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In Vivo Reconstitution of *Saccharomyces cerevisiae* DNA Polymerase ϵ in Insect Cells

PURIFICATION AND CHARACTERIZATION*

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Rajiv Dua‡, Daniel L. Levy§, Caroline M. Li, Peter M. Snow, and Judith L. Campbell¶

From the Braun Laboratories, California Institute of Technology, Pasadena, California 91125

DNA polymerase ϵ (pol ϵ) is a multiple subunit complex consisting of at least four proteins, including catalytic Pol2p, Dpb2p, Dpb3p, and Dpb4p. Pol ϵ has been shown to play essential roles in chromosomal DNA replication. Here, we report reconstitution of the yeast pol ϵ complex, which was expressed and purified from baculovirus-infected insect cells. During the purification, we were able to resolve the pol ϵ complex and truncated Pol2p (140 kDa), as was observed initially with the pol ϵ purified from yeast. Biochemical characterization of subunit stoichiometry, salt sensitivity, processivity, and stimulation by proliferating cell nuclear antigen indicates that the reconstituted pol ϵ is functionally identical to native pol ϵ purified from yeast and is therefore useful for biochemical characterization of the interactions of pol ϵ with other replication, recombination, and repair proteins. Identification and characterization of a proliferating cell nuclear antigen consensus interaction domain on Pol2p indicates that the motif is dispensable for DNA replication but is important for methyl methanesulfonate damage-induced DNA repair. Analysis of the putative zinc finger domain of Pol2p for zinc binding capacity demonstrates that it binds zinc. Mutations of the conserved cysteines in the putative zinc finger domain reduced zinc binding, indicating that cysteine ligands are directly involved in binding zinc.

DNA polymerase epsilon (pol ϵ)[1] purifies from yeast as a four-subunit holoenzyme. The subunits are Pol2p (256 kDa), Dpb2p (80 kDa), Dpb3p (34 kDa), and Dpb4p (29 kDa), and the genes encoding all four have been identified (1–5). Pol2p encodes the polymerase catalytic activity and is essential for the viability of yeast. Unlike the other essential polymerases, the polymerization function of pol ϵ can actually be performed by another polymerase during DNA replication because deletion of the catalytic portion of the protein is not lethal (6). However, a point mutation in the polymerase active site is lethal, suggesting that normally the polymerase does function during DNA replication and that the presence of an inactive molecule is inhibitory. In *Xenopus*, pol ϵ is also essential for DNA replication (7). Our previous work showed that Pol2p and Dpb2p expressed in insect cells form a heterodimer that itself forms a tetramer $(Pol2p/Dpb2p)_2$ mediated by self-interaction of the Dpb2p subunits. In addition, we found that Dpb3 and Dpb4 form a dimer that can interact with Pol2p/Dpb2p heterodimers. We identified a motif in the COOH-terminal, zinc finger domain of Pol2p (256 kDa) which was essential for interaction with Dpb2p and for dimerization, and we showed that this motif was essential for viability. The same region mediates interaction with Dpb3p/Dpb4p. Despite these advances in studying pairwise interactions and even interactions between the two heterodimers, Pol2p/Dpb2p and Dpb3p/Dpb4p, we were not able to reconstitute a holoenzyme containing all four subunits with the same stoichiometry as the holoenzyme isolated from yeast. Such reconstitution is critical for the next step in our planned analysis, namely, to investigate how the holoenzyme interacts with its accessory factors, RF-C and PCNA in primer binding and processivity, as well as how it interacts at the mechanistic level with other proteins in replication, recombination, and repair.

Our results suggested that failure to reconstitute the holoenzyme was the result of poor expression of the large catalytic subunit compared with the other subunits. We describe a new expression vector that allows efficient expression of a tagged version of Pol2p in insect cells. Using this new virus, along with the previously validated vectors encoding the remaining subunits (8), we have devised a rapid purification of a highly active four-subunit holoenzyme. We show that this preparation is suitable for studying the interaction between pol ϵ and other proteins by demonstrating that it is stimulated by the accessory proteins RF-C and PCNA, in just the same way as the enzyme from yeast (9–11). Both the RF-C complex and PCNA have been overexpressed by others, dramatically facilitating purification of large amounts for our studies (12, 13). The fact that pol ϵ, unlike pol δ, is very processive even in the absence of PCNA and RF-C raises the question of the physiological significance of RF-C and PCNA stimulation. To investigate this issue, we present a genetic study of the interaction between PCNA and the catalytic subunit using site-directed mutagenesis to define the interaction site and its physiological significance. We show that mutation of a conserved motif corresponding to a putative PCNA interaction site in the Pol2p catalytic subunit leads to sensitivity to MMS. Similarly, we show that there is a zinc binding domain in the COOH-terminal portion of the protein and that it is important for growth in the presence of MMS. A separate motif, located in the midst of the zinc-binding amino acids, defined by us previously as essential for

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‡ Present address: Hyseq Inc., 670 Almanor Ave., Sunnyvale, CA 94086.

§ Present address: Dept. of Biochemistry and Biophysics, Box 0448, University of California, San Francisco, CA 94143.

¶ To whom correspondence should be addressed: Braun Laboratories 147-75, California Institute of Technology, 1200 East California Blvd., Pasadena, CA 91125. Tel.: 626-395-6053; Fax: 626-405-9452; E-mail: jcampbel@cco.caltech.edu.

[1] The abbreviations used are: pol ϵ, DNA polymerase ϵ; PCNA, proliferating cell nuclear antigen; RF-C, replication factor C; MMS, methyl methanesulfonate; MBP, maltose-binding protein; RPA, replication protein A.

dimerization of Pol2p by Dpb2p (6, 14), is apparently not involved in zinc binding but is important for viability.

EXPERIMENTAL PROCEDURES

Materials—Plitmus39, M13KO7, restriction enzymes, T4 DNA ligase, and Klenow large fragment were from New England Biolabs. *Escherichia coli* CJ236 and mutagenesis kit were obtained from Bio-Rad. $^{65}ZnCl_2$ was from PerkinElmer Life Sciences. 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 from Invitrogen. Anti-FLAG M2 affinity gel, anti-FLAG M2-alkaline phosphatase antibody, and 3xFLAG peptide were from Sigma. pMal-P2X vector and amylose resin was from New England Biolabs. Oligonucleotides were synthesized by the oligonucleotide facility at California Institute of Technology. Plasmid minipreparation kits were from Qiagen. Western blotting reagents and nitrocellulose membrane were from Amersham Biosciences, Inc. Polyclonal antibody for the pol ϵ complex was initially provided by Dr. Akio Sugino (Osaka University, Osaka, Japan). RPA was purified using vectors provided by Richard Kolodner, Ludwig Institute, La Jolla, CA).

Construction of POL2, DPB2, DPB3, and DPB4 Recombinant Baculoviruses—The NH_2 terminus of *POL2* was amplified by PCR. A putative viral ribosomal binding site and FLAG epitope sequence in-frame with the NH_2 terminus of *POL2* were also added. The sequences of the primers used for PCR are as follows: FE1, 5′-GATCCTCGAGCCTATAAATATGGACTACAAGGACGACGATGACAAAATGATGTTTGGCAAGAAAAAA-3′ FE2, 5′-GTTGATAAGCTTGAACCATC-3′.

