THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 32, Issue of August 6, pp. 22283–22288, 1999

Printed in U.S.A.

Analysis of the Essential Functions of the C-terminal Protein/Protein Interaction Domain of *Saccharomyces cerevisiae* pol ϵ and Its Unexpected Ability to Support Growth in the Absence of the DNA Polymerase Domain*

(Received for publication, April 22, 1999, and in revised form, June 9, 1999)

Rajiv Dua, Daniel L. Levy, and Judith L. Campbell‡

From the Braun Laboratories 147-75, California Institute of Technology, Pasadena, California 91125

As first observed by Wittenberg (Kesti, T., Flick, K., Keranen, S., Syvaoja, J. E., and Wittenburg, C. (1999) *Mol. Cell* 3, 679–685), we find that deletion mutants lacking the entire N-terminal DNA polymerase domain of yeast pol ϵ are viable. However, we now show that point mutations in DNA polymerase catalytic residues of pol ϵ are lethal. Taken together, the phenotypes of the deletion and the point mutants suggest that the polymerase of pol ϵ may normally participate in DNA replication but that another polymerase can substitute in its complete absence. Substitution is inefficient because the deletion mutants have serious defects in DNA replication. This observation raises the question of what is the essential function of the C-terminal half of pol ϵ. We show that the ability of the C-terminal half of the polymerase to support growth is disrupted by mutations in the cysteine-rich region, which disrupts both dimerization of the POL2 gene product and interaction with the essential *DPB2* subunit, suggesting that this region plays an important architectural role at the replication fork even in the absence of the polymerase function. Finally, the S phase checkpoint, with respect to both induction of *RNR3* transcription and cell cycle arrest, is intact in cells where replication is supported only by the C-terminal half of pol ϵ, but it is disrupted in mutants affecting the cysteine-rich region, suggesting that this domain directly affects the checkpoint rather than acting through the N-terminal polymerase active site.

In *Saccharomyces cerevisiae*, three DNA polymerases participate in chromosomal DNA replication, pol[1] α, pol δ, and pol ϵ. pol α is primarily involved in the initiation of DNA replication and priming of Okazaki fragments (2), whereas pol δ and pol ϵ are required for completion of synthesis of both the leading and lagging strands. The precise reactions performed by pol δ and pol ϵ on leading and lagging strands, however, have not yet been delineated. In an interesting contrast to yeast chromosomes, simian virus 40 DNA replication does not require pol ϵ. Instead, pol α and pol δ are sufficient for viral DNA replication (3). Thus, there appears to be some plasticity in the eukaryotic replication fork.

Pol ϵ is a multi-subunit complex consisting of Pol2p, Dpb2p, Dpb3p, and Dpb4p (4). The Pol2p is the catalytic subunit, and it is encoded by the *POL2* gene (5). The Pol2p is a class B polymerase, characterized by a series of conserved domains, called domains I–VI, containing the exonuclease subdomains and the DNA polymerase active site residues in the N-terminal half of the protein (Fig. 1*A*) (6, 7). Mutations M643I and P710S (the *pol2-9* and *pol2-18* alleles, respectively) within the polymerase domain in *POL2* result in temperature sensitivity (8). The remaining half of *POL2* consists of a long region that is conserved in pol ϵ from all organisms but is not found in any other class B polymerase. An interesting feature of the extreme C terminus is a cysteine-rich stretch of amino acids containing two *putative* zinc fingers, ZF1 and ZF2 (Figs. 1*A* and 2) (9). Although all of the essential polymerases have a cysteine-rich domain in a similar location, the specific amino acids are better conserved among pol ϵ proteins from different organisms than between pol α, pol δ, and pol ϵ from the same species (10). The C terminus of *POL2* has been shown to have a dual role (11). Unlike the N-terminal *pol2-9* and *pol2-18* mutants, which have defects only in DNA replication, *pol2-11* and *pol2-12*, which are non-sense mutations about 30 amino acids from the termination codon, have defects in both DNA replication and in the cellular response to DNA damage during S phase, known as the S/M checkpoint (11–13). In investigating the molecular basis for these defects, we showed that the C-terminal half of *POL2* is important in the assembly of the pol ϵ holoenzyme, a finding supported by suppressor studies, synthetic lethal tests, and purification of various pol ϵ subassemblies from yeast (4, 14, 15). We discovered that pol ϵ can dimerize and that a 10-amino acid deletion between ZF1 and ZF2 that results in a replication and checkpoint defect both abolishes self-interaction and reduces the ability of Pol2p to interact with Dpb2p (9). In addition to mediating essential protein/protein interactions, the C-terminal 100-kDa portion of Pol2p might also contribute to the binding of single-stranded or other non-B form DNA (16). One unanswered question is whether the C-terminal portion of the protein acts independently or whether it modifies some function of the N-terminal polymerase domain.

