which encodes *DPB4*,[2] was synthesized by PCR using yeast genomic DNA as template and cloned at *Bam*HI/*Xho*I in pfastbac1 and pfastbacHTb vectors. (The Western blot in Fig. 3 confirms that the gene we cloned actually encodes Dpb4p.) The *DPB2*, *DPB3*, and *DPB4* genes in pfastbacHTb have a His_6 epitope and 16 additional amino acids at the N terminus. The oligonucleotides used for PCR cloning of *DPB3* and *DPB4* are as follows: DPB31, 5′-C AAC CGT GTT GGA TCC AAA ATG TCC AAC TTA G-3′; DPB32, 5′-CA ATA ATC TCG AGA TCA CTA AGG ATC GGT G-3′; DPB41, 5′-GAC CAT ATA TTT GGA TCC ACG ATG CCA CC-3′; and DPB42, 5′-TAG ACA GTT TCC CTC GAG GGG TTA CGT TTG-3′.

The C terminus of *POL2* was prepared by PCR (25) and cloned at *Sac*I/*Xho*I sites in pfastHT1 and pfastHA baculovirus vectors. pfastHT1 and pfastHA were made by inserting oligonucleotide cassettes shown below into pfastbac1. The pfastHT1 and pfastHA vectors contain His_6 and HA epitopes at *Bam*HI/*Eco*RI sites, respectively. The oligonucleotides used are as follows: HT1, 5′-GA TCC ATG TCG CAT CAT CAT CAT CAT CAT GGT G-3′; HT2, 5′-AA TTC ACC ATG ATG ATG ATG ATG ATG CGA CAT G-3′; HA1, 5′-GA TCC ATG TAC CCA TAC GAC GTC CCA GAC TAC GCT G-3′; HA2, 5′-AA TTC AGC GTA GTC TGG GAC GTC GTA TGG GTA CAT G-3′; K1, 5′-G CCC TCG GAG AGA GCT CTC ATG GAC GAG G-3′; K2, 5′-CA TTG AAC CTC GAG TTA TAT ACT G-3′.

The preparation of baculoviruses was performed using the Bac to Bac system from Life Technologies Inc. Briefly, recombinant pfastbacI or pfastHTb vectors were transformed into *E. coli* DH10bac cells. Bacmid DNA was prepared according to the manufacturer's instructions and immediately transfected into log phase Sf9 insect cells using Cellfectin reagent. The culture supernatant containing the baculovirus particles was collected after 3 days, amplified twice, and stored in darkness at 4 °C. As a control, pfastbacI vector plasmid was used to prepare wild type baculovirus.

Expression and Purification of the pol ϵ Subunits—For expression of proteins, 70×10^6 log phase Sf9 insect cells were layered on four 175-cm^2 tissue culture flasks. After cells were attached (1 h), supernatant was removed and cells were infected with viral supernatant at multiplicity of infection of 10 for *POL2* (or *pol2-F* mutant), 5 for *DPB2*, 5 for *DPB3*, and 5 for *DPB4*. The cells were incubated for 2 h at room temperature with gentle rocking. Then, 5 ml of additional Grace's complete medium was added and cells were incubated at 27 °C. After 2 days, cells were harvested, washed once with cold 1× Tris-buffered saline, flash frozen in liquid N_2, and stored at −70 °C. For pairwise coinfections, Sf9 cells were infected with the corresponding baculoviruses as described above and incubated at 27 °C for 2 days. To purify various pol ϵ subunits, the frozen cells ($70–100 \times 10^6$ cells) were thawed once on ice and then resuspended in 2 ml of buffer A (20 mM Tris, 100 mM NaCl, 5 mM EGTA, 20% glycerol, pH 7.9) containing leupeptin (20 μg/ml) and pepstatin A (20 mg/ml). After lysis, cells were kept on ice for 15 min and centrifuged at 12,000 rpm in a microcentrifuge for 10 min and supernatant was recovered. 300 μl of Ni^{2+}/NTA-agarose beads (50:50 in buffer A) were added to the supernatant, and the mixture was kept at 4 °C with end to end rotation for 2 h. All further purification steps were carried out at 4 °C unless otherwise stated. After binding of the proteins, beads were centrifuged at 1000 rpm using a swinging bucket centrifuge and the unbound protein fraction was collected. The beads were extensively washed (8 × 0.8 ml) with buffer A. To elute proteins, beads were mixed with 350 ml of buffer B (20 mM Tris, 100 mM NaCl, 20% glycerol, pH 7.9) containing 0.3 M imidazole with rotation for 30 min. The mixture was centrifuged, and the supernatant containing the eluted proteins was collected. Samples were stored at −70 °C. The total protein, unbound protein, and eluted protein fractions were analyzed using 10% SDS-PAGE and Western blotting. The protein concen-

tration was determined using Bradford assay using bovine γ-globulin as a standard.

Gel Filtration Analysis—The SMART system (Amersham Pharmacia Biotech) and precision column Superdex 200 PC (2.4 ml) (Amersham Pharmacia Biotech) were used for the analysis. The column was equilibrated at 4 °C with 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 M KCl, and 10% glycerol. 50 μl (50 μg) of the protein eluted from the Ni^{2+}/NTA column was injected on the column. The flow rate was set at 40 μl/min, and protein fractions (40 μl) were collected. The fractions were analyzed by 10% SDS-PAGE and assayed for DNA polymerase activity using poly(dA)/oligo(dT) as a DNA template as described below. Marker proteins (Amersham Pharmacia Biotech) were chromatographed in a similar manner. To cleave the N-terminal His_6 sequence, the proteins (20–30 μg) were incubated with rTEV protease enzyme as described in the manufacturer's instructions (Life Technologies, Inc.). The cleaved peptide and the rTEV protease were removed by adding Ni^{2+}/NTA beads to the protein mixture and collecting the unbound fraction.

Yeast Two-hybrid Protein/Protein Interactions—The *lexA* DNA binding domain (LEX A-BD) plasmid BTM116, provided by Stanley Fields (University of Washington, Seattle, WA), and *Gal4* activation domain plasmid pACT2 (Matchmaker two-hybrid system; CLONTECH) were used for cloning the C-terminal fragment of *POL2* (aa 1265–2222), *DPB2*, *DPB3*, *DPB4*, and the N-terminal fragment of *POL2* (aa 1–1265). Construction of the pACT2/C terminus *POL2*, *DPB2*, and *DPB3* were described previously (21). pACT2/N terminus *POL2* and *DPB4* were cloned by generating a PCR product using yeast genomic DNA as a template and primers to give flanking *Sma*I/*Xho*I sites (5′-CTGCCCGGGCATGTTTGGCAAGAAAAAAAAACAACGG-3′ and 5′-GGTCGCCTCGAGCTAGTCCATGGAAGGAATCTCCG-3′) and *Nco*I/*Bam*HI sites (5′-CATATTTTTTCCATGGCGATGCCACCA-3′ and 5′-TACTAGACAGGATCCATAGCGGG-3′).

Plasmid pBTM116BD-C terminus *POL2* was cloned by generating a PCR product using pK1 (21) with primers generating flanking *Sma*I/*Bam*HI sites (5′-AGATTCCTTCCCCGGGCGAGGACTATGT-3′ and 5′-CGTTATATACTGGATCCTCATATGGTCAAATC-3′).

pBTM116BD-N-terminus *POL2*, *DPB2*, *DPB3*, and *DPB4* were cloned via the univector plasmid-fusion system (UPS) (25). UPS uses Cre recombinase to facilitate a lox*P* site-specific recombination event. *DPB2*, *DPB3*, and *DPB4* were excised from previously isolated pAS2–1 vectors (21) and ligated into the pUNI15 vector at the *Nco*I and *Bam*HI sites, downstream of the *loxP* site, 5′-ATAACTTCGTATAGCATACATTATACGAAGTTAT-3′. The N terminus *POL2* was cloned into pUNI15 downstream of the *loxP* site after PCR with *Sma*I primers 5′-CTGCCCGGGCATGATGTTTGGCAAGAAAAAAAAACAACGG-3′ 5′-GGTCGCGCCCGGGTAGTCCATGGAAGGAATCTCCG-3′. A *loxP* site was inserted into the polylinker of pBTM116 at *Sma*I/*Pst*I, designated pBTM116loxP. The respective pUNI15 plasmids were recombined with pBTM116loxP, and the pBTM116BD-N-terminus *POL2*, *DPB2*, *DPB3*, and *DPB4* plasmids were isolated as described (25). P1 Cre recombinase was either from Novagen or purified from GST-Cre-expressing pBL21DE3plys/pQL123 (25). (pUNI15 and pQL123 plasmids were supplied by the Elledge laboratory, Baylor College of Medicine, Houston, TX).

