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DNA polymerase II, the probable homolog of mammalian DNA polymerase ϵ, replicates chromosomal DNA in the yeast *Saccharomyces cerevisiae*

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Two temperature-sensitive DNA polymerase II mutants (*pol2-9* and *pol2-18*) of the yeast *Saccharomyces cerevisiae* were isolated by the plasmid shuffling method. DNA polymerase II activity partially purified from both mutants was thermolabile, while DNA polymerase I and III activities remained thermotolerant. At the restrictive temperature, the *pol2* mutants were defective in chromosomal DNA replication and exhibited the dumbbell terminal morphology typical of DNA replication mutants. The *POL2* transcript accumulated periodically during the cell cycle, peaking at the G_1/S boundary in the same manner as the transcripts of more than 10 other DNA replication genes. These results indicate that DNA polymerase II participates in nuclear DNA replication. The similarities in structure and activities between the DNA polymerases of yeast and mammals make it likely that mammalian DNA polymerase ϵ too is required for chromosomal DNA replication.

Key words: cell cycle/chromosomal replication/DNA polymerase/*Saccharomyces cerevisiae*

Introduction

Two DNA polymerases, α and δ, are thought to be required for DNA replication in higher eukaryotes. The requirement for DNA polymerase α has been demonstrated by genetic and antibody microinjection experiments (Eki *et al.*, 1986; Kaczmarek *et al.*, 1986). Both DNA polymerases α and δ as well as PCNA, an auxiliary protein of DNA polymerase δ, have been identified as host factors required for SV40 DNA replication *in vitro* (Murakami *et al.*, 1986; Bravo *et al.*, 1987; Prelich *et al.*, 1987; Lee *et al.*, 1989; Weinberg and Kelly, 1989). *In vitro* reconstitution studies of SV40 DNA replication have led to the proposal of a two DNA polymerase model for eukaryotic DNA replication (Prelich and Stillman, 1988; Tsurimoto and Stillman, 1989; Tsurimoto *et al.*, 1990; Weinberg *et al.*, 1990). *Saccharomyces cerevisiae* DNA polymerases I and III are structurally and biochemically homologous to mammalian DNA polymerases α and δ, respectively (reviewed in Burgers *et al.*, 1990). The genes for both polymerases have been cloned and their nucleotide sequences determined, and conditionally lethal mutants have been isolated (Johnson *et al.*, 1985; Budd and Campbell, 1987; Pizzagalli *et al.*, 1988; Boulet *et al.*, 1989; Sitney *et al.*, 1989). These mutant cells exhibit temperature-sensitive chromosomal DNA replication (Budd and Campbell, 1987; Conrad and Newlon, 1983), indicating that DNA polymerases I and III are required for normal chromosomal DNA replication in yeast. Yeast genetic evidence thus supports the belief that both DNA polymerases α and δ are required for chromosomal DNA replication in higher eukaryotes.

S.cerevisiae, however, has another essential DNA polymerase, DNA polymerase II (Morrison *et al.*, 1990). DNA polymerase II can be purified as a complex which consists of four different subunits (Hamatake *et al.*, 1990; Araki *et al.*, 1991b). This polymerase is biochemically similar to mammalian DNA polymerase ϵ (Hamatake *et al.*, 1990; Syväoja *et al.*, 1990; Bambara and Jessee, 1991; Linn, 1991), which has been suggested to function in DNA repair in human cells (Nishida *et al.*, 1989). We have suggested that DNA polymerase II is required for yeast chromosomal DNA replication (Morrison *et al.*, 1990). In a previous study, we isolated a temperature-sensitive mutant of the *DPB2* gene which encodes the second largest subunit of DNA polymerase II, and showed that chromosomal replication in the mutant cells is partially defective at the restrictive temperature, suggesting that DNA polymerase II participates in yeast genome replication (Araki *et al.*, 1991a). However, the formal possibility that the *DPB2* gene product is required for chromosomal replication independently of the catalytic subunit of DNA polymerase II was not eliminated. In order to demonstrate directly its requirement for chromosomal replication, we report here the isolation and characterization of temperature-sensitive DNA polymerase II mutants and we examine expression of the gene in the cell cycle. We show that these mutants have a temperature-sensitive DNA polymerase II activity and exhibit temperature-sensitive chromosomal DNA replication, showing definitively that DNA polymerase II functions in chromosomal DNA replication. The strong conservation in structure and activity of the DNA polymerases of yeast and mammals makes it appear likely that mammalian DNA polymerase ϵ is also required for chromosomal DNA replication.

Results

Isolation of temperature-sensitive pol2 mutants

We isolated thermosensitive *pol2* mutants by the plasmid shuffling method (Budd and Campbell, 1987; Boeke *et al.*, 1987, and Figure 1). From seven mutants isolated, the two with the most easily observed temperature-sensitive phenotype (*pol2-9* and *pol2-18*) were selected for further

