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Genetics

Temperature-sensitive mutations in the yeast DNA polymerase I gene

(*Saccharomyces cerevisiae*/DNA replication/DNA polymerase α/plasmid mutagenesis)

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ABSTRACT **Seven mutations that yield thermolabile DNA polymerases have been isolated in the DNA polymerase I gene, *POL1*, of *Saccharomyces cerevisiae*. Strains carrying the mutant genes are temperature sensitive for growth. The *pol1* mutants were identified by a method that has general applicability for identification of both temperature-sensitive and null mutations. A plasmid containing a mutagenized *pol1* gene was transformed into a strain in which the only functional copy of the *POL1* gene was carried on an unstable plasmid. The genes conferring temperature-sensitive growth were detected after elimination of the unstable plasmid containing the wild-type gene. DNA polymerase I isolated from each of the mutants is defective at both 23°C and 36°C. DNA synthesis is deficient *in vivo* at 36°C in all the mutants, while RNA synthesis is normal in all but one of the mutants. The terminal phenotype of *pol1* temperature-sensitive mutants is dumbbell-shaped cells in which the nucleus has migrated to, but apparently not entered, the isthmus separating the mother and the daughter. The *POL1* gene is located on chromosome XIV ≈2 centimorgans away from *met4*.**

To understand the proteins involved in eukaryotic DNA replication, several different approaches are possible in the yeast *Saccharomyces cerevisiae*. The genetic approach was initiated by Hartwell (1) and continued by other groups (2–4). These workers identified strains with physiological defects in DNA replication by screening for blocks at specific points in the cell cycle or by screening random temperature-sensitive (ts) mutants. Mutants in DNA ligase, *cdc9* (5), thymidylate kinase, *cdc8* (6, 7), and thymidylate synthetase, *cdc21* (8, 9), have been identified. However, no mutants for genes encoding proteins directly involved in DNA replication have been found. Furthermore, the biochemical defects in most of the mutants have never been identified.

An alternative approach to identifying components of the replication apparatus has been recently undertaken. This involves purifying replication proteins, isolating the genes encoding them, and constructing the appropriate mutant from the cloned gene. We have cloned the gene for the catalytic core subunit of DNA polymerase I from a λgt11 expression library using antibody against the protein (10). The complete *POL1* gene was then isolated from a YEp24 library (YEp, yeast 2-μm plasmid) and is contained in a 9-kilobase (kb) *Bam*HI fragment. DNA polymerase I activity is overproduced in extracts of cells containing the *POL1* gene cloned into the high copy number vector YEp24 (see ref. 10). The *POL1* gene is present in a single copy and is essential for mitotic growth, as shown by Southern analysis and gene disruption experiments. Spores carrying a disrupted *POL1* gene germinate but arrest after a few cell divisions with a dumbbell morphology and with the nucleus in the isthmus connecting the mother and daughter cell. This is a phenotype typical of a defect in medial nuclear division and DNA replication. Interpretation of the terminal phenotype, however, is complicated by the possible presence of functional polymerase in a spore with a disrupted polymerase gene. Furthermore, the lethal phenotype does not allow detailed analysis of the role of DNA polymerase I in the cell.

Since DNA polymerase I is an essential gene, it should be possible to construct conditionally lethal mutants that would yield more information as to cellular processes requiring DNA polymerase I. Such mutants should allow definitive determination of terminal phenotype and be useful in further determining the role of DNA polymerase I in replication, repair, mutagenesis, and meiosis. Furthermore, such mutants would permit the isolation of second-site suppressors to identify additional replication functions. The reduced levels of DNA polymerase I in *pol1* ts mutants can also aid in understanding the role of DNA polymerase II, the second nuclear DNA polymerase of yeast, about which almost nothing is known.

Rather than using the method used by Shortle *et al.* (11) for actin, we have identified *POL1* mutants directly on a YCp plasmid (yeast replicating centromere plasmid) carrying the *POL1* gene. The present paper describes the method by which this was accomplished and characterizes seven ts mutants in the *POL1* gene. Terminal phenotype, DNA and RNA synthesis *in vivo*, residual DNA polymerase I levels, and genetic mapping of the *pol1* mutants are described.