The PCR products were cloned directly in PCR Blunt II Topo (Invitrogen). The recombinant plasmid containing the insert was digested with *Xba*I/*Kpn*I, and the *POL2* fragment was cloned in pBacPak8 (CLONTECH) to create pBac-N-POL2. The pfastbacPOL2 (8) was digested with *Bam*HI, and the vector containing the COOH terminus of *POL2* was self-ligated to create pfast-C-POL2. The pBac-N-POL2 was digested with *Bam*HI, and the NH_2-terminal *POL2* fragment was cloned in pfast-C-POL2, which resulted in pfast-FLAGPOL2.

The 6×His-DPB2, DPB3, and DPB4 baculoviruses were prepared as described earlier (8). The preparation of baculoviruses was performed using the Bac to Bac system from Invitrogen as described previously (8). The culture supernatant containing the baculovirus particles was stored in the dark at 4 °C. As a control, pfastbacI vector plasmid was used to prepare wild-type baculovirus. These virus are available from Orbigen (San Diego).

Expression, Purification, and Reconstitution of the Pol ϵ Complex—For expression of proteins, 150×10^6 log phase High-Five insect cells were grown in suspension tissue culture flasks. The cells were infected with viral supernatant at a multiplicity of infection of 10 for *POL2*, 5 for *DPB2*, 5 for *DPB3*, and 5 for *DPB4*. After a 2-day incubation at 27 °C, cells were harvested, washed once with cold 1× Tris-buffered saline, flash frozen in liquid N_2, and stored at −70 °C. The frozen cells were thawed once on ice and then resuspended in 5 ml of buffer A (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 20% glycerol, pH 7.5) containing 20 μg/ml leupeptin. 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. 1 ml of anti-FLAG M2-agarose beads (50:50 in buffer A) was 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 1,000 rpm using a swinging bucket centrifuge, and the unbound protein fraction was collected. Then 1 ml of buffer A was added to the beads, and it was packed on a column. The beads with the bound proteins on the column were washed with 25 ml of buffer A. To elute proteins, 5 × 1 ml of buffer A containing 100 μg/ml 3× FLAG peptide was added, and 0.5 ml fractions were collected. The total protein, unbound protein, and eluted protein fractions were analyzed using 10% SDS-PAGE and Western blotting. The fractions were also analyzed for DNA polymerase activity. 100–200 μg of the eluted protein was diluted with buffer B (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% glycerol) and applied to the Mono Q (5/5) column. The column was washed with 7 ml of buffer A, subjected to a 4-ml linear gradient from 100 to 225 mM NaCl, and then a 25-ml linear gradient from 225 to 430 mM NaCl and 0.5-ml fractions were collected. The fractions were analyzed by 10% SDS-PAGE, silver staining, and also by enzymatic assay analysis. The fractions containing 140 kDa of Pol2p or pol ϵ were combined, and glycerol was adjusted to 35%. The proteins were then aliquoted in 50–100-μl fractions and stored at −70 °C. The concentration of protein was determined using the Bradford assay with bovine γ-globulin as a standard.

Expression and Purification of RF-C and PCNA—The expression of RF-C was performed in *Saccharomyces cerevisiae* using pBL420 expression plasmid containing genes for all five subunits of RF-C complex (12). PCNA was expressed in *E. coli* in a 50-liter culture as described (13). The purification of RF-C and PCNA was carried as described (12, 13). The purified RF-C fractions were shown to contain DNA-stimulated ATPase activity and were also stimulated by PCNA. Gel electrophoresis showed equimolar recovery of all five subunits and greater than 95% purity. Purified PCNA was assayed by the ability to stimulate calf thymus DNA polymerase (15).

DNA Polymerase Assays—DNA polymerase assays were as described, using $(dA)_{300}$·oligo$(dT)_{10}$ (1:20, template to primer chains) as DNA template (4, 5). Reaction mixtures (60 μl) consisted of 50 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 10% glycerol, 8 mM $MgCl_2$, 17 μg/ml $(dA)_{300}$·oligo$(dT)_{10}$, 100 μg/ml bovine serum albumin, and 50 μM [^{3}H]dTTP (100 cpm/pmol). The $(dA)_{300}$·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, twice 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 DNA polymerase activity is defined as 1 nmol of dNMP incorporated per h.

Terminal Removal Assay—A terminal mismatch substrate was prepared by hybridizing 18 bp of TM1 (phosphorylated at the 5′-end by ^{32}P) to 20 bp of TM2 using 1× SSC buffer. The sequences of TM1 and TM2 are as follows: TM1, 5′-CTA GAC TGA TCG ATG CTC-3′; TM2, 5′-TTA AGC ATC GAT CAG TCT AG-3′.

The 25-μl reactions mixture consisted of 50 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 10 mM $MgCl_2$, 50 mM KCl, and 20 pmol of annealed substrate. The reaction was initiated by adding 0.6 unit of pol ϵ or 0.8 unit of Pol2p (140 kDa) at 37 °C. Samples of mixture were removed at 3, 6, 9, 15, and 30 min. The reaction was stopped by adding 1 μl of 0.5 M EDTA, and products were analyzed on a 15% urea-polyacrylamide DNA sequencing gel.

Primed M13 Assay—The 30-μ1 reaction mixture contained 50 mM Tris-HCl, pH 7.5; 8 mM $MgC1_2$; 0.2 mg/m1 bovine serum albumin; 100 μM dCTP, dGTP, and dATP; 25 μM [^{32}P]dTTP (5,000–10,000 cpm/pmol); 0.5 mM ATP; 250 ng of singly primed M13mp18 (the 36-mer primer is complementary to nucleotides 6330–6295); and 1.2 μg of yeast replication protein A. The reaction was started by adding 0.6 unit of pol ϵ or 0.8 unit of Pol2p (140 kDa), and the reaction reaction mixture was incubated for 15 min at 37 °C. The reaction was stopped by adding 8 μ1 of stop buffer (60% glycerol, 50 mM EDTA, 1% SDS, xylene cyanol, and bromcresol green). To assess the effect of RF-C and PCNA, the primed M13 template was incubated with 1.2 μg of RP-A, 20 ng of RF-C, and 200 ng of PCNA. The reaction was started by the addition of either pol ϵ or Pol2p as described above. For salt dependence of the reaction, 25–150 mM KCl was included in the mixture. The products of the reaction were separated on a 1% alkaline agarose gel (15 × 13 × 0.3-cm gel, 23 V, 15 h). The gel was neutralized in 1× TBE buffer for 30–45 min and dried on a gel dryer for 45 min with no heat. The gel was dried further in air for 2–3 h at room temperature and analyzed by phosphorimaging.

