Role of the Putative Zinc Finger Domain of Saccharomyces cerevisiae DNA Polymerase ε in DNA Replication and the S/M Checkpoint Pathway

(Received for publication, June 8, 1998, and in revised form, August 26, 1998)

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It has been proposed that C-terminal motifs of the catalytic subunit of budding yeast polymerase (pol) ε (POL2) couple DNA replication to the S/M checkpoint (Navas, T. A., Zheng, Z., and Elledge, S. J. (1995) Cell 80, 29–39). Scanning deletion analysis of the C terminus reveals that 20 amino acid residues between two putative C-terminal zinc fingers are essential for DNA replication and for an intact S/M cell cycle checkpoint. All mutations affecting the z-finger zinc finger amino acids or the zinc fingers themselves are sensitive to methylmethane sulfonate and have reduced ability to induce RNR3, showing that the mutants are defective in the transcriptional response to DNA damage as well as the cell cycle response. The mutations affect the assembly of the pol ε holoenzyme. Two-hybrid assays show that the POL2 subunit interacts with itself, and that the replication and checkpoint mutants are specifically defective in the interaction, suggesting (but not proving) that direct or indirect dimerization may be important for the normal functions of pol ε. The POL2 C terminus is sufficient for interaction with DPB3, the essential and phylogenetically conserved subunit of pol ε, but not for interaction with DPB3. Neither Dpb3p nor Dpb2p homodimerizes in the two-hybrid assay.

Yeast mutants with defects in S phase progression normally arrest their cell cycles before entering mitosis. This arrest is attributed to a surveillance mechanism, called the S/M checkpoint pathway, that prevents inappropriate segregation of unreplicated or damaged chromosomes by monitoring some as yet ill defined aspect of chromosome structure. In the presence of DNA damage, a signal is generated and transmitted either to the cell cycle apparatus, resulting in inhibition of cell cycle progression, or to the cellular transcription apparatus, resulting in induction of functions that participate in repair of the defect and/or delay cell cycle progression (1). In addition to the S/M checkpoint pathway, there are at least three other checkpoint pathways that have the replication apparatus either as a target of inhibition (G1/S) or as a sensor of aberrant function (S/M) or both (intra-S) (2–4). However, information about the mechanism linking DNA replication and either the cell cycle apparatus or the transcription apparatus in these checkpoints has been limited. Several years ago, it was shown that certain DNA replication initiation mutants, in addition to failing to enter S phase, also fail to establish the S/M checkpoint (5, 6). Such mutants, rather than arresting the cell cycle, progress into mitosis with unreplicated chromosomes, divide and die. It was proposed that assembly of the replication apparatus may be required to establish the checkpoint and that one or more component(s) of the assembled apparatus monitors the completeness of replication and sends an inhibitory signal to the cell cycle apparatus when there is unreplicated (or damaged) DNA (7). In keeping with this model, several mutants affecting proteins in the replisome were recently shown to be defective in the S/M checkpoint: rfc2, rfc5, dpb11, and pol2 (8–12). POL2 encodes the essential DNA polymerase, pol1 ε, and Rfc2p, Rfc5p, and (probably) Dpb11p are polymerase accessory proteins. Thus, an enzyme that was originally thought to have an essential housekeeping function also seems to have an important regulatory function in the cell cycle. This dual function may form an important paradigm for other checkpoint pathways (13, 14).

The first checkpoint-deficient allele of pol2 was identified in a screen for mutants defective in a transcriptional response observed when hydroxyurea is used to block DNA replication (15). (Hydroxyurea blocks DNA synthesis by inhibiting precursor biosynthesis and thus depleting nucleotide pools.) Wild-type cells treated with HU respond by inducing the RNR3 gene, but one mutant, dun2 (damage uninducible), was deficient in induction (9). Cloning of the DUN2 gene showed that it was identical to POL2. Two pol2 mutants temperature-sensitive for DNA replication and DNA repair (16, 17) also turned out to be defective in the transcriptional response to DNA damage (9, 15). What was really interesting, however, was that the latter two mutants were also defective in the cell cycle response to HU inhibition, since they entered mitosis with incompletely replicated chromosomes. Further work showed that the Pol2 DNA damage response pathway appears to function in parallel to the Rad9 pathway and be specific for damage incurred or encountered during S phase (10). POL2 seems to act upstream of RAD53, a protein kinase that is one of the central signal transducers in both the transcriptional and cell cycle responses to DNA damage, and therefore POL2 has been proposed as a sensor of DNA damage and stalled replication forks. We have been interested in identifying new mutants affecting POL2 to further define the mechanism by which pol ε participates in the S/M checkpoint.

pol ε is one of three essential DNA polymerases in yeast (16, 18, 19). pol ε is highly homologous to the other essential DNA polymerases, pol δ and pol α, in the catalytic domains, which have been shown to fall in the N-terminal and central portion