The L40 two-hybrid strain *(MATa his*Δ*200 trp1–901 leu2–3, 112 ade2 lys2–801am URA3::(lexAop)$_8$ -lacZ LYS2::(lexAop)$_4$-HIS*3) (26) was cotransformed with the binding domain and activation domain fusion constructs using the polyethylene glycol/lithium method. Transformants were selected on Leu^-/Trp^-/His^- synthetic agar plates plus or minus 1 mM 3-amino-1,2,4-triazole incubated at 30 °C for 3–5 days. Colonies that grew were tested for β-galactosidase activity using a filter lift assay and then quantified in a subsequent β-galactosidase assay (both as per instructions from CLONTECH). β-Galactosidase units of activity were calculated as β- galactosidase activity = $1000 \times A_{420}/(t \times v \times A_{600})$, where t = time (min) required for the reaction and $v = 0.1 \times$ concentration factor. All quantitative measurements were done in replicates of 4–11n and are reported as an average ± S.D.

Polymerase Assays—The polymerase assays were as described, using poly(dA)/oligo$(dT)_{10}$ (1:20, template to primer chains) as DNA template (4, 5). Reaction mixtures (60 ml) consisted of 50 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 10% glycerol, 8 mM $MgCl_2$, 17 μg/ml poly(dA)/oligo$(dT)_{10}$, 120 mM KCl, 100 μg/ml bovine serum albumin, and 50 μM [^{3}H] dTTP (100 cpm/pmol). The poly(dA) /oligo$(dT)_{10}$ mixture was annealed in 25 mM Hepes, pH 7.1, 60 mM KCl prior to the reaction. The reactions were conducted at 37 °C for 15 min. The reactions were stopped by spotting the reaction mixture on Whatman DE81 filter paper (2.4 cm diameter). The filters were washed (five times) with 0.5 M Na_2HPO_4, two times with water, and then rinsed with 95% ethanol. Filters were dried, 3 ml of scintillation mixture was added, and radioactivity was measured. One unit of polymerase activity is defined as 1 nmol of dNMP incorporated/h.

Mutagenesis and Subcloning—The point mutations in the inter-zinc finger domain of *POL2* were constructed as before (20). The oligonucleotides used for the mutagenesis are as follows: L2146D (5′-G TTT TTC AAT GTC GTG TTC TTG C-3′), L2150D (5′-GAT ATC AGA ACG GTC TTT TTC AAT C-3′), and L2146D,L2150D (5′-GAT ATC AGA ACG GTC TTT TTC AAT GTC GTG TTC TTG CAA C-3′).

The mutations were verified by automated DNA sequencing, and the full-length *POL2* gene was reconstituted in pRS314 as described previously (21).

RESULTS

Interactions between the Essential Subunits, Pol2p and Dpb2p—If two proteins directly contact each other, one of which is tagged with a His_6 peptide, it is expected that the protein without the His_6 tag will co-purify with the protein fused to the His_6 peptide upon Ni^{2+}/NTA chromatography. We have used this as the basis of a strategy to identify putative protein contacts between known subunits of pol ϵ. In order to assess the interaction of Pol2p with Dpb2p subunit, Sf9 cells were infected with Pol2p·His_6-Dpb2p recombinant baculoviruses. After 48 h, cells were harvested, extracts prepared, and proteins purified by Ni^{2+}/NTA affinity chromatography. As shown in Fig. 1*A*, Pol2p coeluted from Ni^{2+}/NTA with His_6-Dpb2p (Fig. 1*A*, *lane 1E*). Pol2p could not be detected in fractions from cells carrying only Pol2p virus without His_6-Dpb2p (Fig. 1*A*, *lane 2E*). Thus, Pol2p interacts directly with Dpb2p.

Previous two-hybrid analysis had suggested that the C-terminal fragment of Pol2p (110 kDa, aa 1265–end) is sufficient for interaction between Pol2p and Dpb2p (21). We confirmed this biochemically by showing that when the truncated HA-Pol2p (aa 1265–end) and His_6-Dpb2p were coexpressed, HA-Pol2p (aa 1265–end) coeluted with His_6-Dpb2p from Ni^{2+}/NTA beads (Fig. 1, *B* (*lane 1*) and *C* (lane 3)).

Two C-terminal Mutations, pol2-E and pol2-F, Which Show Growth Defects, Weaken Interaction between Pol2p and Dpb2p—The Pol2-Fp has a deletion of aa 2153–2162, between the two putative C-terminal zinc fingers (21). Previously, using two-hybrid analysis, we demonstrated that pol2-Fp showed reduced affinity for *DPB2* (21). Here, we further investigated the pol2-Fp/Dpb2p interactions *in vivo* and *in vitro*. Dosage suppression has been used to argue for protein/protein interaction in the past. For example, overexpression of *SLD2* (also known as *DRC1)* suppresses *dpb11* growth defects, and the two proteins have been shown to interact by coimmunoprecipitation from yeast extracts (15, 16). The ability of the *DPB2* gene expressed under the *GAL1,10* promoter in a high copy number plasmid to suppress the temperature-sensitive phenotype of *pol2-F* was tested. As shown in Fig. 2*A*, *pol2-F* was able to grow at 37 °C when *DPB2* was induced by galactose but not when *Gal1,10* was repressed by glucose. This supports the idea that the two proteins may interact and thus stabilize the thermolabile pol2-Fp.

To analyze the interactions of pol2-Fp and Dpb2p biochemically, Sf9 cells were coinfected with pol2-Fp and His_6-Dpb2p recombinant baculoviruses and the ability to form a complex was assessed using Ni^{2+}/NTA beads as above. As predicted, the pol ϵ antibody showed only a very weak pol2-Fp band associated with the His_6-Dpb2p (Fig. 2*B*, *lane 1E*).

Since assays for catalytic activity can be more sensitive than Western blotting, the polymerase activity of the affinity-purified fractions was also used to compare the amount of Pol2p and mutant pol2-F protein copurifying with His_6-Dpb2p. As shown in controls in Table I, no DNA polymerase activity eluted from the Ni^{2+}/NTA column when cells were infected with His_6-Dpb2p. Nor was there any detectable DNA polymerase activity in Ni^{2+}/NTA column fractions when cells were infected with untagged Pol2p virus (Table I). DNA polymerase activity was detected in Pol2p·His_6-Dpb2p and pol2-Fp·His_6-