Are the replication and checkpoint functions of *POL2* separable? The polymerase activity and the checkpoint functions are at least in some sense independent, because the N-terminal *pol2-9* and *pol2-18* mutants appear to be defective in replication but, unlike *pol2-11* and *pol2-12*, proficient in the S/M checkpoint. However, recently, Wittenberg and colleagues (1) have made the somewhat counterintuitive discovery that cells tolerate deletion of the entire polymerase domain of *POL2*, suggesting that the polymerase activity is not the essential

* This work was supported by Public Health Service Grant 25508 and Grant 1153-F12 from the American Heart Association (to R. D.). 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.

‡ To whom correspondence should be addressed. Tel.: 626-395-6053; Fax: 626-405-9452; E-mail: jcampbel@cco.caltech.edu.

[1] The abbreviations used are: pol, DNA polymerase; ZF, zinc finger; HU, hydroxyurea; MMS, methyl methane sulfonate; DAPI, 4′,6-diamino-2-phenylindole; S/M, S phase/M phase checkpoint; YPD, complete growth medium for yeast; PFGE, pulse field gel electophoresis.

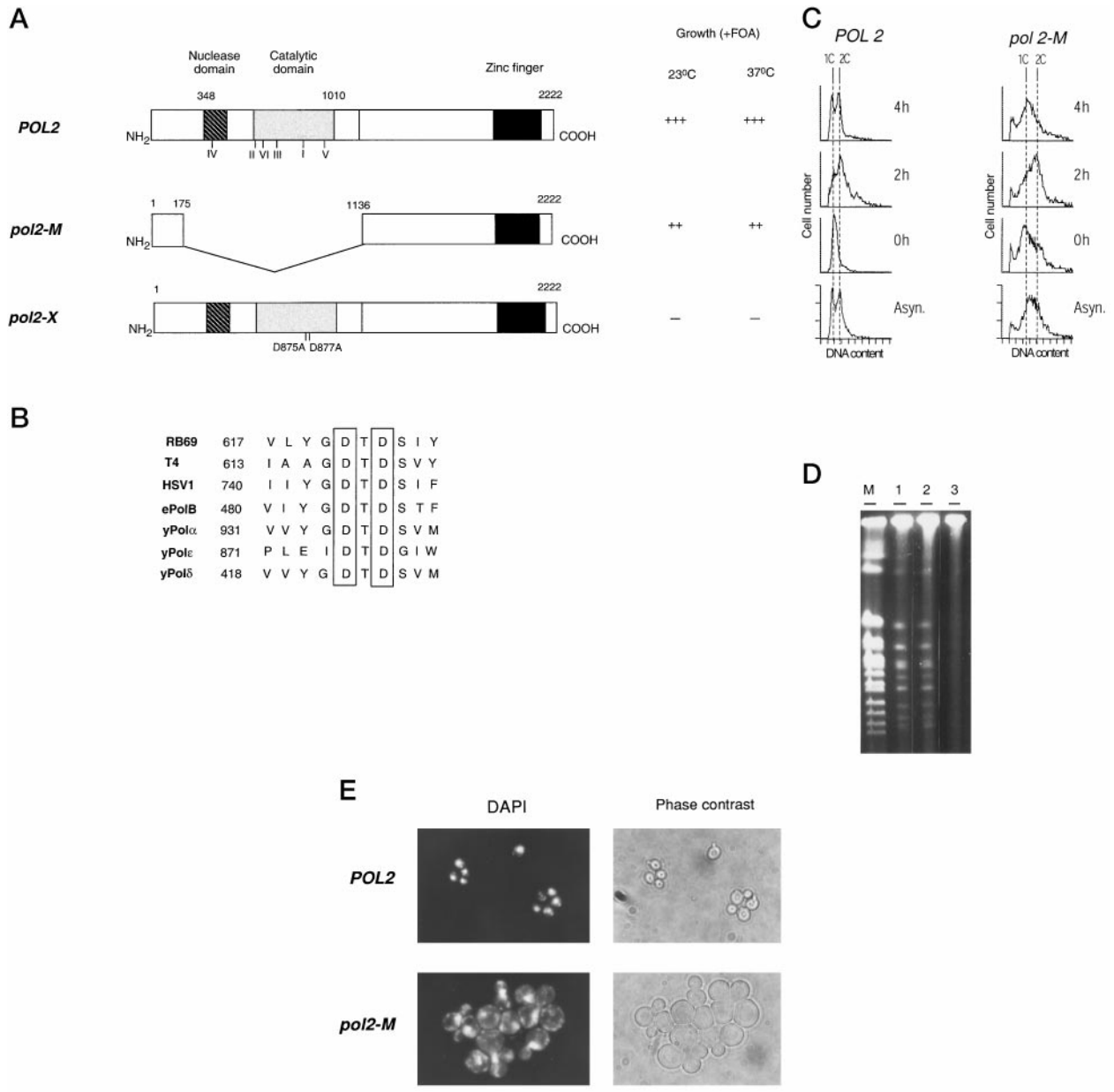

FIG. 1. **Characterization of deletion and point mutations affecting the N-terminal half of pol ϵ.** *A*, site-specific deletion of the polymerase domain of *POL2* and preparation of the *pol2* catalytic site mutant. Amino acids 176–1135 containing the polymerase domain were deleted, and the catalytic site mutant was prepared as described under "Experimental Procedures." The mutant *pol2* genes were subcloned into the pRS314 vector and introduced into strain A1128 *pol2-3::LEU2*(*YEpPOL2*). The transformants were replica-plated to agar plates lacking tryptophan and containing 5-fluoroorotic acid at 24 and 37 °C for 4 days, as described previously (9). The symbols +++, ++, and − refer to normal growth, slow growth, and no growth, respectively, on agar plates. *B*, amino acid alignments showing the conservation of catalytic residues in polymerases. Bacteriophage RB69 and T4, herpes simplex virus (*HSV1*), *E. coli* pol II (*ePolB*), *S. cerevisiae* pol α (*yPolα*), *S. cerevisiae* pol ϵ (*yPolϵ*), and *S. cerevisiae* pol δ (*yPolδ*) polymerases are shown (25). The conserved aspartates are *boxed*. *C*, flow cytometric analysis of *pol2-M*. Mid-log phase *pol2-M* cells were blocked in G_1 with 8 μg/ml α-factor and grown for 4 h at 24 °C. Additional α-factor was added twice at 2-h intervals. The cells were washed with YPD to remove the α-factor, and the culture was transferred to 37 °C. The cells were collected at indicated time intervals, fixed with ethanol, and treated with 20 μg/ml RNase. The DNA was stained with propidium iodide, and at least 10,000 cells were examined by flow cytometry. *D*, PFGE analysis of chromosomes in *pol2-M*. Cells were grown at 30 °C and transferred to 37 °C for 4 h. PFGE was carried out as described previously (29). *Lane 1*, *POL2*; *lane 2*, *pol2-M*; *lane 3*, *pol2-F*; *lane M*, PFGE markers. *E*, nuclear staining. The asynchronous *POL2* and *pol2-M* cells were fixed with ethanol, and the nucleoid was observed using DAPI.

