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Evidence that POB1, a *Saccharomyces cerevisiae* Protein That Binds to DNA Polymerase α, Acts in DNA Metabolism In Vivo

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Potential DNA replication accessory factors from the yeast *Saccharomyces cerevisiae* have previously been identified by their ability to bind to DNA polymerase α protein affinity matrices (J. Miles and T. Formosa, Proc. Natl. Acad. Sci. USA 89:1276–1280, 1992). We have now used genetic methods to characterize the gene encoding one of these DNA polymerase α-binding proteins (POB1) to determine whether it plays a role in DNA replication in vivo. We find that yeast cells lacking *POB1* are viable but display a constellation of phenotypes indicating defective DNA metabolism. Populations of cells lacking *POB1* accumulate abnormally high numbers of enlarged large-budded cells with a single nucleus at the neck of the bud. The average DNA content in a population of cells lacking *POB1* is shifted toward the G_2 value. These two phenotypes indicate that while the bulk of DNA replication is completed without *POB1*, mitosis is delayed. Deleting *POB1* also causes elevated levels of both chromosome loss and genetic recombination, enhances the temperature sensitivity of cells with mutant DNA polymerase α genes, causes increased sensitivity to UV radiation in cells lacking a functional *RAD9* checkpoint gene, and causes an increased probability of death in cells carrying a mutation in the *MEC1* checkpoint gene. The sequence of the *POB1* gene indicates that it is identical to the *CTF4* (*CHL15*) gene identified previously in screens for mutations that diminish the fidelity of chromosome transmission. These phenotypes are consistent with defective DNA metabolism in cells lacking *POB1* and strongly suggest that this DNA polymerase α-binding protein plays a role in accurately duplicating the genome in vivo.

DNA replication in eukaryotes appears to be catalyzed by complex protein machines whose basic architecture is similar to that of the replication complexes found in prokaryotes (1, 2, 15, 22, 31, 33). However, reconstituted eukaryotic replication complexes are unable to synthesize DNA on natural templates at the rate and accuracy observed in vivo, indicating that replication accessory factors remain to be identified (34, 36). While enormous progress has been made toward understanding the replication of eukaryotic viral genomes, a method for preparing cell extracts capable of replicating genomic DNA has not yet been devised, severely limiting the approaches available for identifying replication accessory factors. Broadly interpreted, replication accessory factors could include any components that increase the probability that a cell will produce a faithful copy of its genome. In the standard prokaryotic model, accessory factors are proteins such as DNA helicases, DNA primases, and processivity factors that are needed to enhance the speed, accuracy, and template range of the core DNA polymerase enzyme (1, 15, 22). Even though these accessory factors are not needed to allow the polymerase to add a nucleotide to a suitably primed template, their presence in the cell is essential for progression of replication forks; in the absence of accessory factors, little or no DNA synthesis is observed in vivo (15, 22).

It now appears that eukaryotic replication forks have much in common with prokaryotic machinery; polymerases need the assistance of DNA-binding proteins, DNA primases, and processivity factors in ways strikingly reminiscent of prokaryotic models (3, 7, 34, 36). However, eukaryotic cells have additional layers of complexity with which to contend beyond moving the replication complex down the template in an orderly fashion. The decision to initiate replication must be strictly coordinated with other cell cycle events, multiple origins on multiple chromosomes must be used to accomplish complete replication exactly once per cell cycle, temporal and spatial programs for orderly replication must be maintained, and the adequacy of the genomic copies must be assessed as a prelude to packaging and segregation. In a broad sense, proteins that facilitate these aspects of DNA replication are also accessory factors. Since these functions are more peripheral to actual DNA synthesis than are the roles played by standard accessory factors, the proteins responsible may exert only subtle effects on DNA replication in vitro and in vivo. Therefore, detecting them by standard methods of biochemical analysis would be difficult. In the absence of an in vitro system that reconstitutes the broad range of functions needed for eukaryotic genomic duplication, we have used an alternative strategy for isolating eukaryotic replication accessory factors.

Assuming that proteins that participate in DNA replication bind to one another to form complex protein machines, it should be possible to use one replication protein to identify others. We have therefore used the technique of protein affinity chromatography with yeast DNA polymerase α as the ligand in order to find DNA replication accessory factors (8, 21). Using this technique, we identified six to eight proteins that bind specifically to yeast DNA polymerase α. The validity of this approach for finding replication accessory factors rests on the assumption that binding observed in vitro reflects interaction in vivo. To test this assumption, we must be able to generate cells lacking the putative accessory factors and we must be able to recognize whether these cells are defective in DNA metabolism. Both of these conditions can be met in the yeast *Saccharomyces cerevisiae*.

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Mutations in genes known to be needed for normal DNA replication in *S. cerevisiae* cause a common set of phenotypes. Conditional mutations in DNA polymerases α and δ (*cdc17* and *cdc2* alleles) and in DNA ligase (*cdc9*) cause yeast cells to display elevated levels of both genetic recombination and chromosome loss when grown under semipermissive conditions (12, 23). These mutations also cause a characteristic morphology in which cells fail to progress through mitosis but arrest instead with a single nucleus at the neck of a large bud (26). Surprisingly, even cells with mutations in essential polymerases usually arrest with greater than a normal G_1 DNA content and often with nearly the G_2 content, indicating that cells with temperature-sensitive core replication machinery can complete the bulk of DNA replication (3–5). The arrested morphology of cells with replication-defective genes is dependent on the presence of functional checkpoint genes such as *RAD9*, *MEC1*, and *MEC2* that appear to monitor the quality of the genome before mitosis is allowed to proceed (13, 36a–38). Since mutations in genes known to be needed for DNA replication induce this constellation of phenotypes, new mutations causing these same features are likely to be in genes required for normal DNA replication as well.

We have cloned, sequenced, and disrupted the gene encoding one of the DNA polymerase α-binding proteins, which we have named POB1. Cells lacking the *POB1* gene display a set of phenotypes that are consistent with the interpretation that POB1 protein plays a role in DNA metabolism. This result validates the assumptions underlying our basic approach for identifying replication accessory factors.

Members of the laboratories of Hieter (Johns Hopkins Medical School) and Larionov (Academy of Sciences of Russia, St. Petersburg, Russia) (16–18, 32) have screened yeast cells directly for mutants with elevated genetic recombination and chromosome loss phenotypes. One of these, *CTF4* (*CHL15*), is identical to the *POB1* gene. A comprehensive genetic analysis of the effects of mutations at this locus is presented in the accompanying paper by Kouprina et al. (16), whose results are also consistent with the conclusion that *CTF4* (*CHL15*) plays a role in making usable copies of the genome.

MATERIALS AND METHODS

Media and strains. Synthetic medium (27), YEPD (27), and YM-1 (11) were prepared as described previously. All strains used (Table 1) are isogenic with strain A364a and were derived from the collection of L. Hartwell (University of Washington) by standard genetic methods (27). Strain 7862 is from T. Weinert (University of Arizona); all others are from this laboratory. Since *POB1* and *CTF4* are allelic, future communications will use the *CTF4* designation for this locus, and the Δ*POB1*::*TRP1* allele described in this report will be designated *ctf4-Δ4*.

