A four-subunit DNA polymerase ζ complex containing Pol δ accessory subunits is essential for PCNA-mediated mutagenesis

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Received August 30, 2012; Revised and Accepted September 19, 2012

ABSTRACT

DNA polymerase ζ (Pol ζ) plays a key role in DNA translesion synthesis (TLS) and mutagenesis in eukaryotes. Previously, a two-subunit Rev3–Rev7 complex had been identified as the minimal assembly required for catalytic activity in vitro. Herein, we show that Saccharomyces cerevisiae Pol ζ binds to the Pol31 and Pol32 subunits of Pol δ, forming a four-subunit Pol ζ4 complex (Rev3–Rev7–Pol31–Pol32). A [4Fe-4S] cluster in Rev3 is essential for the formation of Pol ζ4 and damage-induced mutagenesis. Pol32 is indispensable for complex formation, providing an explanation for the long-standing observation that pol32Δ strains are defective for mutagenesis. The Pol31 and Pol32 subunits are also required for proliferating cell nuclear antigen (PCNA)-dependent TLS. Pol ζ as Pol ζ2 lacks functional interactions with PCNA. Mutation of the C-terminal PCNA-interaction motif in Pol32 attenuates PCNA-dependent TLS in vitro and mutagenesis in vivo. Furthermore, a mutant form of PCNA, encoded by the mutagenesis-defective pol30-113 mutant, fails to stimulate Pol ζ4 activity, providing an explanation for the observed mutagenesis phenotype. A stable Pol ζ4 complex can be identified in all phases of the cell cycle suggesting that this complex is not regulated at the level of protein interactions between Rev3–Rev7 and Pol31–Pol32.

INTRODUCTION

DNA polymerase ζ (Pol ζ) is a B-family DNA polymerase participating in DNA translesion synthesis (TLS) and plays a predominant role in both spontaneous and damage-induced mutagenesis in all eukaryotes (1–3). Pol ζ bypasses a variety of DNA lesions and readily extends mismatched primer-template termini (4,5). Pol ζ was initially identified as a heterodimeric complex of the catalytic Rev3 subunit with the accessory Rev7 subunit that is also required for DNA polymerase activity (6). Mutations in REV3 or REV7 result in a severe decrease of induced mutagenesis. The rev3Δ and rev7Δ strains are also spontaneous antimutators, suggesting that Pol ζ acts to bypass naturally occurring damage or other structural blocks (7–9). Deficiency in the Rev3 catalytic subunit leads to embryonic lethality in mice (10). In humans, alterations in Pol ζ expression are associated with cancer, chromosome instability and cisplatin resistance (11).

All four eukaryotic B-family DNA polymerases, Pol α, δ, ε, and ζ, contain two conserved cysteine-rich metal-binding motifs, CysA and CysB, in the C-terminal domain (CTD) of their catalytic subunits [reviewed in (12,13)]. The four cysteine residues of CysA form a classical zinc ribbon motif. In the case of Pol δ, where the role of both CysA and CysB in metal binding has been studied most extensively, the four-cysteine motif of CysB coordinates a [4Fe-4S]2+ cluster (14). However, the other catalytic subunits have also been shown to bind [4Fe-4S] clusters. Indeed, expression of the CTD of Rev3 in Escherichia coli also indicated the presence of a [4Fe-4S] cluster in this domain (14). In Pol δ, the [Fe-S] cluster is required for stable binding of Pol3 to its second subunit Pol31 (14,15), which in turn binds to Pol32 (16–18). The CysB motif of the catalytic subunit of Pol α also coordinates interactions with its second subunit (19,20).

Therefore, an arrangement analogous to that determined for Pol δ may also hold for Pol α and for Pol ε.