replication function of the POL2 gene product. It now appears that the only nonredundant essential function of pol ϵ lies in its C terminus, which has no known catalytic activity. This finding is in keeping with the nonviability of C-terminal deletions (9, 11) and helps explain the fact that the *pol2-18* mutant can be complemented partially by overproduction of a large C-terminal fragment entirely lacking the polymerase domain (11) but not by C-terminal mutants (9). To further define the relative contributions of the polymerase domain and the C-terminal half of the protein to events at the replication fork and in the checkpoint, we have made an N-terminal deletion mutant that, in accord with the results of Wittenberg (1), is viable and has an intact S/M checkpoint. We have extended that work by pinpointing, via site-directed mutagenesis, regions essential for function.

EXPERIMENTAL PROCEDURES

Materials—Plasmid PTZ18 was from Bio-Rad. Plasmid pLitmus39 and M13 phage M13KO7 were from New England Biolabs, as were the

TABLE I
Effect of small C-terminal deletions and Cys-to-Ala mutations in ZF1 and ZF2 on the growth of the pol2-M polymerase domain deletion mutant

Mutants	Mutations (aa)	Growth	Growth
		23 °C	*37 °C*
POL2		+++	+++
pol2-M	Δ176–1135	++	++
Small deletions			
pol2-M/A–I; K	deletions A–I; K[a]	–	–
pol2-M/J		++	++
pol2-M/L		++	++
Zinc finger 1			
pol2-M/N	C1 + C2*	–	–
pol2-M/O	C3 + C4	–	–
pol2-M/P	C1–C4	–	–
Zinc finger 2			
pol2-M/Q	C5 + C6	++	–
pol2-M/R	C7 + C8	++	++
pol2-M/S	C5–C8	++	–

[a] Deletions A–L, 10 amino acid deletions spanning the C-terminal 120 amino acids of *POL2*, as described previously (Ref. 9; see Table II) and in Fig. 2, were combined with *pol2-M* as described under "Experimental Procedures" to give *pol2-M/A*, etc. C1, etc. refer to the consecutive cysteines in ZF1 and ZF2. The cysteine-to-alanine mutations, designated N-S, were also combined with the *pol2-M* mutation as described under "Experimental Procedures" to give *pol2-M/N*, etc.

restriction enzymes, T4 DNA ligase, and Klenow fragment. CJ236 *Escherichia coli* strain and the mutagenesis kit were obtained from Bio-Rad. DH5α bacterial strain used for routine subcloning was from Life Technologies Inc. All oligonucleotides were synthesized by the oligonucleotide facility at the California Institute of Technology. Plasmid purification was done from Qiagen miniprep kits. ECL Western blotting reagents were obtained from Amersham Pharmacia Biotech. The reagents for Western blotting using the alkaline phosphatase method were from Roche Molecular Biochemicals. The polyclonal antibody for the pol ϵ holoenzyme complex was provided by Dr. Akio Sugino from Osaka University, Japan.

Mutagenesis and Subcloning—The deletion of amino acids 176–1135 (polymerase domain) in pol ϵ was as follows. The pK series of plasmids containing the *POL2* or mutant *pol2* genes (9) were digested with *Bgl*II. The resulting linearized plasmids were self-ligated and transformed into DH5α cells, which resulted in an in-frame deletion of the *Bgl*II fragment (amino acids 176–1135) in each plasmid as confirmed by DNA sequencing. The resulting series of plasmids is designated the pL series.