† This work was supported by PHS grant GM25508 and American Heart Association Research Fellowship Award 1153-F12. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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The abbreviations used are: pol, DNA polymerase; 5-FOA, 5-fluoroorotic acid; aa, amino acid; HU, hydroxyurea; MMS, methylmethane sulfonate; ZFI, 4-cysteine motif between aa 2103 and aa 2133; ZF2, 4-cysteine motif, between aa 2163 and aa 2183; HA, hemagglutinin; PCR, polymerase chain reaction.
of the proteins (20). The temperature-sensitive mutants, pol2–9 and pol2–18, have mutations in the conserved catalytic polymerase domain, M643I and PT10S, while mutations at Asp-290 and Glu-292 reduce the exonuclease proofreading activity and affect the fidelity of the polymerase (19). The Dun−, DNA damage response-defective pol2 mutants described above all map to the C terminus, while the N-terminal pol2–9 and pol2–18 mutants are proficient in the checkpoint, pol1 and pol3 mutants, defective in DNA polymerases α and δ, respectively, have constitutively elevated expression of the damage inducible genes, rather than a defect in induction (15).

What is special about the pol ε C terminus that could be important for both DNA replication and DNA damage sensing? Interestingly, the C-terminal region of pol ε is essential in vivo, though far removed from the catalytic domains (9). In vitro, however, the C-terminal mutants show nearly normal DNA polymerase activity (16). The catalytic site mutants, pol2–9, pol2–11, and the C-terminal mutants, defective in DNA polymerases α and δ, respectively, have constitutively elevated expression of the damage inducible genes, rather than a defect in induction (15).

### EXPERIMENTAL PROCEDURES

**Materials**—plasmids expressing pol1 or pol2 were from New England Biolabs. pRS314 TRP1 and his3 were from the laboratory collection (21). pZZ2, carrying RNR3-lacZ was provided by Dr. Stephen Elseed (Baylor College of Medicine, Houston, TX). Restriction enzymes, T4 DNA ligase, Vent DNA polymerase, and Klenow large fragment were obtained from New England Biolabs. Mutagenesis kit was from Bio-Rad. All the oligonucleotides were synthesized by the oligo- nucleotide facility at Caltech. anti-Myc and anti-His antibodies were obtained from ICN Corp. Polyclonal antibody for the hemagglutinin epitope were prepared at Caltech. Monoclonal 12CA5 antibodies for the hemagglutinin epitope were prepared by Dr. Akio Sugino (Osaka University, Osaka, Japan).

**Bacterial and Yeast Strains**—Escherichia coli C3236 dUT-1, ung-1, thi-1, relA1; pCJ105(Cm+) was provided in the Bio-Rad mutagenesis kit. For routine cloning, the DH5α bacterial strain was obtained from Life Technologies, Inc. Yeast strains are listed in Table 1.

**Mutagenesis and Subcloning**—pSEY18 vector containing the full-length POL2 gene cloned at the SacI site (16) was digested with BglI, and the C-terminal POL2 fragment (~3 kb) was purified. The purified C-terminal fragment was subcloned at the BglI site in the pLITMUS vector (19). The linearized pSEY18 vector containing the N terminus of the POL2 was self-ligated and digested with SnaI, and the POL2 fragment was purified. It was then subcloned in the pRS314 vector (pRPOL2), pLITPOL2 was transformed into C3236 E. coli strain, and transformed cells were infected with M13KO7 phage. Uracil-phagemid DNA template was prepared, and mutagenesis was performed according to instructions of the supplier (Bio-Rad) with some modifications. Briefly, the mutagenic oligonucleotide was annealed to the uracil-containing DNA template by first heating at 70 °C for 3 min and then slowly cooling to 30 °C for 1 h. The polymerization mix, T4 DNA ligase and T7 DNA polymerase were added to the annealed template and incubated on ice for 5 min, at room temperature for 5 min, and then at 37 °C for 4 h. The reaction was stopped by adding 17 μl of 1× TE buffer. 3 μl of the final mixture was transformed into DH5α cells. The DNA was prepared from the transformants and sequenced using automated sequencing at the Caltech DNA sequencing facility. The oligonucleotides used for the mutagenesis are as follows: 2213–2222, 5′-GGC TTA TAT ACT GCT TAC CTA TAT ATC AAA ACC GTA ATA CTT G-3′; 2205–2212, 5′-ATC AGC AAT ACA ACT CA A TAA CCT

