MOLECULAR AND CELLULAR BIOLOGY, Jan. 1993, p. 496–505
0270-7306/93/010496-10$02.00/0

Vol. 13, No. 1

DNA Polymerases δ and ε Are Required for Chromosomal Replication in *Saccharomyces cerevisiae*

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Received 29 July 1992/Returned for modification 15 September 1992/Accepted 19 October 1992

Three DNA polymerases, α, δ, and ε, are required for viability in *Saccharomyces cerevisiae*. We have investigated whether DNA polymerases ε and δ are required for DNA replication. Two temperature-sensitive mutations in the *POL2* gene, encoding DNA polymerase ε, have been identified by using the plasmid shuffle technique. Alkaline sucrose gradient analysis of DNA synthesis products in the mutant strains shows that no chromosomal-size DNA is formed after shift of an asynchronous culture to the nonpermissive temperature. The only DNA synthesis observed is a reduced quantity of short DNA fragments. The DNA profiles of replication intermediates from these mutants are similar to those observed with DNA synthesized in mutants deficient in DNA polymerase α under the same conditions. The finding that DNA replication stops upon shift to the nonpermissive temperature in both DNA polymerase α- and DNA polymerase ε- deficient strains shows that both DNA polymerases are involved in elongation. By contrast, previous studies on *pol3* mutants, deficient in DNA polymerase δ, suggested that there was considerable residual DNA synthesis at the nonpermissive temperature. We have reinvestigated the nature of DNA synthesis in *pol3* mutants. We find that *pol3* strains are defective in the synthesis of chromosomal-size DNA at the restrictive temperature after release from a hydroxyurea block. These results demonstrate that yeast DNA polymerase δ is also required at the replication fork.

Three nuclear DNA polymerases, α, δ, and ε, have been purified from the yeast *Saccharomyces cerevisiae* (12), and all three are essential for viability (5, 12, 20, 26, 42). However, are all three DNA polymerases required for DNA replication, or is one or more dedicated to some other essential process such as the DNA repair carried out by *RAD3* (19, 30)? The answer is unambiguous only for DNA polymerase α. Chromosomal DNA replication is completely blocked at the nonpermissive temperature in yeast strains carrying a thermolabile DNA polymerase α, as shown by alkaline sucrose gradient analysis (9). Since DNA polymerase α can be isolated as a complex with DNA primase (12, 33, 41), it presumably initiates Okazaki fragment synthesis. Mammalian DNA polymerase α is required for Okazaki fragment synthesis in the simian virus 40 (SV40) in vitro DNA replication system (29). Thus, the essential role of DNA polymerase α in DNA replication is well established.

While it seems abundantly clear that at least one additional polymerase is essential for eukaryotic replication, the problem comes in deciding which one or how many. DNA polymerase δ is encoded by the yeast *CDC2* (*POL3*) gene (5, 42). In contrast to yeast *pol1* mutants, temperature-sensitive *pol3* mutants replicate 70% of the genome at the restrictive temperature, and chromosomal-length DNA is synthesized (14). This level of residual replication is more than would be expected if DNA polymerase δ carried out extensive synthesis, such as that on the leading strand, during DNA replication. On the other hand, in Brij 58-permeabilized cells, an in vitro replication system capable of elongation, *pol3* mutants are totally defective in replication at 36°C but not at 23°C (21), suggesting that the in vivo results may be due to leakiness of the allele used in the in vivo studies. Indeed, biochemical experiments with the SV40 in vitro replication system argue, albeit indirectly, that DNA polymerase δ is involved in leading-strand DNA replication (23, 24, 34, 35). Nevertheless, there is no experimental explanation as yet for the extensive synthesis in *pol3* mutants.

Even less is known about DNA polymerase ε. This third polymerase received much less attention until disruption of the *POL2* gene in yeast cells showed that DNA polymerase ε was also required for viability (26). Temperature-sensitive DNA polymerase ε mutants have recently been isolated (2), and metabolic labeling of *pol2-18* strains at the restrictive temperature shows a reduction in DNA synthesis. However, in the *pol2-18* strains, chromosomal DNA synthesis stopped gradually at the restrictive temperature, which could be due to a secondary effect rather than a direct disruption of the replication fork. Araki et al. (2) suggested that DNA polymerase α or δ may continue to replicate DNA in the absence of DNA polymerase ε, which is certainly plausible. Alternatively, the mutants might retain residual DNA polymerase ε activity. Replication intermediates have not yet been characterized in these mutants. Strains temperature sensitive for the *DPB2* gene product, a possible subunit of DNA polymerase ε, synthesize DNA at 36°C at half the rate seen at 23°C (1). Another circumstantial piece of evidence for a replicative role for DNA polymerase ε is that mutations in the exonuclease domain cause a mutator phenotype, as is also true of analogous *pol3* mutants (27, 40). Yet the mutator phenotype alone does not imply an obligatory role in replication, since strains mutant in *RAD51* (*mut5-1*), which encodes a homolog of prokaryotic RecA protein and not a replication protein, also show a mutator phenotype (28, 38). The SV40 in vitro replication system has not helped clarify the matter in this case. Although yeast DNA polymerase ε can substitute for human DNA polymerase δ in a reconstituted SV40 in vitro replication system, no unique role for human DNA polymerase ε has been demonstrated (24).

To understand the role, if any, of DNA polymerases δ and ε at the replication fork, we have carried out further genetic

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and physiological analysis of yeast *pol2* and *pol3* mutants. Using the plasmid shuffle strategy (4, 7), we have isolated two additional strains mutant in the DNA polymerase ε gene. The optimum way to strongly implicate a polymerase in replication is by demonstrating a physical aberration in replication intermediates synthesized in the absence of the DNA polymerase. Therefore, the DNA synthesized at the nonpermissive temperature in these two mutants has been characterized by using alkaline sucrose gradients. In *pol2* mutants, the only DNA synthesized at the restrictive temperature consists of small fragments of less than 10 kb. We find also that *cdc2-1*, a mutant allele of *POL3*, displays a substantial reduction in the size and extent of DNA synthesis at high temperatures. The defect is more pronounced when *cdc2-1* cells are arrested with hydroxyurea (HU) and DNA synthesis is analyzed during a subsequent release at 38°C. These results resolve some of the ambiguity in previous studies and strengthen the notion that there are actually three DNA polymerases at the eukaryotic replication fork.