To construct the catalytic site mutations, the N terminus of *POL2* from pRPOL2 (9) was subcloned into the pTZ18 vector at the *Sac*I site. The uracil-phagemid was prepared and mutagenesis was performed as described (9). After mutations were confirmed by DNA sequencing, the *POL2* gene was reconstituted by subcloning the *Bgl*II fragment into the pL1 of the pL series plasmids. The mutagenic oligonucleotide for the D875A,D877A double mutation was 5′-CA CCA AAT ACC A*GC* AGT A-*G*C TAA TTC TAA TG-3′.

For mutagenesis of ZF1 and ZF2, pLitPOL2, which contains the C terminus of *POL2* (9), was used to prepare the uracil-phagemid template, and mutagenesis was performed according to the instructions of the supplier (Bio-Rad). After mutations were confirmed by DNA sequencing, the *POL2* gene was reconstituted by subcloning into the pRPOL2 vector containing the N terminus of *POL2* as described previously for the pK series (9) The oligonucleotides used for the mutagenesis were as follows: C2108A,C2111A, 5′-C AGA AAT GAA AAA GGC GTA-TTC GGC TAA AAA ATC CG-3′; C2130A,C2133A, 5′-GGC TTT GTG-GGC TCT GAC GGC TGA AAA AAT AG-3′; C2164A,C2167A, 5′-CAC-TTT ATG GGC TCT GGA GGC TCT CAA ATC-3′; and C2179A, C2181A, 5′-CGC GCC GGC GGC TGG GGC GTG GGC ACT C-3′.

RESULTS

Deletion of Amino Acids 176–1135, the Catalytic Domain of pol ϵ—To further define the replication and S/M checkpoint function of the C-terminal half of pol ϵ, we deleted the catalytic polymerase domain of the Pol2p. Maki and colleagues (16) recently showed that Pol2p amino acids 191–1270 are sufficient for the polymerase activity of pol ϵ. We removed most of this region, amino acids 176–1135, including conserved domains I–VI (see Fig. 1*A*), to produce mutant *pol2-M*. *pol2-M* was introduced by plasmid shuffling into a strain containing a complete deletion of the resident *POL2* gene, as described previously (9), and the ability of *pol2-M* to support growth was assessed (Fig. 1, Table I). The *pol2-M* mutant was viable at both 23 and 37 °C, although the doubling time was increased even at 23 °C, and cells were mostly "dumbbells" and extremely enlarged at 37 °C, suggesting a defect in cell cycle progression (Fig. 1*E*). Similar observations have been reported by Wittenberg (1).

FIG. 2. **Schematic diagram of the cysteine-rich region.**

To scrutinize the defect in the cell cycle, *pol2-M* cells were grown at 23 °C to log phase, blocked in G_1 with α factor, and then released from pheromone block at 37 °C. Samples were collected at various intervals, and their DNA content was analyzed by flow cytometry (Fig. 1*C*). *pol2-M* cells proceeded through the cell cycle, but more slowly than wild type, as seen best in the 4-h sample. A comparison of the asynchronous wild-type and *pol2-M* cells grown at 23 °C, before the addition of pheromone, revealed that the wild-type population is evenly divided between 1C and 2C DNA content, but the *pol2-M* cells are mostly in S phase, with a DNA content between 1C and 2C, also indicating a slowing of S phase. The state of the DNA in the asynchronous culture was probed by pulsed field gels. As shown in Fig. 1*D*, the bulk of the *pol2-M* DNA enters the gel, the same as for wild type. By contrast, DNA in a *pol2-F* mutant, which contains a temperature-sensitive mutation in the C-terminal region, does not enter the gel, as expected if most of the cells contain chromosomes with activated but stalled replicons (17). Thus, Fig. 1, *C* and *D,* shows that DNA replication is delayed but ultimately completed in *pol2-M* cells. Microscopic examination of the log phase *pol2-M* cells shows that they are greatly enlarged compared with wild type, and DAPI staining shows many large-budded cells with a nucleus that is undivided, again suggesting an S phase delay (Fig. 1*E*). The cell cycle delay observed in these experiments suggests that there may be some damage occurring and that the S/M checkpoint is functioning normally in *pol2-M* strains, which is investigated further below.

Effect of Point Mutations in the Polymerase Catalytic Site—The defects in *pol2-M* suggest that the polymerase domain of pol ϵ does indeed normally participate in DNA replication but that in the absence of the polymerase domain another polymerase can carry out its function, albeit less efficiently. Supporting this idea, when the highly conserved, putative catalytic residues of the polymerase active site (*region I*, Fig. 1*B*), Asp-875 and Asp-877, were changed to alanines, the double point mutation was lethal (Fig. 1*A*, mutant labeled *pol2-X*). We propose that when the polymerase domain is present but catalytically dead, it blocks any other polymerase from compensating. A similar argument might explain why *pol2-18*, a point mutation mapping to region II, part of the nucleoside triphosphate substrate binding site in *POL2*, is temperature-sensitive for growth (8).