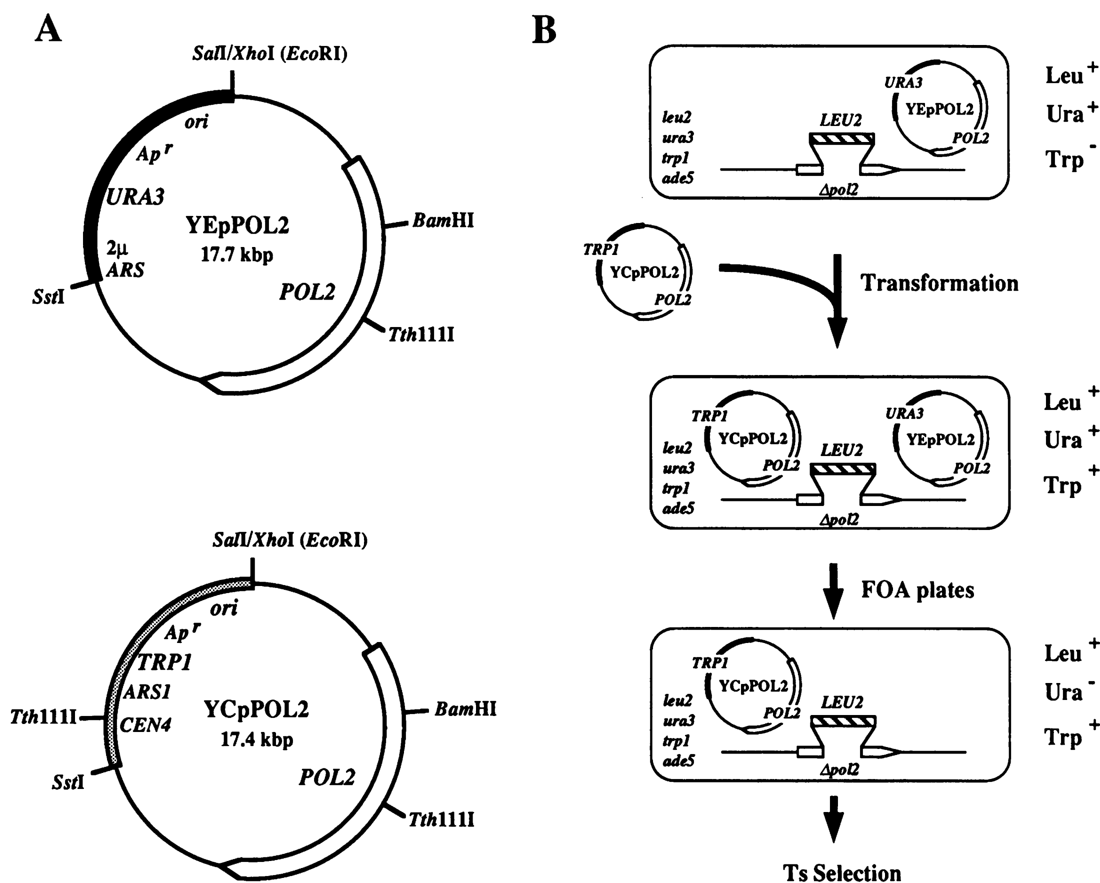

Fig. 1. Isolation of temperature-sensitive *pol2* mutants by the plasmid shuffling method. **A.** structures of the *POL2* plasmids. The open arrow and thin line in the figure are the *POL2* gene and its flanking yeast DNA. The shaded area shows vector DNA. **B.** The plasmid shuffling method. The yeast strain YHA21 carrying *pol2-3::LEU2* on the chromosome and *POL2* on the plasmid YEpPOL2 is shown in the upper panel. YHA21 transformed with the mutagenized YCpPOL2 DNA is shown in the middle panel. Leu^+ Trp^+ Ura^- cells were selected by plating on medium containing FOA (bottom panel) and screened for temperature-sensitivity.

study. To exclude the possibility that a mutation(s) occurring in the vector DNA, rather than in *POL2*, was responsible for thermosensitivity, the 1.8 kb *Bam*HI–*Tth*111I fragment (Figure 1) from the *pol2* mutant plasmids was excised and used to replace the corresponding fragment of YCpPOL2. The resulting plasmids were introduced into the yeast YHA21, and subsequent loss of plasmid YEpPOL2 was selected for by growth on 5-fluoroorotic acid (FOA) containing plates at 25°C. Cells grown on FOA plates at 25°C did not grow at 37°C. Therefore, the mutations resulting in temperature-sensitive cell growth were within the *Bam*HI–*Tth*111I fragment of the *POL2* gene. Neither *pol2-9* nor *pol2-18* mutant cells exhibited any detectable sensitivity to X-rays, UV or methylmethane sulfonate at the permissive or semi-restrictive temperatures (data not shown).

Mutation site of the temperature-sensitive pol2 mutants

The nucleotide sequence of the entire region of the *POL2* open reading frame (ORF) as well as at least 200 bp upstream and downstream of the ORF of *pol2-9* and *pol2-18* plasmid DNAs was determined. Only a single base-pair change in the *POL2* gene was identified in each mutant. As shown in Figure 2, each single-base change leads to an amino acid substitution: for *pol2-9*, Met643Ile in the conserved region II of aphidicolin-sensitive DNA polymerases (Wang *et al.*, 1989); and for *pol2-18*, Pro710Ser between regions II and VI (Wang *et al.*, 1989).

DNA polymerase II activity from temperature-sensitive mutant cells

DNA polymerase II activity was partially purified from YHA21 and the mutant cells were grown at 25°C by chromatography on S-Sepharose and Mono Q columns as described previously (Morrison *et al.*, 1990; Araki *et al.*, 1991a). The activity eluted in the expected two peaks, the first corresponding to the proteolyzed catalytic subunit of DNA polymerase II, and the second to DNA polymerase II holoenzyme (Hamatake *et al.*, 1990). The total activity from the temperature-sensitive mutant cells was much lower than that from wild-type cells. Aliquots of fractions from both DNA polymerase II peaks (indicated by arrows in Figure 3A) were preincubated at 45°C for various time before measurement of the remaining DNA polymerase II activity at 25°C. As shown in Figure 3B, both forms of the polymerase II from the mutant cells were inactivated much faster at 45°C than enzyme from wild-type cells. The half-lives of the activities from *pol2-18* and *pol2-9* mutant cells were <1 min and 4 min, respectively, while the wild-type activity had a half-life of >20 min at 45°C. Conversely, DNA polymerase I and III activities from the mutant cells showed the same thermosensitivities at 45°C as the respective