MATERIALS AND METHODS

Strains. The following strains were used: GC378/GC379 [**a**/α *ade5/ade5 LEU2 (pol2-3)/leu2-3,112 trp1-289/trp1-289 ura3-52/ura3-52 can/can1*] (obtained from Akio Sugino [26]), GC378.5-1A [*ade5 LEU2 (pol2-3) trp1-289 ura3-52 can1* (pSEY18pII)], TC102-2D (α *leu2 ura3-52 can1*ρ^0) (10), TC102-2-11 (α *pol2-11 leu12 ura3-52 can1*ρ^0), TC102-2-12 (α *pol2-12 leu2 ura3-52 can1*ρ^0), SS111 (**a** *trp1-289 ura3-1,2 ade2-101 gal2 can1*), SS111-2-11 (**a** *pol2-11 trp1-289 ura3-1,2 ade2-101 gal2 can1*), SS111-2-12 (**a** *pol2-12 trp1-289 ura3-1,2 ade2-101 gal2 can1*), A354A (**a** *ade1 ade2 ura1 his7 lys2 tyr1 gal1*), and ts370 (**a** *cdc2-1 ade1 ade2 his7 lys2 tyr1 ura1 gal1*).

The mutant alleles, *pol2-11* and *pol2-12*, were introduced into strain TC102-2D by using the gene replacement technique of Scherer and Davis (37). The plasmid used in the gene replacement had a 13-kb *pol2*(Ts) fragment cloned into the *Sac*I site of pRS306 (39). The alleles *pol2-11* and *pol2-12* segregate 2:2 in crosses with *POL2* strains. Strain TC201-2D was chosen because when diploid it sporulates at a significantly higher temperature (37°C) than do most yeast strains and thus appeared to be a good candidate for high-temperature studies.

Plasmids. The following oligonucleotides were used to clone the DNA polymerase ε gene:

631-654 CCACTGCAAGATATTCAGCTGGCA
3170-3194 TGGCGAGAGCTTTAGTAGAAAGGG
4072-4111 CCAGCAGCATTACAAGGGGTTTCCAATCCTGTTCCAAGGG
7276-7295 CGCGAGGTTCAATGGCCTCT

The oligonucleotides are numbered from the sequences of Morrison et al. (26).

To isolate the entire DNA polymerase ε gene, a yeast genomic DNA was digested to completion with *Sac*I and ligated to the *Sac*I site of pSEY18S. The library was screened with the *Hin*dIII fragment (bp 1080 to 2110) obtained from a genomic clone that contained 5.3 kb of the DNA polymerase ε coding sequence. Southern blots of the resulting clone, pSEY18pII, were rescreened with oligonucleotides 631–654, 3170–3194, 4072–4111, and 7276–7295.

Plasmid pSEY18pII complemented the DNA polymerase ε (*pol2-3*) gene disruptant GC378/GC379 (26). A *LEU2* (*pol2-3*) segregant, GC378.5-1A, was used in the mutagenesis. The 13-kb *Sac*I DNA polymerase ε-containing fragment was cloned into the *Sac*I site of YCp19S to yield YCp19pII.

Mutagenesis. Temperature-sensitive mutants were isolated by plasmid shuffling. The plasmid shuffle was carried out as described previously (4, 7). YCp19pII was mutagenized with 1.0-, 1.5-, 2-h treatments with 1 M hydroxylamine. Transformation efficiencies relative to that of untreated DNA were 7, 1, and 0.14%, respectively. The frequency of *trp1* auxotrophy was 0.6% at the 1-h time point. Strain GC378.5-1A was transformed with DNA from the three mutagenized populations; 20,000 transformants were picked and screened for growth at 22 and 37°C on 5-fluoro-orotic acid (5-FOA) plates. Two transformants, *pol2-11* and *pol2-12*, grew at 22°C but not at 37°C on 5-FOA plates. They grew at both temperatures with plasmid pSEY18pII. When both plasmids were isolated from GC378.5-1A and used to repeat the plasmid shuffle protocol, the resulting transformants exhibited temperature-sensitive growth on 5-FOA medium but grew at both 22 and 37°C on uracil-deficient medium. The mutated 13-kb *Sac*I DNA polymerase ε fragments were cloned into the *Sac*I site of pRS306 (39) and integrated into strains SS111 and TC102-2D by the method of Scherer and Davis (37) to create isogenic temperature-sensitive *pol2* strains. Plasmid pSEY18pII complemented the temperature-sensitive phenotype of strains TC102-2-11, TC102-2-12, SS11-2-11, and SS111-2-12.

Sucrose gradient sedimentation. DNA synthesis was analyzed as previously described (9), using a modification of the method of Resnick et al. (36). Cells were grown overnight in 1% yeast extract–2% peptone–2% glucose, harvested in early log phase, resuspended at 10^7 cells per ml in YPD medium, and allowed to grow for an additional 30 to 50 min. Then 80 μCi of [^{3}H]uracil was added to the culture, and cells were incubated at 22, 37, 38, 39, or 40°C for 3 h. *cdc2-1* experiments were carried out at both 37 and 38°C. Cells were harvested, washed with 0.1 M Tris HCl (pH 8.5)–0.01 M EDTA, incubated with 2% β-mercaptoethanol–0.1 M Tris HCl–0.01 M EDTA (pH 8.5) for 10 min, washed with 0.01 M KPO_4–0.01 M EDTA (pH 7.0), and resuspended in 150 μl of 0.01 M KPO_4–0.01 M EDTA. The cells were transferred to 5-ml polyallomer tubes, and 20 μl of a 10-mg/ml solution of zymolyase and 20 μl of 10% Nonidet P-40 were added. After 10 min at 37°C, a 15 to 30% sucrose gradient containing 0.3 M NaOH, 0.7 M NaCl, and 0.03 M EDTA was pumped into the bottom of the tube. Gradients were spun at 12,000 rpm for 16 h; 0.2-ml fractions were collected, neutralized with 1 M Tris (pH 7.5), and precipitated with 1 M HCl–0.1 M sodium pyrophosphate. To determine RNA, 5% of the sample was saved, and 50% of that was directly precipitated with 1 M HCl–0.1 M sodium pyrophosphate. Experiments measuring RNA incorporation involved harvesting cells in early log phase, resuspending them at 10^7 cells per ml, adding 25 μCi of [^{3}H]uracil per ml, and acid precipitating 100-μl fractions.

The molecular weight of DNA is computed according to the equation (16)

$$\frac{M_1}{M_{T4}} = \left(\frac{d_1}{d_{T4}}\right)^{2.63}$$

where M is molecular weight, d is distance sedimented, and M_{T4} = 160 kb.