Effect of Mutations in the Cysteine-rich Domain of pol2-M—The small deletion mutations that we described previously, A through L, spanning amino acids 2103 to the end, as well as the *pol2-11* mutation (Fig. 2), were introduced into the *pol2-M* gene (9). In addition, we created new point mutations in the cys-

TABLE II
Effect of Cys-to-Ala mutations in ZF1 and ZF2 in intact POL2 on growth

Mutant	Amino acid changed	Growth	Growth
		23 °C	*37 °C*
Zinc finger 1			
pol2-N	C1 + C2[a]	+++	++
pol2-O	C3 + C4	+++	++
pol2-P	C1–C4	+++	++
Zinc finger 2			
pol2-Q	C5 + C6	+++	+++
pol2-R	C7 + C8	+++	+++
pol2-S	C5–C8	+++	+++

[a] C1, etc. refer to the consecutive cysteines in ZF1 and ZF2.

teines of ZF1 and ZF2 of *pol2-M*: mutations *N* (C2108A, C2111A), *O* (C2130A,C2133A), *P* (C2108A,C2111A,C2130A, C2133A) in ZF1; and mutations *Q* (C2164A,C2167A), *R* (C2179A,C2181A), *S* (C2164A,C2167A,C2179A,C2181A) in ZF2. The mutants were first examined for their ability to support growth by the plasmid shuffling assay (9). Table I shows that deletions A through K each abolishes growth. Because only deletions E and F had serious growth defects in the full-length *POL2* gene, the deletions are more deleterious in the C-terminal peptide, as might have been expected from the growth defect in *pol2-M* itself. Interestingly (because we had previously shown that deletions within ZF1 in the intact polymerase were viable), cysteine-to-alanine single amino acid changes in ZF1 of *pol2-M* also abolish growth (Table I). Mutations that affect the cysteines of ZF2 allow slow growth at 23 °C but are lethal at 37 °C. Thus, not only the inter-zinc finger region but also the zinc fingers themselves contribute to the essential function of the C terminus. ZF1 appears to be more critical than ZF2. These mutations confirm that the C terminus has an essential function in DNA replication independent of the polymerization of nucleotides.

We reconstituted the intact *POL2* gene carrying the cysteine-to-alanine mutations described above. All of the full-length cysteine-to-alanine mutants supported growth at all temperatures (Table II), although the mutations affecting ZF1 grew more slowly at the nonpermissive temperature. (Cysteine-to-serine mutants were also viable). A comparison of the results in Tables I and II leads to the conclusion that the presence of the N terminus alters the contribution of the C terminus to DNA replication, again suggesting that the polymerase portion of the protein is important for replication when it is present.

The Role of the Zinc Fingers in the S/M Checkpoint—The viability of cysteine-to-alanine mutants was analyzed in the presence of HU to check their ability to activate the cell cycle checkpoint in response to the replication block by HU. (Because of the low viability of the *pol2-M* ZF mutants in the presence of HU, these experiments were carried out with ZF mutants in the full-length *POL2* gene). The mutants were synchronized in the G_1 phase by α-factor at 23 °C and then released from the pheromone block in the presence of HU at 37 °C. Samples were collected at various time intervals to determine viability. Mutations in ZF1 cysteines caused loss of viability, with only 20% remaining viable after 8 h (Fig. 3). The ZF2 mutants were comparable with *POL2*, having 60% viability after 8 h. The results shown in Table II and Fig. 3 indicate that putative ZF1 plays a role in both DNA replication and the S/M checkpoint. ZF2 is less important for both functions but also appears to contribute. The results are consistent with our previous study in which deletion mutants in ZF1 showed increased sensitivity to MMS and HU and reduced damage-induced *RNR3* transcription (9).

DNA damage inducible transcription, another branch of the S/M checkpoint, which we have shown previously to be defective in *pol2-F* strains, was then measured in *pol2-M* and *pol2-M/Q,* a viable ZF2 mutant (Fig. 4). (Because the ZF1 mutants