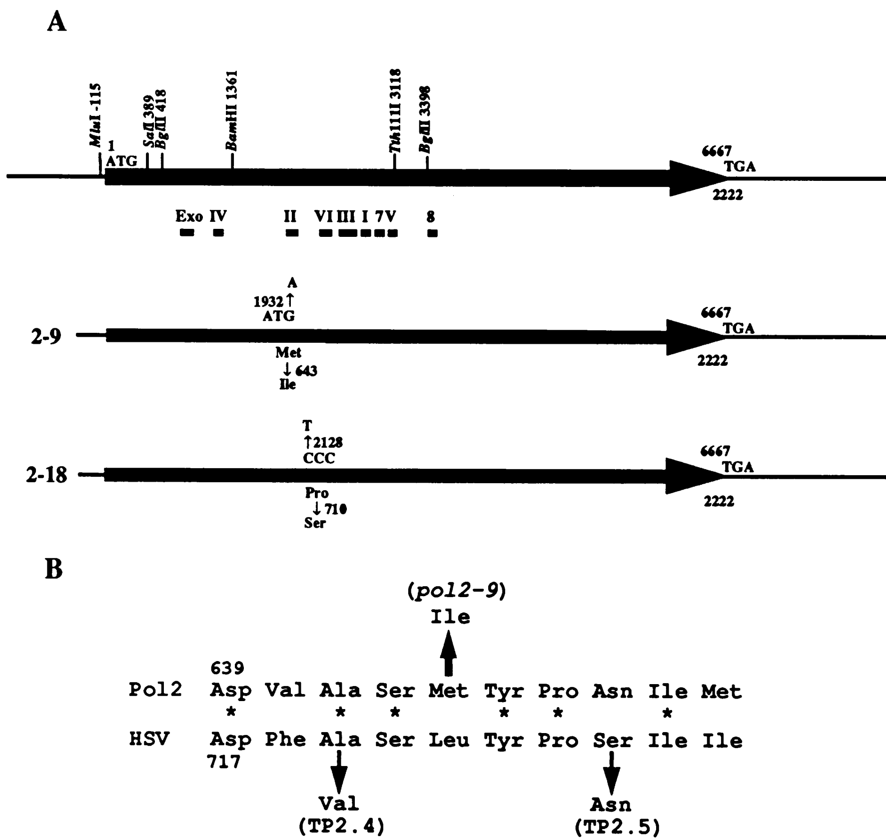

Fig. 2. Sequencing the *pol2-9* and *pol2-18* mutations. **A.** *pol2-9* (YHA301) and *pol2-18* (YHA302) mutant cells were grown in YPDA at 24°C and total DNA was prepared as described (Sherman *et al.*, 1986). Plasmid DNA was recovered from total DNA by transformation of *E.coli* DH5α by electroporation. Synthetic DNA primers were used to determine the nucleotide sequence of the *pol2* region of the recovered plasmid DNAs as described (Morrison *et al.*, 1990). The figure shows the *POL2* gene, indicating several regions conserved among aphidicolin-sensitive DNA polymerases (Wang *et al.*, 1989) and the single-base changes and the resulting amino acid substitutions observed in *pol2-9* and *pol2-18*. **B.** Amino acid alignment between the region of the *pol2-9* mutation and the region of phosphonoacetic acid-resistant mutations of HSV DNA polymerase. An arrow indicates amino acid change in the mutation and asterisks indicate matched amino acids. The numbers are the amino acid numbers of the DNA polymerases.

activities from wild-type cells (data not shown). DNA polymerase II has an intrinsic 3′→5′ exonuclease activity (Hamatake *et al.*, 1990) and this activity was not changed in these mutant cells (data not shown). These results correlate the thermosensitive cell growth with a thermolabile DNA polymerase II activity.

pol2-9 and *pol2-18* mutants exhibit cell cycle arrest with the terminal morphology typical of DNA replication mutants

Yeast cells conditionally defective in DNA replication exhibit a dumbbell shape, with the nucleus in or near the isthmus, on arrest under restrictive conditions (Pringle and Hartwell, 1981). We have shown previously that cells harboring the deleted *pol2* or *dpb2* genes typically display this dumbbell terminal morphology (Morrison *et al.*, 1990; Araki *et al.*, 1991a). As shown in Figure 4, >80% of *pol2-18* cells incubated at the nonpermissive temperature arrested with a dumbbell shape with the nucleus adjacent to the isthmus between mother and daughter cells. *pol2-9* cells also exhibited the same terminal morphology at 37°C as *pol2-18* cells, although they were less temperature-sensitive than *pol2-18* cells (Figure 5). These observations suggest that the *POL2* gene is required for chromosomal DNA replication.

Chromosomal DNA replication in the *pol2* mutants is defective at the restrictive temperature

We investigated macromolecular synthesis in the *pol2* mutants at the restrictive temperature. Cells grown at 25°C in medium containing [^{3}H]uracil were shifted to 37°C, and RNA and DNA synthesis were measured. Concurrently, cell numbers were counted, and protein synthesis was monitored by labeling the cells with ^{14}C-labeled amino acids. In the wild-type cells, cell division continued and the rate of DNA synthesis increased after temperature shift up. Cell division (Figure 5, left) and DNA synthesis (Figure 5, right) of the *pol2-18* mutant cells stopped within 4 h after temperature shift. In *pol2-9*, the cell number gradually increased for >10 h after temperature shift, while DNA synthesis ceased within 5 h. In comparison the *pol1-17* mutant, which is defective in DNA polymerase I (Budd and Campbell, 1987; Budd *et al.*, 1989), ceased both cell division and DNA synthesis within 3 h after temperature shift. Synthesis of both RNA and protein, however, in any of these mutant cells were not significantly affected for up to 10 h after shift to the restrictive temperature (data not shown).