In contrast to the three replicative DNA polymerases, interactions between the Rev7 subunit of Pol ζ with the catalytic subunit Rev3 have been mapped to the N-terminal region of human Rev3 rather than its CTD (6,21). The possibility then exists that the [4Fe-4S]-containing CTD of Rev3 might provide interactions with other factors that function in mutagenesis. Indeed, two recent articles report on the interaction between Rev3 and Pol31. One interaction study was carried out in...
slysed by vortexing with glass beads on ice. Cell lysates were collected, resuspended in lysis buffer (50 mM Hepes (pH 7.4), 800 mM NaCl, 5% glycerol, 1 mM DTT, 0.1% Tween 20, 0.01% NP40, 1 mM peptatin A, 0.5 mM PMSF) and boiled for 2 min in 80 μl of 2× sodium dodecyl sulfate (SDS) sample buffer.

**Cell cycle analysis and exposure to DNA-damaging agents**

Cells containing GST-REV3 on plasmid pBL1813 were grown in 125 ml of selective medium with 2% raffinose to an O.D$_{660}$ = 0.5. Protein expression was induced by 2% galactose for 8 hours. Cells were arrested in G1 phase by α-factor (20 μg/ml for 2 h), in G2/M phase with nocodazole (15 μg/ml for 2 h) and in S phase by hydroxyurea (200 mM for 90 min). Then cells were treated with 4NQO (1 μg/ml) or methylmethane sulfonate (0.05%) for 30 min at 30°C. The cells from 200 μl of culture were fixed, stained with propidium iodide and DNA content was measured by flow cytometry. The remaining cultures were harvested, and extract preparations and GST-pull down were performed as described earlier.

**Western blot and antibodies**

Western blot analysis was performed to detect the presence of GST-Rev3, Rev7, Pol3, Pol31, Pol32 and Rev1 proteins in purified Pol ζ preparations and after pull-down experiments. To detect the Rev1, Rev3 and Rev7 proteins, rabbit polyclonal antisera were raised against purified yeast Rev1 and Pol ζ antibodies. Rabbit anti-Pol3, -Pol31 and -Pol32 antibodies were immunopurified. Detection was carried using alkaline phosphatase–conjugated secondary antibody (Sigma) and a BCIP/TNB chromogenic substrate (Sigma).

**DNA polymerase and translesion synthesis assays**

Three different assays were used. (i) Measurement of basal DNA polymerase activity: This measures polymerase activity on activated calf thymus DNA, for 45 min at 30°C, as described (29). (ii) DNA replication assay on circular ssDNA: The assay on primed ssDNA (pSKII) performed as described previously (24). The reactions containing 5 nM of 3 kb circular ssDNA, 500 nM RPA, 3 nM RFC and 10 nM of Pol ζ were incubated at 30°C for 50 min with increasing PCNA as shown in legends to figures. (iii) In vitro DNA translesion bypass assay: Sequences of the 107-nt template (with or without a model abasic site) and the primer are given in Supplementary Table S1.
CysB region, and an intact iron–sulfur cluster is required for interaction. This is the same binding specificity as observed between Pol3 and Pol31 (14).

We next analyzed these interactions by pull-down experiments using GST-Rev3 trapping. We overexpressed GST-REV3 and REV7 and assayed for Rev3–Rev7–associated factors by glutathione chromatography (Figure 1C). Significant levels of Pol31 and Pol32 were detected, when compared with controls (Figure 1D, lane 3 vs. 1 and 2). When POL31 and POL32 were also overexpressed, a strong interaction signal was detected (lane 4). However, when the same experiment was carried out in a pol32A strain, Pol31 was undetectable after affinity co-purification (lane 7 vs. lane 3). This defect was rescued by providing back overexpressed POL32 (lane 8). These data strongly suggest the existence of a four-subunit Rev3–Rev7–Pol31–Pol32 complex called Pol $\xi_5$. Importantly, unlike Pol $\delta$, in which a Pol3–Pol31 complex is a stable assembly (30), Pol32 is required to stabilize the interactions between Rev3 and Pol31. These important differences in polymerase complex stabilities between Pol $\delta$ and Pol $\zeta$ explain why pol32A mutants are viable, but defective for mutagenesis (16).