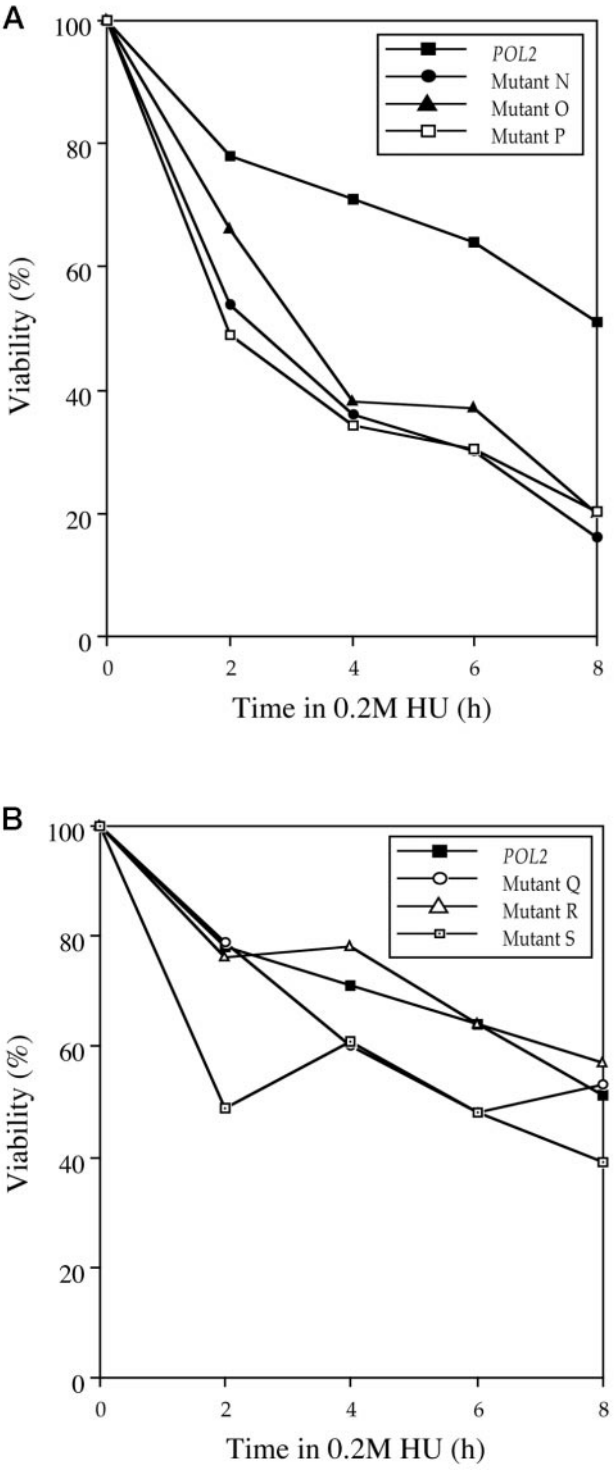

FIG. 3. **Checkpoint defects in mutants affecting the cysteine-rich putative zinc finger in the C terminus of *POL2*.** *A*, viability of *pol2* ZF1 mutants synchronized in G_1 and released into S-phase at 37 °C. Mid-log phase cells were grown in YPD (pH 3.9) and treated with 8 μg/ml α-factor at 24 °C. After 2 h, additional α-factor at 4 μg/ml was added, and incubation was continued for 2 h at 24 °C. After 4 h, the α-factor was removed by washing the cells in YPD, cells were released into S-phase in the presence of 0.2 M HU, and the culture was transferred to 37 °C. Viability was determined at various time intervals by plating cells on YPD at 24 °C. *B*, viability of *pol2* ZF2 mutants as described in *A*.

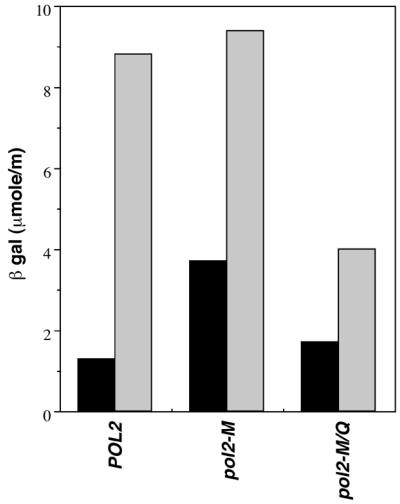

FIG. 4. ***RNR3* expression by *pol2-M* mutants in the presence of MMS.** *pol2-M* and viable derivatives were transformed with the *RNR3-lacZ* reporter, and levels of β-galactosidase were determined after treatment with MMS as described previously (9). *Filled bars*, no MMS; *shaded bars*, plus MMS.

were nonviable, they could not be tested.) Levels of *RNR3* were significantly induced by treatment of *pol2-M* with MMS, showing that the checkpoint is intact. However, when a mutation was introduced into ZF2, *pol2-M/Q*, the level of induction was significantly reduced. Thus, ZF2 is required for setting up a functional checkpoint, even in the absence of the polymerase domain. The low level of damage-induced induction of *RNR3* in *pol2-M/Q* is presumably because of cells that were not in S phase at the time of MMS treatment, as previously observed for *pol2-11* and *pol2-F* (9, 12). The *pol2-M* strain seemed to have slightly higher constitutive levels of *RNR3* transcription in the absence of damage than strains carrying the intact DNA polymerase. Elevated constitutive levels of *RNR3* expression have also been observed in *pol1* and *pol3* mutants (11, 18).