Protein sequencing. POB1 protein was isolated from yeast whole cell extracts of strain 7208-12 essentially as described previously (21). Briefly, extracts were prepared from a protease-deficient yeast strain that had been grown either in synthetic medium supplemented with [^{35}S]methionine or in rich medium (YM-1). These extracts were chromatographed in low-salt buffer (50 mM KCl in buffer) on an Affi-Gel 10 (Bio-Rad) column containing covalently attached yeast DNA polymerase α catalytic subunit and were eluted with buffer containing 800 mM KCl. Fractions containing eluted proteins were concentrated as described previously (21), and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the gel were blotted to a ProBlott polyvinylidene difluoride membrane (Applied Biosystems), the membrane was rinsed first in H_2O and then in methanol, and proteins were detected by staining with Coomassie blue dye (0.1% in 40% methanol–1% acetic acid) and destaining in 50% methanol. The band containing POB1 protein was excised and submitted to Edman degradation, using an Applied Biosystems 477A protein sequencer. Several trials were attempted; all trials were consistent for the initial amino acids, and one trial gave enough signal to read the entire 20 amino acids reported in Fig. 1.

Cloning the *POB1* gene. Since the 20 amino acids of protein sequence did not contain any regions whose nucleotide coding sequence could be inferred unambiguously, two pools of 23-base oligonucleotides were synthesized that together contained 384 sequences representing one-half of the sequences that could possibly encode the amino acids DKLVFDFG (omitting the ambiguous third position for G; Fig. 1). Since valine is usually encoded in yeast messages by GUU or GUC codons (6), the possible oligonucleotides with GUA or GUG were omitted from the pools. An exact match to a 23-mer should dissociate at a temperature near 58°C in the hybridization solution used (6, 28).

The oligonucleotides were 5′ end labeled with ^{32}P for use as hybridization probes (28). Yeast genomic DNA was digested with restriction endonucleases; fragments were separated by agarose gel electrophoresis and then transferred to Nytran membranes (Schleicher & Schuell) essentially as described previously (28). Membranes containing these fragments were probed under the conditions given elsewhere (6) at 44, 48, 52, or 56°C and then washed at the same temperature. Under these conditions, oligonucleotide pool 2 hybridized to a 7.5-kb *Eco*RV fragment even at 56°C. Oligonucleotide pool 1 also hybridized to this same fragment but dissociated at about 52°C. This finding indicated that a single gene in the yeast genome hybridized at high stringency with a member of oligonucleotide pool 2.

A genomic library of approximately 10-kbp fragments obtained by partial *Sau*3A digestion of yeast DNA was prepared by Hua-Ming Wang and David Stillman (University of Utah) in the *Bam*HI site of vector pTF63 (a derivative of YEplac195 [10] in which the *Pvu*II fragment containing the polylinker region was substituted with the *Pvu*II polylinker fragment from the pBSII KS+ vector [Stratagene]). The library was transformed into *Escherichia coli* DH5α by electroporation (model T100 apparatus [Biotechnologies and Experimental Research, Inc., San Diego, Calif.], used according to the manufacturer's instructions) and plated onto selective medium. Duplicate impressions of about 20,000 bacterial colonies were made on Nytran membranes, cells were lysed, and DNA was denatured essentially as described previously (6). These membranes were probed with the labeled oligonucleotide pool 2. The hybridization and washes were performed at 52°C. Positive colonies were retested, and one colony that retained a positive signal at high stringency was recovered. The region of the clone containing homology to the oligonucleotide was determined by probing blots of restriction digests of this plasmid with the oligonucleotide pool (28). The homologous region was subcloned into pBSII KS+ and sequenced.

DNA sequencing. Nested sets of deletions were generated by the exonuclease III method (14), and plasmids were sequenced in both directions by the dideoxy-chain termination method, using the Sequenase enzyme and protocols (U.S. Biochemical). A FASTA search of GenBank se-

TABLE 1. Strains used

Strain	Genotype
7208-12	*MATa/MATα his7/his7 ura3-52/ura3-52 trp1/trp1 pep4-3/pep4-3 prb1-1122/prb1-1122 can1/can1*
7236	*MATa/MATα his3/HIS3 his7/HIS7 can1/CAN1 cyh2/CYH2 ura3-52/ura3-52 leu2/leu2 trp1/trp1*
7373-3-3	*MATα trp1 leu2 ura3 his3 ΔRAD9::LEU2*
7373-10-2	*MATα trp1 leu2 ura3 his3 ΔRAD9::LEU2 ΔPOB1::TRP1*
7380-3-2	*MATa trp1 leu2 ura3 his3 can1 cyh2 ΔPOB1::TRP1*
7380-6-1	*MATa trp1 leu2 ura3 his3 can1 cyh2*
7380-6-4	*MATa trp1 leu2 ura3 his3 can1 cyh2 ΔPOB1::TRP1 mec1-1*
7380-19-3	*MATa trp1 leu2 ura3 his3 can1 cyh2 mec1-1*
7390-5-1	*MATα trp1 leu2 ura3 his3 his7 cdc17-1*
7390-5-2	*MATa trp1 leu2 ura3 his3 his7 cdc17-1 ΔPOB1::TRP1*
7390-7-2	*MATa trp1 leu2 ura3 his3 his7 can1 ΔPOB1::TRP1*
7391-1-1	*MATa trp1 leu2 ura3 his3 cdc17-2*
7391-4-2	*MATa trp1 leu2 ura3 his3 cdc17-2 ΔPOB1::TRP1*
7404	*MATa/MATα trp1/trp1 leu2/leu2 ura3/ura3 his3/HIS3 HIS7/his7 CYH2/cyh2 can1,hom3/CAN1,HOM3 ΔPOB1::TRP1/ΔPOB1::TRP1*
7407	*MATa/MATα trp1/trp1 leu2/leu2 ura3/ura3 his3/HIS3 HIS7/his7 CYH2/cyh2 can1,hom3/CAN1,HOM3 ΔPOB1::TRP1/POB1*
7411-10-3	*MATa trp1 leu2 ura3 his3 ΔPOB1::TRP1*
7862	*MATa/MATα TRP1/trp1 leu2/leu2 ura3/ura3 his3/HIS3 HIS7/his7 can1,hom3/CAN1,HOM3*

quences (24) revealed that *POB1* is adjacent to *MSS18*; *POB1* is the URF identified by Seraphin et al. (29) that appears to produce a 2.8-kb mRNA.

Deletion of the *POB1* gene. A 591-bp DNA fragment containing the 3′ end of the *POB1* gene from the *Pst*I site (at +2691 relative to the initiating AUG) to the *Eco*RI site (at +3282) was inserted into *Pst*I- and *Eco*RI-digested pRS304 (30) to produce pJM16. Another DNA fragment from an *Eco*RI site about 1,800 bp upstream of the *POB1* open reading frame (ORF) to the *Hin*dIII site 55 bp upstream of the *POB1* ORF was cloned into *Eco*RI- and *Hin*dIII-digested pJM16 to produce pJM17. After digestion with *Eco*RI, pJM17 was used to transform yeast cells by the lithium acetate method essentially as described previously (27), forming a so-called γ deletion (30).