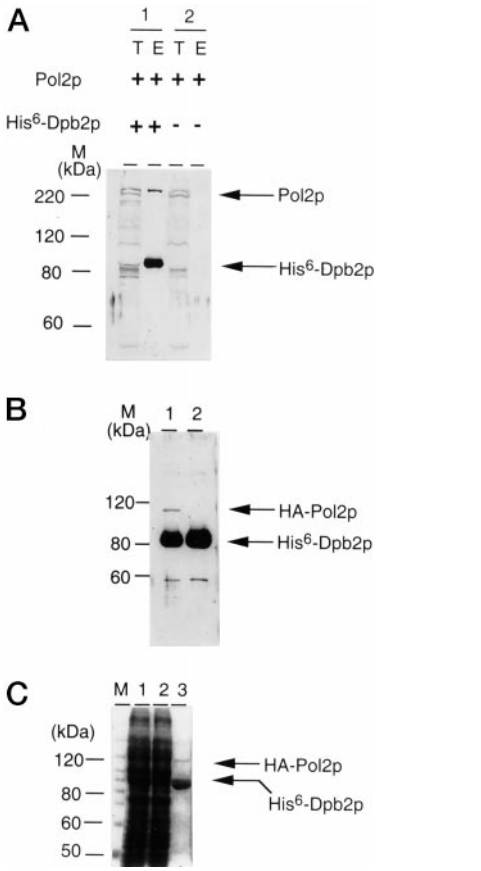


FIG. 1. **Pol2p/Dpb2p interactions in pol complex.** *A*, Western blots of affinity-purified proteins. Sf9 insect cells (40×10^6) were coinfected with Pol2p and His_6-Dpb2p baculoviruses and expression and purification of proteins was carried out as described under "Experimental Procedures." Proteins were analyzed by Western blotting using pol ϵ antibody. *Lane 1T*, total cell protein for cells infected with Pol2p and His_6-Dpb2p virus; *lane 1E*, protein from cells infected with Pol2p and His_6-Dpb2p bound to Ni^{2+}/NTA and eluted with imidazole; *lane 2T*, total cell protein for cells infected with Pol2p virus; *lane 2E*, protein from cells infected with Pol2p virus only that bound to Ni^{2+}/NTA and eluted with imidazole. *B* and *C*, interaction of 110-kDa Pol2p fragment with Dpb2p. *B*, Western blot of HA-Pol2p (aa 1265–end) eluted from Ni^{2+}/NTA after coinfection with His_6-Dpb2p. HA-Pol2p (aa 1265–end)·His_6-Dpb2p coinfection and the His_6-Dpb2p infection were analyzed by Western blotting using pol ϵ antibody. *Lane 1*, eluted protein from HA-Pol2p (aa 1265–end)·His_6-Dpb2p coinfection; *lane 2*, control showing eluted protein from His_6-Dpb2p infection. *C*, protein fractions of cells infected with HA-Pol2p (aa 1265–end) and His_6-Dpb2p were separated on 10% SDS-PAGE and bands were visualized by staining with Coomassie Brilliant Blue. *Lane 1*, total cell protein (150 mg); *lane 2*, protein not bound to the Ni^{2+}/NTA column; *lane 3*, protein bound to Ni^{2+}/NTA and eluted in the presence of 0.3 M imidazole (8 mg).

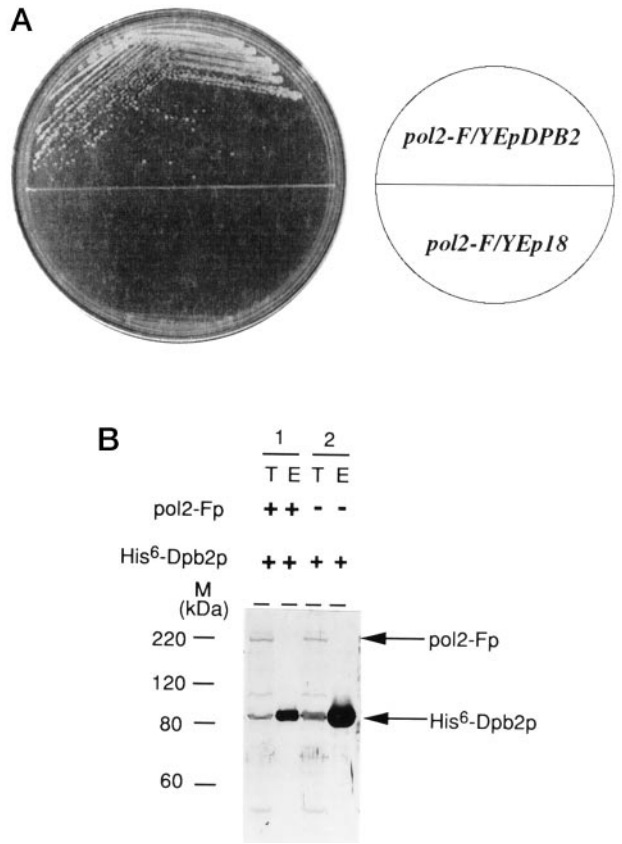


FIG. 2. **Interaction of pol2-Fp with Dpb2p.** *A*, suppression of *pol2-F* by *DPB2*. The *pol2-F* yeast strain, harboring YEp18-DPB2 or YEp18 only, was streaked on minus-tryptophan, minus-uracil minimal medium plate containing raffinose and 2% galactose and incubated at 37 °C for 3 days. *B*, interaction between pol2-Fp and Dpb2p; Sf9 insect cells (40×10^6) were coinfected with pol2-Fp·His_6-Dpb2p, and proteins were purified by Ni^{2+}/NTA affinity chromatography as described under "Experimental Procedures." The various protein fractions as shown in the *top* of the figure were separated on 10% SDS-PAGE and analyzed by Western blotting using pol ϵ antibody. As indicated, *lane 1* is from the coinfection and *lane 2* is a control in which pol2-Fp was not expressed. *Lane 1T*, total protein loaded on Ni^{2+}/NTA column; *lane 1E*, protein bound to and eluted from Ni^{2+}/NTA column; *lane 2T*, total protein; *lane 2E*, bound and eluted protein.

Dpb2p coinfections, however. As shown in Table I, the activity of pol2-Fp·His_6-Dpb2p was 10-fold reduced compared with wild-type Pol2p·His_6-Dpb2p. However, the pol2-F complex polymerase activity was still significantly (50-fold) higher than the control lacking any Pol2p (Table I). We conclude that there is residual but reduced interaction between pol2-Fp and Dpb2p. Taken together with the Western blot, we attribute the reduction in activity to reduction in the amount of pol2-F protein present, although we cannot rule out that the polymerase activity itself may also be reduced. Thus, the pol2-F mutation reduces but does not abolish Pol2p/Dpb2p interaction. A similar reduction in recovery of Pol2p using the His_6-Dpb2 was observed with a second mutant protein, pol2-Ep, carrying a deletion of aa 2143–2152 that fails to support growth at any temperature (data not shown) (21).

Interactions between Pol2p and the Non-essential Subunits Dpb3p and Dpb4p—To determine if the catalytic subunit also interacted with Dpb3p and/or Dpb4p, Pol2p was expressed in the presence of either His_6-Dpb3p or His_6-Dpb4p and Ni^{2+}/NTA chromatography was performed. Western blotting failed to detect Pol2p copurifying with either His_6-Dpb3p or His_6-Dpb4p alone (data not shown). However, the affinity-purified Pol2p and His_6-Dpb3p or Pol2p and His_6-Dpb4p complexes did contain DNA polymerase activity (Table I). This activity was 50 times higher than the background levels in His_6-Dpb3p or His_6-Dpb4p affinity-purified from cells that did not express recombinant yeast Pol2p (Table I). Thus, there may also be direct interactions between Pol2p and Dpb3p and Dpb4p (Table I). These interactions with Pol2p are weaker than Pol2p/Dpb2p interaction, both as measured by Western blotting and DNA polymerase activity recovered (Table I) (see also two-hybrid analysis, Fig. 7).

Direct Interactions between Dpb2p, Dpb3p, and Dpb4p in the Absence of Pol2p—Sf9 insect cells were coinfected with pairwise combinations of His_6-Dpb2p·Dpb3p, His_6-Dpb2p·Dpb4p, His_6-Dpb3p·Dpb4p, and Dpb3p·His_6-Dpb4p baculoviruses. Ni^{2+}/NTA affinity-purified proteins were monitored by Western blotting using pol ϵ antibody. The mobilities of the respective tagged species are shown in Fig. 3 (*lanes 5–7*). Both untagged Dpb3p and Dpb4p, which migrate slightly faster than the tagged proteins in

TABLE I

Enzymatic activity of purified wild type and mutant pol ϵ complexes or subcomplexes by Ni^{2+}/NTA affinity chromatography

The polymerase activity of the complexes was measured using poly(dA)/oligo(dT)$_{10}$ (1:20) as DNA template. The assay conditions are described under "Experimental Procedures." The purifications were done in parallel as were the assays, and 1–2 μg of protein was used in the polymerase determination. Determinations were made a minimum of three times, and the average is reported.