Construction of MalE-POL2 Gene Fusions—POL2 fragments (nucleotides 6663–6942, amino acids 2100–2190) were generated by PCR using pRS314-POL2, pRS314-pol2-F (2153–2162), and pRS314-pol2-P (14) as DNA templates. *Bam*HI/*Sal*I restriction enzyme sites were created in the design of the oligonucleotide primers. The sequences of the oligonucleotides are as follows: MAL3, TTC AAA GAT CCA GGA TCC AGT CTC GTC GTG-3′; MAL4, TAA CTT TTG AAG TCG ACT TTA TCA GGG GAG AGT TC-3′.

The fragments were purified, digested by *Bam*HI/*Sal*I, and then purified from agarose gels. The trimmed fragments were cloned in-frame with MalE between the *Bam*HI and *Sal*I sites of pMal-P2X vector.

Expression and Purification of MalE-POL2 Fusion Proteins—BL21(DE3) *E. coli* cells were transformed with pMal-P2X recombinant plasmids containing the *MalE-POL2* gene fusions. As a control, cells were also transformed with pMal-P2X vector only. Cells harboring the plasmids were grown in 1 liter of 2× YT medium at 37 °C until the A_{600} reached 0.8. Isopropyl-1-thio-β-D-galactopyranoside was added to 0.5 mM, and the culture was grown for another 4 h. The cells were harvested and centrifuged at 12,000 rpm. The pellet was resuspended in 22 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM β-mer-

captoethanol, 0.05% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μM pepstatin, 5 μM leupeptin, and 10% glycerol). The mixture was sonicated (6 × 30 s at 50% setting). To the cell extract, polymin P was added to 0.3% and was tumbled at 4 °C for 1 h to precipitate the DNA. The mixture was centrifuged at 18,000 rpm for 30 min, and the supernatant was collected and combined with 3 ml of hydrated amylose resin (equilibrated in buffer M (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM β-mercaptoethanol, 0.05% Triton X-100, l mM EDTA)). The mixture was tumbled at 4 °C for 2 h, centrifuged at 1,200 rpm, and unbound protein fraction was collected. The resin containing the bound proteins was packed into a gravity column. The resin was washed with 40 ml of buffer M, and MalE fusion proteins were eluted with buffer N (buffer M containing 50 mM maltose). Fractions (8 × l ml) were collected and analyzed by 10% SDS-PAGE and Coomassie staining. The protein concentration was determined using the Bradford assay with bovine γ-globulin as standard. Typically, 20–30 mg of MalE fusion protein was obtained from l liter of culture.

Zinc Blotting Assay—The assay was performed as described (16). Briefly, MalE-POL2 fusion proteins were separated by 10% SDS-PAGE and electroblotted on a nitrocellulose membrane. The membrane was incubated in metal-binding buffer T (100 mM Tris-HCl, pH 7.0, 50 mM NaCl, 5 mM dithiothreitol) for 1.5 h. The proteins were denatured for 30 min in the same buffer containing 6 M guanidine HCl. The guanidine HCl was removed, and the membrane was washed (3 × 15 ml) with buffer T and then incubated for 1 h with 7 μCi/ml $^{65}ZnCl_2$ in 10 ml of buffer T. The membrane was washed with 3 × 15 ml of buffer T, dried briefly in air, and analyzed by phosphorimaging. After phosphorimaging, quantification of the proteins was carried out by staining the membrane with 0.1% Amido Black in 45% methanol and 10% acetic acid and densitometry analysis.

RESULTS

In Vivo Reconstitution of Pol ϵ in Insect Cells—Although we were previously able to achieve high expression levels of Dpb2p, Dpb3p, and Dpb4p in insect cells, the large catalytic subunit Pol2p was expressed poorly (8, 14). To increase the Pol2p recovery level, the Pol2p was tagged with a FLAG epitope at its amino terminus, allowing affinity purification by anti-FLAG resin. Insect cells were simultaneously infected with four viruses, each expressing one of the four subunits. The Dpb2p was tagged with His_6 at its NH_2 terminus for the current studies. Tagging resulted in higher solubility of the Dpb2p as compared with the untagged version, and attempts to purify the four-subunit enzyme using virus expressing untagged Dpb2p have failed to yield the holoenzyme. Dpb3p and Dpb4p were expressed without tags. Western blotting of extracts of cells expressing these four viruses using anti-FLAG or α-Pol2p antibody showed a 10× increase in Pol2p in extracts compared with our previous expression strategy (data not shown) (8). Pol ϵ was then purified using an anti-FLAG affinity column. As shown in Fig. 1*A*, all four subunits (FLAG-Pol2p, His_6-Dpb2p, Dpb3p, and Dpb4p) eluted together, suggesting that these polypeptides had formed the pol ϵ holoenzyme complex *in vivo*. As observed during purification of pol ϵ from yeast, in addition to the pol ϵ holoenzyme, a 140 kDa truncated form of Pol2p was also present in the purified fraction (Fig. 1*A*). To resolve the pol ϵ holoenzyme from the truncated Pol2p (140 kDa), the mixture was fractionated further on a Mono Q anion exchange column, and the fractions were assayed for DNA polymerase activity and analyzed by gel electrophoresis. As shown in Fig. 1*B*, the polymerase activity was resolved into two distinct peaks. SDS-PAGE analysis of the corresponding fractions indicated that fractions 20–23 (eluting at 0.26 M NaCl) contained Pol2p (140 kDa), whereas fractions 25–28 (eluting at 0.29 M NaCl) contained the four-subunit pol ϵ holoenzyme (FLAG-Pol2p (256 kDa), His_6-Dpb2p, Dpb3p, and Dpb4p) complex. Identity of the bands was confirmed by Western blotting (not shown). This elution profile is similar to that obtained when pol ϵ was purified from yeast cells (1). Scanning of either Coomassie- or silver-stained gels indicates that Pol2p:Dpb2p:Dpb3p:Dpb4p are in 1.3:1:3:4 stoichiometric ratio in the holoenzyme preparation, which is consistent with the composition of the holoenzyme purified for yeast. The specific activities of the pol ϵ holoenzyme and Pol2p (140 kDa) were 25,000 and 65,000 units/mg, respectively. The yield of the holoenzyme was 15–20 μg/100 ml of infected cells. The copurification of the four subunits of the pol ϵ holoenzyme in the same stoichiometric amounts as found in the native enzyme purified from yeast combined with the high enzymatic activity demonstrates that we have reconstituted the functional polymerase holoenzyme.