Fixing cells for microscopy and flow cytometry. For microscopy, cells were grown in YM-1 to logarithmic phase (A_{600}s of 0.2 to 0.4 for haploids and 0.2 to 0.6 for diploids), fixed in 3.7% formaldehyde for 30 to 60 min, washed in methanol (10 min, −20°C) and acetone (5 min, room temperature), rinsed with H_2O, suspended in phosphate-buffered saline (PBS; 10 mM potassium phosphate [pH 7], 150 mM NaCl), sonicated for 5 to 10 s, and stained with 0.1 μg of DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) per ml. Cells were mounted on polylysine-coated glass slides essentially as described previously (25).

For flow cytometry, cells were grown to logarithmic phase, collected by centrifugation, suspended in 70% ethanol for 60 min, washed in 0.2 M Tris-Cl (pH 7.5)–20 mM Na_3EDTA, and then suspended in the same buffer containing 1 mg of RNase A per ml. The samples were incubated for 2 to 4 h at 37°C, washed in PBS, and then stained in PBS containing 50 μg of propidium iodide per ml for 1 h at room temperature. The samples were then diluted 10-fold with PBS, sonicated for 5 to 10 s, and analyzed by flow cytometry.

Chromosome stability assay. Diploids heterozygous for mutations in *hom3* and *can1* and either homozygous wild

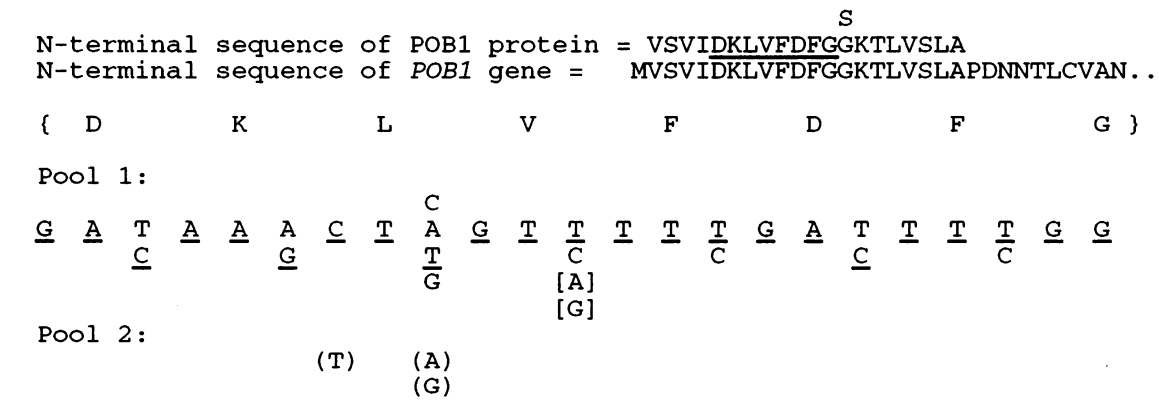

FIG. 1. Amino acid sequence of POB1 protein and oligonucleotides used to clone the gene. The underlined portion of the amino acid sequence of POB1 protein was used to direct the sequence of two pools of 23-base oligonucleotides. (The amino acid sequence was ambiguous at position 13.) Pool 1 contained the degenerate sequence shown at the top; pool 2 was the same except that the bases in parentheses were substituted for those above them. The bases in brackets would have given complete coverage of the possible oligonucleotide sequences but were not included in either pool. Underlined bases correspond to the sequence of the *POB1* gene. The N-terminal amino acid sequence of the gene cloned using these oligonucleotides as a probe is shown below the protein sequence.

type at the *POB1* locus, homozygous for deletions of *POB1*, or heterozygous at this locus were constructed by using standard genetic methods (27). The stability of chromosome V was measured in these strains by fluctuation analysis by the method of the median as described elsewhere (12, 19).

Cell viability and UV sensitivity. To assess cell viability, cultures were grown to logarithmic phase, sonicated for 5 s, diluted, and plated to YEPD. Fields were viewed microscopically after 16 and 40 h of growth at room temperature to determine the total number of cells plated. Foci containing less than about 10 cells after 40 h were counted as inviable. Sensitivity to UV light was assessed by applying the same log-phase cultures to YEPD plates and then placing the plates facedown with the covers removed on a UV light box for various times (Fotodyne FotoPrep I, preparative setting; UV fluence was approximately 3 $J/m^2/s$ according the manufacturer's specifications; because of the age of the device, the absolute fluence may not be accurate). Plates were incubated in the dark but were otherwise exposed to ambient visible light and may have experienced some photoreactivation. CFU were counted after 3 days at room temperature.

Nucleotide sequence accession number. The sequence data shown in Fig. 2 have been deposited in GenBank (accession number M94769).

RESULTS

Isolation and sequencing of the *POB1* gene. *S. cerevisiae* DNA polymerase α was overexpressed, purified, and covalently attached to an agarose matrix. This matrix was used to chromatograph extracts of yeast cells (21). Using this procedure, we identified an approximately 115-kDa protein that bound to polymerase α but not to control columns. We named this protein POB1, for polymerase one-binding protein. From the total amount of POB1 protein present in an extract, we calculate that a diploid yeast cell contains 1,000 to 2,000 copies of POB1 protein. Since a diploid yeast cell also contains about 1,000 copies of DNA polymerase α, this level of POB1 protein is consistent with a role in vivo in a stoichiometric complex with the polymerase α protein.

The eluates from multiple passages over a DNA polymerase α column were pooled, concentrated, and subjected to SDS-PAGE. Proteins in the gel were blotted to membranes, stained, excised, and sequenced by Edman degradation as described in Materials and Methods. The N-terminal 20 amino acids were identified as shown in Fig. 1. Two oligonucleotide pools with about a 75% probability of containing the coding sequence for a seven-amino-acid region were synthesized (Fig. 1). These oligonucleotide pools were used as hybridization probes to screen a library of yeast genomic sequences. A single clone was found to hybridize at high stringency with pool 2. The region containing the homology was subcloned, and its nucleotide sequence was determined (Fig. 2). An ORF capable of encoding a 105-kDa protein was identified; with the exception of the initiating methionine, the sequence of the first 20 amino acids of this ORF was identical to the 20-amino-acid sequence determined for POB1 protein (Fig. 1). Only seven residues of POB1 protein sequence were used to generate the oligonucleotide probe, yet all 20 amino acids of POB1 protein match the conceptual translation of the nucleotide sequence's ORF, which is strong evidence that we have cloned the gene encoding POB1 protein. POB1 protein appears to be processed to remove the initiating methionine in vivo.

A search of GenBank with the *POB1* gene nucleotide sequence revealed no homologous proteins. A search of a library including unpublished sequences by Goebl (10a) indicated a match between *POB1* and *CHL15*. *CHL15*, which was previously shown to be an allele of *CTF4*, was identified independently in the laboratories of Larionov and Hieter in screens for genes which when mutated cause elevated levels of chromosome loss (17, 18, 32). The accompanying report (16) details the genetic characterization of the *CTF4* gene. We found that the *POB1* gene appears to reside on chromosome XVI by probing filters containing separated whole yeast chromosomes (data not shown); *CHL15* has been more precisely mapped by Kouprina et al. (16) to a locus on the right arm of chromosome XVI. Since both genes appear to be present in single copy, as judged by hybridization analysis of genomic DNA blots (data not shown), we conclude that *POB1*, *CHL15*, and *CTF4* are identical. By mutual agreement, future communications will use the *CTF4* designation for this gene.