Enzyme	Polymerase activity
	units/mg of total protein
Pol2p only	2.3
His$_6$-Dpb2 · 6xHis-Dpb3 · Dpb4	2.2
His$_6$-Pol2p	1289[a]
Pol2p · His$_6$-Dpb2p	764
Pol2p · His$_6$-Dpb3p	97
Pol2p · His$_6$-Dpb4p	110
Pol2p · His$_6$-Dpb2p · His$_6$-Dpb3p · Dpb4p	1338
Pol2-Fp · His$_6$-Dpb2p	126
Pol2-Fp · His$_6$-Dpb2p · His$_6$-Dpb3p · Dpb4p	548

[a] Please note that the specific activity is calculated based on the total protein in the preparation. Thus, the apparently small difference in specific activity between Pol2p and the four subunit complex is an underestimate of the difference between them. Based on the concentration of the Pol2p peptide alone, we estimate that the specific activity is at least 5-fold higher in the four-subunit complex. The His$_6$-Pol2p is estimated to be only 10% pure, is unstable, and has a molecular mass by Western blotting of only 165 kDa, making comparison of the single-subunit enzyme with the other preparations difficult. However, it is presented for completeness. The lower extent of purification efficiency for His$_6$-Pol2p may be due to lower effective expression and instability of the intact protein.

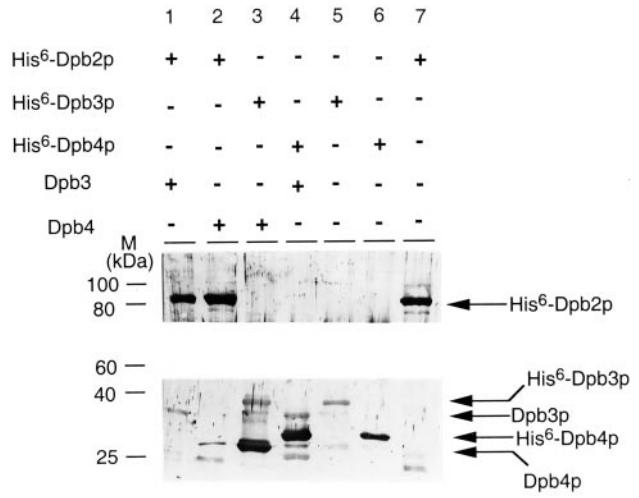


FIG. 3. **Dpb2p, Dpb3p, and Dpb4p interactions.** 40 × 10^6 Sf9 insect cells were infected with various pairwise combinations of His$_6$-Dpb2p, His$_6$-Dpb3p, Dpb3p, His$_6$-Dpb4p, and Dpb4p baculoviruses, and proteins were purified by Ni^{2+}/NTA affinity chromatography as described under "Experimental Procedures." The bound proteins from each infection as indicated were separated on 10% SDS-PAGE and analyzed by Western blot analysis using pol ϵ polyclonal antibody.

lanes 5 and *6*, coeluted with His$_6$-Dpb2p (Fig. 3, *lanes 1* and *2*). Neither untagged Dpb4p nor untagged Dpb3p bound to the column in the absence of a His$_6$ tag (data not shown). Thus, Dpb2p interacts independently with Dpb3p or Dpb4p and interaction doesn't require Pol2p. Dpb3p coeluted with His$_6$-Dpb4p and Dpb4p coeluted with His$_6$-Dpb3p in the coinfections (Fig. 3, *lanes 3* and *4*). This indicates that Dpb3p and Dpb4p form a direct complex without Pol2p or Dpb2p to mediate their interaction. The results in Figs. 1 and 3 suggest that Pol2p and Dpb2p form a complex and that Dpb3p and Dpb4p form a complex. We propose that these two complexes may interact to form the holoenzyme as purified from yeast (4), although further studies will be necessary to verify this hypothesis. This organization is consistent with the recent results obtained with *in vitro* translation with human pol ϵ subunits (27).

The C Terminus of POL2 (aa 1265–end) Does Not Form a Homodimer—Using yeast two-hybrid analysis, we have previously shown that the C terminus of *POL2* (aa 1265–end), lacking the entire polymerase domain, interacts with itself, *i.e.* dimerizes. We also showed that the C terminus is sufficient for interaction with *DPB2* (Ref. 21, and see Fig. 1). To investigate dimerization biochemically, we cloned the C-terminal half of *POL2* (aa 1265–end) in pfastHT1 and pfastHA baculovirus vectors, which fuse *POL2* (aa 1265–end) to the His$_6$ peptide and hemagglutinin (HA) epitope tags, respectively, and thus produce 110-kDa polypeptides when expressed. Cells were coinfected with His$_6$-Pol2p (aa 1265–end) and hemagglutinin epitope-tagged HA-Pol2p (aa 1265–end) recombinant baculoviruses and Ni^{2+}/NTA-purified proteins were analyzed by Western blotting using 12CA5 (for HA epitope) and pol ϵ polyclonal antibodies. Although both HA-Pol2p (aa 1265–end) and His$_6$-Pol2p (aa 1265–end) (Fig. 4, *B* and *C*) were clearly expressed and present in the total cell lysate (Fig. 4*A*, *lane 3T*), the Ni^{2+}/NTA affinity-purified His$_6$-Pol2p fraction did not contain detectable HA-Pol2p (aa 1265–end) (Fig. 4*A*, *lane 3E*). Recovery of His$_6$-Pol2p (aa 1265–end) from the Ni^{2+}/NTA column was verified by SDS-PAGE and Western blotting using pol ϵ antibody (Fig. 4, *B* and *C*). Control cells singly infected with HA-Pol2p (aa 1265–end) or His$_6$-Pol2p (aa 1265–end) or His$_6$-Dpb2 showed efficient expression of HA-Pol2p but no HA-Pol2p in affinity column eluates (Fig. 4*A*, *lanes 1*, *2*, and *4*), as expected. The positive control in Fig. 4*A* (*lane 5*) shows that HA-Pol2p is capable of binding and copurifying with His$_6$-Dpb2p and can be detected in a Ni^{2+}/NTA eluate. The experiment was repeated using various salt concentrations (50–300 mM) during lysate preparation and affinity purification, but the HA-Pol2p (aa 1265–end) was not detected in the eluted protein fraction. Thus, the dimerization of Pol2p detected by the two-hybrid system either is mediated by another protein not present in insect cells or is too weak to survive the isolation procedure used here in the absence of the N-terminal domain.

Dpb2p Forms a Homodimer—Since Pol2p does not itself appear to dimerize but does interact with Dpb2p, it seemed plausible that dimerization might occur through Dpb2p. Although we previously failed to find self interaction for *DPB2* in the two-hybrid system (21), we show below with a more sensitive two-hybrid analysis that Dpb2 dimerization does occur. To test for dimerization biochemically, His$_6$-Dpb2p was purified from the Sf9 insect cells and analyzed by gel filtration. To exclude the possible interference by the N-terminal His$_6$ epitope, we cleaved the epitope by site-specific rTEV protease and the intact Dpb2p was further purified. The protein was then injected onto a Superdex 200 PC gel filtration column. The majority of the protein was eluted at the apparent molecular mass of 160 kDa, as expected of a Dpb2p dimer (Fig. 5*A*). This confirms the two-hybrid results presented below, and we propose that dimerization of Pol2p may therefore occur through Dpb2p.

Further evidence for dimerization of Dpb2p was obtained when we applied a His$_6$-Dpb2p·His$_6$-Dpb3p·Dpb4p affinity-purified mixture, prepared from coinfections of cells with the three corresponding viruses, to the gel filtration column. SDS-PAGE and silver staining of the protein fractions showed that a His$_6$-Dpb2p·His$_6$-Dpb3p·Dpb4p complex eluted at 190 kDa (Fig. 5*B*). The elution position suggests a 2Dpb2p·1Dpb3p·1Dpb4p complex, assuming a globular complex.