MATERIALS AND METHODS

Plasmids and Strains. All plasmids are described in Table 1. The gene disruption experiment was carried out with SEY2112 **a**/α *leu2-3,112/leu2-3,112 ura3-52/ura3-52 ade2/+ his 4-519/+ Δsuc2/Δsuc2* obtained from Scott Emr (California Institute of Technology). The DNA polymerase I mutants were isolated in the strain MB3-8c *pol°:LEU2 trp1-289 his4-519 leu2-3,112* containing pPOL1-7. The mutagenized polymerase fragments were integrated into SS111 **a** *trp1-289 tryl ura3-1 ura3-2 ade2-101 gal2 can1* (Stewart Scherer, California Institute of Technology). Mapping of *pol1* was carried out with strains STX155-9B α *gal1 lys5 his6 ura2 gal2 lys7 prt1 ade2 rad1 met4* (Yeast Genetic Stock Center, Berkeley, CA) and SD2-4 *ade2 ura3-52 his3-11,15 leu2-3, 112 trp1 top2* obtained from Rolf Sternglanz (Stonybrook).

Mutagenesis. Plasmid pPOL1-9 was mutagenized exactly according to the procedure of Busby *et al.* (12).

Other Methods. Terminal phenotype was determined by incubating *pol1* ts strains at 36°C for 4 hr and then staining with 4′,6-diamidino-2-phenylindole as described (10). Yeast extracts and DNA polymerase assays were carried out as described (10). The OFAGE procedure is that of Carle and Olson (13) and Schwartz and Cantor (14) using strain SS327

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Abbreviation: ts, temperature sensitive.

Table 1. Residual DNA and RNA synthesis in *pol* ts mutants

	t, °C	Relative* DNA synthesis	Ratio of DNA synthesis, 36°C/23°C	Relative* RNA synthesis
SS111	23	0.81	1.23	1
	36	1		1
pol1-11	23	0.71	0.56	1.04
	36	0.40		0.77
pol1-12	23	0.56	0.54	0.91
	36	0.30		1.04
pol1-13	23	0.62	0.48	1.14
	36	0.30		1.01
pol1-14	23	0.14	1.6	0.42
	36	0.23		0.77
pol1-15	23	0.91	0.55	1.18
	36	0.55		1.03
pol1-16	23	0.71	0.42	1.25
	36	0.42		0.70
pol1-17	23	0.83	0.23	1.27
	36	0.23		1.26

*DNA and RNA synthesis were normalized to the values for strain SS111 at 36°C.

(S. Scherer, California Institute of Technology). Gels were run at 240 V for 16.5 hr at 12°C. Yeast DNA transformations were carried out by the method of Ito *et al.* (15).

***In Vivo* DNA Synthesis.** *In vivo* levels of DNA synthesis were determined by the method of Hartwell (1). Cells were harvested at 10^7 cells per ml, resuspended in synthetic medium and [^{3}H]uracil (7 μCi/ml; 1 Ci = 37 GBq), and incubated for 3 hr. A sample (0.1 ml) was precipitated with 1 M HCl/0.1 M sodium pyrophosphate for RNA synthesis determination. For determination of DNA synthesis, NaOH was added to 0.4 ml of cell suspension after 3 hr at 36°C, to a final concentration of 1 M. Cells were incubated at 39°C for 24 hr, followed by precipitation with 1 M HCl/0.1 M sodium pyrophosphate. For strain SS111, cpm of [^{3}H] DNA were ≈1.5% the cpm of [^{3}H] RNA. Samples were analyzed in triplicate and the experiments were repeated four or five times for each strain.

RESULTS

Construction of ts *poll* Mutations. The cloned copy of a unique essential gene can be used in several ways to produce conditional lethal mutations useful for structural and functional analyses. In this study, ts mutants were isolated by a three-step procedure based on a plasmid exchange strategy. Since we wished to identify the mutant gene on a plasmid and since the gene is essential and single copy, a partial diploid was constructed whose only functional copy of the *POL1* gene was on an unstable autonomously replicating plasmid. Thus, the strain contained a lethal chromosomal disruption of the *POL1* gene and a plasmid encoding the wild-type *POL1* gene. A different plasmid, also carrying the *POL1* gene, was then mutagenized with hydroxylamine and introduced into the partial diploid by transformation. Finally, the nonmutagenized plasmid was exchanged for the mutagenized plasmid by counterselection against the nonmutagenized plasmid. Resulting transformants were screened for inability to grow at 36°C, which indicated a ts mutation in the *POL1* gene on the plasmid. We are grateful to B. Patterson (University of California, San Francisco) for suggesting the general idea of plasmid exchange for isolation of mutants.