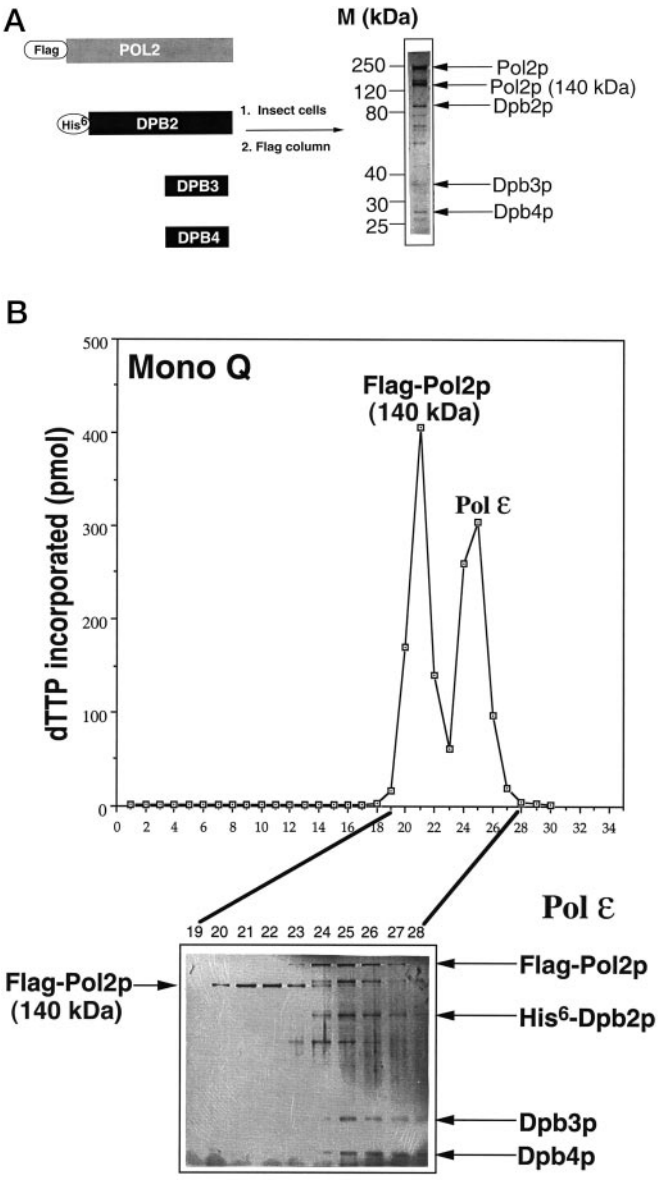

FIG. 1. **Reconstitution and purification of pol ϵ in Sf9 insect cells.** *Panel A*, Sf9 cells (150 × 10^6) were coinfected with FLAG-POL2, His_6-DPB2, DPB3, and DPB4, and proteins were purified using anti-FLAG M2 as described under "Experimental Procedures." The fractions eluted with FLAG peptide were pooled and analyzed by Coomassie staining after 10% SDS-PAGE. *Panel B*, the protein was fractionated on a 1-ml Mono Q column as described under "Experimental Procedures." The column was washed with 5 column volumes of buffer A, and then a 20-ml linear gradient was applied from 100 to 500 mM NaCl in buffer A. Fractions were analyzed on 10% SDS-PAGE and visualized by silver staining. The fractions were also assayed for polymerase activity using poly(dA)·oligo(dT). Fractions 20–23 containing the Pol2p (140 kDa), pol ϵ complex (fractions 25–28), and Pol2p (140 kDa) + pol ϵ (fraction 24) were pooled.

There are two bands present in the reconstituted holoenzyme which warrant further explanation. The additional band below 80 kDa is an unrelated contaminant because it does not copu-

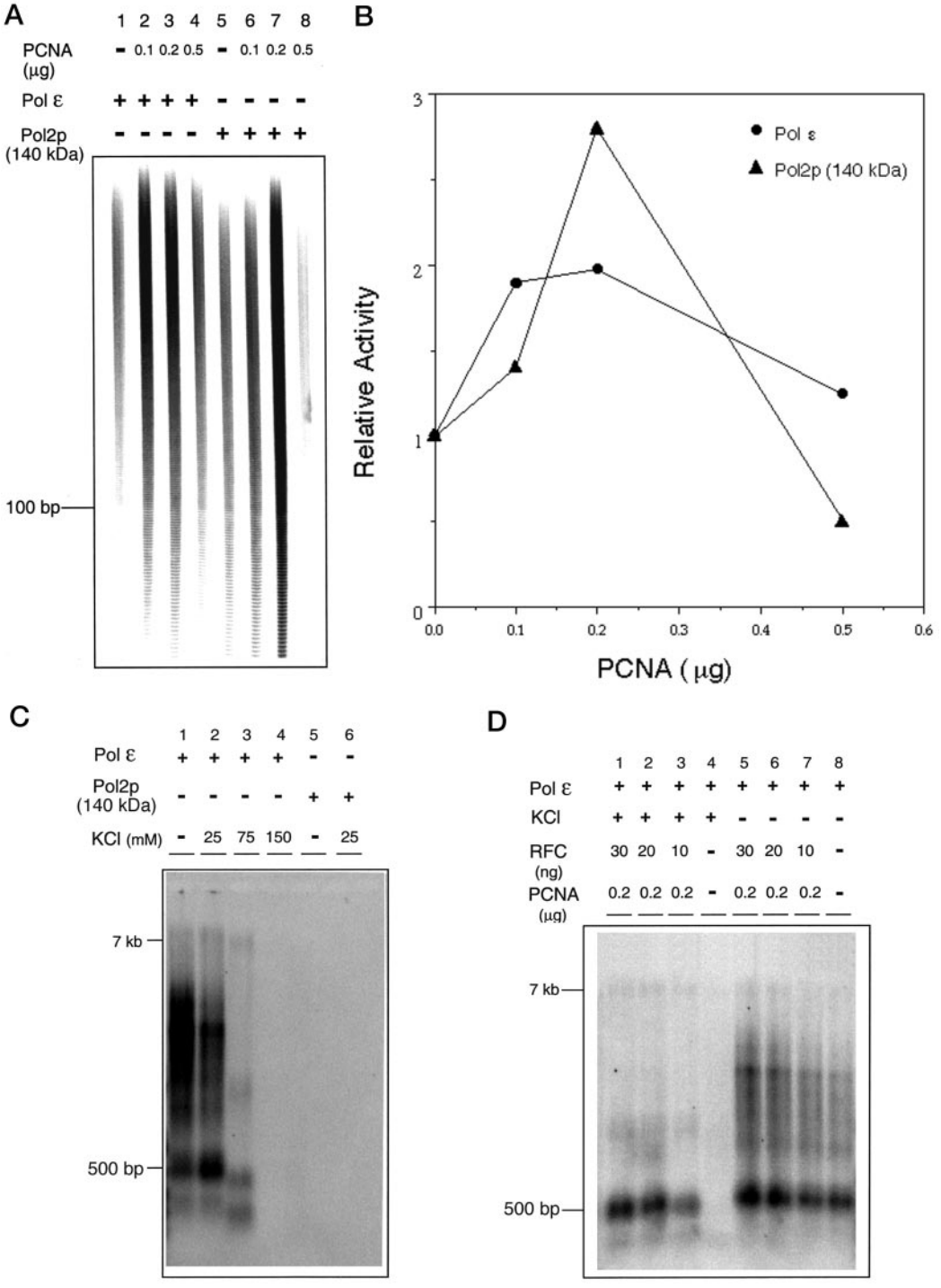

FIG. 2. **Processivity of pol ϵ and Pol2p (140 kDa).** *Panel A*, processivity of 0.1 unit of pol ϵ and 0.05 unit of Pol2p (140 kDa). Processivity was measured using poly(dA)·oligo(dT) (1:20), which was present in excess to ensure that only one molecule of polymerase was present per template-primer terminus. Samples were run on a denaturing urea-acrylamide gel (6%). *Panel B*, relative stimulation of pol ϵ or Pol2p (140 kDa) by PCNA. Stimulation was determined by quantitating *lanes 1–8* shown in *panel A* using Image Quant. *Panel C*, replication of circular RPA-coated M13mp18 DNA by 0.6 unit of pol ϵ or 0.8 unit of Pol2p (140 kDa). The enzymes were assayed in the absence or presence of varying concentrations of KCl as described under "Experimental Procedures." *Panel D*, PCNA·RF-C-mediated replication of circular RPA-coated M13mp18 DNA by 0.6 unit of pol ϵ in the absence or presence of 75 mM KCl as described under "Experimental Procedures."