### TABLE I

| Strain   | Genotype                                      | Source     |
|----------|-----------------------------------------------|------------|
| YHA1     | MATa/MATa POL2/pol2–3::LEU2 LEU2–3, 112/LEU2–3, 112 ura3–52 trp1–289 ade5–11/ADE5 | (19)       |
| A1128    | MATa ade5–1 leu2–3, 112 ura3–52 trp1–289 pol2–3::LEU2 [YEp101] | This study |
| YHA301   | MATa ade5–1 leu2–3, 112 ura3–52 pol2–3::LEU2 [YCpop2–18] | (19)       |
| YRD1     | A1128/pk1                                     | This study |
| YRD2     | A1128/pk2                                     | This study |
| YRD3     | A1128/pk3                                     | This study |
| YRD4     | A1128/pk4                                     | This study |
| YRD5     | A1128/pk5                                     | This study |
| YRD6     | A1128/pk7                                     | This study |
| YRD7     | A1128/pk8                                     | This study |
| YRD8     | A1128/pk9                                     | This study |
| YRD9     | A1128/pk10                                    | This study |
| YRD10    | A1128/pk11                                    | This study |
| YRD11    | A1128/pk12                                    | This study |
| YRD12    | A1128/pk13                                    | This study |
| PJ69–1A  | LYS2::GAL1-HIS3, GAL2-ADE2, met2::GALT7-lacZ  | (29)       |
| SS111–2–11| MATa, trp1–289, ura3–12, ade2–101, gal2, can1, pol2–11 | (16)       |
| SS111–2–12| MATa, trp1–289, ura3–12, ade2–101, gal2, can1, pol2–12 | (16)       |
| TC102–2–11| MATa, leu2, ura3–52, can1, pol2–11             | (16)       |
| TC102–2–12| MATa, leu2, ura3–52, can1, pol2–12             | (16)       |
Table II  

| Plasmid | Relevant genotype | Mutant | Base plasmid |
|---------|-------------------|--------|--------------|
| pk1     | TRP1 ARS4 CEN6 POL2 | A       | pkRS154     |
| pk2     | TRP1 ARS4 CEN6 pol21/Δ2103-2112) | A       | pkRS154     |
| pk3     | TRP1 ARS4 CEN6 pol21/Δ2113-2123) | B       | pkRS154     |
| pk4     | TRP1 ARS4 CEN6 pol21/Δ2153-2163) | C       | pkRS154     |
| pk5     | TRP1 ARS4 CEN6 pol21/Δ2163-2173) | D       | pkRS154     |
| pk6     | TRP1 ARS4 CEN6 pol21/Δ143-2152) | E       | pkRS154     |
| pk7     | TRP1 ARS4 CEN6 pol21/Δ153-2162) | F       | pkRS154     |
| pk8     | TRP1 ARS4 CEN6 pol21/Δ163-2172) | G       | pkRS154     |
| pk9     | TRP1 ARS4 CEN6 pol21/Δ173-2182) | H       | pkRS154     |
| pk10    | TRP1 ARS4 CEN6 pol21/Δ2183-2192) | I       | pkRS154     |
| pk11    | TRP1 ARS4 CEN6 pol21/Δ193-2197) | J       | pkRS154     |
| pk12    | TRP1 ARS4 CEN6 pol21/Δ2003-2212) | K       | pkRS154     |
| pk13    | TRP1 ARS4 CEN6 pol21/Δ2213-2222) | L       | pkRS154     |
| pk14    | TRP1 ARS4 CEN6pol2F2093-TAA) | M       | pkRS154     |
| pk15    | TRP1 ARS4 CEN6pol22151-TAA) | N       | pkRS154     |
| pk16    | URA3 ARS4 CEN6pol21/△1548) | O       | pkRS154     |

The full-length POL2 gene was reconstituted by subcloning the mutant pol 2 C terminus fragments into the pRPOL2 vector at the BarG1 site. The recombinants were screened using colony hybridization. The orientation of the cloned insert was confirmed by hybridization to DNA sequencing. pol2-11, pol2-12, and pol2-18 were made by site-directed mutagenesis to ensure that the phenotype of the original mutations was due to the single base pair changes found in the original mapping. The plasmids are listed in Table II. The size of the expressed proteins was determined by Western blotting after subcloning the C terminus fragments into the pRPOL2 vector (with BamHI sites). pK series plasmids (see Table II) containing the full-length wild-type and mutant pol2 genes were made with MMS with varying concentration. The presence of 5-FOA at 24 °C and 37 °C. Samples (5 x 10⁶ cells) of each dilution were placed on the MMS plates and incubated at 24 °C for 4 days. Induction of RNR3 Expression in the Presence of MMS—The pol 2 yeast strains were transformed with pZZ2, which carries RNR3-lacZ (15). The yeast strains containing the RNR3-lacZ reporter were grown at 24 °C in selective medium until the A600 reached 0.6. The culture was divided in two equal halves. To one part, MMS was added to a final concentration of 0.01%. Both cultures were grown for another 6 h at 24 °C. The cells were washed once with water, and then β-galactosidase assays were carried out as per instructions (CLONTECH Matchmaker two-hybrid system manual).