Fig. 1 illustrates how we implemented this strategy. We first constructed a strain containing the chromosomal gene disrupted by a plasmid containing the yeast *LEU2* gene (16). A 3-kb fragment from plasmid YEp13 (17) containing the

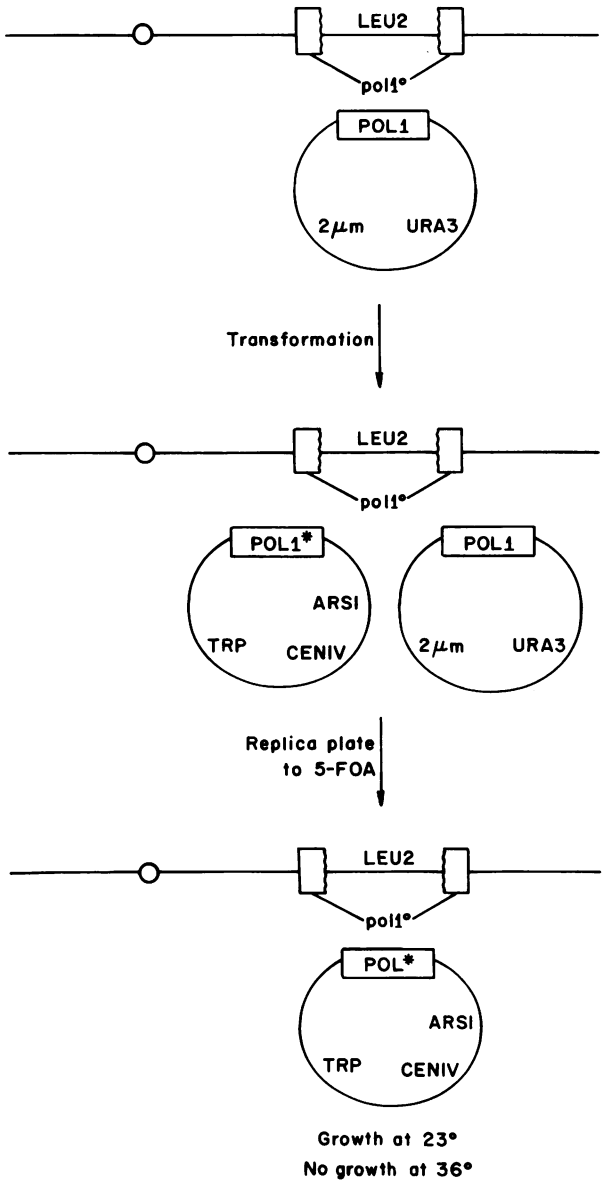

FIG. 1. Strategy for construction of ts mutants. 5-FOA, 5-fluoroorotic acid.

LEU2 gene was inserted at the *Pst* I site in the *POL1* gene of pPOL1-3 (10), a plasmid containing a 1.4-kb fragment of the *POL1* gene inserted into the *Eco*RI and *Sal* I sites of pBR322. The resulting plasmid, pPOL1-4, was cut with *Xba* I, which cleaves within the *POL1* gene, and the diploid strain SEY2112 was transformed with this DNA. LEU2$^+$ transformants were selected and stable transformants were sporulated. Dissection of the resulting tetrads yielded the previously observed 2 viable/2 inviable spore segregation pattern diagnostic of a disruption of an essential gene. The viable spores were all *leu2* auxotrophs. To obtain a partial diploid, strain SEY2112 (*POL$^+$/pol°:LEU2*) was transformed with the plasmid pPOL1-7. This plasmid contained a 2-μm origin of replication so that it would be mitotically unstable and easily exchangeable in the final step of the mutant construction. The plasmid also carries the *URA3* selectable marker, which allows for positive selection of plasmids on medium lacking uracil. *URA3* was chosen here because it also allows counter-selection on medium containing the drug 5-fluoroorotic acid (see below). The plasmid pPOL1-7 contains a 4.6-kb *Bgl* II/*Bam*HI fragment encoding a functional *POL1* gene and was constructed by BAL-31 deletion of pPOL-1 at the *Sph* I site (unpublished data). When this diploid trans-

formant was sporulated and dissected, the resulting spore segregation was predominately 4 viable/0 inviable, indicating that the plasmid complemented the DNA polymerase gene disruption. The partial diploid was designated MB3-8c.