rify with the polymerase peak and does not react with Pol2p antibody. Western blotting using a polyclonal antibody to the entire Pol2p expressed in *E. coli*[3] revealed that the additional band above the Pol2p (140 kDa) is a proteolytic product of the FLAG-Pol2p. We have now demonstrated that its presence can be minimized by growing the infected cells for 60 h rather than 48 h, the conditions under which the enzyme in Fig. 1 was prepared (data not shown).

Processivity of the Pol ϵ Holoenzyme and Pol2p (140 kDa)—Both pol ϵ and Pol2p (140 kDa) have been shown to possess high intrinsic processivity (10–12, 17). To characterize our enzyme preparations, we measured the processivity of pol ϵ and Pol2p (140 kDa) using the $(dA)_{300}$·oligo$(dT)_{10}$ template (18). As shown in Fig. 2*A*, both pol ϵ holoenzyme and Pol2p were inher-

[3] C. Li and J. Campbell, unpublished observations.

ently processive and able to synthesize at least 200 bases/synthesis cycle. The addition of PCNA, however, stimulated both the extent of synthesis by 2–3-fold (Fig. 2*B*) and the processivity of both pol ϵ holoenzyme and Pol2p (140 kDa) (Fig. 2*A*). The extent of stimulation is similar to that reported by others (19). As described previously, large amounts of PCNA were inhibitory (9). This level of PCNA may compete with the template for the polymerase.

Processivity was also measured using single primed M13 template. As shown in Fig. 2*C*, pol ϵ holoenzyme copied the M13 template generating an almost full-length (approximately 7210 bp) product, as well as some shorter species (see below). An equivalent number of units of Pol2p, as measured by activity on $(dA)_{300}$·oligo$(dT)_{10}$, was not able to copy the M13 efficiently (Fig. 2C, *lanes 5* and *6*). As reported previously, however, when Pol2p (140 kDa) was used at a higher concentration (5-fold), synthesis of full-length DNA was evident (not shown). This suggests that although Pol2p (140 kDa) can copy the M13 template, it is not as efficient as the pol ϵ holoenzyme.

Processivity was measured in the presence of KCl because the enzyme purified from yeast is very sensitive to salt. Pol ϵ holoenzyme showed reduced DNA synthesis in the presence of KCl (Fig. 2*C*, *lanes 2*, *3*, *4*, and *6*). Pol ϵ holoenzyme was almost completely inhibited by 150 mM KCl and more than 95% inhibited by 75 mM KCl.

Because pol ϵ is far more processive than pol δ, even without accessory proteins, there has been some question as to the significance of the ability of PCNA and RF-C to stimulate pol ϵ. Therefore, the effects of PCNA and RF-C on pol ϵ holoenzyme were tested. As shown in Fig. 2*D*, using primed M13 coated with replication factor A as template pol ϵ is inhibited drastically in the presence of 75 mM KCl (compare *lanes 4* and *8*). The addition of yeast PCNA and RF-C stimulated synthesis in the presence of KCl dramatically (Fig. 2*D*, *first four lanes*), as observed previously using human PCNA, RF-C, and single-stranded DNA-binding protein (10) or yeast accessory proteins and pol ϵ purified from yeast (9). Also as observed previously (11), synthesis was semiprocessive. Much of the product was only 500 nucleotides in length, but some product extended to full length with the highest concentrations of RF-C used. This 500-bp product has been described previously when M13 is used as template for pol ϵ (9, 11) and has been shown to be caused by polymerase pausing and lability of the PCNA·RF-C·pol ϵ complex compared with a PCNA·RF-C·pol δ complex (11). When the activity was measured in the absence of KCl, the addition of PCNA and RF-C increased processivity, and this increase was proportional to the amount of RF-C added (Fig. 2*D*, *fifth* through *eighth lanes*). We conclude that PCNA and RF-C stimulate the extent of synthesis and the processivity of reconstituted pol ϵ *in vitro* in a manner mechanistically similar to native pol ϵ. Thus, this preparation has all of the properties of the enzyme purified from yeast.

Exonuclease Activity of the Purified Protein—The 3′–5′ exonuclease assay was evaluated using a primer-template with a 3′-terminal mismatch on the primer (Fig. 3*A*) to compare further the new preparation of pol ϵ with previously published results obtained using the enzyme purified from yeast (1). The activity shown in Fig. 3*B* and quantified in Fig. 3*C* was similar to that found previously. The mismatched terminus is removed efficiently, with a half-life of less than 2 min (Fig. 3, *B* and *C*) by both reconstituted pol ϵ and Pol2 (140 kDa), and the resulting matched 17-mer is comparatively stable, with 80% remaining after a 30-min incubation. One minor difference from previous results is that there is a greater preference for the mismatched terminus than the subterminal base paired nucleotides in this experiment than in that carried out with the enzyme from yeast, but this activity might vary from preparation to preparation and with different primer-template sequences. The major result of this experiment is that both enzymes showed significant exonuclease activities.

Effect of Mutations in a Consensus PCNA Interaction Domain in POL2—PCNA interacts with many proteins of DNA metabolism, and a consensus sequence constituting the PCNA interaction domain conserved in many of these proteins has been defined (20). The PCNA interaction motif is of the form **Q*XX*h*XX*aa** (Fig. 4), where **h** and **a** are hydrophobic and aromatic residues, respectively. Because PCNA stimulates both Pol2p (140 kDa) and pol ϵ holoenzyme, we searched the *POL2*, *DPB2*, *DPB3*, and *DPB4* gene sequences for potential PCNA interaction sites using BLAST. We observed that the catalytic *POL2* subunit contains a consensus PCNA interaction domain from amino acids 1193 to 1201. This region is included in the Pol2p (140 kDa), whose most COOH-terminal amino acid has been estimated to be 1,270 amino acids (19). It falls within a larger region, amino acids 1136–1263, which we have shown to be essential for viability.[3] Strain *pol2-M* contains amino acids 1136–2222 of the Pol2p and can support the growth of yeast even in the absence of the catalytic domain. When we deleted amino acids 1136–1263 from the pol2-M protein, pol2-M was no longer able to support growth.[3] We constructed a series of mutants in full-length *POL2* genes carrying specific mutations within the PCNA consensus (amino acids 1193–1201), using the site-directed mutagenesis approach we described previously for mutations in the zinc finger region (14). The phenotypes of the resulting mutants are shown in Fig. 4. Although the PCNA binding motif falls in the essential 1136–1263 amino acid region, all of the mutants shown in Fig. 4 were viable with only a marginal, if any, growth defect. Even mutant *pol2-305*, carrying a deletion of the entire consensus motif, grew apparently normally.