RESULTS

Amino Acid Residues between ZF1 and ZF2 in the C Terminus of pol ε Are Essential for Viability—In order to better understand the molecular details of the C terminus of pol ε required for its role in DNA replication and damage sensing, we constructed a panel of deletion mutants covering the C-terminal 120 aa of the Pol2p protein and studied various phenotypes. For clarity, we will refer in the text to the amino acid motifs in the region as they are defined in Fig. 1, although there are no structural data to support the designation “zinc finger” as yet. Each mutant (Fig. 1, A–L) contains a deletion of 10 amino acids. Mutants A, B, C, and D have deletions in ZF1; mutants E and F have deletions between ZF1 and ZF2; mutants G and H have deletions in ZF2; and mutants I, J, K, and L have mutations in the extreme C terminus of pol ε. The previously studied pol2–11 and pol2–12 nonsense alleles fall in the region covered by deletion J. In addition to the deletion mutations, pol2–11, pol2–12, and pol2–18 were made by site-directed mutagenesis to ensure that the phenotype of the original mutations was due to the single base pair changes found in the original mapping. We first examined the ability of the mutants to complement a pol2Δ strain using a plasmid shuffling assay (16, 22). The mutant genes, cloned on a plasmid carrying a TRP selection marker, were transformed into a pol2Δ yeast strain, which was kept alive by the presence of a wild-type POL2 gene on a URA plasmid. Transformants were analyzed for growth at 24 °C and 37 °C on synthetic agar medium containing 5-FOA, which is toxic to Ura- cells and therefore toxic against cells carrying the wild-type POL2 plasmid (Fig. 1). Among the mutants studied, mutants E and F showed a drastic growth defect when present as the only copies of the pol2 gene in the cells. Mutant E was unable to support growth at any temperature, while mutant F was viable at 24 °C but inviable at 37 °C. Mutant F also grew slower than wild-type and was enlarged at 24 °C. Mutants A, B, C, and D showed a...
marginal decrease in viability while mutants G, H, I, J, K, and L had no detectable growth defect. The results show that the mutations between ZF1 and ZF2 are critical for the essential role of pol in the cell.

Mutants pol2–18 and pol2–11, with N- and C-terminal mutations, respectively, show interallelic complementation of their growth defect (9). Mutant F was therefore tested for complementation of pol2–18 (and pol2–11) strains. Plasmid pk16 (pol2–18) was transformed into strain A1128/pk7 (pol2–F). Transformants were obtained but grew slowly at 37 °C, producing cultures that consisted of about 70% viable cells. Thus, pol2–F and pol2–18 showed partial complementation. Neither mutant E nor mutant F complemented strain SS111 pol2–11. Mutants A to D and G to L complemented pol2–11, as expected, since they themselves had only slight growth defects.

**Mutant F Is Defective in the S/M Checkpoint**—Cells defective in the S/M phase checkpoint progress into mitosis with incompletely replicated DNA resulting in rapid loss of cell viability. To assess the role of the region between ZF1 and ZF2 in the S/M checkpoint, mutant F was examined for viability, DNA content, and nuclear and spindle morphology after incubation at the nonpermissive temperature. Wild-type and mutant F cells were grown to log phase at 24 °C and transferred to 37 °C, and samples of cells were collected at various time intervals and plated at 24 °C. As expected for a checkpoint defect, mutant F showed a 70% loss in viability compared with wild-type after 2 h at 37 °C, and 90% of the cells were dead after 4 h (Fig. 2A). The DNA content of the cells was also monitored at each time point. Mutant F cells accumulate in S phase (70%) and G2 (30%) at 24 °C, which indicates a defect in S phase progression even at the permissive temperature (Fig. 2B, 0 h). After 2 h at 37 °C, when the cell viability had dropped drastically, most cells revealed a DNA content between 1C and 2C, indicating a block in S phase. Nevertheless, about 40% of the cells had fragmented DNA or nuclear bodies (Fig. 2C). After 4 h, some cells had apparently undergone an abnormal mitosis as cells were present with both 1C and greater DNA content and again spindles were elongated and nuclei fragmented (Fig. 2C). Loss of viability and spindle elongation in the presence of unreplicated DNA are two phenotypes associated with a defect in the S/M checkpoint.