There is a single *Mlu*I site near the 5′ end of the *POB1* ORF (Fig. 2). This sequence has been associated with genes involved in DNA replication in yeast cells and has been found to confer cell cycle-dependent regulation of transcription (20, 35). Other features of POB1 protein are discussed by Kouprina et al. (16).

Deletion of the *POB1* coding region. To assess the role played by *POB1* in vivo, we constructed plasmid pJM17 for deleting the *POB1* gene (Fig. 3). In this construction, vector sequences replace 97% of the *POB1* ORF, from a site 55 bp upstream of the initiating AUG to a site 90 bp upstream of the terminus. Linear pJM17 molecules were used to transform a diploid yeast strain (see Materials and Methods). Independent isolates were checked for correct replacement by using Southern blot analysis; then these diploids were sporulated and haploid derivatives were isolated.

Haploids lacking the *POB1* gene are viable and form colonies at about the same rate as do wild-type cells. This result shows that either the role played by *POB1* is not essential to the cell or some other gene is capable of at least partially playing that role. (Although haploids are viable, to our surprise we have not been successful at obtaining cells with the correct replacement by direct transformation of haploids.) However, the colonies carrying the deletion are made up of cells with an unusual morphology (Fig. 4 and 5). These cells are somewhat larger than normal, are more likely than wild-type cells to be large budded (Fig. 6), and are more likely than wild-type cells to have a single nucleus at the neck of the bud. The *POB1*-deleted cell cultures also contain a significant proportion of anomalous conglomerates seen only rarely in wild-type cultures, such as doubly budded cells, cells with either two or no nuclei, and cells with apparently fragmented nuclei or aggregated mitochondria (Fig. 5). We interpret this morphology as indicating an inability to produce genomic copies suitable for segregation, causing cells to delay before mitosis. The cells continue to grow during this delay, so that they become larger than normal, allowing the eventual segregants to experience a minimal G_1 and instead proceed directly through start. A similar phenotype was observed for cells lacking *CTF1* (9).

The DNA content of Δ*POB1* cells is altered. The DNA content of cells either lacking or containing the normal *POB1* gene was analyzed by flow cytometry (Fig. 7). This analysis revealed that cells lacking the *POB1* gene have a higher DNA content than do wild-type cells and that the distribution between apparent G_1 and G_2 populations is shifted toward the G_2 cells. The higher DNA content is due to the increased size of the cells lacking *POB1*, since these cells have more mitochondrial DNA (data not shown). This view

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ACAAGGCTTATCCATATTCGTATATATCTTCATAATGTTTCATAATTGCTTCAGAAAATTATTCAAAACGTCTTGTGATATTCATTAGGT  90
GAAATCGTAGTTTGCCTGATACTTGAAAGAATCTCACCTGGAGAATGGGTGTTTTCACTAAGATTCGTTAATTTCCACTAATGATTAATT 180
GTTAACTGTTTAACGCGACGCGTAATAAAGTTTCCTGAATACGCCAACATATGGGAACATATAGATTAAATTAATAAGAAAGCTTGGGAA 270

                                                    M  V  S  V  I  D  K  L  V  F  D  F  G   13
AAATAATTGAGAAGGGCAAGAAGTGACGTAAATATACTAGACGTACTATTATGGTTTCAGTTATAGACAAGCTTGTTTTTGACTTTGGTG 360

G  K  T  L  V  S  L  A  P  D  N  N  T  L  C  V  A  N  K  N  G  L  T  K  I  L  K  T  N  N   43
GGAAAACTCTGGTCTCTCTCGCACCAGATAATAATACTTTGTGTGTAGCCAATAAAAATGGCTTAACCAAGATTCTGAAGACAAATAACC 450

P  E  E  E  P  E  T  L  D  S  S  K  L  V  S  S  I  K  C  Y  S  N  S  H  F  L  M  T  T  M   73
CAGAAGAAGAGCCAGAGACTTTAGATTCTTCCAAATTGGTCTCGTCTATAAAATGCTATTCAAACTCGCACTTTCTGATGACTACAATGC 540

Q  G  D  A  L  R  Y  N  I  D  S  S  Q  E  E  L  L  A  R  F  A  L  P  L  R  D  C  C  V  I   103
AAGGTGATGCTCTTAGGTATAACATCGATTCTAGTCAAGAAGAATTATTGGCTAGATTTGCTTTACCCCTACGTGACTGCTGTGTTATTC 630

H  S  G  K  M  A  V  F  G  G  D  D  L  E  L  I  L  L  E  L  D  D  E  T  H  K  K  H  A  I   133
ATTCAGGTAAAATGGCCGTATTTGGAGGAGACGACTTAGAACTAATCCTTTTGGAATTGGACGATGAAACACACAAAAAACATGCCATTA 720

K  I  D  E  Q  V  S  Q  I  S  Y  N  S  Q  M  N  I  L  A  V  S  M  I  N  G  K  V  Q  I  F   163
AAATCGATGAACAAGTTTCTCAAATTTCTTACAATTCACAGATGAATATTTTAGCAGTTTCAATGATAAATGGTAAGGTACAAATTTTTT 810

S  L  T  S  T  I  P  N  K  V  H  E  L  N  D  Y  I  V  A  N  S  Y  D  D  T  H  R  D  K  I   193
CTCTGACATCTACTATTCCAAACAAAGTTCATGAGTTAAATGATTACATAGTGGCCAATTCATATGATGATACACACAGAGATAAGATAC 900

L  S  N  M  M  D  D  I  D  K  D  N  D  N  D  L  S  E  T  A  D  P  D  E  N  N  V  A  D  P   223
TCTCGAATATGATGGATGATATAGATAAAGACAATGATAATGACCTGAGTGAAACGGCTGATCCAGATGAGAACAATGTAGCTGATCCAG 990

E  F  C  A  A  N  R  I  C  T  R  V  A  W  H  P  K  G  L  H  F  A  L  P  C  A  D  D  T  V   253
AATTCTGTGCTGCTAATAGAATTTGCACGAGAGTGGCTTGGCATCCGAAGGGTCTGCATTTTGCGCTACCATGTGCAGATGATACAGTAA 1080

K  I  F  S  I  K  G  Y  S  L  Q  K  T  L  S  T  N  L  S  S  T  K  A  H  F  I  D  L  Q  F   283
AAATATTCTCTATAAAGGGATATTCCCTACAAAAGACGTTGTCCACAAATCTCTCATCAACAAAGGCTCATTTCATTGATTTGCAATTTG 1170

D  P  L  R  G  T  Y  I  A  A  V  D  L  N  N  K  L  T  V  W  N  W  E  T  S  E  I  H  Y  T   313
ACCCGTTACGTGGAACTTACATTGCGGCAGTAGATTTAAATAATAAGTTAACGGTATGGAATTGGGAAACTTCCGAGATCCACTACACCA 1260