Because pol ϵ is implicated in repair, the mutants were also analyzed for sensitivity to MMS, a DNA-alkylating agent, as described previously (14). As shown in Fig. 3, the *pol2-305* mutant, with deletion of the entire consensus PCNA interaction motif, was 1,000-fold more sensitive to MMS than wild type, in fact, just as sensitive as the *po2-11* mutant described previously (14). Because the *pol2-305* mutant strain grows normally in the absence of MMS, the sensitivity is most likely the result of specific disruption of a protein-protein interaction site, rather than a gross structural change caused by the removal of nine amino acids. However, we cannot rule out that a minor structural change makes the polymerase less efficient and the resulting strain more sensitive to DNA damage. To increase confidence that we were affecting a protein-protein interface, point mutations were also investigated. Mutation of the conserved hydrophobic group (L1196A) increased MMS sensitivity 10-fold, whereas a double point mutant, F1199A/F1200A, was 100-fold more sensitive to MMS. The phenylalanine aromatic residues in the PCNA interaction motifs of a number of other proteins involved in DNA metabolism have been shown to be important for PCNA binding.

MalE Fusion Protein of Putative Zinc Finger of POL2 Binds Zinc—Significant differences between the pol ϵ holoenzyme and the Pol2p (140 kDa) such as those reported here and elsewhere may be the result of another interesting but incompletely characterized motif in the Pol2p. The full-length catalytic Pol2p subunit of the pol ϵ holoenzyme contains a putative zinc finger of type $CX_2CX_{18}CX_2CX_{30}CX_2CX_{11}CXC$ located at the COOH terminus (amino acids Cys-2108 to Cys-2181). Evidence to date suggests that it plays a critical role in protein-protein interactions and single stranded DNA sensing during DNA replication and S phase cell cycle checkpoint signaling (14, 19). Given the importance of the zinc finger region, it will

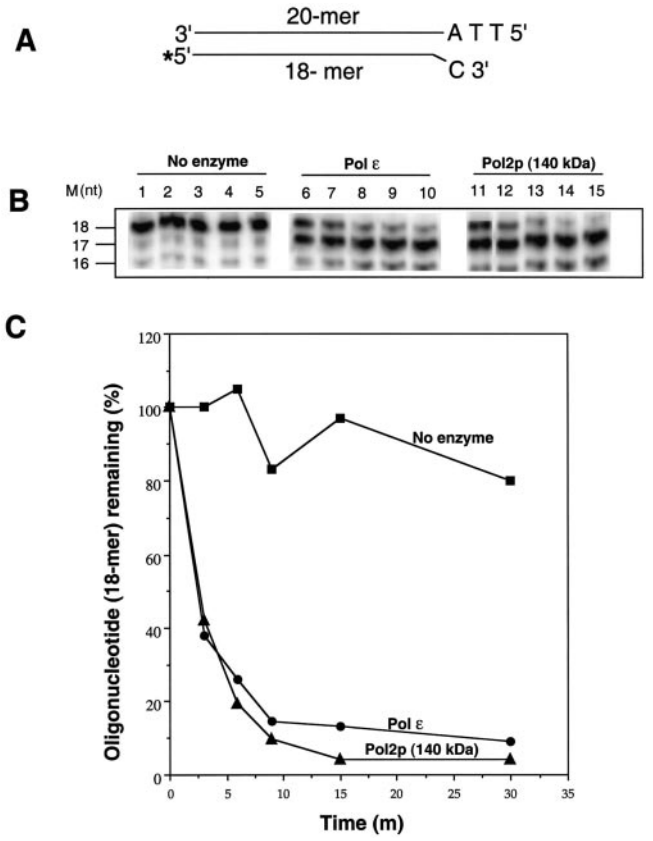

FIG. 3. **Terminal mismatch excision by the 3′–5′ exonuclease activity of pol ϵ and Pol2p (140 kDa).** *Panel A*, schematic of the oligonucleotide cassette used in the assay. *Panel B*, mismatch excision reaction. The reactions were performed as described under "Experimental Procedures." Samples were taken at different time intervals and analyzed on a 15% urea-polyacrylamide gel and scanned on a PhosphorImager. *Lanes 1*, *6*, and *11*, 3 min; *lanes 2*, *7*, and *12*, 6 min; *lanes 3*, *8*, and *13*, 9 min; *lanes 4*, *9*, and *14*, 15 min; and *lanes 5*, *10*, and *15*, 30 min. *Panel C*, the amount of the 18-mer oligonucleotide remaining was quantitated using Image Quant and plotted.

be interesting to determine the NMR or crystal structure of this domain. Toward this end, we have expressed and purified milligram quantities of MalE fusions of *POL2* zinc finger (amino acids 2100–2190 of Pol2p) and characterized the domain for zinc binding capacity. The MalE-POL2 fusion protein was purified, blotted to nitrocellulose membrane, denatured in guanidine HCl, renatured, and probed for zinc binding using $^{65}ZnCl_2$. As shown in Fig. 5*A*, the MalE-POL2 zinc finger binds zinc (*lanes 5* and *6*) as well as control proteins alcohol dehydrogenase (*lane 2*) and serum albumin (*lane 1*). MalE protein with no *Pol2* zinc finger did not bind zinc (*lanes 3* and *4*), which indicates that it is the *POL2* zinc finger that is responsible for zinc binding. Previously, we had shown that the *pol2-F* mutant with a 10-amino acid deletion between Cys-2153 and Cys-2162 is defective in interaction with pol ϵ subunits and is temperature-sensitive for growth. To understand the effect, if any, of the deletion on zinc binding, we overproduced and expressed the MalE-pol2-F fusion protein and assessed its zinc binding capacity. As shown in Fig. 5*A* (*lanes 7* and *8*), the MalE-pol2-F fusion protein binds zinc in a manner similar to that of the wild-type MalE-POL2. Amido Black staining of the membrane used in Fig. 5*B* showed that equal amounts of the MalE-POL2 and MalE-pol2-F fusion proteins were present. The data indicate that the deletion of 10 amino acid residues within the inter-zinc finger amino acids has little effect on zinc binding capacity, although it abolishes Pol2 activity *in vivo*.

A central feature of the zinc finger is the tetrahedral coordination of the Zn(II) by cysteine or histidine ligands. Some metal ions such as Cu(II), Co(II), and Cd(II) have the capacity to replace Zn(II). A competition with other divalent metal ions for zinc binding capacity was carried out. The results are shown in Fig. 5*C*. The addition of Cd(II) and Cu(II) but not Co(II) at 1 mM concentration interfered with the zinc binding, whereas other metal ions Mg(II), Mn(II), Ca(II), and Ni(II) at a 10 mM concentration had little effect. This suggests that Cd(II) and Cu(II) can substitute for Zn(II) binding.