Since HU prevents cells from completing replication, HU can be used to monitor S/M checkpoint defects. Cells with an intact checkpoint arrest with short spindles and do not enter mitosis when treated with HU, thus recovering when HU is removed. Checkpoint mutants are supersensitive to HU because they enter mitosis with unreplicated DNA. The mutant strains were streaked on YPD plates containing 150 mM HU and incubated at 24 °C. Mutant F and pol2–11 were inviable in the presence of HU even at 24 °C. Among the other mutants, A through D showed growth retardation, while the remaining pol2 mutants did not show a significant growth defect relative to wild-type.
Because of its supersensitivity to HU, mutant F was further examined for viability, DNA content, and nuclear and spindle morphology after treatment with HU at the nonpermissive temperature. Wild-type and mutant F cells were arrested in the G1 phase using α-factor. They were then shifted to 37°C, and half of the culture was incubated in the continued presence of pheromone while the other half was released from the G1 block in the presence of HU (26). Fig. 3A shows that mutant F incubated at the nonpermissive temperature during a sustained G1 block retains viability. Thus, entry into S phase seems to be required to observe loss of viability (compare Figs. 2A and 3A). In contrast, mutant F cells released from the G1 block and allowed to proceed into a synchronous S phase in the presence of HU at the nonpermissive temperature show a significant loss of viability compared with wild-type (Fig. 3B). Flow cytometric analysis of mutant and wild-type cells treated in the same fashion showed that both wild-type and mutant F cells were efficiently blocked in the S phase (DNA content between 1C and 2C) 4 h after release from the α-factor block into HU (Fig. 3C). Indirect immunofluorescence demonstrated that 60–70% of the mutant F cells had partially elongated spindles after 4 h in HU at the nonpermissive temperature (Fig. 3D), despite failure to complete replication (Fig. 3C), whereas wild-type cells showed no spindle separation (Fig. 3D).

Asynchronous cultures of mutant F treated with HU also lose viability (Fig. 3B). Thus, there is a correlation between spindle elongation and loss of viability, indicative of a defective S/M checkpoint. This defect is observed only in cells incurring damage during S phase, but not in cells that are arrested in G1, consistent with the results obtained for pol2–11 (10). When released from α-factor block at the permissive temperature, mutant F did not show a significant loss in viability, even in the presence of HU (data not shown), which is consistent with the fact that the previously studied pol2–11 also has a greater defect in the S/M checkpoint at elevated temperature (10). We conclude that mutant F is defective in the checkpoint pathway that monitors damage due to reduction of nucleotide pools as well as damage due to a thermolabile DNA polymerase at the nonpermissive temperature.

Mutations in ZF1 and between ZF1 and ZF2 Are Sensitive to Growth in the Presence of MMS—In order to study the effect of the pol2 mutations on the DNA damage response, ability to tolerate MMS damage was monitored by examining growth of pol2 mutants during chronic exposure to MMS. The mutants were grown to saturation at the permissive temperature, and various dilutions were placed on plates containing different concentrations of MMS. pol2–11 and mutants A, B, D, and F showed greater sensitivity to 0.025% MMS in comparison to wild-type and mutants G and H at the permissive temperature (Fig. 4A). At 37°C, mutants G and H also displayed MMS sensitivity (Fig. 4B). The results suggest that ZF1 and amino acid residues between ZF1 and ZF2 play a primary role in MMS sensitivity, while ZF2 may have a secondary role. It should be noted that the N-terminal pol2–18 mutant was also sensitive to MMS, which may reflect a defect in the repair machinery itself, since pol2–18 mutants have been shown previously to be proficient in the checkpoint (Ref. 9; see also Fig. 5).

Mutations in ZF1 and between ZF1 and ZF2 Show Reduced Inducibility of RNR3 Expression in the Presence of MMS—To investigate whether the MMS sensitivity of the C-terminal mutants was due to a checkpoint defect, we studied the ability of the pol2 mutants to induce transcription of RNR3 in the presence of MMS (9). The mutants were transformed with an RNR3-lacZ reporter plasmid, and the expression of RNR3 was assayed. Mutants A, C, and F and pol2–11 showed a reduced level of RNR3 expression in the presence of MMS (Fig. 5), while mutants G, H, and L showed a similar level of RNR3 expression as compared with wild-type POL2. pol2–18 also showed wild-type levels of induction, as expected. The residual transcription induction observed in mutants A, C, and F and pol2–11 is expected and is probably due to the Rad9 pathway.
studies have recently dissected the damage response into two different branches and revealed that the Pol2 pathway responds to damage only during S phase, whereas Rad9 responds to damage in other phases of the cell cycle (10). The results support the earlier proposal that the zinc fingers in the C terminus of pol ε act as a damage sensor and indicate that ZF1 may play a more important role than ZF2.

Since ZF1 showed greater MMS sensitivity while the interzinc finger mutants E and F showed greater sensitivity to HU, it seemed possible that the two different motifs might recognize different types of lesions. However, further examination of mutant A, which deletes two cysteines of ZF1, showed that it responded to HU with loss of viability at 37 °C just like mutant F (compare mutants A and F in Fig. 3B). Thus, ZF1 is not specific for MMS-induced damage.

Yeast Two-hybrid Assay of Protein Interactions in the C Terminus of POL2 with DPB2 and DPB3—The question now is how these motifs contribute to the checkpoint. Zinc finger domains have been inferred to play a critical role in protein-protein interactions (27). To test if the cysteine-rich C terminus of Pol2p is important for protein-protein interactions in pol ε, we employed the yeast two-hybrid assay (28) and investigated interactions among the subunits of pol ε: Pol2p, Dpb2p, and Dpb3p. The results are shown in Fig. 6 and Table III and are summarized in Table IV.