R  E  F  K  R  K  I  T  N  I  A  W  K  I  Q  A  D  S  K  T  L  D  L  V  L  G  T  W  S  G   343
GGGAATTCAAAAGAAAAATTACTAATATTGCCTGGAAAATCCAAGCGGACTCAAAAACTCTAGACCTTGTTTTAGGTACCTGGTCCGGTA 1350

S  I  A  I  V  Q  N  L  A  E  S  V  V  S  N  I  P  D  Q  S  V  A  E  S  S  T  K  H  G  L   373
GTATAGCCATTGTCCAAAATTTGGCAGAGTCCGTAGTATCTAATATACCTGACCAATCTGTTGCTGAATCTTCAACTAAACATGGGCTTT 1440

F  V  D  S  E  S  D  L  E  N  L  E  G  N  D  D  I  N  K  S  D  K  L  F  S  D  I  T  Q  E   403
TTGTAGACTCCGAATCTGACTTGGAAAACTTAGAGGGAAATGATGATATAAACAAAAGCGATAAGCTATTTTCAGATATTACTCAAGAAG 1530

A  N  A  E  D  V  F  T  Q  T  H  D  G  P  S  G  L  S  E  K  R  K  Y  N  F  E  D  E  E  D   433
CGAATGCGGAAGATGTGTTCACTCAAACACACGACGGCCCCAGTGGATTAAGTGAAAAGAGAAAATACAACTTCGAAGATGAAGAAGACT 1620

F  I  D  D  D  D  G  A  G  Y  I  S  G  K  K  P  H  N  E  H  S  Y  S  R  V  H  K  T  H  S   463
TTATTGATGATGACGATGGTGCTGGTTATATTAGTGGCAAAAAACCACATAATGAACATTCTTATTCGAGAGTACACAAGACTCATTCGT 1710

F  P  I  S  L  A  N  T  G  K  F  R  Y  M  P  F  S  P  A  G  T  P  F  G  F  T  D  R  R  Y   493
TTCCAATCAGTTTGGCAAACACAGGAAAATTTCGTTATATGCCTTTTTCTCCAGCGGGAACACCTTTTGGCTTTACTGACAGGCGTTATT 1800

L  T  M  N  E  V  G  Y  V  S  T  V  K  N  S  E  Q  Y  S  I  T  V  S  F  F  D  V  G  R  F   523
TGACAATGAATGAAGTGGGCTACGTATCTACTGTCAAGAATAGTGAGCAATACAGCATAACTGTCTCTTTTTTTGATGTTGGACGTTTTA 1890

R  E  Y  H  F  E  D  L  F  G  Y  D  L  C  F  L  N  E  K  G  T  L  F  G  Q  S  K  T  G  Q   553
GAGAATACCATTTCGAGGACTTATTTGGCTACGATTTATGCTTCCTAAATGAAAAAGGCACTTTATTTGGCCAATCCAAAACTGGGCAGA 1980

I  Q  Y  R  P  H  D  S  I  H  S  N  W  T  K  I  I  P  L  Q  A  G  E  R  I  T  S  V  A  A   583
TACAATATAGGCCACACGATAGCATACATTCAAACTGGACCAAGATTATTCCTTTGCAAGCTGGTGAGAGAATAACAAGTGTGGCAGCCA 2070

T  P  V  R  V  I  V  G  T  S  L  G  Y  F  R  S  F  N  Q  F  G  V  P  F  A  V  E  K  T  S   613
CCCCGGTTCGCGTTATTGTTGGTACATCATTAGGCTATTTCAGAAGTTTCAATCAATTCGGGGTTCCATTTGCTGTTGAAAAGACATCCC 2160

P  I  V  A  L  T  A  Q  N  Y  R  V  F  S  V  H  Y  S  Q  F  H  G  L  S  Y  S  L  S  E  L   643
CAATTGTAGCGCTTACTGCTCAGAATTATAGGGTTTTTTCAGTACATTATTCGCAGTTTCATGGCCTTTCATACTCTTTATCTGAATTGG 2250

G  T  S  S  K  R  Y  Y  K  R  E  C  P  L  P  M  S  L  P  N  I  N  S  D  M  K  K  D  A  N   673
GTACTTCTAGTAAAAGGTACTATAAAAGAGAGTGTCCACTTCCAATGAGTTTACCAAACATTAATTCTGATATGAAAAAAGACGCAAATC 2340

L  D  Y  Y  N  F  N  P  M  G  I  K  S  L  F  F  S  S  Y  G  D  P  C  I  F  G  S  D  N  T   703
TTGACTACTACAATTTTAATCCGATGGGCATCAAAAGTTTGTTCTTTTCAAGCTACGGAGATCCATGCATTTTTGGGTCCGACAACACGC 2430

L  L  L  L  S  K  W  R  S  P  E  E  S  K  W  L  P  I  L  D  S  N  M  E  I  W  K  M  S  G   733
TTCTATTGTTATCAAAGTGGAGATCACCAGAAGAAAGTAAATGGCTCCCTATTCTAGATAGCAACATGGAAATATGGAAGATGTCAGGAG 2520

G  K  E  T  T  D  I  H  V  W  P  L  A  L  A  Y  D  T  L  N  C  I  L  V  K  G  K  H  I  W   763
GGAAGGAAACGACAGATATACATGTCTGGCCCTTGGCTTTGGCGTATGACACATTGAATTGTATCTTAGTTAAGGGCAAGCATATATGGC 2610
```

FIG. 2. Nucleotide sequence of the *POB1* gene. An *Mlu*I site near the 5′ end of the *POB1* gene is highlighted.

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P  E  F  P  L  P  L  P  S  E  M  E  I  R  M  P  V  F  V  K  S  K  L  L  E  E  N  K  A  I    793
CCGAGTTTCCCCTTCCGTTGCCATCCGAAATGGAGATTAGAATGCCAGTATTTGTTAAGAGTAAATTACTAGAGGAAAACAAAGCTATAT  2700

L  N  K  K  N  E  I  G  A  D  T  E  A  E  E  G  E  E  D  K  E  I  Q  I  P  V  S  M  A  A    823
TAAATAAAAAGAACGAAATTGGAGCTGACACCGAAGCGGAAGAAGGGGAAGAAGACAAAGAAATACAGATTCCTGTTTCTATGGCGGCGG  2790

E  E  E  Y  L  R  S  K  V  L  S  E  L  L  T  D  T  L  E  N  D  G  E  M  Y  G  N  E  N  E    853
AAGAAGAGTATCTGCGCAGCAAGGTTTTGTCAGAGCTGTTGACAGATACACTCGAAAATGACGGTGAAATGTACGGCAACGAAAATGAGG  2880

V  L  A  A  L  N  G  A  Y  D  K  A  L  L  R  L  F  A  S  A  C  S  D  Q  N  V  E  K  A  L    883
TATTGGCAGCATTGAACGGTGCATATGATAAGGCTTTGTTACGTTTATTTGCGTCTGCATGCTCAGACCAAAATGTTGAAAAGGCTCTTT  2970

S  L  A  H  E  L  K  Q  D  R  A  L  T  A  A  V  K  I  S  E  R  A  E  L  P  S  L  V  K  K    913
CGCTTGCTCATGAATTAAAACAAGATAGAGCACTTACTGCAGCCGTTAAGATATCAGAAAGAGCTGAGCTGCCCTCTCTCGTTAAAAAAA  3060

I  N  N  I  R  E  A  R  Y  E  Q  Q  L  K                                                    927
TTAATAATATAAGGGAAGCTAGATATGAACAGCAATTGAAATAAAAAAATGTAAAATATATATACGCAAGAGACAATTATTTGATACCTG  3150
TTCATTTGTTCAAATCATCGTTCAAAATGCTCCCACATTAACATATATTTCTTTGAATGCGACGATAATAAAGAGTTACGTTGAACATGA  3240
TCTGAGCTGGAAGCGTCCTTGCGAATTTTCGCGAAAGGAAAAAGTGAAAAAATATGAAAAAAAAAAAAAAGAGATGACATTTTATAGATGA  3330
GCTTTGATTAAACAGTGTAACACCTGGTCCTGTACCGTAAATATTCAAATAAGCATCAATTCAAAGTTCTTTATTTCTTCTTTAGCATTT  3420
CAAAATTATAGAAAATCTAGAAAAGATGTCAGATGTTACCCAACAGAAAAAGAGGAAAAGATCCAAAGGAGAAGTTAATCCATCAAAACC  3510
CACAGTAGACGAAGAAATCACAGATCCATCCTCAAACGAAGATGAACAACTAGAAGTTTCGGATGAAGAAGACGCATTGGAATCTGAAGA  3600
AGAATTCGAGGGTGAAAATCCAGCCGATAAAAGAAGGAGGCTTGCAAAGCAATATCTGGAGAATCTGAAAAGCGAACGTAATGACATTTT  3690
AACTGATAATAGGAACGCTGAAGAAAAA                                                                3718
```