Effects of Mutation Which Alter Zinc Binding—In a previous study, we described a number of cysteine to alanine point

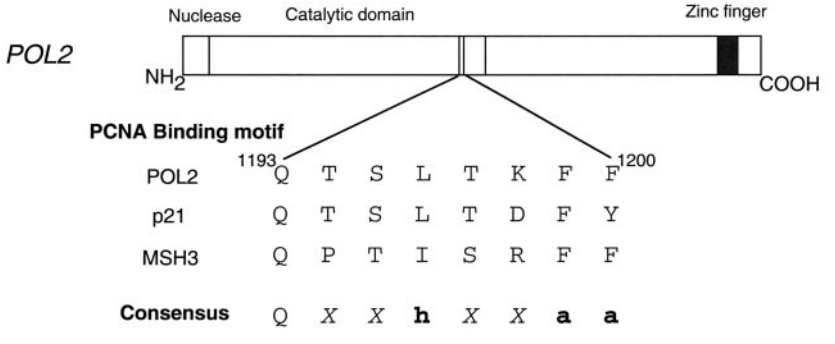

FIG. 4. **Characterization of point mutations in the PCNA interaction motif in *POL2*.** The point mutants were prepared as described previously (14) and under "Experimental Procedures." The mutant genes were subcloned in pRS314 vector and introduced into the A1128*pol2–3::LEU2(YEpPOL2*) as described (14). The transformants were selected on agar plates lacking tryptophan and containing 5-fluoroorotic acid at 24 °C. The viable colonies were grown in YPD, and the saturated culture was serially diluted. 5 μl of each dilution, containing 10^4, 10^3, 10^2, and 10 cells, respectively, as determined by hemocytometer counting, was plated on YPD plates. The plates were kept at 24 and 37 °C for 4 days. For measuring the MMS sensitivity, 0.02% MMS was added in the YPD plates. 5 μl of each dilution was plated, and the plates were kept at 24 °C for 4 days. **++++,** wild type resistance; +++, 10-fold more sensitive; ++, 100-fold more sensitive; +, 1,000-fold more sensitive.

Mutant	Mutations	Growth		Growth on MMS (0.02%)
		23°C	37°C	%
POL2		++++	++++	100
pol2-301	Q1193A	++++	++++	100
pol2-302	L1196A	++++	++++	10
pol2-303	F1200A	++++	++++	100
pol2-305	Δ1193-1200	++++	++++	0.1
pol2-306	F1199A	++++	++++	100
pol2-307	F1199A; F1200A	++++	++++	1

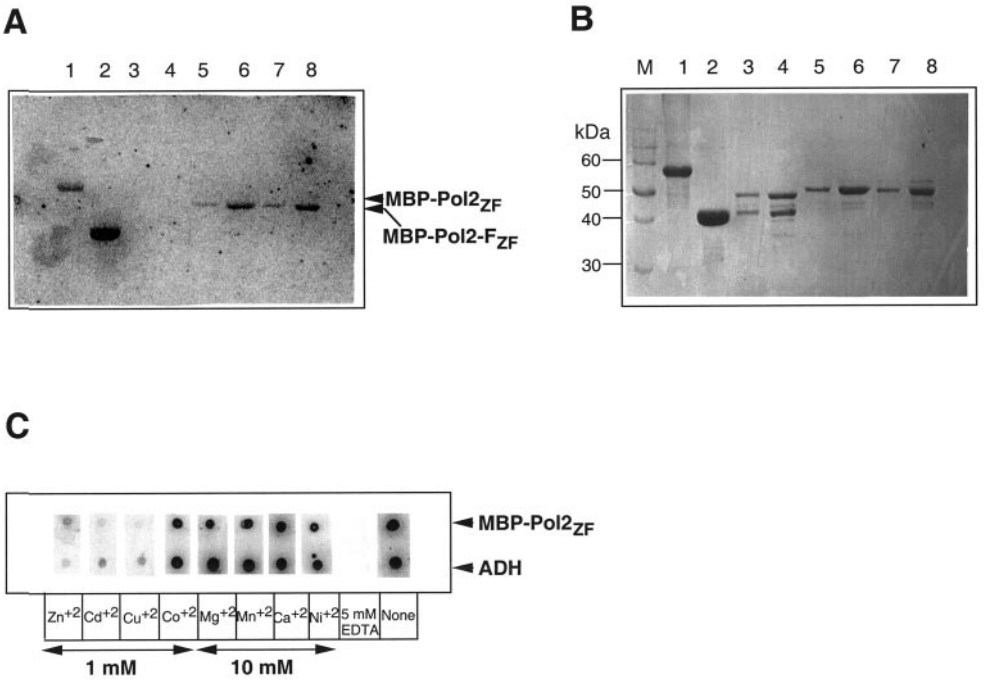

FIG. 5. **Characterization of the putative zinc finger of *POL2* for zinc binding.** *Panel A*, the putative zinc finger of *POL2* (amino acids 2100–2190) and *pol2-F* (amino acids 2100–2190, Δ 2153–2162) (14) were fused with maltose-binding protein (MBP), and the protein was expressed and purified as described under "Experimental Procedures." The purified proteins were separated on 10% SDS-PAGE and were electroblotted on a nitrocellulose membrane. The proteins on the membrane were denatured using 6 M guanidine HCl, renatured by quick washes, and the membrane was incubated with 7 μCi/ml $^{65}ZnCl_2$ and analyzed by phosphorimaging as described under "Experimental Procedures." *Lane 1*, bovine serum albumin; *lane 2*, alcohol dehydrogenase; *lane 3*, 0.5 μg of MBP; *lane 4*, 1.0 μg of MBP; *lane 5*, 0.5 μg of MBP-POL2; *lane 6*, 1.0 μg of MBP-POL2; *lane 7*, 0.5 μg of MBP-pol2-F; *lane 8*, 1.0 μg of MBP-pol2-F. *Panel B*, after phosphorimaging, the proteins on the membrane were visualized by Amido Black staining to confirm that the same quantities of protein were transferred to membrane. *Panel C*, competition of various divalent metal ions for zinc binding. The MBP-Pol2p was spotted on nitrocellulose membrane. The denaturation-renaturation step was omitted, and the proteins were probed for 1 h with $^{65}ZnCl_2$ in 0.5 ml of metal-binding buffer in the presence or absence of competing ions as indicated. The filters were washed and analyzed by phosphorimaging. Alcohol dehydrogenase (*ADH*) was used as a positive control for zinc binding.

mutations in the zinc binding domain of *POL2*. The *pol2-P* (C2108A, C2111A, C2130A, C2133A) and *pol2-S* (C2164A, C2167A, C2179A, C2181A) mutants grew well at all temperatures but were MMS-sensitive (6). To correlate the function with zinc binding, MalE-pol2-P and MalE-pol2-S fusion proteins were expressed and purified and analyzed by the zinc blotting assay. The ^{65}Zn binding was reduced at least 10-fold for the *pol2-S* mutant (Fig. 6*A*, *lane 5*), whereas it was reduced about 2-fold for *pol2-P* (Fig. 6*A*, *lane 4*), indicating that cysteine residues are involved in coordination with zinc, but they do not contribute equally. Fig. 6*B* shows the Amido Black staining of the membrane shown in Fig. 6*A*. The amino acids between position 2153 and 2162 are important for viability because *pol2-E* and *pol2-F* mutants are defective in DNA replication. However, the results presented here suggest that zinc binding to both cysteine clusters is not essential for formation of the essential Cys-2153 to Cys-2162 conformation.