DNA polymerase ε purification. Cells (20 g) were lysed by the protocol of Sitney et al. (42), with the following modification. After zymolyase spheroplasting and lysis with 0.6 M $(NH_4)_2SO_4$, the extracts were diluted to 0.2 M $(NH_4)_2SO_4$ with 0.04 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.1)–0.01 M $MgCl_2$–0.1% Triton X-100, and then 40 μl of 10% polyethyleneimine (pH 7.5) per

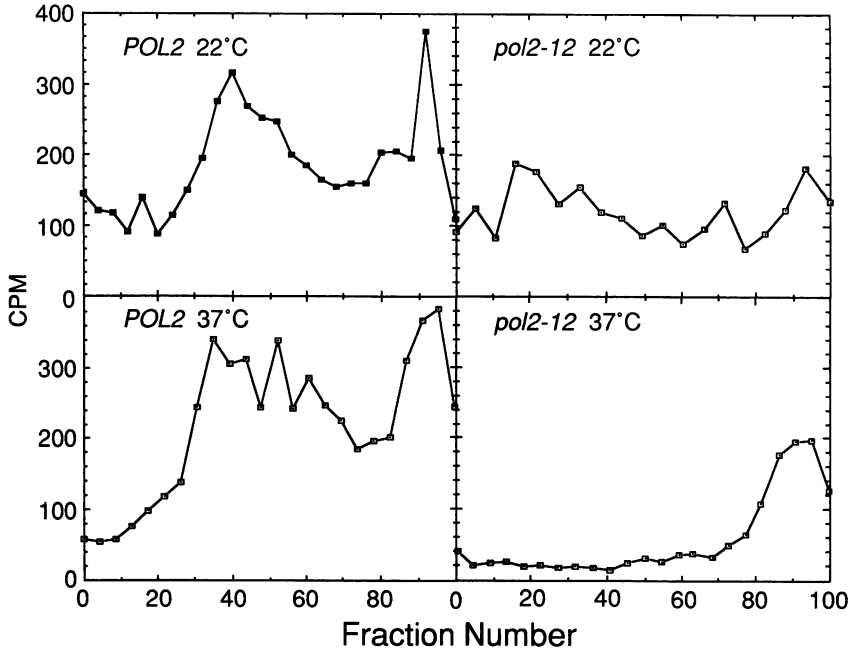

FIG. 1. Alkaline sucrose gradient analysis of DNA synthesis in strain TC102-2D (*POL2*$^+$) and in strain TC102-2-12 (*pol2-12*). Cells were labeled with 25 μCi of [^{3}H]uracil for 3 h at 22 and 37°C and analyzed as described in Materials and Methods. The horizontal axis represents normalized fraction number, with 0 as the bottom and 100 as the top of the gradient. T4 phage sediments at 77. The vertical axis represents acid-precipitable ^{3}H counts per minute.

ml of extract was added. After 15 min at 4°C, extracts were spun at 25,000 rpm in a Beckman Ti45 rotor for 20 min; 0.31 g of $(NH_4)_2SO_4$ was added per ml of extract. Wild-type *POL2* and mutant *pol2* strains were processed in parallel. After dialysis against 25 mM KPO_4–10% glycerol–1 mM EDTA–0.2 mM EDTA–5 mM β-mercaptoethanol, extracts were fractionated on a phosphocellulose column as previously described (42). Active fractions were pooled, dialyzed into 0.025 M KPO_4–10% glycerol–1 mM EDTA–0.2 mM EDTA–5 mM β-mercaptoethanol and loaded onto a 1-ml Mono Q fast protein liquid chromatography (FPLC) column equilibrated with the dialysis buffer. Protein was eluted with a 0.0 to 0.5 M KCl gradient. DNA polymerase and exonuclease assays were carried out as previously described (8).

Isolation of ρ^0 mutants. To isolate ρ^0 mutants, cells were grown to mid-log phase, resuspended in SD medium at 2 × 10^5 cells per ml, grown for 20 h in the presence of 25 μg of ethidium bromide per ml, and then plated on YEPD plates. This treatment induced 100% petite cells when assayed by replica plating on 1% yeast extract–2% peptone–2% ethanol–3% glycerol plates. To check for the ρ^0 phenotype, i.e., absence of mitochondrial DNA, DNA from ethidium bromide-treated strains was run on 4′,6-diamidino-2-phenylindole–cesium chloride gradients, using a ρ$^+$ strain as a control (49).

RESULTS

Identification of temperature-sensitive *pol2* mutants. As described in Materials and Methods, new temperature-sensitive *pol2* alleles were identified by a plasmid shuffle. Of 20,000 colonies screened, 2 colonies exhibited no growth at 37°C and were shown to harbor plasmids carrying the temperature-sensitive mutations. Strains carrying chromosomal copies of these alleles, designated *pol2-11* and *pol2-12*, were constructed, and their phenotypes were analyzed. After a 3-h incubation at 37°C, both strains arrested growth and exhibited the dumbbell morphology characteristic of DNA replication mutants. The growth arrest was reversible for up to 6 h at the nonpermissive temperature, indicating that the cells were viable during arrest. Both mutants also showed somewhat defective growth at 22°C. The *pol2-12* cells were significantly enlarged at 22°C, and the *pol2-11* strain was larger than a *POL2* strain although smaller than the *pol2-12* strain.

Analysis of DNA synthesis in *pol2* mutants. We next analyzed the DNA synthesized at the restrictive temperature. Since the *pol2-11* and *pol2-12* alleles have been integrated into the genome of TC201-2D, all three strains are identical with the exception of the *POL2* locus. Preliminary alkaline sucrose gradient analysis of newly replicated DNA from ρ$^+$ strains of *POL2*, *pol2-11*, and *pol2-12* strains immediately revealed that synthesis of chromosomal-size DNA was blocked at 37°C in ρ$^+$ *pol2-12* strains but that there was a small amount of low-molecular-weight DNA near the top of the gradient (Fig. 1). The same result was obtained with ρ$^+$ *pol2-11* strains at 37°C. At 22°C, although the rate of DNA replication in *pol2-11* and *pol2-12* strains was 40% of that in *POL2* strains, the size of DNA synthesized was not different from the size of the DNA synthesized in *POL2* strains.

These interesting findings prompted us to repeat the experiments in ρ^0 derivatives of the *pol2* mutants to eliminate contributions from mitochondrial DNA replication to our gradients. We also found that strain TC102-2D grew normally at temperatures as high as 39°C, which allowed us to use a higher restrictive temperature to eliminate any synthesis that might be due to leakiness of the *pol2-11* or *pol2-12* mutation at 37°C. Temperature-sensitive mutants are intrinsically leaky since they have an essential function in the cell, and one wishes to use the highest temperature at