FIG. 2—*Continued*

is consistent with the excess of punctate staining observed in the Δ*POB1* cells (Fig. 4). Attempts to derive cell lines lacking mitochondrial DNA so that the nature of the excess DNA could be unambiguously determined have not been successful.

Chromosome stability is affected by deletion of the *POB1* gene. Mutations in genes involved in DNA replication display elevated levels of both genetic recombination and chromosome loss. This feature distinguishes them from mitotic defects that show only elevated chromosome loss. We have used an assay that detects both mitotic recombination and aneuploid monosome production at chromosome V in *S. cerevisiae* (12) to measure the rates of these events in wild-type and *POB1*-deleted diploids. We find that cells lacking the *POB1* gene undergo recombination and also experience chromosome loss ~10 times more frequently

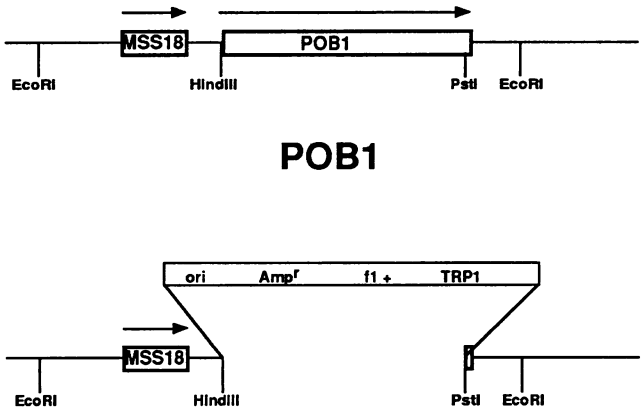

FIG. 3. Deletion of the *POB1* gene. pJM17 was constructed from pRS304 (30) and used to delete the *POB1* gene as described in Materials and Methods. The expected structure of the *POB1* locus after integration of pJM17 is shown. The adjacent gene *MSS18* encodes a mitochondrial protein which was described by Seraphin et al. (29).

than do wild-type cells (Table 2). Using a broader spectrum of assays, Kouprina et al. (16) have also documented elevated levels of chromosome loss and genetic recombination in cells containing mutations in *CTF4*.

Genetic interactions with other mutations. The morphology of the *POB1*-deleted cells suggests that these mutants experience difficulty progressing from S phase into mitosis, similar to known DNA replication mutants. Weinert and Hartwell have shown that yeast cells pause in G_2 to repair radiation-induced damage only in the presence of normal checkpoint gene products (13, 37, 38). Weinert (36a) and Weinert and Hartwell (13, 37, 38) have elucidated both apparent S-phase (*MEC1* and *MEC2*) and G_2 (*RAD9*) controls that appear to assess the quality of the products of replication prior to mitosis. One explanation for the accumulation of *POB1*-deleted cells as large, large-budded cells with the nucleus poised for entry into mitosis is that they contain replication errors that invoke the control of such a checkpoint. We have found that double mutants with the *POB1* deletion and either a *mec1-1* mutation or a deletion of *RAD9* have distinct phenotypes.

Haploids containing both the *mec1-1* and Δ*POB1* mutations exhibit a more severely distorted cellular morphology than does either single mutant (Fig. 4), and cultures of this double mutant contain a high proportion of dead cells (about 40%; Table 3). Since the penetrance of the *mec1-1* allele is not complete (36a), it is possible that *MEC1* is the only checkpoint responsible for sensing the defect in cells lacking *POB1*; it is certainly at least partially responsible for arresting cells with damage induced by the lack of *POB1*, since the double-mutant cells die at a high frequency. We interpret this sensitivity to the *mec1-1* mutation as strong evidence that the cells lacking *POB1* are defective in DNA metabolism.

Haploids containing both the Δ*POB1* and Δ*RAD9* mutations are morphologically similar to single Δ*POB1* mutant strains, but they exhibit higher sensitivity to UV than does either single mutant (Fig. 8). Therefore, while *RAD9* is not needed to cause the *POB1*-deleted cells to accumulate in G_2, some damage induced by UV that is normally repaired before the *RAD9* checkpoint persists for a longer time in cells lacking *POB1*.

Since *POB1* was isolated as a gene encoding a protein that binds to the yeast DNA polymerase α, we determined