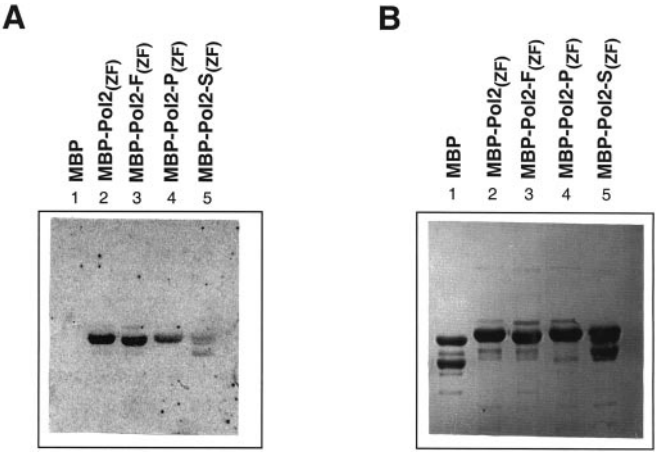

FIG. 6. **Cysteine to alanine point mutations in the putative zinc finger of *POL2* inhibit zinc binding.** *Panel A*, the putative zinc fingers (amino acids 2100–2190) in *pol2-P* (C2108A, C2111A, C2130A, C2133A) and *pol2-S* (C2164A, C2167A, C2179A, C2181A) and *pol2-F* (Δ 2153–2162) were fused to MBP, and the proteins were expressed, purified, and analyzed for zinc binding as described in the legend of Fig. 5*A* and under "Experimental Procedures." In each of *lanes 1–5* 5 μg of protein was used. The MBP served as a control (*lane 1*). *Panel B*, the proteins on the membrane shown in *panel A* were visualized by Amido Black staining to confirm that same amounts were transferred to the membrane.

DISCUSSION

Pol ϵ is one of the three essential polymerases in yeast. It has been shown to play crucial roles in replication, repair, and cell cycle checkpoint signaling. However, the precise role of pol ϵ in DNA replication is still unclear. To gain better understanding of the biochemical properties that might provide clarification of the specific *in vivo* reactions mediated by pol ϵ, we attempted to reconstitute and purify significant amounts of the pol ϵ complex. The data presented in this study show that we have reconstituted the pol ϵ complex in baculovirus-infected insect cells. This preparation offers unlimited usefulness for characterization of the enzymatic properties of this enzyme, which should aid in clarification of its enigmatic biological activity.

The holoenzyme has the same stoichiometry of subunits as in yeast. Interestingly, the 140-kDa, presumed proteolytic product of the *POL2* gene observed in yeast, is also recovered from insect cells (Pol2p (140 kDa)). This species may be produced by a caspase-like enzyme in human cells (21), although its origin in yeast and possible physiological role remain undefined.

Characterization of the properties of reconstituted pol ϵ, such as inherent processivity, mismatch removal, salt sensitivity, and stimulation by PCNA, demonstrated that it is similar to its native counterpart. We also showed that both pol ϵ complex and truncated Pol2p (140 kDa) show similar processivity on poly(dA)·(oligo)dT templates. On the M13 templates, pol ϵ showed strong pausing, indicating dissociation and association of the complex. In the presence of 75–100 mM KCl, pol ϵ lost significant activity on the M13 template. However, in the presence of a PCNA·RF-C complex, it regained its activity. Loss of activity could be the result of destabilization of the pol ϵ in the presence of salt and addition of PCNA·RF-C complex may stabilize the complex at a template-primer terminus. Interestingly, Pol2p (140 kDa) showed a reduction in activity compared with holoenzyme on M13 templates, further indicating the importance of the stabilization of the pol ϵ complex at the template-primer terminus. This preparation should be even more useful in further investigation of this question given the significant recent advances in characterizing the RF-C mechanism (22–25). Recent work has also shown variants of RF-C which are proposed to participate in specialized aspects of DNA replication, such as sister chromatid cohesion and DNA repair; the pol ϵ preparation will also be useful for working out their roles (26, 27).

To extend our previous work on the analysis of *POL2* structure/function, we studied the consensus PCNA interaction domain motif in *POL2*. Surprisingly, the deletion of the entire motif (amino acids 1193–1200) resulted in no loss of viability, possibly indicating that the observed *in vitro* stimulation of pol ϵ by PCNA is not required for its replication function. However, we cannot rule out the possibility of another PCNA binding site in Pol2p and/or other subunits. In fact, we have identified a second putative PCNA binding motif in *DPB2* (amino acids 219–226; QMFLTKYY), but its role has not yet been investigated. The deletion mutant and point mutants affecting the PCNA consensus motif of Pol2p were very sensitive to MMS, suggesting a role for the Pol2p-PCNA interaction in DNA repair. The MMS sensitivity of these mutants suggests that amino acids 1193–1200 may constitute a *bona fide* PCNA interaction site, and, if so, that PCNA is more important for recruiting pol ϵ to sites of DNA repair than for processivity during DNA replication. Alternatively, this site represents the ability to be stimulated by PCNA but not a direct interaction site, as has recently been shown for yeast FEN-1 (28).

Previously, our work showed that the putative zinc finger domain in Pol2p plays dual roles in DNA replication and checkpoint signaling (6, 8, 14). We showed, surprisingly, that the inter-zinc finger domain is essential for viability but that the conserved cysteines are not (6, 8, 14). The data in this report demonstrate that the putative zinc finger of Pol2p has zinc binding capacity. Furthermore, the mutations in the conserved cysteines resulted in loss of zinc binding capacity, indicating the direct participation of the cysteine ligands. As reported previously, the cysteine mutants show defects in repair of MMS-induced DNA damage (6). Because the inter-zinc finger mutation in Pol2p, which is essential *in vivo*, had no effect on zinc binding and because the cysteine mutations that reduce zinc binding are viable, full zinc binding is not essential for viability. The precise function of the zinc remains to be determined, but it may be required for maintaining the structural integrity of the zinc finger domain. Determination of the structure of the Pol2p zinc finger domain will provide further insights for the functional importance in protein-protein interactions and single stranded DNA sensing.

The efficient strategy to reconstitute the pol ϵ complex described in this report should enable us to take a more rigorous approach to understand the biochemical mechanisms of binding of pol ϵ to the template-primer terminus, interactions with PCNA·RF-C complexes and other replication proteins during DNA replication, recombination and repair, and ultimately with checkpoint signaling molecules. The similarity of the enzyme produced in insect cells to the enzyme described from yeast is striking. This similarity increases confidence that studies using the enzyme produced in insect cells to investigate higher levels of biochemical integration will have biological significance.

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