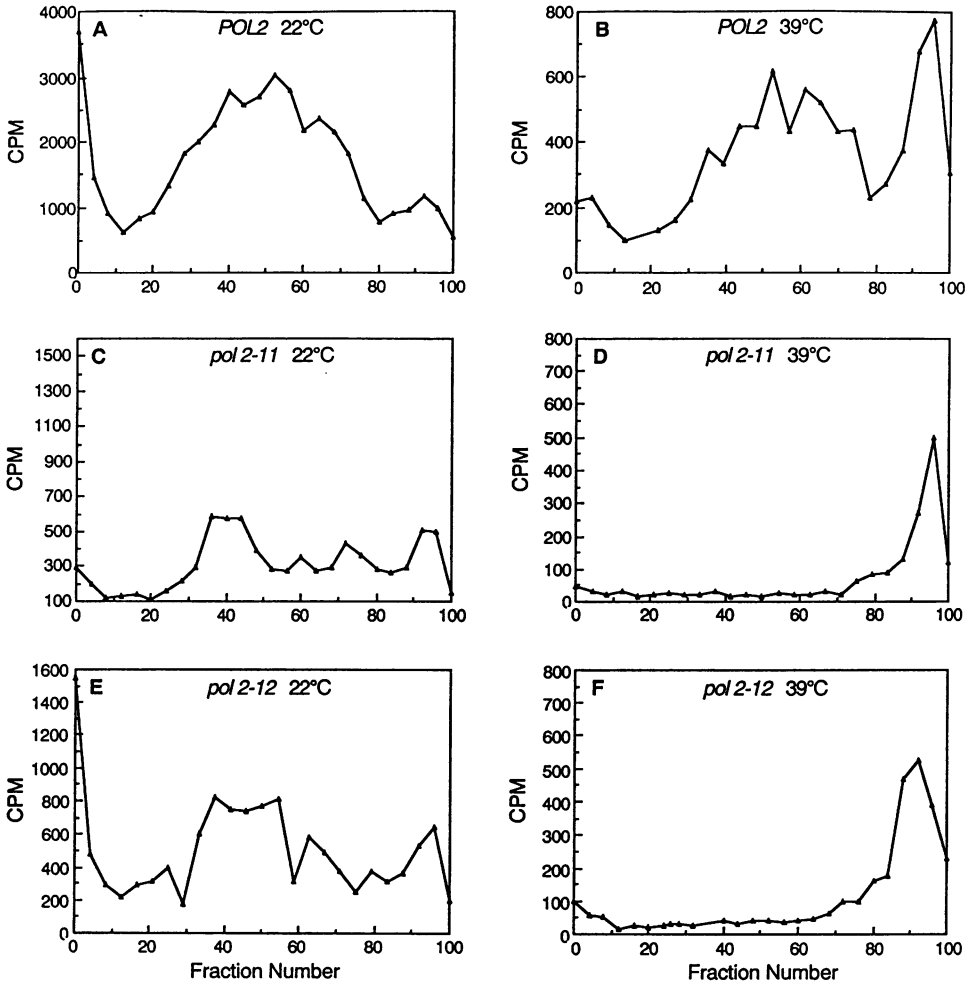

FIG. 2. Alkaline sucrose gradient analysis of residual DNA synthesis in TC102-2D, TC102-2-11, and TC102-2-12 cells at 22 and 39°C. For details, see Materials and Methods.

which the wild type grows as the restrictive condition for testing phenotype. The results with the ρ^0 strains at 38 or 39°C were similar to those shown in Fig. 1 except that the difference between the wild-type and mutant chromosomal peak at 38 and 39°C was more pronounced than at 37°C. Figure 2 illustrates a 15 to 30% alkaline sucrose gradient of DNA labeled for 3 h at 22 and 39°C with ρ^0 derivatives of *POL2*, *pol2-11*, and *pol2-12*. Phage T4 DNA sediments at position 77, and if one assumes the molecular weight relation of the equation given in Materials and Methods, then chromosomes of length 1,650 kb should appear at position 45, those of 400 kb should appear about position 62, and small chromosomes of 250 kb should appear at position 73. Chromosomal-size DNA is synthesized in the *POL2* strain at 39 and 23°C, although the amount is lower at 39°C than at 23°C (Fig. 2A and B). The average reduction of counts for four experiments is about 50%. *POL*$^+$ cells appear to synthesize a greater percentage of low-molecular-weight DNA at high temperatures. In contrast to the wild-type strains, *pol2-11* and *pol2-12* strains synthesize no chromosomal-size DNA at 39°C (Fig. 2D and F). The bulk of the newly synthesized DNA in both mutants is in the top 12% of the gradient, consistent with a size of less than 10 kb. This low-molecular-weight DNA is resistant to RNase and is not mitochondrial DNA, since ρ^0 strains are used. The quantity is less 20% that found in the *POL2* strain and much less than would be expected if the *pol2-11* and *pol2-12* strains were synthesizing Okazaki fragments at the same rate as *POL2* strains were. We conclude that leading-strand DNA synthesis is blocked and lagging-strand DNA synthesis is defective at the restrictive temperature in both DNA polymerase ε mutants. Thus, *pol2* mutants are defective in the synthesis of chromosomal-size DNA and show a quick-stop phenotype with respect to DNA synthesis, suggesting a role for DNA polymerase ε in elongation.

Several control experiments were performed. The size of the DNA synthesized at 22°C in *pol2-11* and *pol2-12* strains is the same as in *POL2* strains (Fig. 2C and E). However, the amount of DNA synthesis is an average of 40% lower (data from six experiments) in both *pol2-11* and *pol2-12* strains than in *POL2* strains. This result is consistent with the observation that both *pol2-11* and *pol2-12* strains grow more slowly than *POL2* strains. (We also found that *cdc2-1* strains synthesize less DNA at 22°C than does strain A364A *CDC2* [see Fig. 4].) Finally, DNA prelabeled at the permissive temperature was not broken after incubation at the nonpermissive temperature in any of the strains used (data not shown).

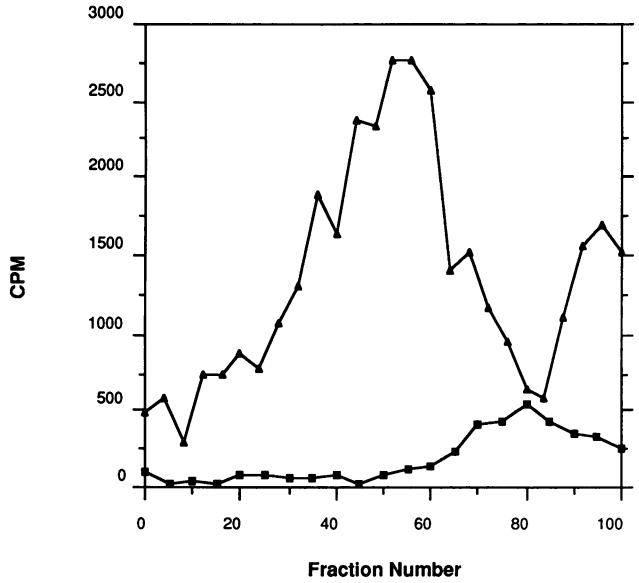

FIG. 3. Alkaline sucrose gradient analysis of DNA synthesis at 37°C in strain A364A *CDC2* (triangles) and ts370 *cdc2-1* (squares) cells. Cells were labeled with 50 μCi of [^{3}H]uracil for 3 h at 37°C as described in Materials and Methods.