We also carried out flow cytometric analysis to determine the DNA content of *pol2-18* cells. Cells were grown in YPDA medium to early logarithmic phase and treated with α-factor to synchronize the cell population. The cells were

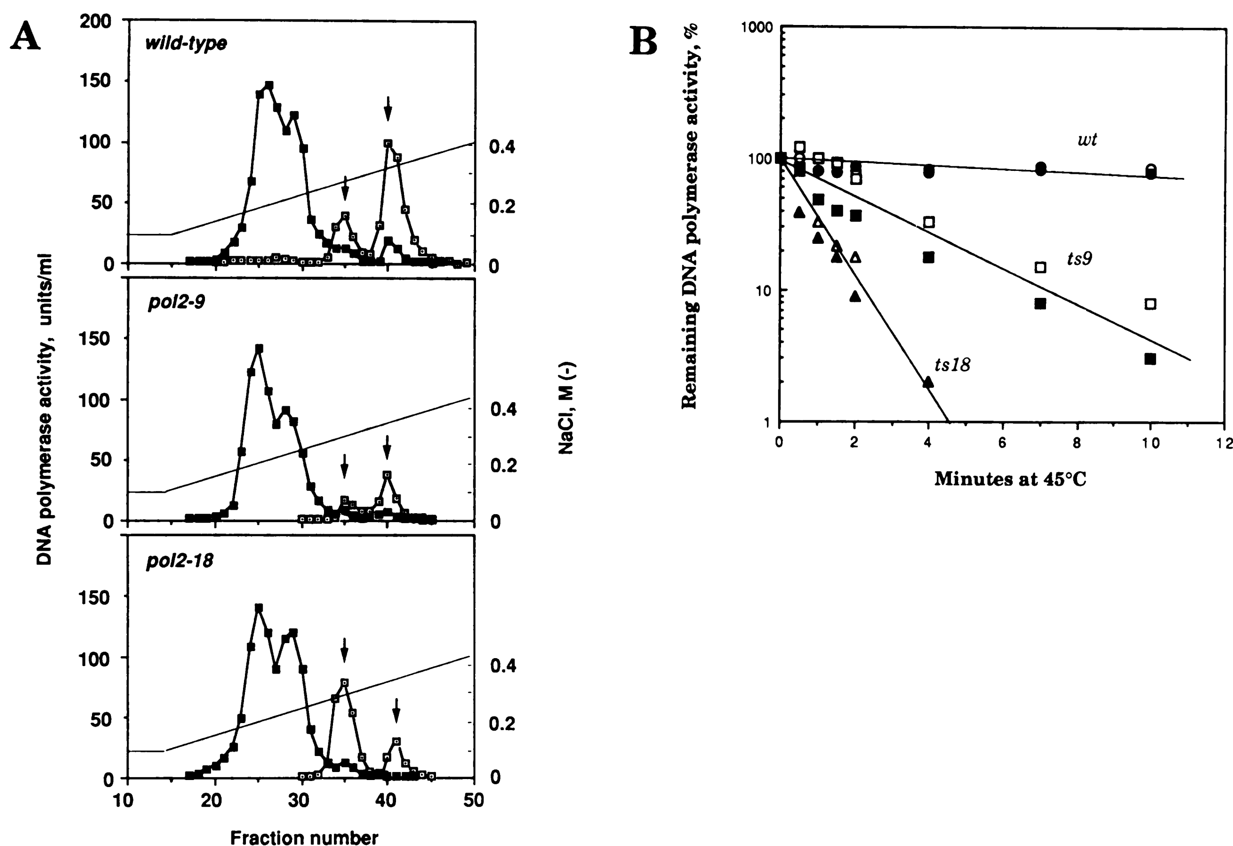

Fig. 3. Heat inactivation of DNA polymerase II from *pol2-9* and *pol2-18* cells. **A.** Mono Q profiles of the DNA polymerase activity are shown. Closed symbols represent DNA polymerase activity on activated calf thymus DNA as template. This represents mainly DNA polymerases I and III activities (Hamatake *et al.*, 1990; Morrison *et al.*, 1990). Open symbols represent the DNA polymerase II activity (Morrison *et al.*, 1990). The fractions indicated by the arrows were used for the heat-inactivation experiments. **B.** Heat inactivation of DNA polymerase II from *pol2-9* and *pol2-18* cells. The Mono Q fractions (each 0.1 ml) indicated in panel A were incubated at 45°C and, at various times, 5-μl aliquot were assayed for DNA polymerase II activity at 25°C for 15 min. Circles, squares and triangles represent the DNA polymerase activity from wild-type, *pol2-9* and *pol2-18* cells, respectively. Closed and open symbols represent the proteolyzed catalytic subunit of DNA polymerase II (yPolII) and the intact holoenzyme form (yPolII*), respectively (Hamatake *et al.*, 1990). 100% represents the activity without preincubation at 45°C.

then washed, resuspended in the same volume of fresh medium and divided into two portions. One portion was incubated at 25°C and the other at 37°C for various times. After harvesting the cells, DNA was stained with propidium iodide and the DNA content of the cells was analyzed by flow cytometry. As shown in Figure 6, the mutant cells did not progress through the cell cycle at 37°C, but stayed at or near G_2/M (2N), while at 25°C the cells underwent normal progression through the cell cycle. As a control, *pol1-17* were processed in the same way as the *pol2-18* cells. The result with *pol1-17* was very similar to that with *pol2-18,* suggesting that the shift of the fluorescence peak in *pol2-18* cells toward G_2/M may have resulted from the contribution of mitochondrial DNA. Thus, we can conclude that the *pol2* mutants have a primary defect in chromosomal DNA replication at the restrictive temperature.