Gel Filtration Analysis of Pol2p·Dpb2p Complexes Reveals a 600-kDa Heterotetrameric Form—To assess whether Dpb2 dimerization might mediate Pol2p dimerization, we performed gel filtration analysis of complexes isolated from cells coin-

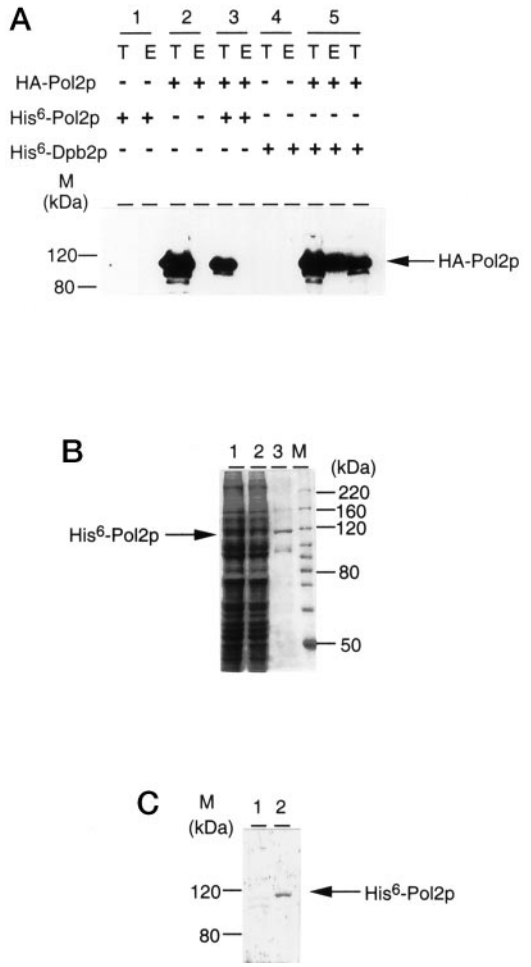


FIG. 4. **Failure of the 110-kDa C terminus of *POL2* (aa 1265–end) to dimerize.** *A–C*, biochemical test for dimerization of the 110 kDa fragment. *A*, Western blot using anti-HA 12CA5 monoclonal antibody. Sf9 insect cells (40×10^6) were singly infected or coinfected in pairwise combination with baculoviruses for His_6-Pol2p (aa 1265–end), HA-Pol2p (aa 1265–end), and His_6-Dpb2p as indicated. After cell lysis, the proteins were purified using Ni^{2+}/NTA affinity chromatography as described under "Experimental Procedures." Protein from the total cell lysate (*T*) and bound protein fractions eluted with imidazole (*E*), were separated on 10% SDS-PAGE and subjected to Western blotting using 12CA5 anti-HA epitope monoclonal antibody. *B*, protein from the cells infected with His_6-Pol2p (aa 1265–end) were separated on 10% SDS-PAGE and the gel was stained with Coomassie Brilliant Blue. *Lane 1*, total cell protein (150 mg); *lane 2*, protein that failed to bind to the Ni^{2+}/NTA beads; *lane 3*, bound proteins (4 mg) eluted in the presence of 0.3 M imidazole. *C*, Western blot of extract and Ni^{2+}/NTA-bound fractions using pol ϵ antibody. *Lane 1*, unbound protein; *lane 2*, bound protein eluted in the presence of 0.3 M imidazole.

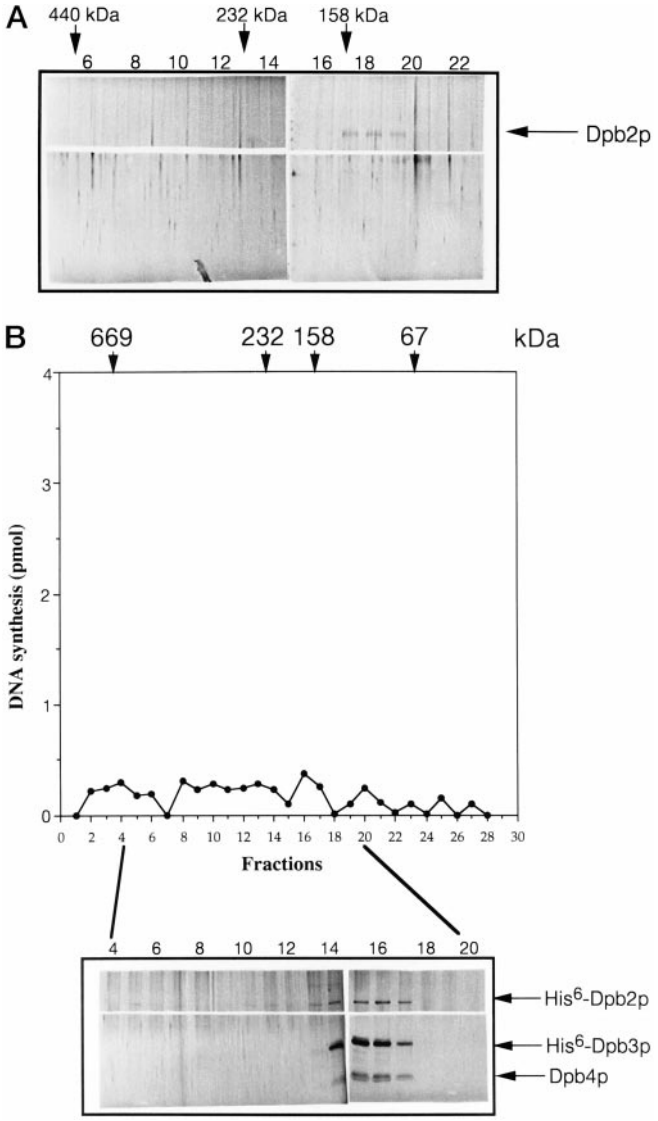


FIG. 5. **Dpb2p forms a homodimeric complex.** *A*, gel filtration analysis of affinity-purified His_6-Dpb2p. The N-terminal His_6 sequence in His_6-Dpb2p was cleaved by rTEV protease as described under "Experimental Procedures." 10 μg of the purified Dpb2 was injected on the Superdex 200 PC column, and protein fractions were separated by 10% SDS-PAGE and silver staining. *B*, gel filtration analysis of affinity-purified protein from a His_6-Dpb2p·His_6-Dpb3p·Dpb4p coinfection. 50 μg of the protein eluted from Ni^{2+}/NTA resin was injected on Superdex 200 PC column. The protein fractions were separated on 10% SDS-PAGE and silver-stained. The polymerase activity was determined using poly(dA)/oligo(dT) as described under "Experimental Procedures." Size markers were thyroglobulin (669 kDa), catalase (232 kDa), aldolase (158 kDa), and bovine serum albumin (67 kDa).

fected with Pol2 and His_6-Dpb2. A substantial amount of DNA polymerase activity eluted in fractions 8–10, corresponding to 600 kDa. Both Pol2p and Dpb2p were present in these fractions (Fig. 6*A*). Given that Pol2 is a 256-kDa protein and Dpb2 is an 80-kDa protein, the most likely composition of the protein in this 600-kDa peak is a heterotetramer consisting of two Pol2p·Dpb2p heterodimers. Additional polymerase activity also eluted at 210 kDa. Since this is smaller than Pol2p (256 kDa) itself, this may represent proteolyzed fragments and we have not analyzed this (these) species in detail. It is worth pointing out that pol ϵ activity has previously been isolated from yeast and shown to contain a 120-kDa form of the Pol2p, and there is a 120-kDa band in this fraction. Unlike here, that form was not shown to be complexed with Dpb2p, Dpb3p, or Dpb4p (4, 5). This polymerase activity was due to some form of Pol2p, however, since no polymerase was observed if His_6-Dpb2p alone or His_6-Dpb2p·His_6-Dpb3p·Dpb4p was applied to the column (Fig. 5). In fact, controls showed that no polymerase activity was observed in any of the gel filtration experiments shown in this work when Pol2p was omitted.

It was possible, since these experiments were carried out in the absence of Dpb3p·Dpb4p complexes, that the putative 600-kDa complex might be an artifact and might not form in their presence. To test this possibility, Sf9 insect cells were coinfected with Pol2p, His_6-Dpb2p, His_6-Dpb3p, and Dpb4p recombinant baculoviruses. All four proteins were recovered from the affinity column, as revealed by Western blots (data not shown). Gel filtration revealed a DNA polymerase peak at 600 kDa, with the same mobility and composition (Fig. 6*B*) as the complex formed in the absence of Dpb3p and Dpb4p (Fig. 6*A*). Although there was no detectable Dpb3 or Dpb4 in the 600-kDa