DNA synthesis in temperature-sensitive *pol3* mutants. The synthesis of only a small amount of low-molecular-weight DNA in *pol2-11* and *pol2-12* mutant strains is similar to the situation in *pol1-17* strains at 36°C (9). Thus, DNA polymerases α and ε are essential at the replication fork for elongation. However, *S. cerevisiae* has a third DNA polymerase required for viability, DNA polymerase δ. DNA polymerase δ is missing in extracts from *pol3* (*cdc2*) strains (5, 42). In a previous study (14), *cdc2-3* mutants replicated two-thirds of their genomes and also synthesized chromosomal-size DNA at 37°C. A second study also found high-molecular-weight DNA synthesized at 37°C in a *cdc2* mutant (21). Therefore, *cdc2* mutants did not exhibit an obvious defect in elongation at the replication fork, which is at variance with a large body of data for the SV40 in vitro replication system suggesting that DNA polymerase δ is required for coordinated leading and lagging-strand DNA synthesis.

We reinvestigated the residual synthesis at the nonpermissive temperature in a strain carrying a different *cdc2* allele, *cdc2-1* (ts370). This strain was shown to carry a single temperature-sensitive allele that cosegregated in genetic crosses with *cdc2* (data not shown). Six alkaline sucrose gradients were carried out to examine residual DNA synthesis in *cdc2-1* mutants at 37 or 38°C. The amount of DNA synthesis at 37 or 38°C varied from experiment to experiment in *cdc2-1* strains. The ratios of total *cdc2-1* incorporation at 37 or 38°C to total *CDC2* incorporation at 37°C were 0.19, 0.20, 0.44, 0.27, 0.54, and 1.2 for the six experiments. What is very clear, however, is that even for the experiment in which recovery of total counts per minute was the same in the wild type and mutant, the state of the DNA was very different. In no case was there a peak of chromosomal-length DNA, and more than 80% of the DNA sedimented instead in the top 20% of the gradient. A profile from one experiment carried out at 37°C is shown in Fig. 3. Clearly, there is a gross defect in chromosomal DNA synthesis, though there is some residual synthesis.

If the residual DNA synthesis seen in *cdc2* mutants is due to partially functional DNA polymerase δ, then it might be possible to perturb the cell in a manner that further inactivates the mutant DNA polymerase δ but not wild-type enzyme. Since the parent strain, A364A, grew at 38°C, we carried out a more complete analysis at this more restrictive temperature (Fig. 4 and 5). Wild-type A364A synthesized chromosomal-size DNA at 38°C (Fig. 4B), although the rate of synthesis was twofold lower than that at 22°C (Fig. 4A). In contrast, the chromosomal peak was absent in *cdc2-1* strains at 38°C (Fig. 4D), and the bulk of the residual synthesis sedimented in the top 20% of the gradient. Careful analysis of the size of newly synthesized DNA in six *cdc2-1* experiments revealed that an increase in ^{3}H counts above background began around positions 40 to 50 in the sucrose gradients, corresponding to a molecular size of about 10^6 bp. Although the amount of newly synthesized DNA at this position was small and the peak of newly synthesized DNA occurred at position 80, one would not expect replication forks lacking a functional DNA polymerase δ to synthesize DNA of 10^6 bp. Thus, there may be some residual DNA polymerase δ activity in the strain, or another polymerase can partially compensate.

An HU arrest was used to further investigate *cdc2-1* residual DNA synthesis. HU blocks DNA synthesis and reversibly arrests cells in S phase (43). It has been shown previously that the *cdc2* step cannot be completed during an HU arrest at the permissive temperature (18); that is, *CDC2* is required for completion of the cell cycle after release from an HU block. When A364A was incubated with [^{3}H]uracil in the presence of HU, the number of acid-precipitable counts was less than 10% of that of cells incubated without HU (6a). The small amount of incorporation was analyzed by sucrose gradient sedimentation (data not shown). The counts concentrated in the top 40% of the gradient. HU arrest was reversible, since the amount of DNA synthesis seen after HU was washed out was the same as in cells not treated with HU (6a).

A second protocol was designed to circumvent potential leakiness in the *cdc2* alleles. The original idea was to arrest cells at 37°C with HU to give the polymerase time to fully denature in the absence of DNA synthesis and then to measure DNA synthesis at the nonpermissive temperature after washing out the HU. Residual DNA synthesis in *cdc2-1* mutants at 37°C was indeed further reduced after a 3-h 37°C HU arrest. Interestingly, in a control experiment, a 22°C HU arrest followed by labeling at 38°C had the same effect (Fig. 5). (The same result was observed with a 22°C HU arrest followed by labeling at 37°C.) The data of Fig. 5B and D compare incorporation of [^{3}H]uracil label by *cdc2-1* cells at 38°C with and without a 3-h pretreatment with HU at 22°C. A 3-h arrest with HU at 22°C followed by washing out of the HU eliminated most of the residual DNA synthesis at 38°C. The increase in ^{3}H counts at positions 40 to 50 upon incubation of *cdc2-1* strains at 38°C (Fig. 4D and Fig. 5B) is not observed when these cells are preincubated with HU (Fig. 5D). The [^{3}H]DNA appeared only in the top 20% of the gradient. The effect of a HU arrest on residual DNA synthesis was repeated in three other experiments. In two of the experiments, HU pretreatment was effective in reducing high-molecular-weight DNA synthesized at 38°C beyond the reduction seen in the *cdc2-1* control without HU. In one experiment, HU pretreatment did not further reduce total synthesis in *cdc2-1*, perhaps because of an incomplete block or recovery from HU before the temperature block was invoked. Nevertheless, the HU block did shift the DNA to a