Abundance of the POL2 transcript during the cell cycle

DNA replication genes of *S.cerevisiae* have an ACGCGT or similar nucleotide sequence in their 5′-upstream sequences, and their transcripts accumulate periodically during the cell cycle, peaking at the G_1/S boundary (Johnston *et al.*, 1991). Since *POL2* also has an ACGCGT sequence in its 5′-upstream region (Morrison *et al.*, 1990), we analyzed the abundance of its transcript during the cell cycle. Cells were synchronized by treatment with α-factor and, after washing away α-factor, were incubated in YPDA. At various times before and after α-factor release, RNA was extracted and the RNA hybridizing to the *POL2* DNA was determined by Northern blotting. As shown in Figure 7, the abundance of the 7.5 kb *POL2* RNA fluctuated during the cell cycle and peaked at the G_1/S boundary, in the same manner as *POL1*. The levels of a control transcript that was not regulated in the cell cycle confirmed that these were real fluctuations in amount (data not shown). Similar results were obtained from cells synchronized by two other methods, the feed-starved protocol and the centrifugal elutriation method (data not shown). It appears likely from this result that expression of the *POL2* gene is regulated by the same mechanism as other DNA replication genes (Johnston *et al.*, 1991).

Discussion

In this paper, we have used conditional mutants of *pol2* to demonstrate directly that DNA polymerase II is required for chromosomal DNA replication in *S.cerevisiae*. Two mutants in *pol2* were temperature-sensitive for cell growth, expressed temperature-sensitive DNA polymerase II activity and were temperature-sensitive for chromosomal DNA synthesis. Furthermore, at the restrictive temperature these mutant cells

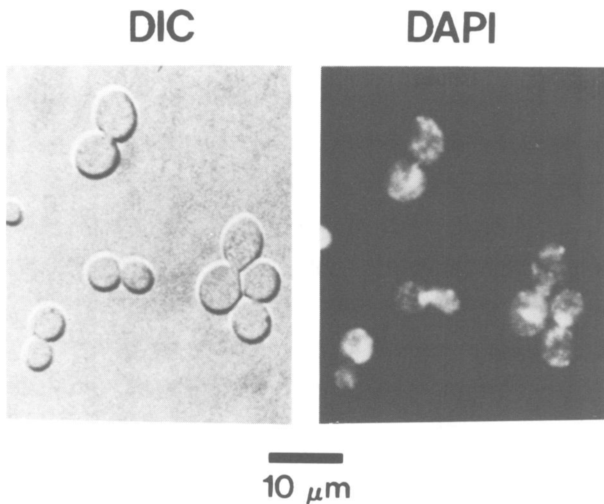

Fig. 4. Terminal morphology of *pol2-18* cells. Cells grown at 25°C in YPDA to a density of 2×10^6 cells/ml were incubated at 37°C for 6 h. Cells were fixed, treated with 4′,6-diamino-2-phenylindole (DAPI) to stain DNA, and observed by differential interference contrast (DIC) or epifluorescence (DAPI) microscopy.

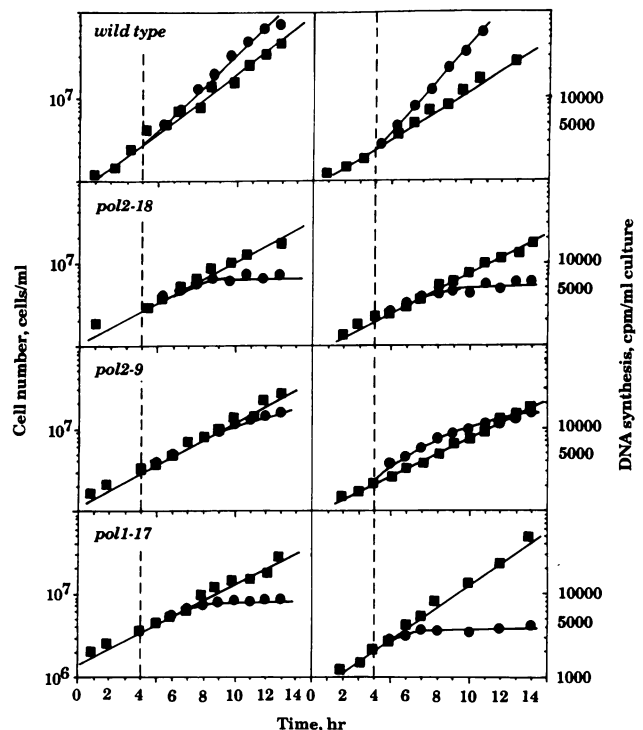

Fig. 5. Cell growth and *in vivo* DNA synthesis in *pol2-9* and *pol2-18* cells at the restrictive temperature. Cells were grown at 25°C in YPDA containing [^{3}H]uracil (10 μCi/ml) for 4 h and the cell culture was divided into two equal portions. One portion was continued in its incubation at 25°C and the other was incubated at 37°C. At the indicated times, 1 ml aliquots were withdrawn and the radioactivity incorporated into RNA and DNA was measured as described by Eberly *et al.* (1989). Cell numbers were counted using a hemocytometer. The left and right panels show cell number and DNA synthesis, respectively. Closed circles and squares refer to 37°C and 25°C, respectively. No significant differences were observed in RNA synthesis measured at 25°C or 37°C in wild-type or mutant cells (data not shown).

exhibited the dumbbell terminal morphology typical of mutants defective in DNA replication, and the *POL2* transcript showed the same periodic accumulation at the G_1/S boundary as >10 other DNA replication genes (Lowndes *et al.*, 1991; Johnston *et al.*, 1991). It has thus been established genetically that three DNA polymerases I, II and III, participate in chromosomal DNA replication in yeast. Each DNA polymerase plays a unique role that cannot be substituted for by either of the other polymerases. It is unlikely that this situation is specific to *S.cerevisiae*, because many yeast DNA replication proteins have mammalian homologs identified as DNA replication factors in the reconstituted SV40 DNA replication system. These homologous proteins are similar not only functionally, but also in amino acid sequence. In addition to mammalian DNA polymerases α and δ, the homologs of yeast DNA polymerases I and III, respectively, examples are yRF-C (Burgers, 1991), yPCNA (Bauer and Burgers, 1990) and ySSB, which consists of three distinct polypeptides (yRF-A) (Brill and Stillman, 1989). It is, therefore, not inconceivable that DNA polymerase ϵ, the probable homolog of yeast DNA polymerase II, participates in mammalian chromosomal DNA replication. In human cells, it has been suggested that DNA polymerase ϵ participates in DNA repair (Nishida *et al.*, 1988). None of the *pol2*, *dpb2* or *dpb3* mutants thus far isolated exhibit any sensitivity to either UV, X-rays or methylmethane sulfonate (this study and our unpublished results). However, these data do not exclude the possibility that yeast DNA polymerase II participates in DNA repair.