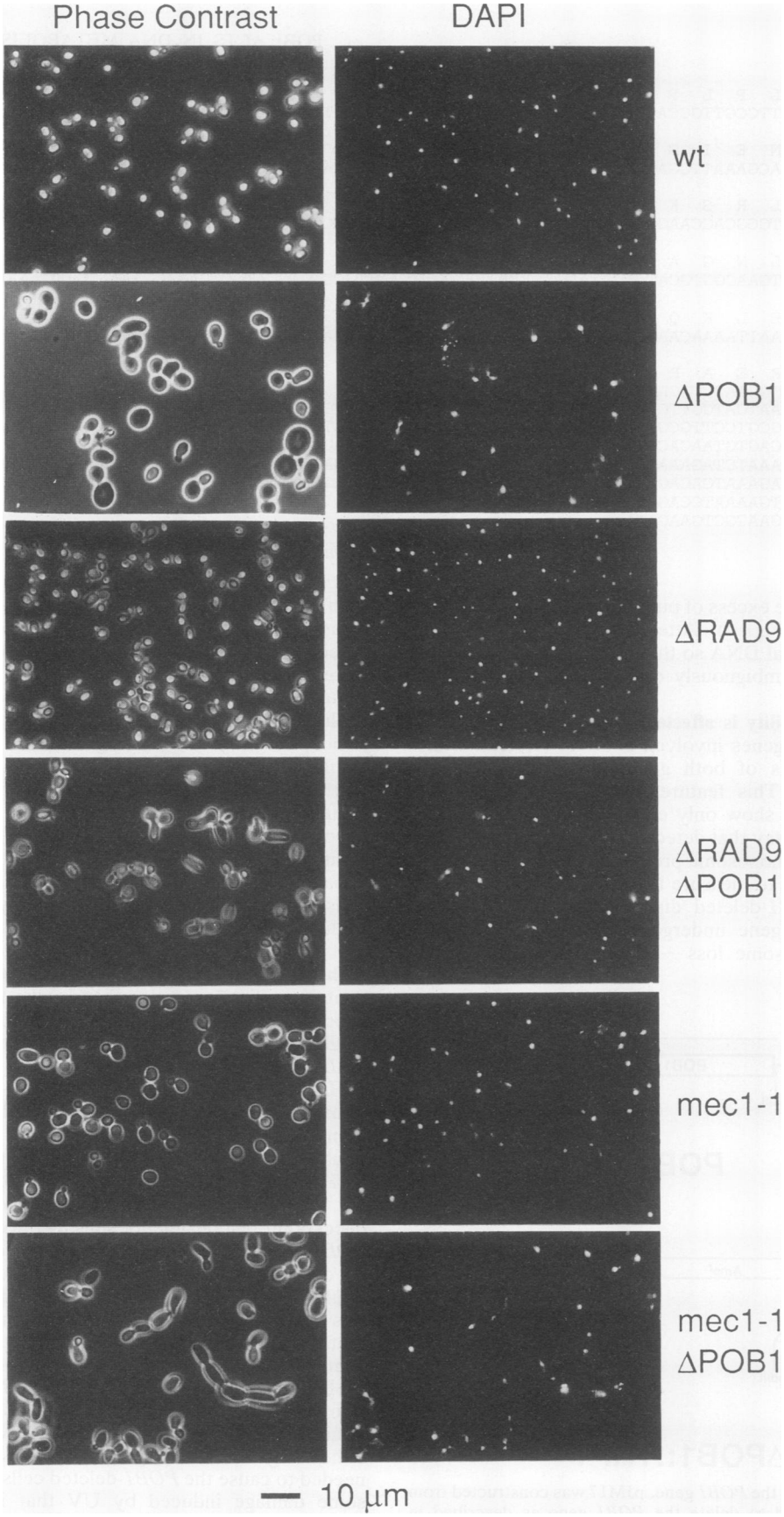

FIG. 4. Morphologies of multiple mutants lacking the *POB1* gene. Cells were fixed and stained as described in Materials and Methods. Cells were photographed by using phase-contrast optics with visible light (Phase Contrast) or by using epifluorescence with UV illumination and appropriate filters to detect DNA (DAPI); all photographs are reproduced at the same magnification. The strains shown are the same as those in Table 3.

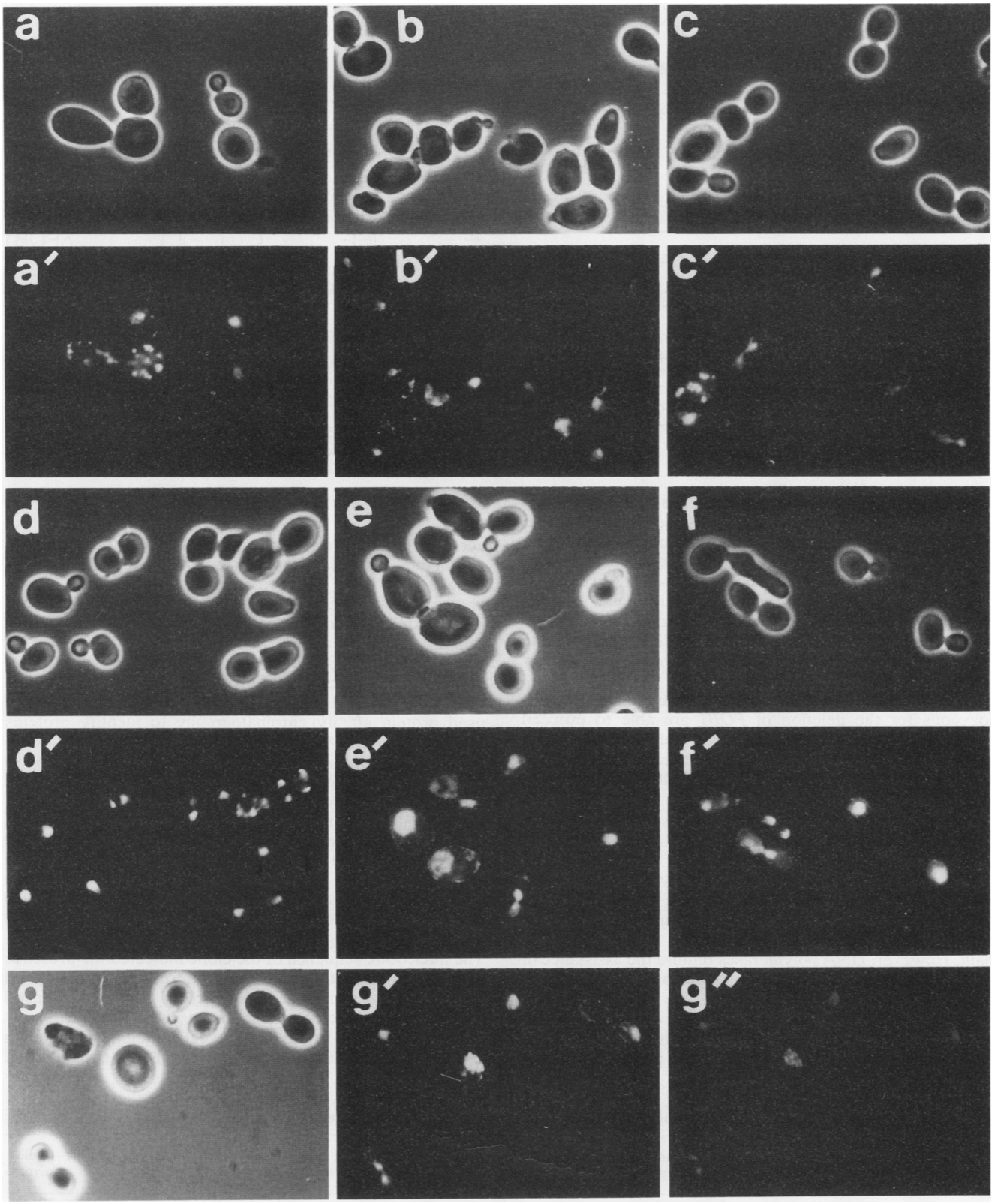

FIG. 5. Comparison of the morphologies of cells lacking *POB1*. Cells of strain 7380-3-2 were fixed, stained, and photographed as for Fig. 4, using phase-contrast optics (a to g) or DAPI staining (a′ to g′ and g″). Panels a to g represent different anomalous cells, indicating the range of morphologies observed.