Since it had previously been proposed that pol ε might dimerize through the C terminus, we first checked self-interaction (9). We subcloned the C terminus (aa 1265-end) into two yeast expression vectors, one such that C terminus was in-frame with the Gal4 binding domain (pAST2–1) and another such that it was in-frame with Gal4 acidic activation domain (pACT2). The pACT2 activation domain vector also has an HA epitope toward the C terminus of Gal4. In both vectors, the C terminus region lacks the nuclear localization signal.

Interaction was assayed by the ability to transcribe three different reporters,
ADE2, HIS3, and lacZ, controlled by three different promoters, as described under “Experimental Procedures.” The expression levels of the Gal4 fusion proteins were confirmed by Western blotting using anti-Gal4 binding domain and anti-HA 12CA5 antibodies. As shown in Fig. 6A, interaction was detected when the C terminus of pol ε was present on both binding domain and activation domain vectors. The C terminus of pol ε was unable to activate transcription on its own or in combination with control proteins p53 and T-antigen, suggesting that the self-association of pol ε through C terminus is specific. To localize the site of self-association, we subcloned various C-terminal mutants of pol ε in both binding domain and activation domain vectors and assayed for the protein-protein interactions. Deletion of the entire zinc finger region and the extreme C terminus (Δ2103-end) abolished interaction, while deletion of ZF2 and the extreme C terminus (Δ2163-end) did not, localizing the effect to the region containing ZF1 and amino acids between the two zinc fingers. Mutants A and H, with deletions within ZF1 and ZF2, respectively, showed interaction; but the replication/checkpoint-defective mutants E and F, with deletions between ZF1 and ZF2, failed to show any interaction. (Western blotting confirmed that the proteins were expressed efficiently (Fig. 6B).) Thus, there is a striking correlation between the mutants that affect replication and the S/M checkpoint and those that affect interaction as measured by the two-hybrid assay. With these experiments it is impossible to determine if the interaction documented is direct or indirect, however.

We next investigated interaction of Pol2p with the other pol ε subunits. We found that the C terminus interacts strongly with DPB2 but is not sufficient for interaction with DPB3 (Tables III and IV). Mutants E and F interact with Dpb2p, suggesting that the deletions do not cause a catastrophic disruption of the structure of the entire C-terminal region. However, quantitation of the interaction by β-galactosidase assay (Table III) suggests the interaction is weakened by the mutations. Deletions of the extreme C terminus such as are found in pol2–11 and pol2–12 also fail to abolish interaction with Dpb2p, but reduce the level of β-galactosidase activity. However, deletion of 30 amino acids between mutant F and mutant J, which includes ZF2, abolishes interaction. This may be due to a large structural perturbation, however, since mutants G, H, and I each appear to interact with Dpb2p. The interaction between the POL2 C terminus and Dpb2p has been verified by expression in recombinant baculovirus infected insect cells, as has the reduced affinity between pol2 mutant E and Dpb2p. Since mutants E and F interact with Dpb2p, this may suggest that the effects on checkpoint are directly due to the mutations in POL2 rather than indirectly due to failure to interact with another protein in the holoenzyme complex. On the other hand, the inefficient interaction with mutant E and Dpb2p may suggest the checkpoint defect reflects faulty assembly of the holoenzyme.

Neither Dpb2p nor Dpb3p appear to self-interact by the two-hybrid assay (Table IV).

**DISCUSSION**

Yeast DNA polymerase ε plays an essential role in chromosomal replication (16, 18, 19). In recent years, additional roles of pol ε in repair and cell-cycle regulation have emerged (9, 10, 17). Structure-function analysis has suggested that the C ter-

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minus of pol ε, although far removed from the catalytic domain, is essential for replication, for repair, and for DNA damage-induced transcription and cell cycle arrest. It has been proposed that pol ε could be involved in the initial recognition and processing of the DNA damage. How pol ε recognizes and communicates the checkpoint signal downstream to other member(s) of the transduction machinery is unknown. In this study, we have begun to illuminate the structural basis for the checkpoint function of pol ε by site-specific deletion mutagenesis of the C terminus. We demonstrate that amino acid residues (mutants E and F) between ZF1 and ZF2 in the C terminus are essential for viability, indicating a role in DNA damage-processing of the DNA damage. How pol ε recognizes and communicates the checkpoint signal downstream to other member(s) of the transduction machinery is unknown. In this study, we have begun to illuminate the structural basis for the checkpoint function of pol ε by site-specific deletion mutagenesis of the C terminus. We demonstrate that amino acid residues (mutants E and F) between ZF1 and ZF2 in the C terminus are essential for viability, indicating a role in DNA damage.