We have proposed a model to explain how the three DNA polymerases might synthesize DNA at a replication fork (Morrison *et al.*, 1990). In the model, we proposed that the DNA polymerase I−primase complex functions as a primosome similar to the primosome in *Escherichia coli* (Kornberg, 1988), making RNA−DNA primers on both lagging and leading strands for initiation of Okazaki fragment and leading strand synthesis, respectively. Subsequently, the second and third DNA polymerases continue synthesis of either lagging or leading strand of chromosome. Currently, we favor the model in which DNA polymerase II synthesizes the leading strand of the chromosome, while DNA polymerase III completes the lagging strand to form matured Okazaki fragments, but a reversal of these roles would be equally feasible. An efficient SV40 DNA replication system has been reconstituted using either one DNA polymerase (α) or two polymerases (α and δ) along with other replication factors (Ishimi *et al.*, 1988; Tsurimoto *et al.*, 1989; Weinberg *et al.*, 1990). Based on this system, the currently proposed model holds that lagging and leading strands of chromosomal DNA are synthesized by DNA polymerases α and δ, respectively (Tsurimoto *et al.*, 1990; Weinberg *et al.*, 1990). It has been shown recently, however, that at least two DNA polymerases are required for formation of Okazaki fragments on the lagging strand in SV40 DNA replication both in isolated nuclei (Nethanel and Kaufman, 1990) and in crude extracts from HeLa cells (Bullock *et al.*, 1991). Furthermore, leading strand synthesis does not show high specificity for catalysis by DNA polymerase δ, which can be substituted for by prokaryotic DNA polymerases having a high processivity (Matsumoto *et al.*, 1990; Tsurimoto *et al.*, 1990). Although the SV40 replication system has provided many important advances in our understanding of the mechanism of chromosomal replication in eukaryotes, it is conceivable that, by analogy to the bacteriophage M13, ϕX174 and G4 systems (Kornberg,

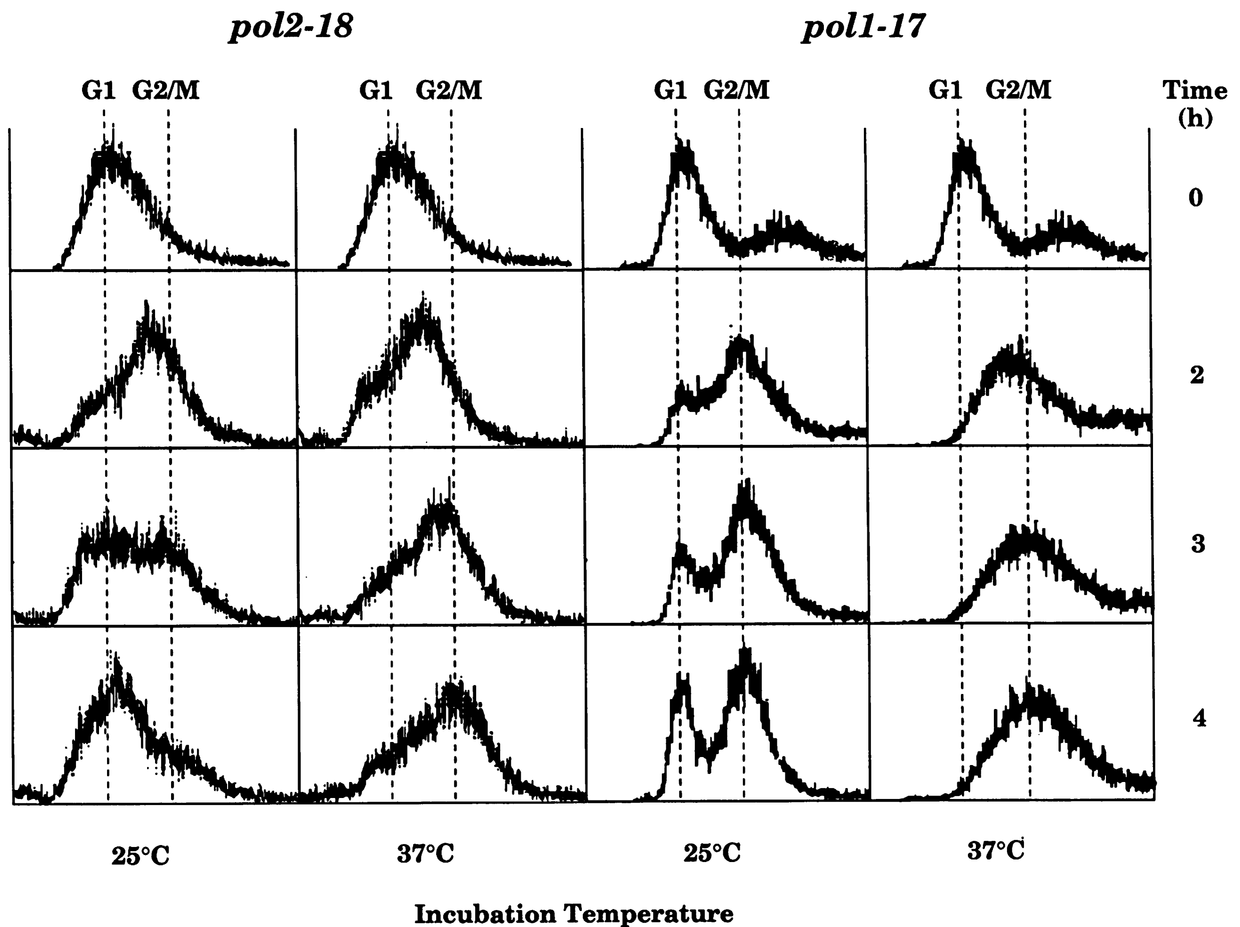

Fig. 6. Flow cytometric analysis of *pol2-18* and *pol1-17* cells synchronized by treatment with α-factor. Synchronized mutant cells were incubated at either 25°C or 37°C for the times indicated. The cells were fixed, their DNA stained with propidium iodide and their DNA content analyzed using a FACS analyzer. The dotted lines represent the DNA content of G_1 (N) and G_2/M (2N) phase cells.