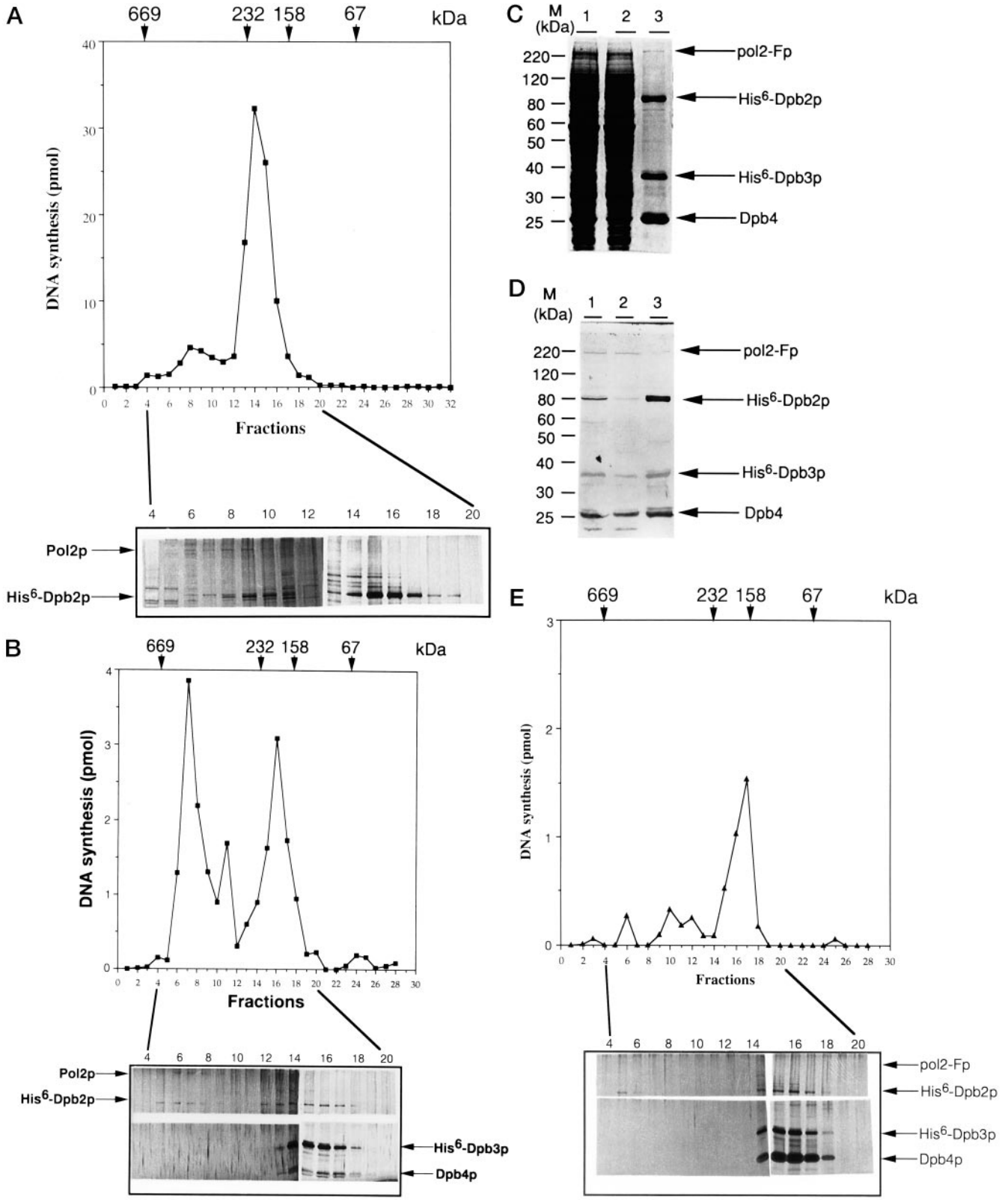


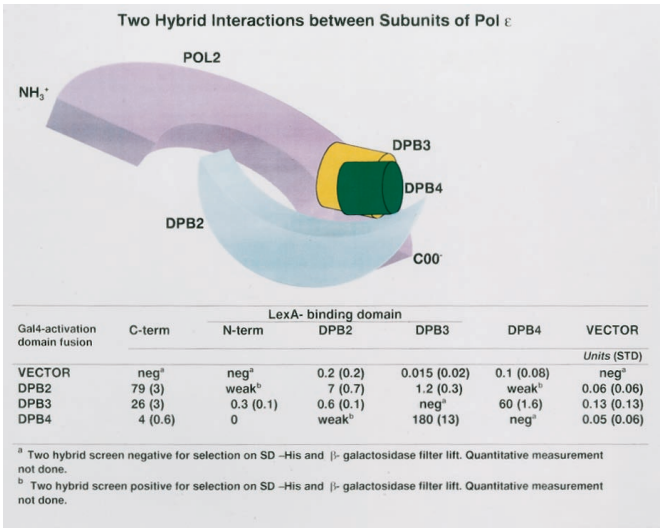
FIG. 6. **Gel filtration analysis of Pol2p·Dpb2 complexes reveals a heterotetrameric form.** 80 μg of Pol2p·His_6-Dpb2p of the protein eluted from the Ni^{2+}/NTA was injected onto a Superdex 200 PC column, and proteins were separated on 10% SDS-PAGE and silver-stained. The polymerase activity of the protein fractions was determined using poly(dA)/oligo(dT) as described under "Experimental Procedures." The size markers were thyroglobulin (669 kDa), catalase (232 kDa), aldolase (158 kDa), and bovine serum albumin (67 kDa). *B*, gel filtration analysis of the Pol2p·His_6-Dpb2p·His_6-Dpb3p·Dpb4p affinity-purified sample. 50 μg of the protein eluted from the Ni^{2+}/NTA was injected on Superdex 200 PC column, and proteins were separated on 10% SDS-PAGE and silver-stained. The polymerase activity of the protein fractions was determined using poly(dA)/oligo(dT) as described under "Experimental Procedures." *C*, stabilization of pol2-Fp·Dpb2p complex by Dpb3p and Dpb4p. Sf9 cells (70×10^6) were coinfected with pol2-Fp·His_6-Dpb2p·His_6-Dpb3p·Dpb4p, and proteins were purified by Ni^{2+}/NTA affinity chromatography. The various protein fractions were subjected to 10% SDS-PAGE, and the resulting gel was stained with Coomassie Blue. *Lane 1*, total cell protein before addition of Ni^{2+}/NTA-agarose (200 μg); *lane 2*, protein not bound to the beads; *lane 3*, proteins eluted in the presence of 0.3 M imidazole (20 μg). *D*, Western blotting of pol2-Fp·His_6-Dpb2p·His_6-Dpb3p·Dpb4p. Protein fractions as described in Fig. 6*C* were analyzed by Western blotting using

peak, there was more of the 600-kDa form compared with the 210-kDa (presumably) degraded form seen also in Fig. 6*A*. Although it is not clear how Dpb3p and Dpb4p increase the amount of the heterotetrameric peak, these data show at the least that the heterotetramer form can be a major reproducible form of this Pol2p·Dpb2p subassembly. We speculate that the apparent relative stabilization of the 600-kDa form by Dpb3 and Dpb4p may be related to the fact that coexpression of the latter two subunits with Pol2p·Dpb2p also results in an increase in recovery of overall DNA polymerase activity (Table I). It is noteworthy that, if Dpb3 and Dpb4 are not tagged with His_6, they do not copurify from extracts with His_6-Pol2p nor with Pol2p·His_6-Dpb2p (data not shown).

We next asked if the pol2-Fp mutant could form heterotetramers with Dpb2p, even though its interaction with Dpb2p was weak compared with wild-type Pol2p (see Fig. 2*A*). Sf9 cells were coinfected with pol2-Fp, His_6-Dpb2p, His_6-Dpb3p, and Dpb4p recombinant baculoviruses, and protein complex was purified using Ni^{2+}/NTA chromatography. Coexpression of the His_6-Dpb3p and Dpb4p proteins appeared to stabilize the pol2-Fp·Dpb2p complex since pol2-Fp now clearly coeluted with His_6-Dpb2p (compare Fig. 2*B* (*lane 1E*) with Fig. 6*C* (*lane 3*)). This effect of Dpb3p and Dpb4p was reflected in increased recovery of DNA polymerase activity (Table I). The increased recovery allowed us to analyze the pol2-Fp·His_6-Dpb2p complex by gel filtration. In contrast to the results with wild type Pol2p·His_6-Dpb2p (Fig. 6*A*), the polymerase activity of pol2-Fp mutant eluted in a single peak at an apparent molecular mass of 190 kDa (Fig. 6*E*), suggesting severe degradation. The striking reduction of the amount of the 600-kDa Pol2p·Dpb2p complex containing pol2-Fp demonstrates that the inter-zinc finger mutation destabilizes the Pol2p·Dpb2p complex and reduces formation of the heterotetramer. (We note parenthetically that a small amount of polymerase may be found in fraction 6. Multimeric His_6-Dpb2p is also observed in fractions 5 and 6 (Fig. 6*E*). A small amount of pol2-Fp is presumably present but below the level of detection by silver staining in these fractions.)