In agreement with the yeast two-hybrid experiments, we found that Pol31 and Pol32 fail to bind the CysB mutant of GST-Rev3, independent of overexpression (Figure 1E, lanes 5 and 8). In contrast, the CysA mutant of GST-Rev3 pulled down Pol31–Pol32 with the same efficiency as wild-type (compare lane 3 with 4 and 6 with 7).

### Results

#### Damage-induced mutagenesis assays

The rev3A strain BY4741 (rev3::KanMX4) contained empty vector or plasmid pBL811 (GST-REV3) or mutants of REV3 as shown in Table 1. Strains were grown for 2 days to saturation in selective minimal media. The cells were washed with sterile water and grown for 2 days to saturation in selective minimal 80 $\mu$g/ml canavanine and either irradiated or not irradiated with 30 J/m$^2$ of UV light. The plating efficiencies and the percent of UV survival were measured on plates without canavanine. Spontaneous frequencies to canavanine resistance were measured on unirradiated canavanine plates, and UV-induced frequencies to canavanine resistance were measured on irradiated canavanine plates. Colonies appearing after 3 days of growth at 30°C were counted. Frequencies of mutation to canavanine resistance were corrected for the UV survival percentage. The experiments were carried out on three independent cultures, and in duplicate, and the results are presented in Table 1.

#### Purification and characterization of two forms of Pol $\zeta$: Pol $\xi_2$ and Pol $\xi_4$

To obtain a Pol $\xi_4$ complex containing an intact [4Fe-4S] cluster, we overexpressed all four genes from galactose-inducible promoters (Figure 1C) and modified the purification protocol of Pol $\zeta$ that was described previously (28). Overexpression was carried out in a rev1A strain to eliminate trace contamination of the purified preparation with Rev1 (see below). The modified procedure made use of two affinity purification tags, an N-terminal GST tag on Rev3 and an N-terminal His7-
tag on Pol32. First, the extract, after an ammonium sulfate precipitation step, was subjected to glutathione-affinity chromatography. The resistance of the Pol γ complex to ammonium sulfate precipitation indicates that the interaction between Rev3–Rev7 and Pol31–Pol32 is very strong and specific. This procedure yielded a preparation that was slightly substoichiometric for Pol31–Pol32 (80–90% in three purifications). Next, after cleavage of the GST-tag by rhinoviral 3C protease, the complex was further purified by Ni-chelate affinity chromatography with 100% stoichiometry (Figure 2A). The Pol32-His6 tag did not influence the activity of the Pol γ complex (data not shown).

In agreement with the yeast two-hybrid analysis and pull-down experiments, Pol31 and Pol32 were present in affinity-purified preparations of Pol γ with mutations in the CysA cluster (Rev3-CC1401,1407SS or Rev3-CC1401,1407AA) and CysB (CC1449,1473SS) mutants. (B) Yeast two-hybrid analysis. REV3, rev3-cysA or rev3-cysB was fused to the GAL4 DNA-binding domain. REV7, POL31 or POL32 was fused to the GAL4 AD. Empty vector pACT2 was the negative control. Analysis was in two-hybrid indicator strain P69-4A. Cells were grown on His-selective medium. (C) Scheme for overexpression of GST-REV3, REV7, POL31 and POL32 and affinity pull down of complexes. (D) Pull down of Pol31 and Pol32 with GST-Rev3. GST-Rev3-Rev7 complex was overexpressed alone or together with Pol31–Pol32 subunits in either wild-type or Apol32 yeast. Cell extracts were incubated with glutathione sepharose beads and washed extensively. GST-Rev3 and Pol31 and Pol32 were detected by western analysis. –, gene deleted; +, native level; ++, overexpression. (E) Analysis of the interaction between Pol31–Pol32 and GST–Rev3 mutants by GST-pull down. Details are as in (D).