1982), SV40 utilizes a different combination of DNA polymerases and replication factors from that of chromosomal DNA replication. Alternatively, it is possible that some factor(s) is still missing from the reconstituted SV40 DNA replication system, and that one of the missing components is DNA polymerase ε. Consistent with this is the possibility that DNA polymerase II (or ε) has a role in maturation, perhaps filling a gap left by removal of RNA primers. Another formal possibility, that the requirement for DNA polymerase II during yeast chromosomal replication might be at a specific region such as telomeres, seems less likely: *pol2* mutants expressing a 3'→5' exonuclease-deficient DNA polymerase II exhibit a spontaneous mutator phenotype at a variety of genetic markers scattered widely throughout the genome (Morrison *et al.*, 1991; A.Morrison, unpublished results), suggesting that DNA polymerase II normally replicates all these chromosomal sites.

In *pol2-9* and *pol2-18* cells, chromosomal DNA synthesis ceased gradually at the restrictive temperature (Figure 5) and this slow shut-off may indicate a possibility that one strand synthesis catalyzed by DNA polymerase I and/or DNA polymerase III continues for a while even after the other strand synthesis is stopped at the restrictive temperature. Alternatively, when DNA polymerase II formed a replication complex, it might be more heat resistant than the free enzyme and slow stop of DNA synthesis may be a reflection of

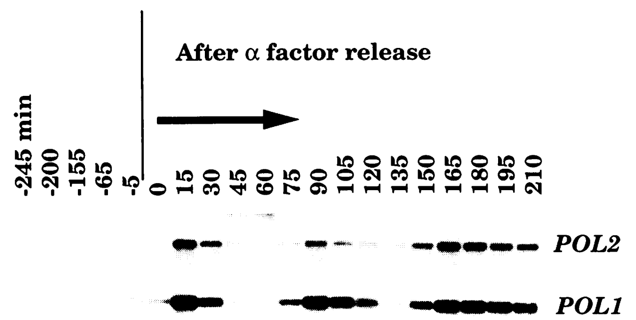

Fig. 7. Abundance of the *POL2* transcript during the cell cycle. L115-1B cells grown in a synthetic complete medium were synchronized by treatment with α-factor as described (White *et al.*, 1986). Total RNA was extracted and separated by agarose gel electrophoresis. RNA was transferred onto a GeneScreen filter which was probed with the ^{32}P-labeled, 2.1 kb *Hin*dIII fragment of the *POL2* gene (Morrison *et al.*, 1990). Following autoradiography of the filter, the radioactivity was removed and the filter was reprobed with a ^{32}P-labeled *POL1* as before (Johnston *et al.*, 1987).

dissociation of DNA polymerase II from the replication complex.

The mutation site of *pol2-9* was Met643Ile in region II of DNA polymerase II, indicating that this region has a

crucial function during the polymerization reaction. Region II has been implicated as a site involved in dNTP binding (Larder *et al.*, 1987; Gibbs *et al.*, 1988; Hall *et al.*, 1989). In region II of herpes simplex virus DNA polymerase, two mutations (TP2.4 and TP2.5) conferring resistance to phosphonoacetic acid have been mapped (Figure 2B) (Larder *et al.*, 1987). The Tyr254Phe change of ϕ29 DNA polymerase (corresponding to Tyr722 of HSV DNA polymerase and Tyr644 of yeast DNA polymerase II) lowers its processivity as well as decreasing protein priming activity (Blanco *et al.*, 1991). Furthermore, Jung *et al.* mutated the Asp220 of Region II of PRD1 DNA polymerase to Val (correspond to DNA polymerase II Asp639), resulting in no DNA polymerase activity (Jung *et al.*, 1990). The Pro710Ser substitution in *pol2-18* potentially alters tertiary structure more drastically than the Met643Ile change of *pol2-9*, since a proline residue is often located at a kink in a polypeptide chain. This might explain why DNA polymerase II activity from the *pol2-18* mutant cells was much more heat-labile than that from the *pol2-9* mutant cells.

Materials and methods

Yeast and E.coli strains

E.coli strains used were DH5α (Hanahan, 1983), for plasmid transformation and preparation of plasmid DNA, and MC1066 (Casadaban *et al.*, 1983). *S.cerevisiae* strains were YHA1 (*MATa/MATα POL2/pol2-3::LEU2 leu2-3,112/leu2-3,112 ura3-52/ura3-52 trp1-289/trp1-289 ade5-1/ADE5*), YHA21(*MATa ade5-1 leu2-3,112 ura3-52 trp1-289 pol2-3::LEU2* [YEp*POL2*]), YHA300 (the same as YHA21, but [YCp*POL2*]), YHA301 (the same as YHA21, but [YCp*pol2-18*]), YHA302 (the same as YHA21, but [YCp*pol2-9*]), a *pol1-17* derivative of strain 488 (*MATα trp1 leu2 ura3-52 his1-7 can1 pol1-17*) (Budd *et al.*, 1989) and L115-1B (Chapman and Johnston, 1989).

Plasmids

The plasmids used for the isolation of temperature-sensitive *pol2* mutants are shown in Figure 1. YCplac22 and YEplac195 were described previously (Gietz and Sugino, 1988).