**FIG. 6. Yeast two-hybrid analysis.** A, interactions. The C terminus (aa1265-STOP) of POL2 was prepared by PCR and cloned into pAS2-1 binding domain and pACT2 activation domain vectors as described under "Experimental Procedures." The resulting Gal4 fusion vectors were co-transformed into strain PJ69-4A. Ability to grow on medium lacking adenine or histidine was monitored. The β-galactosidase activity was measured using ortho-nitrophenyl-β-D-galactopyranoside as substrate, as described under "Experimental Procedures." The activity is expressed in β-galactosidase units. B, Western blots of extracts of cells expressing mutant E in the binding domain and activation domain vectors. Fusions in the binding domain vector were detected with Gal4 antibody, and fusions in the activation domain vector were detected with 12CA5 anti-hemagglutinin antibody as described under "Experimental Procedures." The band corresponding to mutant E migrates just above the 104-kDa marker.

**TABLE III**

Two-hybrid interaction between DPB2 and the POL2 C terminus

| Bait       | Prey       | Ade  | His  | β-Gal units |
|------------|------------|------|------|-------------|
| DPB2       | Vector     | –    | –    | –           |
| DPB2       | DPB2       | –    | –    | ND          |
| POL2(WT)   | DPB2       | +    | +    | 28          |
| DPB2       | POL2(WT)   | +    | +    | 30          |
| pol2(I)    | DPB2       | +    | +    | ND          |
| pol2(G)    | DPB2       | +    | +    | ND          |
| pol2(I)    | DPB2       | +    | +    | ND          |
| pol2–11    | DPB2       | +    | +    | 20          |
| pol2(Δ1263-STOP) | DPB2 | –    | –    | ND          |

**TABLE IV**

Summary of two-hybrid interactions between the C terminus of POL2 and its subunits, DPB2 and DPB3

| Bait          | Prey          | Prey          | Prey          |
|---------------|---------------|---------------|---------------|
| POL2(1265–2222) | DPB2          | DPB3          |
| WT (1265–2222) | +             | –             | –             |
| A             | +             | –             | –             |
| E             | –             | +             | –             |
| F             | –             | –             | +             |
| G             | +             | –             | +             |
| H             | +             | –             | +             |
| I             | +             | –             | +             |
| 2192-STOP(pol2–11) | +            | +             | +             |
| 2163-STOP    | –             | ND            | –             |
| 2103-STOP    | –             | –             | ND            |
| DPB2         | +             | –             | –             |
| DPB3         | –             | –             | –             |

* All of the POL2 genes used as prey correspond to the mutants used as bait; thus, mutant A was tested against mutant A, etc. See also Fig. 6.

† Interactions involving Dpb2p were carried out with DPB2 cloned in both binding domain and activation domain vectors. Dpb2p interaction with Pol2p mutants was only tested with DPB3 in the activation domain vector, since there was no interaction between Dpb3p and wild-type Pol2p in either orientation.

contrary to expectations from previous studies of the effect of HU on *S. pombe* has a functional S phase checkpoint in the complete absence of pol ε. To explain this behavior, it was proposed that the checkpoint in the presence of DNA damage by HU might be different than the checkpoint for the coordination between the S and M phase in a normal cell cycle. This is entirely possible, since there are clearly numerous subsets of checkpoint pathways yet to be defined.

The S/M checkpoint cell cycle arrest pathway and damage-sensitive transcription induction pathway appear to share many common regulatory elements. In *S. pombe*, the cell cycle arrest in response to DNA damage is mediated by the checkpoint protein RFC5, which is required for both the replication and repair pathways. RFC5 mutants are defective in recognizing MMS-induced DNA damage. Rfc5 mutants have reduced damage-induced transcription and a defective S/M checkpoint. In the current study, we show that pol2-12 mutants were sensitive to MMS and showed reduced RNR3 induction in the presence of MMS, in addition to the cell cycle

Recent studies of the effect of HU on *S. pombe* have shown that pol ε recognizes and communicates the checkpoint signal downstream to other member(s) of the transduction machinery is unknown. In this study, we have begun to illuminate the structural basis for the checkpoint function of pol ε by site-specific deletion mutagenesis of the C terminus. We demonstrate that amino acid residues (mutants E and F) between ZF1 and ZF2 in the C terminus are essential for viability, indicating a role in DNA damage-processing of the DNA damage. How pol ε recognizes and communicates the checkpoint signal downstream to other member(s) of the transduction machinery is unknown. In this study, we have begun to illuminate the structural basis for the checkpoint function of pol ε by site-specific deletion mutagenesis of the C terminus. We demonstrate that amino acid residues (mutants E and F) between ZF1 and ZF2 in the C terminus are essential for viability, indicating a role in DNA damage.

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effects discussed. Mutants A, B, C, and D, with deletions in ZF1, were less sensitive to growth on HU-containing plates than mutant F, but they were more sensitive to MMS and showed equivalent reduced expression of RNR3. The sensitivity toward MMS could also arise due to a direct defect in the DNA repair machinery, although it seems less likely since pol2–11 and pol2–12 mutants are proficient in the repair of MMS-induced damage (32).