Pol31 by Coomassie staining after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and by western analysis (Figure 2A).

Overexpression of REV3 and REV7 in wild-type yeast without concomitant overexpression of POL31 and POL32 yielded affinity-purified preparations that were severely substoichiometric for Pol31 and Pol32, with abundances ranging from 3 to 15% (Figure 2A). We had previously noted that different Pol γ preparations were quite variable in activity, but because of the extreme difficulty in purifying the enzyme and the very low yields, it had not been feasible to investigate those issues further at that time (28). We now think that the variations in activity were due to the variable presence of low levels of Pol31–Pol32 that escaped detection. With improved expression and purification methodologies and increased yields, we re-investigated the protein composition of our purified preparations. First, because Rev1 is known to interact with Pol γ through Rev7 (31), we probed Pol γ preparations for the presence of Rev1 by western analysis. Both Pol γ2 and Pol γ4 complexes, as well as all preparations of Pol γ mutants, contain similar levels of Rev1 (~1–2% compared with Rev3,
Supplementary Figure S1B). Therefore, we have purified Pol ζ2 and Pol ζ4 from a rev1Δ mutant strain without loss of complex stability, indicating that Rev1 is not required for the formation of the Pol ζ4 complex (data not shown). Second, because Pol31 interacts with the catalytic subunit of Pol δ, we investigated the possibility of the presence of Pol3 by western analysis. However, none of the Pol ζ preparations contained Pol3 at detectable levels (detection limit is ~0.1%), suggesting that Pol31 binds either Pol3 or Rev3, but not both catalytic subunits (Supplementary Figure S1C). Therefore, we conclude that our current forms of Pol ζ4 and Pol ζ2 contain the expected subunits without contamination by other proteins that may function in TLS and mutagenesis.

Expression in E. coli of the entire CTD of Rev3, containing both CysA and CysB motifs, yielded a yellow-brown preparation that after reduction by dithionite was converted into an electron spin resonance (EPR) active form with the spin signal of that of a [4Fe-4S]1+ cluster (14). This suggests that, like Pol3, Rev3 has a [4Fe-4S]2+ cluster. Indeed, similar to Pol δ, purified Pol ζ4 has a UV-spectral signature that is indicative of the presence of an iron–sulfur cluster (Figure 2B). Unfortunately, we were unable to obtain sufficiently high quantities of the CysB mutant form to query whether the iron–sulfur cluster was eliminated in the mutant, but on the basis of the strong sequence homology between Pol3 and Rev3 CTD, we accept this as a likely explanation.

**Pol31 and Pol32 are essential for functional interactions between PCNA and Pol ζ**

The presence of Rev7 is required for DNA polymerase activity of Rev3 (6). We measured basal DNA polymerase activity of Pol ζ preparations on activated DNA in the absence of PCNA. The presence of the Pol31 and Pol32 subunits in Pol ζ4 enhanced the activity 5- to 10-fold compared with the Pol ζ2 preparations, which were either obtained by purification from a pol32Δ strain or by mutation of the CysB motif in REV3 (Supplementary Figure S2A).

To determine the role of PCNA in TLS by Pol ζ, we used an oligonucleotide-based replication system with defined template damage. The substrate is incubated with RPA to coat the ssDNA regions, and PCNA is loaded by RFC and ATP. To prevent sliding of PCNA off the DNA, biotin-streptavidin bumpers are added to the 5′- and 3′-termini of the template (Figure 3A). We first assayed the replication by Pol ζ2 on an undamaged template-primer. Pol ζ2 activity on this template was much less efficient compared with the Pol ζ4 complex (Figure 3B). In addition, the presence of PCNA had no detectable effect on DNA replication by Pol ζ2. Because of the robust activity of Pol ζ4 on this DNA substrate, PCNA stimulation could not be detected under these conditions. However, PCNA stimulation of Pol ζ4 on undamaged DNA was readily detected using primed single-stranded plasmid DNA substrates (Supplementary Figure S2C).