The experiments in Figs. 5 and 6 have been repeated at least twice, using at least two different infections; therefore, we believe the amounts of activity and protein are reproducible. We conclude that the inter-zinc finger domain stabilizes interaction between Pol2p and Dpb2p and therefore may be involved in dimerization.

Semi-quantitative Analysis of Subunit Interactions Using the Yeast Two-hybrid Assay—Although the fusion of proteins to Gal4 required for implementation of the two-hybrid assay can interfere with normal protein/protein interactions, nevertheless the approach has been successful in many cases in estimating affinities between different proteins. To compare the strength of the various protein/protein interactions described above, we used a two-hybrid assay in which the reporter strain has eight *lexA* operators in tandem controlling the *lacZ* gene and four *lexA* operators controlling the *HIS3* gene. Strong protein/protein interactions show high levels of β-galactosidase expression, but even weak interactions are detectable. The *POL2*, *DPB2*, *DPB3*, and *DPB4* genes were fused both to the *lexA* DNA binding domain and to the *Gal4* activation domain as described under "Experimental Procedures." The C-terminal and N-terminal portions of *POL2* were tested individually. The strength of the interactions between the various proteins was quantitated by measuring β-galactosidase activity in all possible pairwise combinations of the proteins. The results are collated in Fig. 7. Supporting the biochemical evidence, the C terminus of Pol2p interacted with Dpb2p, Dpb3p, and Dpb4p (Figs. 1*A* and 3). The strength of the interactions was Dpb2>Dpb3≫Dpb4. Although we had previously failed to detect *DPB2* self-interaction in a two-hybrid assay with a less sensitive reporter strain (21), with the current assay the interaction was easily detectable, as supported by the gel filtration analysis that showed a homodimer (Fig. 5*A*). Although we have shown that the C-terminal half of Pol2p is sufficient for interaction with Dpb2p, a fusion containing the N-terminal half of Pol2p also showed weak interactions with Dpb2p, consistent with the fact that high copy plasmids expressing high levels of *DPB2* suppress N-terminal temperature-sensitive *pol2–18* mutant (10). Dpb3p·Dpb3p and Dpb4p·Dpb4p homodimerization was not observed under the same conditions, but there was a very strong interaction between Dpb3p and Dpb4p, confirming that they form a stable heterodimer (Fig. 3). Dpb2p·Dpb3p and Dpb2p·Dpb4p showed very weak but detectable interaction, again consistent with the recovery of co-complexes from the Ni^{2+}/NTA column (Fig. 3).



Gal4-activation domain fusion	LexA- binding domain C-term	N-term	DPB2	DPB3	DPB4	VECTOR
						Units (STD)
VECTOR	neg[a]	neg[a]	0.2 (0.2)	0.015 (0.02)	0.1 (0.08)	neg[a]
DPB2	79 (3)	weak[b]	7 (0.7)	1.2 (0.3)	weak[b]	0.06 (0.06)
DPB3	26 (3)	0.3 (0.1)	0.6 (0.1)	neg[a]	60 (1.6)	0.13 (0.13)
DPB4	4 (0.6)	0	weak[b]	180 (13)	neg[a]	0.05 (0.06)

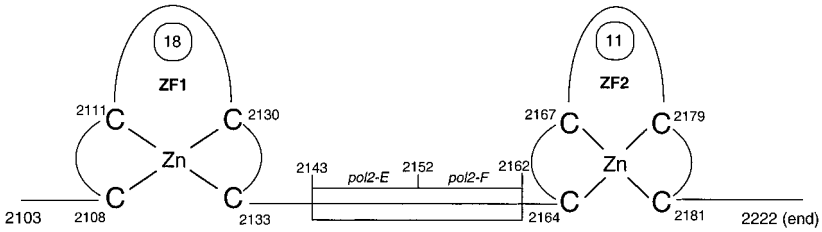
[a] Two hybrid screen negative for selection on SD –His and β- galactosidase filter lift. Quantitative measurement not done.
[b] Two hybrid screen positive for selection on SD –His and β- galactosidase filter lift. Quantitative measurement not done.

FIG. 7. **Yeast two-hybrid analysis.** The L40 two-hybrid yeast strain was used for the assay. *C-term* corresponds to amino acids 1265–2222, and *N-term* corresponds to amino acids 1–1265 of Pol2p. The strain was transformed with the LexA DNA binding domain plasmid and Gal4 activation domain plasmid, and the colonies were selected on Leu^-/Trp^-/His^- synthetic agar plates containing 1 mM 3-amino-1,2,4-triazole. The colonies that grew were further analyzed by X-gal filter lifts, and interactions were quantitated by β-galactosidase assays. The measurements were done in replicates of 4–11 samples, and the data are reported as an average. *Numbers* in *parentheses* are standard deviation.

An overview of these data is provided in the model shown in Fig. 7. In summary, the C terminus of Pol2p interacts with Dpb2p, Dpb3p, and Dpb4p. Dpb3p and Dpb4p directly interact with each other in the absence of Pol2p and Dpb2p. Dpb2p forms a homodimer and interacts with the N terminus of Pol2p as well as the C terminus. The dimerization of the Pol2p C terminus seen in the two-hybrid system is probably indirect and may occur through interaction of two Pol2p subunits with a Dpb2 dimer.

Point Mutations in the C-terminal Inter-zinc Finger Domain of POL2—In our previous study, we showed that the *pol2-E* and *pol2-F* mutants just described showed defects in DNA replication and the S/M checkpoint (Fig. 8*A* and Ref. 21). Now

pol ϵ antibody. *E*, gel filtration analysis of pol2-F·His_6-Dpb2p·His_6-Dpb3p·Dpb4p. 50 μg of the eluted protein from Ni^{2+}/NTA resin was injected on Superdex 200 PC column. The protein fractions were separated on 10% SDS-PAGE and silver-stained. The polymerase activity was determined using poly(dA)/oligo(dT) as described under "Experimental Procedures."



S. Cerevisae pol ε	^{2143}Q	E	H	**L**	I	Q	K	**L**	R	S^{2152}
S. Pombe pol ε	^{2117}E	E	T	**L**	V	D	N	**L**	Y	H^{2126}
Human pol ε	^{2171}E	M	T	**L**	V	E	V	**L**	Q	K^{2180}

Mutant	Mutation	Growth (+5 FOA)	
		25°C	37°C
Wild type		+++	+++
pol2-E	Δ 2143-2152	-	-
pol2-F	Δ 2153-2162	+/-	-
pol2-310	L2146D	+++	+++
pol2-311	L2150D	+++	+++
pol2-312	L2146D, L2150D	-	-

FIG. 8. **Characterization of the point mutations affecting the putative inter-zinc finger domain of *POL2*.** *A*, schematic diagram of putative zinc finger of *POL2*. The *pol2-E* and *pol2-F* mutants each containing site-specific 10 consecutive amino acid deletion is highlighted in a *box* (21). *B*, amino acid alignment showing the conservation of leucine amino acid residues in pol ϵ from various sources. *C*, point mutations in the conserved leucine. The mutants were prepared as described under "Experimental Procedures." The mutant *pol2* genes were subcloned in the pRS314 vector and introduced into the A1128 *pol2–3::LEU2(YEpPOL2)* as described before (21). The transformants were replica plated on agar plates lacking tryptophan and containing 5-fluoroorotic acid (*5-FOA*) at 24 °C and 370 °C for 4 days.

we have provided further evidence that the defect lies in interaction with other subunits. However, it is not clear whether the specific amino acid residues between the zinc fingers play an essential role or if the deletions cause a structural change adjacent to the amino acid residues. To investigate specific amino acids, we made point mutations in two conserved leucines (L2146D and L2150D) in the inter-zinc finger domain (Fig. 8, *A* and *B*). The mutant proteins were analyzed for complementation of a *pol2*Δ strain using a plasmid shuffling assay as described previously (21). The *pol2–310* and *pol2–311* mutants, with L2146D or L2150D mutations, respectively, showed no detectable growth defect. However, the *pol2–312* mutant, with an L2146,L2150D double mutation, was inviable at any temperature (Fig. 8*C*). These results strengthen the conclusion that specific amino acids covered by the *pol2-E* mutation are essential for cell viability, although it does not prove that there is no structural change.