Isolation of temperature-sensitive pol2 mutants

The *POL2* gene on plasmid P2A7 (Morrison *et al.*, 1990) was cloned as an *Xho*I−*Sst*I fragment of 12.1 kb (the *Xho*I site is in the polylinker) into the *Sal*I and *Sst*I sites of both YCplac22 and YEplac195, creating plasmids YCpPOL2 and YEpPOL2, respectively (Figure 1). The diploid strain YHA1, one of whose chromosomal *POL2* genes is disrupted by insertion of the *LEU2* gene (Morrison *et al.*, 1990), was transformed with YEpPOL2 and the resulting Ura^+ transformants were sporulated and dissected. From among the dissected spores, one Ura^+Leu^+ haploid segregant, YHA21, was selected. YCpPOL2 was treated with 1 M hydroxylamine at 75°C for 1.5 h to mutate the *POL2* gene. More than 100 000 ampicillin-resistant *E.coli* MC1066 transformants were obtained from the mutagenized plasmid. Plasmid DNA was extracted from them and used for YHA21 transformation. About 1200 transformants grown at 37°C on Ura^- Trp^- plates (SD-UT plates) were isolated. Each colony was plated onto two sets of SD plates containing FOA (Boeke *et al.*, 1987); one set of plates was incubated at 25°C, and the other at 37°C, for 3 days. Seven colonies grew at 25°C but not at 37°C. To exclude the possibility that a mutation(s) occurring in the vector DNA, rather than in *POL2*, conferred thermosensitive cell growth, a 1.8 kb *Bam*HI−*Tth*111I fragment from the plasmids carrying the *pol2-9* or *pol2-18* mutations was used to replace the corresponding fragment of YCpPOL2. The resulting plasmid was used for YHA21 transformation, and loss of YEpPOL2 was selected for on FOA plates at 25°C. Strains YHA301 and YHA302 grew on FOA plates at 25°C but not at 37°C.

Flow cytometric analysis

YHA21 cells (10 ml) harboring YCp*pol2-18* and *pol1-17* cells (10 ml) were grown in YPDA medium (pH 4.0) at 25°C to 2 x 10^6 cell/ml and synchronized by treatment with 3 μg/ml mating pheromone α-factor (Peptide Institute, Inc., Osaka, Japan) for 5 h at 25°C. After removing α-factor by filtration, the cells were resuspended in 10 ml of YPDA medium (25°C) and divided into two equal portions. One portion was incubated at 25°C and the other at 37°C. At various times, 0.5 ml of the culture was withdrawn and the cells were fixed in 70% ethanol and stored overnight at 4°C. The fixed cells were washed with 0.2 M Tris−HCl, pH 7.4 and suspended in 0.5 ml of Tris−HCl, pH 7.4 containing 1 mg/ml RNase A. After incubation at 37°C for 3 h, the cells were washed with 0.2 M Tris−HCl, pH 7.4 and resuspended in 0.25 ml of 0.2 M Tris−HCl, pH 7.4 containing 50 μg/ml propidium iodide, followed by incubation on ice for 15 min. 0.1 ml of 0.2 M Tris−HCl, pH 7.4 was added to the cell suspension and the stained cells were then analyzed for nuclear DNA content by a FACScan analyzer (Becton−Dickinson, CA).

Northern blots

Total RNA was prepared from *S.cerevisiae* cells synchronized either by mating pheromone α-factor, a feed-starve procedure or centrifugal elutriation as described (White *et al.*, 1986). The RNA (5 μg) was denatured with glyoxal, separated by agarose gel electrophoresis and transferred to a GeneScreen membrane (DuPont). The filters were hybridized with a ^{32}P-labeled 2.1 kb *Hin*dIII fragment of the *POL2* gene (Morrison *et al.*, 1990). After autoradiography of the filter, the ^{32}P label was stripped off and the filter was reprobed with a ^{32}P-labeled 3.2 kb *Hin*dIII−*Sal*I fragment of the *POL1* gene (Johnston *et al.*, 1987).

Partial purification of DNA polymerase II

YHA300 (wild-type), YHA301 (*pol2-9*) and YHA302 (*pol2-18*) cells were grown in 60 l YPDA medium in a fermenter at 24°C to a density of 5×10^7 cells/ml, harvested and resuspended into 10% sucrose, 50 mM Tris−HCl, pH 7.5, 10 mM EDTA, and cell extracts were prepared as described (Hamatake *et al.*, 1990). The lysate was applied onto a 400 ml S-Sepharose column (Pharmacia) equilibrated with buffer A (Hamatake *et al.*, 1990) containing 0.1 M NaCl, the column was washed with same buffer and the proteins retained on the column were eluted with buffer A plus 0.5 M NaCl. Ammonium sulfate (0.45 g/ml) was added to the 0.5 M NaCl eluate, the precipitate was collected by centrifugation, resuspended in buffer A and dialyzed against buffer A plus 0.1 M NaCl for 4 h. The dialyzed sample was applied onto a Mono Q FPLC column (HR16/10) equilibrated with buffer A plus 0.1 M NaCl, and DNA polymerases were separated by a 0.1−0.5 M NaCl linear gradient in buffer A as described (Hamatake *et al.*, 1990). For assay of the activity of DNA polymerases I and III, activated calf thymus DNA was used, while $poly(dA)_n \cdot oligo(dT)_{10}$ (40:1) and 0.12 M KCl were used for assay of DNA polymerase II activity (Morrison *et al.*, 1990). We did not detect any contamination of either DNA polymerase I or III in the DNA polymerase II fractions.

Other materials and methods

5-FOA was obtained from PCR Inc. (Gainsville, FL). Other methods used in this study were previously described (Morrison *et al.*, 1990; Araki *et al.*, 1991a,b; Morrison *et al.*, 1991).

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