To study the role of PCNA in DNA damage TLS, we used the oligonucleotide assay system with a model abasic site at the +2 position after the primer terminus. We observed that Pol ζ2 readily extended the primer by one nucleotide but did not insert a nucleotide opposite the abasic site, and PCNA did not enhance this activity (Figure 3C). In contrast, the Pol ζ4 complex bypassed the abasic site damage even in the absence of PCNA. Remarkably, a dramatic stimulation of damage bypass synthesis was detected in the presence of PCNA. These data indicate that formation of the Pol ζ4 complex is essential for both efficient damage bypass in the absence of PCNA and stimulation of Pol ζ-mediated TLS in the presence of PCNA. Therefore, we conclude that functional interactions between Pol ζ and PCNA require its PCNA-dependent TLS-promoting activity through Rev1 (32).

The observation that interactions with Pol31–Pol32 enhanced the PCNA-dependent activity of Pol ζ raised the possibility that the PCNA-binding motif is localized in the Pol31 or Pol32 subunit. Previously, we have identified a C-terminal PCNA-binding motif in Pol32...
Deletion of this motif only marginally affected processive DNA replication by Polδ; however, the pol32-PIP mutant showed a substantial reduction in the efficiency of damage-induced mutagenesis, particularly at higher loads of DNA damage. We purified a mutant Polζ containing a truncated form of Pol32 that lacks its PCNA-binding motif (Polζ-ΔPIP). Although the basal activities of Polζ and Polζ-ΔPIP were comparable, PCNA stimulation of the mutant complex was substantially reduced (Figure 3D and Supplementary Figure S2A and S2C). We conclude that the PCNA BD of Pol32 contributes to the functional interaction between Polζ and PCNA.

DNA replication by Polδ requires an intact CysA motif, as CysA mutants are severely compromised for PCNA-dependent replication (14). In contrast, the CysA mutant form of Polζ demonstrated close to wild-type basal DNA polymerase activity (Supplementary Figure S2A). Although its TLS activity was slightly reduced (~60% of wild-type), PCNA stimulated this TLS activity to the same degree as it did wild-type Polζ (Figure 3D). This lack of a strong in vitro phenotype is consistent with the lack of a damage-induced mutagenesis phenotype of the same CysA mutations in yeast (Table 1).

The pol30-113 mutant of PCNA shows severe defects in damage-induced mutagenesis, without affecting the efficiency of a proper DNA damage response through PCNA ubiquitination at Lys164 (27,33). Pol30-113 has mutations at Glu113 and Leu151 near the monomer–monomer interface of PCNA. Previously, we showed that this mutant form of PCNA was defective for PCNA-mediated TLS in vitro (27). With our increased knowledge of the assembly state of Polζ, we assume that the previous preparations of Polζ contained low levels of Pol31–Pol32 that drove the observed PCNA stimulation. Indeed, the stoichiometrical Polζ complex was unable to perform processive replication with pcna-113 (Supplementary Figure S2C).

The Polζ complex is stable throughout the cell cycle

To test whether the formation of the Polζ complex is subject to either cell cycle or DNA damage control, we prepared synchronized cell populations and determined co-purification of Pol31 and Pol32 with GST-Rev3 on glutathione sepharose beads. For this experiment, we used the GST-REV3 expression plasmid, however, omitted galactose induction to maintain Rev3 at low levels. Under the same growth conditions, this construct fully complemented the mutagenesis defect of a rev3D mutant (data not shown). POL31 and POL32 were not overexpressed in these experiments. Cells were arrested in G1 phase with α-factor, in S phase with hydroxyurea and in G2/M phase with nocodazole. About 80–95% of cells were arrested in the appropriate phase of the cell cycle in our experiments (Figure 4A). Synchronized cells were also treated with MMS or 4NQO to induce the DNA damage response. After affinity purification on glutathione beads, the presence of Rev7, Pol32 and Rev1 was monitored by western analysis (Figure 4B). The data indicate that Polζ can exist as a four-subunit complex in all phases of the cell cycle. Furthermore, treatment with DNA-damaging agents did not alter the formation or stability of the complex. Interestingly, Rev1 association with Polζ is highest in G2 phase. This study addressed the question
Figure 4. Stability of Pol \( \xi_4 \) during the cell cycle. (A) Fluorescence-activated cell sorting analysis of the DNA content of cells. Cells expressing low levels of GST-REV3 and REV7, and POL31 and POL32 at native levels, were arrested in G1, S, or G2 phase, followed by treatment with MMS or 4NQO. (B) Extracts were prepared from arrested cells, and Pol32, Rev7 and Rev1 were detected by western analysis after GST-Rev3 pull down with glutathione sepharose beads. Control: Western analysis of extracts made from cells lacking GST-Rev3 and subjected to glutathione affinity purification.