DISCUSSION

DNA polymerases are large molecular machines that play an integral role in chromosomal replication. Polymerase ϵ plays a major role in the elongation stage of the replication of chromosomal DNA. Recently, a number of studies have indicated an additional role of pol ϵ in S/M checkpoint regulation, recombination, and transcriptional silencing (16, 19, 21, 28–31). By site-specific deletion analysis in the putative zinc finger domain of *POL2*, we showed that the amino acid residues in the inter-zinc finger domain are essential for DNA replication, DNA repair, and the S/M checkpoint. We have also shown that the putative zinc finger participates in protein/protein interactions. In this study we have extended our analysis of the inter-zinc finger domain of *POL2* and its protein/protein interactions. By point mutations, we showed that the two conserved leucines in the inter-zinc finger domain are essential for the cell viability. The essential nature of the leucine amino acid residues suggests that protein/protein interactions mediated through the inter-zinc finger could be mainly hydrophobic in nature, although we cannot completely rule out ionic and hydrogen bond interactions, given the sequence shown in Fig. 8*B*. A recent crystal structure analysis of a cocrystal containing the phage RB69, "PCNA"-like sliding clamp and the C-terminal, 11-residue peptide of RB69 DNA polymerase showed that the "PCNA" is bound in a hydrophobic pocket through Leu-897, Met-900, and Phe-901 amino acid residues of the polymerase peptide (32). The C-terminal 11 amino acid residues of the polymerase have also been shown to be essential for the interaction of T4 DNA polymerase with the T4 sliding clamp (33). It is possible that another subunit of pol ϵ, Dpb2p, uses a similar mode of interaction with Pol2p, as suggested by our comprehensive genetic and biochemical analysis of the pairwise protein/protein interactions between the various subunits of pol ϵcomplex.

Studies of contacts between four of the subunits of pol ϵ gave the following picture of their organization. Pol2p forms a stable complex with Dpb2p. The human homolog of Dpb2p also forms a very tight complex with the mammalian Pol2p catalytic subunit (12), suggesting a structural conservation between human and yeast pol ϵ complexes. Dpb3p and Dpb4p also form a strong complex, and we propose that the Pol2p·Dpb2p dimer interacts with the Dpb3p·Dpb4p dimer to form the holoenzyme isolated from yeast by Sugino and colleagues (4). The proteins in the Pol2p·Dpb2p heterodimer can make multiple contacts with proteins in the Dpb3p·Dpb4p dimer. Pol2p interacts independently with Dpb3p and Dpb4p, but the interactions are weaker than in the presence of Dpb2p. The Pol2p/Dpb3p and Pol2p/Dpb4p interactions are also much weaker than the Pol2p/Dpb2p interaction (both biochemically and by two-hybrid analysis). Dpb2p also interacts independently with Dpb3p and Dpb4p (Fig. 3), but both purification of complexes from insect cells infected with baculoviruses carrying all four subunits and two-hybrid analysis suggests this interaction may be weak (Fig. 7). The C terminus of Pol2p is sufficient to interact with Dpb2p. A model for the interaction of the subunits is shown in Fig. 7.

Several studies based on purification of pol ϵ activity from yeast cells support the contacts suggested here. Pol ϵ purified from a *dpb2-ts* mutant lacked Dpb2p but contained Dpb3p (10). This is consistent with our observation of a direct Pol2p/Dpb3p

contact. When pol ϵ was purified from a *dpb3*Δ yeast strain, Dpb2p copurified with Pol2p but Dpb4p was not present (11). This can be explained if Dpb3p and Dpb4p interact more strongly with each other than with Pol2p or Dpb2p and if Dpb3p interacts more strongly with Pol2p than does Dpb4p alone, as our results suggest (Fig. 7). By the same token, it is possible that a *dpb4*Δ mutation would result in the dissociation of Dpb3p from the pol ϵ complex, although the interaction between Dpb3p and Pol2p is stronger than between Dpb4p and Pol2p by two-hybrid analysis (Fig. 7). Recent work on the human pol ϵ is also consistent with our findings.

We also attempted to produce the four-subunit pol ϵ complex by coexpressing baculoviruses containing all four subunit genes (data not shown). We were not able to recover all four proteins unless we tagged both Dpb2p and Dpb3p. Since our experiment involved simultaneous coexpression of all four proteins, it is possible that the four subunit complex must assemble in a specific order. For instance, since we have established that Dpb3p and Dpb4p interact with Pol2p, it is possible that their interaction prevents proper association with Dpb2p. Such precise order of assembly has been observed in the *E. coli* replicase, where addition of γ-δ components before the addition of τ prevents τ dimerization (34). Another possibility is that another protein such as Dpb11p or Sld2p, which interact with pol ϵ, might be essential for proper assembly. Yet another alternative is that Dpb3p and Dpb4p function by virtue of their histone-fold motifs to allow entry of pol ϵ into nucleosomes, as recently proposed (27). Thus, Pol2p·Dpb2p efficiently assemble with Dpb3p and Dpb4p only in the context of chromatin. In regard to a role for Dpb3p in chromatin structure, it is intriguing that *dpb3* mutations were identified in a screen for genes involved in rDNA silencing. The structure of silenced chromatin in the rDNA locus may somehow be altered in *dpb3* mutants to allow transcription of the DNA (31). All four subunits of the human enzyme have been shown to coimmunoprecipitate when using an antibody to the Dpb4p homolog, p17, tagged with a FLAG epitope (27).

Our biochemical analysis indicates that Pol2p dimerizes. The dimerization of the Pol2p is most likely not direct (Fig. 4), but instead occurs through homodimerization of the Dpb2p subunit (Figs. 5–7). This situation is somewhat reminiscent of the pol III′ form of the *E. coli* replicase described many years ago by McHenry and O'Donnell laboratories (34), in which the pol III core containing polymerase and proofreading nuclease subunits is dimerized by interaction with a dimeric τ subunit. The pol ϵ inter-zinc finger region is crucial for dimerization, as we proposed previously (21), since gel filtration of the pol2-Fp mutant complex did not show a heterotetrameric polymerase complex, demonstrating that the mutation destabilizes Pol2p interactions with Dpb2p. This was more directly verified by showing that little pol2-Fp coeluted with His_6-Dpb2p from the Ni^{2+}/NTA column. The overexpression of Dpb2p under the *GAL1,10* promoter suppressed the growth defect of the C-terminal *pol2-F* temperature-sensitive mutant at the nonpermissive temperature (Fig. 2), suggesting that we have identified a physiologically significant interaction domain. It will be of interest to isolate and examine the phenotype of *dpb2* mutants that fail to dimerize.

The dimerization of pol ϵ is interestingly different from the dimerization of pol δ. Pol31p, the B subunit of pol δ which is homologous to Dpb2p, does form a heterodimer with the catalytic subunit, Pol3p. However, Pol31p does not form a homodimer. Instead Pol31p interacts with PCNA and with the third pol δsubunit, Pol32p. It is the latter subunit, Pol32p, that homodimerizes and then leads to dimerization of the Pol3p·Pol31p heterodimer (35, 36). Recently, pol δ from *S. pombe* has also been shown to be a dimer of the heterotetramer containing Pol3p, Cdc1p, Cdc27p, and Cdm1p and dimerization was mediated by the essential Cdc27 subunit (37). Mutants of pol δ that fail to dimerize have not been described to date.

Although it is thought that leading and lagging strand elongation are coordinated through the use of two different gene products, pol δ and pol ϵ, one on each strand, obviating the need for dimeric polymerases as are found in prokaryotes, a possible role for dimeric polymerases in eukaryotes could be coordination of the two forks emanating from a single origin. The idea that the two branch points of a bidirectional replication fork are connected into a binary replisome was put forward as a mechanism for preventing rotation of the two forks with respect to one another in order to reduce tangling of the sister chromatids (38). Dimerization of the polymerases could both link the forks physically and ensure synchronous activity at both forks. It has previously been proposed that DNA helicases may also have an organizational role in tethering forks to each other (39).

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