The plasmid to be mutagenized, pPOL1-8, contained the 9-kb *Bam*HI fragment containing the entire *POL1* gene inserted into the *Bam*HI site of YCp19B, a plasmid carrying the three markers *CEN4*, *ARS1*, and *TRP1*. [YCp19B was derived from YCp19 (S. Scherer, California Institute of Technology) by cutting with *Pvu* II and adding *Bam*HI linkers.] The plasmid was treated with hydroxylamine, which deaminates cytosine causing cytosine to thymine transitions, as described in *Materials and Methods*. The mutagenized DNA transformed *Escherichia coli* MC1066 *trpC9830* at a frequency of 1% compared with untreated DNA and the frequency of Trp^+ prototrophs among the transformants was 2%. The frequency of transformation of yeast with mutagenized and unmutagenized pPOL1-8 was ≈1000 transformants per μg of DNA.

The partial diploid MB3-8c was then transformed with the mutagenized pPOL1-8 plasmid and TRP^+ URA^+ cells were selected. This created a strain with two plasmid-encoded copies of the *POL1* gene. We assumed that *pol1* ts mutants would be recessive, since the gene disruption mutations were recessive. Therefore, we wished to remove the plasmid carrying the wild-type copy. However, previous attempts to create ts mutants using cloned genes and transformation have resulted in the isolation of mutations unlinked to the gene of interest (11). To distinguish such unlinked mutations, and thus eliminate nonpolymerase mutants, ≈1000 pPOL1-7/pPOL1-8 transformants were plated on medium lacking uracil and tryptophan and incubated at 23°C. The transformants were replicated and incubated at 36°C. Any transformant that is ts on such medium is not the result of a recessive ts polymerase, but it must be due to a chromosomal mutation unlinked to *POL1* or to a ts mutation in the *TRP1* gene or to ts mutations affecting maintenance of the YCp19B vector. Such ts transformants were discarded at this point so that in the subsequent step all ts transformants observed would be linked to *POL1*. In fact, only one nonspecific ts transformant was observed, and the 999 double transformants that grew at both 23°C and 36°C were used in the next step.

Since cells with mutations at the $ura3^-$ locus are able to grow on medium containing 0.1% 5-fluoroorotic acid, while $URA3^+$ cells are unable to grow on such medium (18, 19), 5-fluoroorotic acid provides a method to select for the loss of a $URA3^+$ plasmid. [Use of 5-fluoroorotic acid was suggested to us by G. Fink (Massachusetts Institute of Technology).] The 1000 double transformants of MB3-8c were replica-plated onto medium containing 5-fluoroorotic acid and lacking tryptophan and allowed to grow at 23°C for 48 hr. After replica plating to medium containing 5-fluoroorotic acid and incubation at 36°C, 12 colonies exhibited ts growth. Approximately one hundred colonies did not grow at either 23°C or 36°C on the 5-fluoroorotic acid plates and are assumed to represent null mutations in the *POL1* gene, since *trp1* and plasmid maintenance mutants were eliminated in the intermediate screen described above. Furthermore, genetic exchanges between various *POL1* genes would give rise to wild type and not be observed in the ts screen. Thus, this is an efficient procedure for isolating both null and ts mutations in a single copy essential gene.

Insertion of *pol* ts Genes into Chromosomes by Gene Replacement. To make chromosomal *pol* ts mutants for genetic analysis, the two-step gene replacement method of Scherer and Davis was used (20). The mutant strains are designated *pol1-11* through *pol1-17*. At 36°C, dumbbell-shaped cells were observed in all the mutants. One mutant, *pol1-16*, was able to grow slowly at 36°C and formed enlarged cells. Transformation of all seven mutants with the plasmid pPOL1-1 restored normal growth at 36°C, showing that the ts phenotype resulted from a mutation in the *POL1* gene.