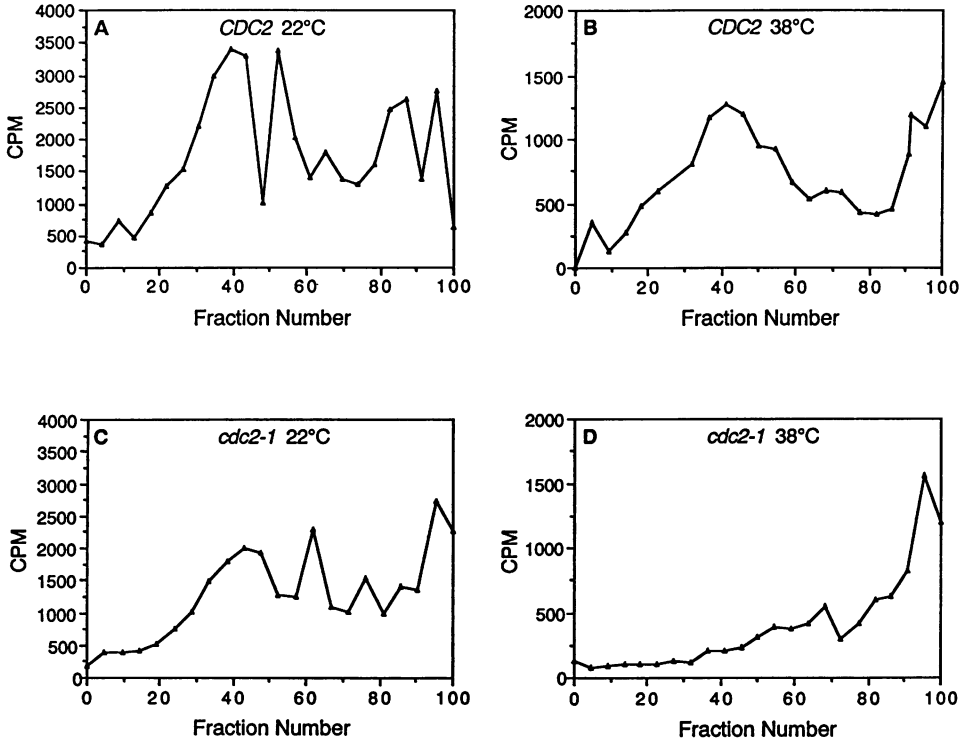

FIG. 4. Alkaline sucrose gradient analysis of residual DNA synthesis in *CDC2* and *cdc2-1* strains at 22 and 38°C. Cells were labeled with 100 μCi of [^{3}H]uracil for 3 h.

lower molecular weight. Thus, residual synthesis in *cdc2-1* strains was reproducibly low at the restrictive temperature after a synchronization with HU. The residual counts, however, were consistently higher than in *pol1* or *pol2* mutants labeled at the nonpermissive temperature (9) (Fig. 4).

A number of control experiments were carried out. A 3-h pretreatment of A364A cells with HU at 22°C did not affect DNA replication during a subsequent incubation at either 38°C (Fig. 5A and C) or 22°C (data not shown). When *cdc2-1* cells were incubated at 22°C with HU and then assayed for replication at 22°C, two experiments showed little or no defect in DNA synthesis, and another experiment showed a 50% reduction in overall recovery of counts relative to cells not incubated with HU (data not shown).

In summary, previous results demonstrating a complete block in DNA replication in *pol1-17* cells at 37°C (9) combined with the alkaline sucrose sedimentation experiments with *pol2-11*, *pol2-12*, and *cdc2-1* strains demonstrate that DNA polymerases α, δ, and ε are at the replication fork. The absence of any one polymerase results in an arrest of elongation.

Further characterization of *pol2* mutants. Figure 6 illustrates an in vivo RNA labeling experiment using *POL2*$^+$, *pol2-11*, and *pol2-12* strains at 37°C. Surprisingly, there appeared to be a slight defect in RNA synthesis in both the *pol2-11* and *pol2-12* strains at the restrictive temperature, with the defect greater in the *pol2-12* strain. This small decrease in RNA synthesis does not account for the defect in DNA synthesis observed in the gradients of Fig. 1 and 2. It occurs after the labeling period, and the cells remain viable for at least 6 h, as evidenced by resumption of the cell cycle after the cells are returned to the permissive temperature. At the permissive temperature, neither the *pol2-11* nor the *pol2-12* strain showed a defect in total RNA synthesis. A defect in total RNA synthesis was also observed with the DNA polymerase α mutant, *pol1-14*, at 23 and 36°C (7). The *pol1-14* strain was the only DNA polymerase α mutant isolated that grew poorly at 23°C.

Mutant DNA polymerase ε proteins show severe defects in vitro. The activity of DNA polymerase ε in the *pol2-11* and *pol2-12* mutants was analyzed by partial protein purification in parallel with *POL2*. Both mutant DNA polymerase proteins were defective. Figure 7 shows an activity profile of *POL2*$^+$ and *pol2-12* extracts eluted from a Mono Q FPLC column. The differential chromatographic properties of the yeast DNA polymerases are well documented. The first peak (0.27 M KCl) represents DNA polymerases α and δ, while the second peak represents DNA polymerase ε (8, 17, 36). DNA polymerase ε elutes at 0.38 M KCl, similar to the behavior of DNA polymerase II* described by Hamatake et al. (17), which elutes at 0.35 M NaCl. Peaks of exonuclease, probably corresponding to the polymerase ε and δ nucleases, also eluted at 0.38 M KCl (data not shown) and 0.22 M KCl. As shown in Fig. 7, the activity of the *pol2-12* protein is reduced more than 90% when assayed with either the favored template, poly(dA)$_{500}$-oligo(dT)$_{10}$, or activated salmon sperm DNA. While there is some residual activity (less than 10% of wild-type activity) on poly(dA)-oligo(dT) (Fig. 7D), this activity is unstable and decays upon freezing and thawing under conditions under which the wild-type protein loses no activity. The heat sensitivity of the *pol2-12* protein was checked by a preincubation at 42°C followed by addition of poly(dA)-oligo(dT) and [^{3}H]dTTP. The half-life did not appear different from that of the *POL2* protein, which is inherently unstable at 42°C (data not shown). The *pol2-12*