**DISCUSSION**

Pol \( \zeta \) is a low-fidelity, B-family DNA polymerase and the sixth eukaryotic DNA polymerase to be described (6). The original article described a form of Pol \( \zeta \) that was overexpressed in yeast, and all subsequent studies, including those from our laboratory, used forms that were also purified from yeast overexpression systems (5, 28,30). Therefore, it is likely that these forms contained low, variable levels of Pol31 and Pol32 in the preparations. Our previous observations that TLS by Pol \( \zeta \) is stimulated by PCNA likely originated from the use of preparations that contained such low levels of Pol31–Pol32, which we now know varied from 3 to 15% over the years. Coupled with the fact that Pol \( \zeta \) has much lower basal polymerase activity than Pol \( \xi_4 \) (Supplementary Figure S2A and Figure S3), the latter species would have contributed more to the observed activity than considerations of abundance suggest. This also explains the variability in activity of different Pol \( \zeta \) preparations that we remarked on several years ago (28).

Previously, we have shown that the catalytic subunits of the yeast B-family DNA polymerases contain an \([4Fe-4S]\)^{2+} cluster, coordinated by the CysB motif in their CTDs, and we and others have suggested that all B-family polymerases are similarly organized (14,20). However, a comparison between the architecture of Pol \( \delta \) and Pol \( \zeta \) reveals some interesting differences that may underlie their different functions in the cell. Both Pol3 and Rev3 bind Pol31 through their CysB motif as mutations in this motif abrogate binding, while mutations in the CysA motif do not. However, Pol3 forms a stable complex with Pol31 alone (34), but Rev3 does not (Figure 1D). As a result, pol32A mutants are viable, but they are defective for damage-induced mutagenesis (16,35). Furthermore, transformation studies with plasmids containing specific DNA damage show that pol32A is defective for the bypass of abasic site damage similar to \( rev3Δ \), but proficient for the bypass of thymine dimers, which is Pol \( \eta \) dependent (36). This is consistent with Pol32 functioning as an integral part of the Pol \( \xi \) complex.

CysA mutations in Pol \( \zeta \) are lethal, most likely because the mutant form of Pol \( \delta \) is severely defective for PCNA-mediated processive replication (14). However, the analogous mutations in the CysA motif of Rev3 show no defect in mutagenesis [Table 1, (22)] nor is the mutant polymerase defective for PCNA-mediated processive replication (Figure 3D). Functional interactions of Pol \( \delta \) with PCNA is imparted by multiple potential PCNA-binding motifs in the various subunits of Pol \( \delta \) (14,24,37–40). In Pol \( \xi_4 \), PCNA interacts through the consensus PIP box in the extreme C-terminus of Pol32 as deletion of this motif reduces TLS in vitro (Figure 3D). This POL3 mutant also has a reduced efficiency in damage-induced mutagenesis (24). The residual PCNA stimulation observed in vitro and mutagenesis in vivo suggests that Pol \( \xi_4 \) contains additional PCNA interaction motif(s). The striking difference in CysA phenotype between Pol \( \delta \) and Pol \( \xi_4 \) suggests a different positioning of the PCNA clamp in relation to this motif in these enzymes. Consistent with this, mutations in PCNA differentially affect its interactions with Pol \( \delta \) versus Pol \( \zeta \). A pcna-113 mutant functions as a processivity clamp for Pol \( \delta \), although its activity is somewhat reduced (27); however it is defective with Pol \( \xi_4 \) (Supplementary Figure S2C). This provides a rational explanation for the mutagenesis defect in this mutant.