Characterization of *pol1-15* DNA Polymerase. All mutant strains showed reduced DNA polymerase activity in extracts both at 23°C and at 36°C. At 36°C, there was <10% of control levels of synthesis observed in wild-type cells. The extent of the defect in each mutant DNA polymerase I was difficult to quantitate because of the low overall amounts of activity detectable and the presence of two other polymerases in *S. cerevisiae* extracts, DNA polymerase II and a polymerase associated with the mitochondria.

To increase the sensitivity of the polymerase I assay, strains carrying the wild-type or mutant genes on high copy number plasmids were prepared. The *pol1* ts alleles were each cloned in YEp24. Even with increased gene dosage, however, there was <10% of wild-type DNA polymerase levels in the mutant.

It is possible that overproduction of thermolabile polymerase might compensate for the *pol* ts mutation and allow growth at 36°C. However, a *pol1-15* strain transformed with pPOL1-15 remained ts. The mutant polymerase activity in the *pol1-15* strain containing pPOL-15 was at most 2-fold greater than in *pol1-15* strains containing YEp24, while POL^+ strains containing pPOL1-1 express ≈6-fold higher levels of polymerase than POL^+ with YEp24 (unpublished work; ref. 10). Thus, a 2-fold overproduction of the mutant polymerase cannot compensate for the replication defect. Similar experiments were carried out for the remaining mutants, but in none of the mutant strains did high copy number of the mutant gene result in overproduction of mutant polymerase sufficient to compensate for the growth defect.

Terminal Phenotype of *pol* ts Mutants. Cell division cycle mutants should arrest with a particular terminal phenotype after incubation at the restrictive temperature (1). After 4 hr at 36°C, >95% of the *pol1* ts cells formed dumbbell-shaped cells, as illustrated in Fig. 2. The nucleus has migrated to but

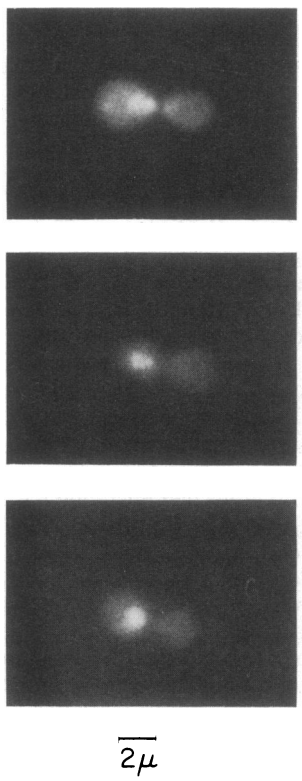

FIG. 2. Terminal phenotype of *pol1-17* strain. *pol1-17* cells were incubated at 36°C for 4 hr, fixed with methanol/acetic acid (3:1), followed by 4′,6-diamidino-2-phenylindole staining.

not entered the isthmus separating the mother and daughter cell. This nuclear morphology is slightly different from that of a 4',6-diamidine-2-phenylindole-stained spore containing a disrupted polymerase gene, in which the nucleus had migrated into the isthmus separating the mother and daughter (10). It is possible that the presence of undegraded polymerase in spores carrying the gene disruption allowed the cells to progress further in the cell cycle than the *pol1* ts mutants at 36°C. The *pol1* ts terminal phenotype is the same as *cdc2* cells, another mutant believed to be defective in replication (4, 21, 22).

There is a progressive loss in viability when logarithmic-phase cultures of *pol1-17* cells are held at 36°C. For short time periods, such as 3 hr, the arrest in DNA synthesis is reversible.

***In Vivo* DNA Synthesis.** DNA synthesis was measured at 23°C and 36°C in the parent strain SS111 and in the *pol1* mutants. Logarithmic-phase cells were labeled with [^{3}H]uracil followed by hydrolysis with alkali. Five of the mutants *pol1-11, -1-13, -1-15, -1-16,* and *-1-17,* exhibit normal DNA synthesis at 23°C (Table 1). The *pol1-14* strain is severely defective in DNA synthesis, even at 23°C. In separate experiments, we have observed that the *pol1-14* mutant grows slowly at 23°C and the cells are enlarged. RNA synthesis is also defective in the *pol1-14* mutant at 23°C, with a value of ≈40% that of SS111. In none of the other mutants is RNA synthesis defective at either 23°C or 36°C. At 36°C, DNA synthesis is defective in all the mutants. *pol1-17* and *pol1-14* mutants have the most severe defects in DNA synthesis at 36°C, with about one-quarter the amount of SS111. Since ≈5% of the DNA synthesis is mitochondrial, the rate of chromosomal DNA synthesis is probably a little less than one-quarter of wild type in these mutants. Strain *pol1-15* has the largest amount of residual DNA synthesis, with a little over one-half the amount of strain SS111. In accordance with this observation, the *pol1-15* mutant does not form terminal phenotypes as fast as the remainder of the mutants.