DISCUSSION

The phenotypes of temperature-sensitive mutants and chromatin cross-linking studies have been interpreted as showing that pol ϵ as well as pol δ and pol α all play essential roles at all replication forks during yeast and mammalian chromosomal replication (8, 13, 19–21). However, the precise function of pol ϵ is not clear. Furthermore, in the SV40 DNA *in vitro* replication system reconstituted from purified proteins, pol ϵ is dispensable, suggesting that the eukaryotic replication fork may have some plasticity. Recently, a demonstration that pol δ has a dimeric structure (22, 23), like the bacterial replicases, raised additional questions about the role of pol ϵ at replication forks, because the dimeric pol δ could presumably coordinate leading and lagging strand synthesis without the aid of pol ϵ. The suggestion was made that pol ϵ might perform an essential function in the maturation of Okazaki fragments (24). The finding by Wittenberg (1) that the polymerase domain of pol ϵ is dispensable, which we have now also observed, at first sight confuses the picture of polymerases at the replication fork further. However, based on our studies of the pol ϵ C-terminal fragment, we propose that the polymerase domain of pol ϵ normally participates in replication but that another polymerase can substitute in its absence. To confirm a role of the N-terminal polymerase domain of pol ϵ in replication, we generated a mutant in which two conserved aspartates in the catalytic active site were changed to alanine, *pol2-X*, and found that the mutation was lethal. The apparent paradox between the viability of the deletion of the entire domain and the nonviability of point mutants in the catalytic residues can be explained if we consider the structure of the polymerase domain. The *E. coli* Klenow fragment, *Thermus aquaticus* polymerase, HIV reverse transcriptase, and bacteriophage RB69 class B polymerase all have a U-shaped polymerase domain that consists of "thumb," "palm," and "finger" subdomains (25). The thumb interacts with the product DNA and template, the palm contains the polymerase active site aspartate residues, and the fingers interact with the template and dNTPs (25, 26). The C terminus, although performing an essential role at the fork, lacks the primer-template binding domain, thus leaving the primer terminus accessible to another DNA polymerase. The *pol2-X* mutant affects the palm but retains the thumb and finger primer-template domains intact and probably can still bind to the primer, blocking accessibility to another polymerase. The behavior of this mutant is in good agreement with the fact that base analog induced mutagenesis studies strongly suggest that the 3′–5′ exonuclease domain of pol ϵ is normally functional at the replication fork (27). It will be interesting to see whether the *pol2-X* allele, with point mutations in domain I, is dominant or recessive to overproduction of the C terminus.

Although the participation of the polymerase domain at replication forks may remain in question, it seems compelling that the C-terminal half of pol ϵ is essential for yeast replication and that it can function independently of the polymerization function. Our results make it seem likely that the domain plays a structural role in organizing other proteins at the replication fork. Using the two-hybrid assay, we showed that the C terminus of pol ϵ can dimerize and that the inter-zinc finger mutations affecting replication and the S/M checkpoint pathway are defective both in the dimerization and interaction with Dpb2p (9). The same mutations inactivate the replication function of the C-terminal fragment in the absence of the polymerase. Our current mutagenesis further refines our understanding of the amino acids that contribute to the essential function of the C terminus by showing that specific cysteines in ZF1 are essential and that mutations of cysteines in ZF2 lead to a temperature-sensitive phenotype. Several mutations are more deleterious when introduced into cells in the C-terminal fragment rather than in the intact polymerase. A comparison of ZF2 mutants G and H (intact polymerase) and *pol2-M/Q* (C-terminal fragment) is of interest. Mutants G and H show no growth defect but are extremely sensitive to MMS at the restrictive temperature, suggesting a defect in repair or damage avoidance (9). *pol2-M/Q* is temperature-sensitive for growth. Because this domain may affect interaction with other pol ϵ subunits, the simplest interpretation of the more serious defect in *pol2-M/Q* is that those subunits may also make contacts with the polymerase domain that stabilize the overall interaction. With respect to induction of *RNR3*, mutant G shows only slightly reduced induction at 23 °C, whereas *pol2-M/Q* shows more complete reduction. This finding might again suggest that proteins that interact with the C terminus also interact with the N terminus, when present, and that these interactions affect the damage response. Regardless of the molecular explanation, the new mutants show that the N-terminal and C-terminal functions of pol ϵ are not totally independent.

Full-length Pol2p, with 10 amino acid deletions in ZF1, still dimerizes and interacts with Dpb2, suggesting that ZF1 is not required for protein/protein interactions. DNA binding is another possible role for ZF1, in keeping with the function of many zinc finger proteins. Because the C-terminal fragment can support replication but lacks the DNA binding residues of the polymerase active site, the C-terminal fragment must bind

to the replication fork either through an independent DNA binding domain or through interaction with another protein that binds there. Supporting the existence of an independent DNA binding domain, Maki and co-workers (16), using a single-stranded DNA trap assay, demonstrated that pol ϵ holoenzyme dissociates from a primer-template 75-fold faster than a pol ϵ preparation consisting of an N-terminal 145-kDa catalytically competent fragment of Pol2p. This enzyme also lacks the three non-catalytic subunits of the holoenzyme (16). It was proposed that the C terminus of pol ϵ and/or other subunits positively modulate a single-stranded binding site in the N terminus or that the C terminus of pol ϵ (and/or other subunits that interact there) has an additional single-stranded binding site. ZF1 may somehow contribute to single-stranded DNA recognition, because ZF1 mutants are defective in the S/M checkpoint. We have reconstituted the Pol2p·Dpb2p·Dpb3p·Dpb4p complex and the pol2-Fp (lacking the inter-zinc finger domain) Dpb2p·Dpb3p·Dpb4p complexes. The pol2-Fp complexes are defective compared with the wild type both in the stability of the complexes and in the sensing of single-stranded DNA, suggesting that there is a single-stranded DNA binding site in the C terminus itself.[2]

It has recently been proposed that the lethal event during replicational stress is the dissociation of replication complexes, leading to an inability to complete replication when conditions become permissive again (28). The role of the checkpoint proteins in the model is therefore to stabilize stalled replication complexes. Because the mutant pol ϵ complexes affect the assembly and structure of the fork, they may be refractory to stabilization by checkpoint proteins, which would account for their checkpoint defects.