Extensive genetic studies and biochemical analysis suggest that the 1200 C-terminal amino acids of POL2 contain sites that stabilize subunit interactions in the pol ε holoenzyme complex (8, 18, 24, 25, 33). The data presented in this study support this by demonstrating interaction between the C terminus of the catalytic subunit and the conserved subunit, Dpb2p, using the two-hybrid assay. The C terminus is not sufficient for interaction with Dpb3p. However, the two-hybrid analysis also suggests that there may be an additional, previously unsuspected interaction involving this domain. Using amino acids 1265 to the stop codon of POL2 as bait in the yeast two-hybrid assay, we found that the C terminus of wild-type pol ε cloned into the activation domain vector can efficiently activate transcription, whereas mutants E and F were unable to do so. Since mutants E and F are defective in replication, the two-hybrid assay results suggest that at least one essential form of pol ε is oligomeric, perhaps a dimer. We do not know if the interaction requires another yeast protein as a bridge, however, since two-hybrid assays with yeast proteins cannot distinguish direct from indirect protein/protein interactions. It is clear, however, that the same mutations that abolish the apparent homodimerization also weaken the interaction of Pol2p with Dpb2p. The proposed oligomerization could explain several other findings. The interallelic complementation of pol2–11 and pol2–18 mutants (9), the interallelic complementation of pol2–18 and pol2–F mutations (this work), partial complementation of pol2–11 by a C-terminal fragment of POL2 (9), and, in S. pombe, the apparently dominant negative effect of expression of a mutant pol ε in cdc20Δ germinating spores (30) could all be explained if pol ε acts as a dimer. The possible dimerization of pol ε is also interesting in view of the fact that the E. coli DNA polymerase III holoenzyme, the essential replicase, forms dimers in solution and acts as a dimer during coordinated synthesis of the leading and lagging strands (34). 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 bubble are connected into a binary replisome was put forward many years ago as a mechanism to prevent rotation of the two forks with respect to each other and thus avoid undue tangling of the daughter chromosomes (35). Dimerization of the polymerases could both serve to link the two forks physically and to ensure synchronous activity at both forks. It has previously been suggested that DNA helicases may also have an organizational role in tethering diverging replication forks to each other (36).

Active replication complexes have been proposed to constitute a device that, once formed, continually sends an inhibitory signal to the checkpoint machinery to prevent mitosis until the replication phase (S phase) is faithfully completed (37). Disassembly after completion of replication would then allow mitosis. At which point in assembly is the surveillance mechanism activated? Does it require unwinding and conversion of the origin into a replication fork? Mutant F, described here, shows a correlation between a defective S/M phase checkpoint and destabilized protein-protein interactions, suggesting that the proper assembly of the pol ε complex within the replication complex could be important for initiating and/or maintaining the S phase checkpoint signal. Pol ε has been shown to be absent from origin replication complexes early in G1 and to associate with origin replication complexes late in G1, so the periodic association of pol ε with origins would be consistent with a role in the checkpoint (38). It is still possible, however, that the protein-protein interactions are required only for the replication function of pol ε. In fact, in only one case have a replication defect and a checkpoint defect been (partially) genetically separated. The overexpression of the POL30 gene, encoding the proliferating cell nuclear antigen was shown to suppress the replication defect of the rfc5 but not its checkpoint defect (11).

Another mystery is how the whole assembly of proteins can sense incomplete DNA replication and DNA damage due to radiation or chemicals such as MMS in chromosomes undergoing replication. Something in the structure of the DNA itself, such as stretches of single-stranded DNA formed during replication and blocks due to DNA damage, has been proposed to act as a signal in the S phase, and the zinc finger of pol ε has been proposed to monitor the signal (9). Single-stranded DNA has been shown to act both as an efficient trap for pol ε and to actually promote dissociation of full-length pol ε but not a pol ε protein missing amino acids 1270 to the end (aa 2222) from primer-templates, suggesting that there is a binding site for single-stranded DNA in the large, non-catalytic C-terminal region (39). The mutant phenotypes described in this work suggest that the zinc finger region may be what is critical for single-stranded DNA sensing in that C-terminal segment. A damage recognition role has been proposed for zinc fingers in two other proteins (40). The new checkpoint-defective mutants will be useful in reconstituting mutant pol ε holoenzymes to further evaluate the physical mechanism of the sensing of DNA damage using in vitro binding studies and kinetic analysis of activity on various model substrates.

Acknowledgments—We thank the members of the Campbell laboratory for helpful discussions and Dr. Julie Archer for help in immunofluorescence studies. We are grateful to Akio Sugino and Hiroyuki Araki for pol ε antibodies and the pol2 knock-out strain, YHA301.

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