Chromosomal Mapping of the *POL1* Gene. Recently, a gel electrophoretic technique has been developed that separates DNA molecules up to 2000 kb by using alternately pulsed perpendicular electric fields (13, 14). Since this technique separates most of the yeast chromosomes, it can be used to map cloned genes. Total yeast DNA was separated on an agarose gel, blotted onto nitrocellulose, and probed using the nick-translated plasmid pPOL1-9, which contains the polymerase gene and CEN4 DNA (23). Comparison of the autoradiograph of the gel in Fig. 3 with the ethidium bromide-stained picture (not shown) showed that the polymerase gene mapped to either chromosome II or XIV. Although the probe pPOL1-9 contains the *URA3* gene, no hybridization to chromosome V was seen in this autoradiograph or in other autoradiographs using different URA3-containing probes. A possible explanation is that the strain SS327 contains the *ura3-52* mutation, a rearrangement of the *URA3* gene. To map the gene more precisely, the *pol1-17* and *pol1-13* mutants were crossed with *met4* and *top2* strains, respectively, followed by sporulation and dissection. *POL1* is ≈2 centimorgans (cM) away from *met4* and ≈12 cM from *top2* (Fig. 3). *pol1* does not map to any previously mapped ts gene on chromosome XIV, although it is close to *pms1* (24).

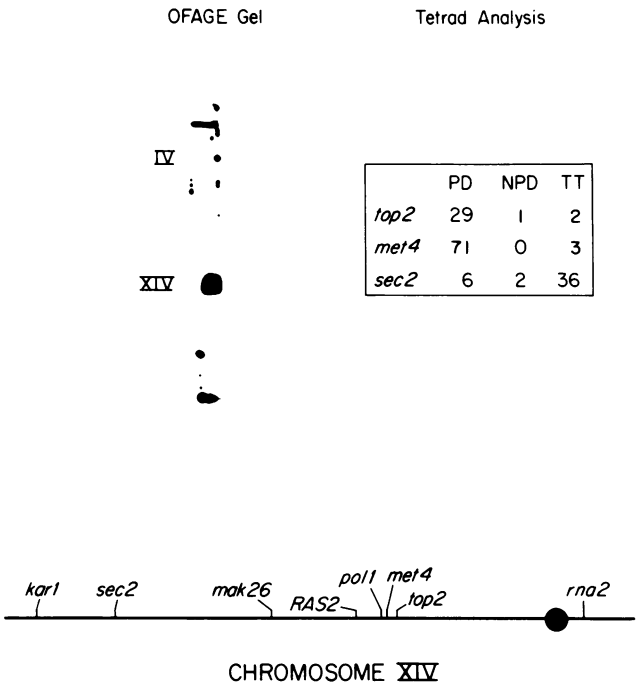

FIG. 3. (*Left*) Chromosomal mapping of the *pol1* gene. Orthogonal field gel electrophoresis of yeast chromosomal DNA was carried out at 240 V for 20.5 hr at 12°C. The gel was blotted onto nitrocellulose and then probed with the plasmid pPOL1-8. The autoradiograph indicates two bands distinct from the origin. One band is chromosome IV containing CEN4 DNA. The other band is chromosome XIV containing POL1 DNA. (*Right*) Results of tetrad analysis. PD, parental ditype; NPD, nonparental ditype; TT, tetratype.