The formation and stability of the Pol \( \xi_4 \) complex was unaffected by the cell cycle or by exposure to DNA-damaged agents (Figure 4). This result suggests that Pol \( \zeta \) mediated mutagenesis can occur throughout the cell cycle. However, other factors, for example, Rev1 and PCNA, show cell cycle and/or DNA damage control, and overall pathway control is likely mediated through those factors. Ubiquitination of PCNA is a key switch in this pathway, and both damage-induced mutagenesis as well as spontaneous mutagenesis in response to replisome dysfunction is dependent on ubiquitination of PCNA (27,41,42). The Rev1 protein, considered to be the scaffold onto which the mutasome assembles through binding of ubiquitinated PCNA on one hand and Pol \( \zeta \) on the other hand, is most highly expressed in G2 phase (43). Indeed, it has been shown that PCNA ubiquitination and mutagenesis can be restricted to the G2 phase of the cell cycle (44,45). We found that Rev1 association with Pol \( \xi_4 \) is also highest during G2 phase (Figure 4). Therefore we suggest that the regulation of Pol \( \xi_4 \)-dependent mutagenesis is likely mediated by the formation of multisubunit complexes of higher order, for example
with Rev1 and ubiquitinated PCNA, but not through the assembly of the Pol ζ4 complex. Finally, the cell cycle kinase CDC7/DBF4 promotes the efficiency of UV mutagenesis (46).

As stated before, two other groups have recently reported that Rev3 interacts with Pol31 and Pol32. The article by Baranovskiy et al. reported the co-purification from E. coli of the CTD of human Rev3 together with human Pol31 and Pol32 (22). Although this approach did not permit functional polymerase studies, it allowed these authors to probe the relevance of the CysA and CysB motifs for complex formation. In agreement with our results in Figure 1E, CysB mutations, but not CysA mutations, abrogated complex formation. Similarly, their genetic studies of the CysA and B motifs in yeast yielded analogous results to ours (Table 1). The second article by Johnson et al. reported the isolation of a Pol ζ4 complex from a yeast overexpression system and is in accord with ours when all four genes are overexpressed (23). However, our conclusion that Pol32 is required for stable complex formation between Rev3 and Pol31 is at variance with theirs when all four genes are overexpressed (23). However, this three-subunit preparation was purified from a wild-type yeast strain rather than a pol32 strain and was highly non-stoichiometric containing predominantly the Pol31 polypeptide, to which the purification tag was fused. Given the low levels of Rev3 in this preparation, and the close migration of Pol31 and Pol32 by SDS-PAGE, low levels of Pol32 may have escaped detection. Unfortunately, a more sensitive western analysis with Pol32 antibodies was not used as a detection method in this study. We think that these are important considerations, because our study indicates that Pol32 is absolutely required for complex formation and thereby provides a logical explanation for the long-standing observation that pol32 strains are defective for damage-induced mutagenesis.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online: Supplementary Text, Supplementary Table 1, Supplementary Figures 1 and 2 and Supplementary Reference [47].

ACKNOWLEDGEMENTS
The authors thank Carrie Stith and Bonnie Yoder for strain and plasmid construction and for help with protein purification, Sandeep Kumar for help with fluorescence-activated cell sorting analysis, and Sara Binz for advice during the course of this study.

FUNDING
Funding for open access charge: National Institutes of Health [GM032431 to P.B.].

Conflict of interest statement. None declared.

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