Acknowledgments—We are grateful to Curt Wittenberg for communicating his results prior to publication and to Laura Hoopes and Elizabeth Bertani for help with PFGE.

[2] R. Dua and J. L. Campbell, unpublished results.

REFERENCES

1. Kesti, T., Flick, K., Keranen, S., Syvaoja, J. E., and Wittenburg, C. (1999) *Mol. Cell* **3,** 679–685
2. Campbell, J. L. (1993) *J. Biol. Chem.* **268,** 25261–25264
3. Waga, S., Bauer, G., and Stillman, B. (1994) *J. Biol. Chem.* **269,** 10923–10934
4. Hamatake, R. K., Hasegawa, H., Clark, A. B., Bebenek, K., Kunkel, T. A., and Sugino, A. (1990) *J. Biol. Chem.* **265,** 4072–4083
5. Morrison, A., Araki, H., Clark, A. B., Hamatake, R. K., and Sugino, A. (1990) *Cell* **62,** 1143–1151
6. Wong, S. W., Wahl, A. F., Yuan, P.-M., Arai, N., Pearson, B. E., Arai, K., Korn, D., Hunkapiller, M. W., and Wang, T. S.-F. (1988) *EMBO J.* **7,** 37–47
7. Morrison, A., Bell, J. B., Kunkel, T. A., and Sugino, A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 9473–9477
8. Araki, H., Ropp, P. A., Johnson, A. L., Johnston, L. H., Morrison, A., and Sugino, A. (1992) *EMBO J.* **11,** 733–740
9. Dua, R., Levy, D. L., and Campbell, J. L. (1998) *J. Biol. Chem.* **273,** 30046–30055
10. Yang, C.-L., Chang, L.-S., Zhang, P., Hao, H., Zhu, L., Toomey, N. L., and Lee, M. Y. W. T. (1992) *Nucleic Acids Res.* **20,** 735–745
11. Navas, T. A., Zhou, Z., and Elledge, S. J. (1995) *Cell* **80,** 29–39
12. Navas, T. A., Sanchez, Y., and Elledge, S. J. (1996) *Genes & Dev.* **10,** 2632–2543
13. Budd, M. E., and Campbell, J. L. (1993) *Mol. Cell. Biol.* **13,** 496–505
14. Araki, H., Hamatake, R. K., Johnston, L. H., and Sugino, A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 4601–4605
15. Araki, H., Leem, S.-H., Amornrat, P., and Sugino, A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 11791–11795
16. Maki, S., Hashimoto, K., Ohara, T., and Sugino, A. (1998) *J. Biol. Chem* **273,** 21332–21341
17. Hennessy, K. M., Clark, C. D., and Botstein, D. (1990) *Genes & Dev.* **4,** 2252–2263
18. Zhou, Z., and Elledge, S. J. (1992) *Genetics* **131,** 851–866
19. Aparicio, O. M., Weinstein, D. M., and Bell, S. (1997) *Cell* **91,** 59–69
20. Zlotkin, T., Kaufmann, G., Jiang, Y. Q., Lee, M. Y. W. T., Vitto, L., Syvajoa, J., Dornreiter, I., Fanning, E., and Nethanel, T. (1996) *EMBO J.* **15,** 2298–2305
21. D'Urso, G., and Nurse, P. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 12491–12496
22. Gerik, K. J., Li, X., Pautz, A., and Burgers, P. M. (1998) *J. Biol. Chem.* **273,** 19747–19755
23. Burgers, P. M. (1998) *J. Biol. Chem.* **273,** 19756–19762
24. Burgers, P. M. J. (1998) *Chromosoma* (*Berl.*) **107,** 218–227
25. Wang, J., Sattar, A. K. M. A., Wang, C. C., Karam, J. D., W. H., K., and Steitz, T. A. (1997) *Cell* **89,** 1087–1099
26. Joyce, C. M., and Steitz, T. A. (1994) *Annu. Rev. Biochem.* **63,** 777–822
27. Scherbakova, P. V., and Pavlov, Y. I. (1996) *Genetics* **142,** 717–726
28. Desany, B. A., Alcasabas, A. A., Bachant, J. B., and Elledge, S. J. (1998) *Genes & Dev.* **12,** 2956–2970
29. Huang, T., and Campbell, J. L. (1995) *J. Biol. Chem.* **270,** 9607–9614