DISCUSSION

Seven ts mutations in the DNA polymerase I gene have been identified using a plasmid exchange screen. One advantage of the screening system chosen here is that the mutant alleles were cloned in plasmids in the initial isolation. Another method, the integrative replacement technique, involves integrating a mutagenized fragment of a gene of interest into the chromosome (11). This technique requires the presence of a unique restriction site close to the 3' or 5' end of the gene. Thus, the integrative replacement strategy requires a physical map of the gene. Although a physical map of the gene is relatively easy to obtain if the cloned gene was obtained by complementation, a physical map of an essential gene that was not cloned by complementation is more time consuming. The plasmid shuffling strategy described here does not require the presence of convenient restriction sites in a gene or the knowledge of the location of the gene on a restriction fragment. This is advantageous since the polymerase gene does not have any convenient restriction sites at either the 3' or 5' end of the gene. Another advantage of this technique is that no carrier DNA is used in the transformation, a possible cause of nonspecific mutants. The technique also allowed the detection of nonpolymerase mutants in the initial screen after transformation with the hydroxylamine-treated plasmid pPOL1-8. This was important, because a number of ts mutations unlinked to the gene of interest had been observed using direct gene replacement to obtain actin mutants (11). Since the transformants have the functional polymerase gene pPOL1-7, any transformant that is ts before replica plating onto 5-fluoroorotic acid-containing medium has a nonpolymerase ts mutation. The number of nonspecific mutations was small (<0.5%), possibly because of the fact that no carrier DNA was used in the experiment. Thus, this is an efficient and specific technique for producing new alleles of a cloned gene. A similar procedure has been described by Boeke *et al.* (19).

Although all seven DNA polymerase I mutants are independent mutants, some may have arisen from identical amino acid changes. Shortle *et al.* (11) have reported that two independently derived ts actin mutants arose from mutations at identical sites. The phenotypes of six of our mutants suggest they are different (data not shown). However, further

work will be necessary to demonstrate whether each of these phenotypes is due to a single point mutation.

DNA synthesis is decreased *in vivo* in all the *pol1* mutants at 36°C without a comparable decrease in the value of RNA synthesis, suggesting that the primary defect is in DNA replication. The extent of the decrease in DNA synthesis in the mutants relative to wild type varies from about one-quarter in the *pol1-14* mutant to one-half in the *pol1-15* mutant. This residual synthesis is greater than might be expected for a mutant that apprently undergoes first cycle arrest of growth. Without further experiments, at least four explanations for the residual synthesis are possible. First, some of the mutants may be leaky, allowing some DNA synthesis but not enough to complete replication. Second, the residual incorporation may all be repair synthesis or mitochondrial DNA replication and may not represent chromosomal replication. This could be evaluated by investigating the nature of the DNA synthesized at the nonpermissive temperature. Third, DNA polymerase I may be responsible for replication of only part of the chromosome and a second polymerase, such as DNA polymerase II, may carry out the rest of the synthesis. For instance, polymerase I might carry out initiation and DNA polymerase II elongation. While current enzymological models favor the idea that the same polymerase is involved in initiation and elongation (25), it may be that DNA polymerase II can elongate when DNA polymerase I is defective. Further studies of the role of the polymerase II enzyme will address this point. Fourth, the function affected in the mutants may be required for initiation but not for elongation of DNA replication. This could arise if the mutants were defective in assembly of replication complexes at the nonpermissive temperature, but not defective in propagation of replication forks already formed at the permissive temperature. A similar idea has been favored to explain the residual synthesis in mouse mutants carrying a ts DNA polymerase α (26).

For the *pol1-14* mutant, there is the unexpected result that DNA synthesis at 23°C is even more defective than DNA synthesis at 36°C. A possible explanation is that DNA polymerase I is part of a replication complex that is stable at 23°C and 36°C in wild-type cells. Although DNA polymerase I is defective at 23°C in *pol1-14* strains, the replication complex is still stable enough to allow chromosomal replication. At 36°C, the DNA polymerase is no more defective than at 23°C; however, at 36°C, the replication complex dissociates, not allowing completion of chromosomal replication. Therefore, this may be an assembly mutant.

The genetic mapping data place the *POL1* gene near *MET4*. Another gene of interest in the region is *PMS1* (24). Mutants in *pms1* have pleiotropic effects on meiosis and mitosis. The mutants exhibit increased postmeiotic segregation frequencies and also have a mitotic mutator phenotype. It will be interesting to see whether *pms1* and *POL1* are allelic.

Many questions remain about the role of DNA polymerase I in recombination and repair, as well as in replication. These mutants provide a key way to study these phenomena.

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