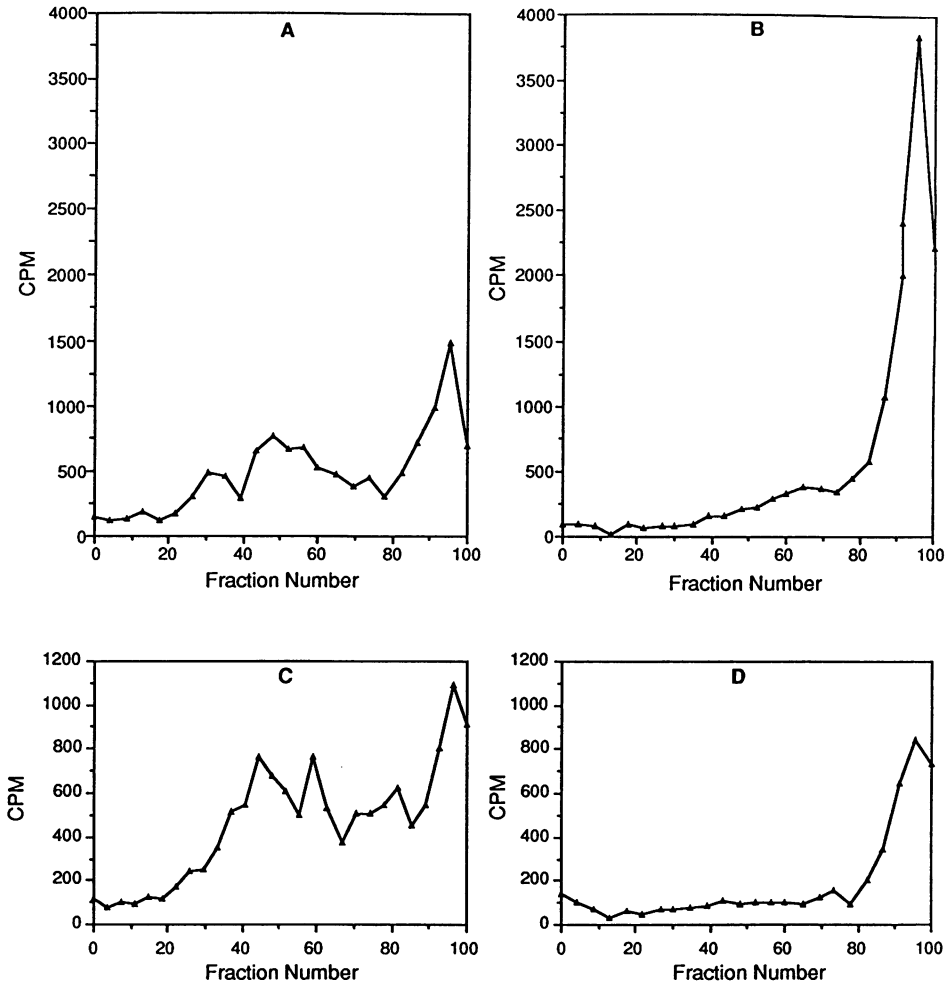

FIG. 5. Alkaline sucrose gradient analysis of a 3-h 22°C HU arrest on residual synthesis in a *cdc2-1* strain at 38°C. In panels A (A364A *CDC2*) and B (cdc2-1), cells were labeled with 80 μCi of [^{3}H]uracil for 3 h at 38°C without prior HU treatment, as for Fig. 4. In panels C (A364A *CDC2*) and D (*cdc2-1*), cells were incubated with HU for 3 h at 22°C and then incubated at 38°C with [^{3}H]uracil for 3 h without HU.

protein is sensitive to pH on poly(dA)-oligo(dT), since it loses one-third of its activity when HEPES buffer at pH 6.5 is substituted for Tris buffer at pH 7.5. The *POL2* protein has the same activity at pH 7.5 and 6.5. The exonuclease activity of the *pol2-12* protein peak is about threefold lower than that of the *POL2* protein peak (data not shown).

The *pol2-11* protein was also partially purified in parallel. There is significantly more *pol2-11* activity than *pol2-12* activity. *pol2-11* protein has about one-half the activity of *POL2* protein on poly(dA)-oligo(dT), activated DNA, and an exonuclease substrate. The protein was not sensitive to pH changes and exhibited the heat sensitivity of the *POL2* protein. Thus, the in vitro assays of the *pol2-11* protein do not suggest a defect that would cause a block in chromosomal DNA replication, as illustrated in Fig. 2. Perhaps a mutation has been made in a protein interaction site which in combination with a moderate loss in activity results in a block in DNA replication, or perhaps the protein is more stable in vitro than in vivo.

DISCUSSION

Velocity sedimentation analysis of newly synthesized DNA from *pol2-11*, *pol2-12*, and *cdc2-1* strains demonstrates

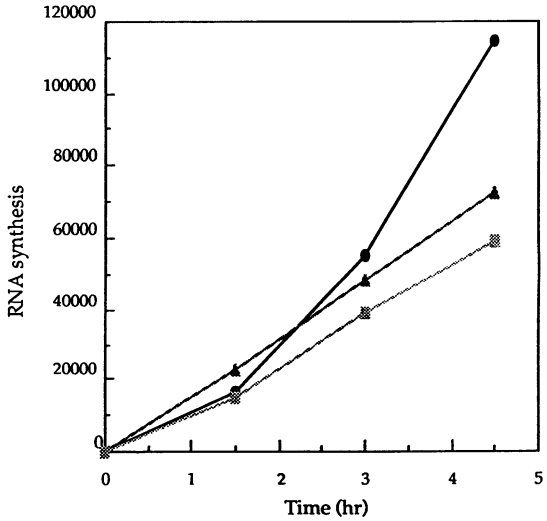

FIG. 6. Incorporation of [^{3}H]uracil into RNA as a function of time at 37°C in *POL2* (SS111; circles), *pol2-11* (SS111-2-11; triangles), and *pol2-12* (SS111-2-12; squares) strains. Cells (10^7/ml) were labeled with 25 μCi of [^{3}H]uracil per ml, and 40 μl was precipitated with 1 M HCl–0.1 M PP_i.

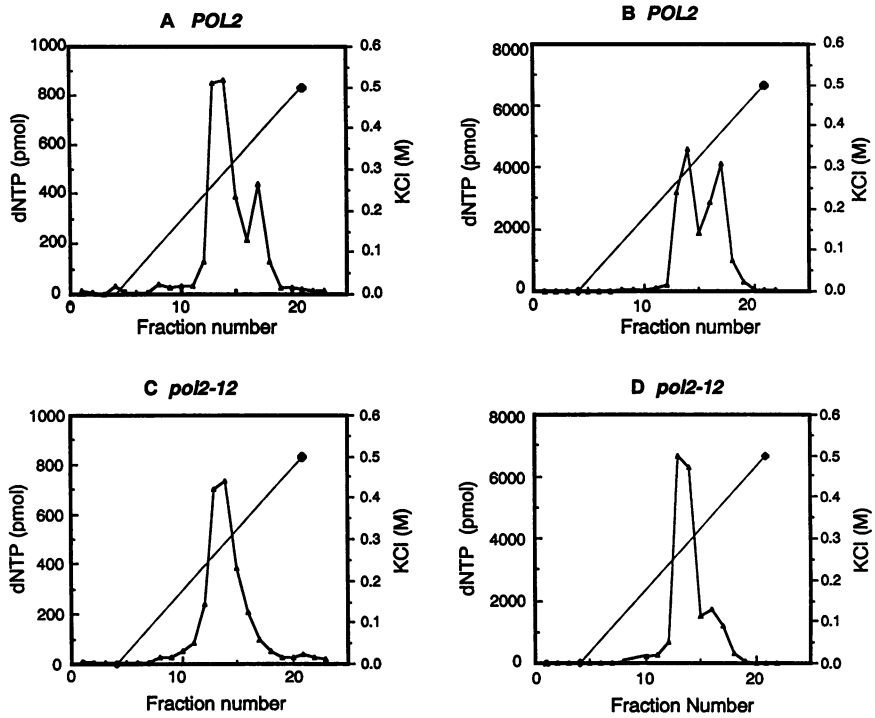

FIG. 7. Mono Q column elution profiles of DNA polymerase activity from *POL2* and *pol2-12* extracts. (A) *POL2* extracts assayed with activated DNA; (B) *POL2* extracts assayed with poly(dA)$_{500}$-oligo(dT)$_{10}$; (C) *pol2-12* extracts assayed with activated DNA; (D) *pol2-12* extracts assayed with poly(dA)$_{500}$-oligo(dT)$_{10}$.

that both DNA polymerases δ and ε are required for the elongation step of DNA replication. The data corroborate and extend findings of Araki et al. (1, 2) that also suggest that DNA polymerase ε is required for DNA replication. The alkaline sucrose gradient profiles of DNA synthesized in *poll1-17* strains (9) and *pol2-11* and *pol2-12* strains at the nonpermissive temperature are very similar. No chromosomal-size DNA is synthesized in either *poll1-17*, *pol2-11*, or *pol2-12* strains, and the only DNA synthesized is in the form of relatively short fragments (≤10 kb), as judged from the position of the T4 marker and the resolution of the gradient. In *pol2* mutants, the short fragments represent, at least in part, aberrant chromosomal DNA synthesis, since they persist in ρ^0 strains, which lack mitochondrial DNA. Since we have not carried out an alkaline sucrose gradient analysis of the DNA from ρ^0 *poll1-17* strains, we cannot be certain how much of the low-molecular-weight DNA derives from mitochondrial DNA.

Alkaline sucrose sedimentation of DNA synthesized in a *cdc2-1* mutant at 37 and 38°C shows severely reduced chromosomal synthesis. In some experiments, there was significant in vivo replication, as reported previously by Conrad and Newlon (14). In contrast to the results of their alkaline sucrose gradient analysis, however, we find that *cdc2* strains synthesize primarily low-molecular-weight DNA at the restrictive temperature. The discrepancy may be explained by the fact that they used a different allele of *cdc2*, *cdc2-3*, for the alkaline sedimentation experiments. Although elongation is clearly defective in *cdc2-1* strains at the restrictive temperature, the amount and size of the residual synthesis are still in excess of what would be expected from a block in leading-strand synthesis. After an HU arrest of *cdc2-1* strains at 22°C, synthesis of chromosomal-size DNA during a subsequent incubation at the restrictive temperature was reduced even further. Synthesis of DNA sedimenting in the top 20% of the gradient still remained. A plausible explanation for the effect of an HU arrest on residual synthesis in *cdc2-1* strains is that the ts370 DNA polymerase δ is more stable at the replication fork than in solution. Upon a shift of *cdc2-1* strains from 22 to 37°C, DNA polymerase δ remains stabilized at the fork but then dissociates over time, leaving a reduced level of chromosomal-size fragments replicated. When *cdc2-1* cells are incubated with HU at 22°C, DNA synthesis is blocked and DNA polymerase δ dissociates from the fork. After removal of HU and incubation of the cells at the restrictive temperature, DNA polymerase δ is unable to reassemble at the fork. The consequence is that chromosomal-size fragments are not synthesized. The data are consistent with the observation that Brij 58-permeabilized *cdc2* cells are defective in replication at 36°C but not at 22°C (22).

The sucrose gradients of *pol2-11*, *pol2-12*, and *cdc2-1* strains combined with previously reported gradients of *poll1-17* strains provide direct evidence that there are three distinct DNA polymerases at the replication fork. Chromosomal replication is blocked in the absence of any one. Sucrose gradients of *pol2-11*, *pol2-12*, and *cdc2-1* strains still show the synthesis of low-molecular-weight DNA at the restrictive temperature. The residual synthesis may indicate that these alleles are leaky or that two polymerases are capable of synthesizing small DNA fragments. Morrison et al. (26) and Araki et al. (2) have proposed that DNA polymerase ε synthesizes the leading strand and that DNA polymerase δ extends lagging-strand primers.

Mutations in acidic residues of the exonuclease I domains of both polymerases δ and ε result in strains with spontaneous mutator phenotypes (27, 40). This observation also indicates that both polymerases play major roles in elonga-

tion. However, mutations in genes unrelated to DNA synthesis, such as *rad51*, also result in spontaneous mutator phenotypes (28, 38).

Prelich et al. (34) have presented evidence for a requirement for two distinct DNA polymerases in eukaryotic replication, with the demonstration that proliferating cell nuclear antigen (PCNA) is required for leading-strand DNA synthesis in in vitro SV40 DNA replication. Since PCNA increases the processivity of purified DNA polymerase δ (45), DNA polymerase δ presumably synthesizes the leading strand. DNA polymerase δ has now been shown to be an essential factor for reconstitution of SV40 replication in vitro (23, 25, 48). The affinity of DNA polymerase δ for a primer recognition complex of RF-C and PCNA capable of highly progressive synthesis further indicates that it synthesizes the leading strand (11, 46, 47). SV40 in vitro systems have suggested a role for a third DNA polymerase, possibly DNA polymerase ε, in replication. Nethanel and Kaufmann (31) have shown that in SV40-infected nuclei, butylphenyl-dGTP inhibits the synthesis of 30-bp DNA primers but does not inhibit as significantly the extension of the primers into Okazaki pieces. Since at this concentration butylphenyl-dGTP inhibits DNA polymerase α and not polymerase δ or ε (15), DNA polymerase α is implicated in primer synthesis, and either DNA polymerase δ or ε may be involved in elongating primers into Okazaki pieces. Bullock et al. (10) have shown that anti-PCNA antibodies inhibit the elongation of 40-bp DNA primers into 300-bp Okazaki fragments during SV40 replication in cytosolic extracts. Addition of purified PCNA reversed the effect. Both DNA polymerases δ and ε interact with PCNA and RF-C at the primer termini, but the DNA polymerase ε complex is less stable than the complex found with DNA polymerase δ (11, 24, 50). In *S. cerevisiae*, PCNA forms a more stable interaction with DNA polymerase δ than with polymerase ε. This conclusion is inferred from the fact that higher concentrations of PCNA are required to stimulate DNA polymerase ε than DNA polymerase δ on singly primed M13 DNA (11, 24, 50). Yeast DNA polymerase ε can substitute for human DNA polymerase δ in the reconstituted SV40 replication system as a leading-strand polymerase; however, human DNA polymerase ε functions less efficiently than DNA polymerase δ as a leading-strand polymerase. It may be that a polymerase ε processivity factor is missing from the reconstituted SV40 system. Thus, studies with SV40 corroborate the work with *S. cerevisiae* in showing a role for DNA polymerases δ and/or ε, but no unique role for these DNA polymerases can be deduced as yet.

Both the *pol2-11* and *pol2-12* proteins exhibit defective activity on poly$(dA)_{500}$- oligo$(dT)_{10}$, activated salmon sperm DNA, and a nuclease substrate. The *pol2-12* protein is significantly more defective than the *pol2-11* protein. In both mutants, chromosomal replication is blocked at 37°C and defective at 22°C. The in vivo defect in replication in *pol2-11* strains may result from factors other than a defect in the catalytic activity. We speculate that at the restrictive temperature, an interaction with a DNA polymerase ε accessory protein is disrupted in *pol2-11* strains, resulting in a block in elongation.

ACKNOWLEDGMENTS

This study was supported by grant GM 25508.

We are greatly indebted to Akio Sugino for the *POL2* gene disruption used in the screening for temperature-sensitive mutants.

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