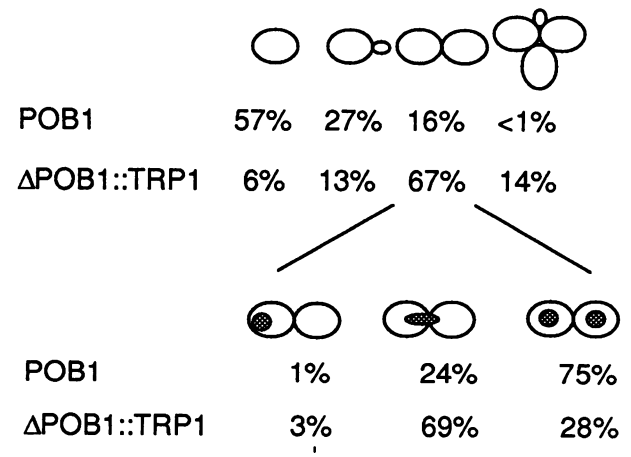

FIG. 6. Quantitation of the morphologies of cells lacking the *POB1* gene. Cells were fixed and stained as described in Materials and Methods. Cells were observed under visible light for observing cellular morphology or UV illumination for scoring nuclear morphology. A minimum of 300 cells were observed to determine the cellular distribution; a minimum of 100 large-budded cells were observed to determine the nuclear distribution.

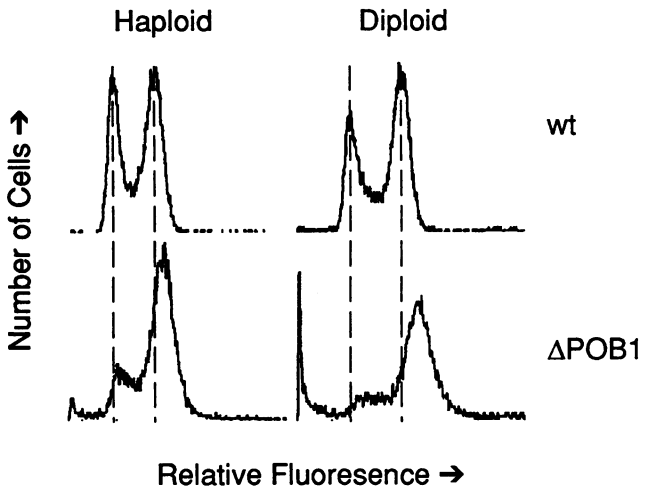

FIG. 7. Flow cytometry. Cells were fixed, stained, and analyzed by flow cytometry as described in Materials and Methods. Haploids are 7380-6-1 (wild type [wt]) and 7380-3-2 (*ΔPOB1*). Diploids are 7407 (*ΔPOB1*/+) and 7404 (*ΔPOB1*/*ΔPOB1*); a homozygous wild-type diploid gave results essentially identical to those for the heterozygote 7407 (not shown). Dashed lines indicate G_1 and G_2 DNA contents for wild-type strains.

whether cells containing a mutation in the *POL1* gene (also known as *CDC17*) that encodes this polymerase would tolerate the loss of the *POB1* gene. Double mutants that lacked the *POB1* gene and also carried a temperature-sensitive mutation in *POL1* (*cdc17-1* or *cdc17-2*) were constructed. While these double mutants were viable at room temperature, each exhibited a lower maximum permissive temperature than did cells carrying the *cdc17* mutation alone (Fig. 9). Other ts mutants tested, such as *cdc4*, did not show a similar effect (data not shown).

***POB1*-deleted cells are defective in meiosis.** Diploids homozygous for the deletion of *POB1* are able to proceed completely through meiosis. However, the spores produced have lower than normal viability (Table 4). The effect is not dramatic (viability is reduced by only about threefold), but it is reproducible in several diploids.

DISCUSSION

DNA replication is catalyzed in vivo by complex protein machines. The central characters in these machines are the DNA polymerases that bind a primer-template and attach the next appropriate nucleotide to the growing nascent chain. However, DNA polymerases require the assistance of a number of accessory factors to perform this job adequately. Since most of these factors are not required for simply observing DNA synthesis in vitro, they have proved somewhat refractory to the traditional tools of biochemistry. While these proteins do not necessarily participate in the actual joining of precursors, the speed, fidelity, and coordination they allow make them an essential part of a robust cell lineage. We have used a combination of protein affinity chromatography and genetics to identify and evaluate candidates for these more peripheral replication accessory functions.

Acting on the assumption that replication-associated proteins form a coherent complex at least partially maintained through a network of protein-protein interactions, we have used protein affinity chromatography to identify proteins that recognize DNA polymerase α in the yeast *S. cerevisiae*. If our assumption is correct, then cells lacking these polymerase-binding proteins should display defects in DNA replication. We have used a series of genetic and morphological criteria to test this prediction for one yeast DNA polymerase α-binding protein that we have called POB1.

The *POB1* gene was cloned by using the amino acid sequence of the N terminus of POB1 protein to direct the synthesis of a pool of oligonucleotides capable of encoding a piece of POB1 protein. This pool was used as a hybridization probe to identify a genomic clone containing the *POB1* gene. The nucleotide sequence of the clone indicated the presence of an ORF of about the size expected for POB1 protein, and the N-terminal amino acid sequence of this ORF matched the entire sequence determined from POB1 protein.

A search of GenBank did not reveal homology with known genes, but a search of unpublished sequences revealed that

TABLE 2. Chromosome loss and genetic recombination frequencies in cells lacking the *POB1* gene

Expt no.	Strain	Relevant genotype	Rate of loss/ mitosis	Fold increase	Rate of recombination/ mitosis	Fold increase
1	7404	*ΔPOB1*/*ΔPOB1*	2.7×10^{-4}	16	2.8×10^{-4}	10
	7407	*ΔPOB1*/+	1.7×10^{-5}		2.9×10^{-5}	
2	7404	*ΔPOB1*/*ΔPOB1*	1.1×10^{-4}	8	1.7×10^{-4}	6
	7862	+/+	1.3×10^{-5}		3.0×10^{-5}	

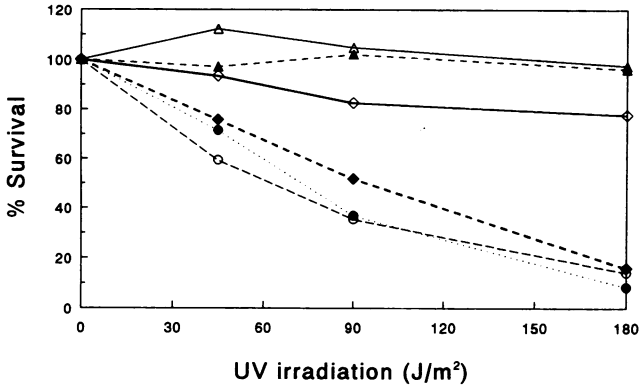

FIG. 8. UV sensitivity. Cells were grown to logarithmic phase, plated, and irradiated with UV as described in Materials and Methods. Open triangles, 7380-6-1, wild type; filled triangles, 7380-3-2, Δ*POB1*; open circles, 7380-19-3, *mec1-1*; filled circles, *mec1-1* Δ*POB1*; open diamonds, 7373-3-3, Δ*RAD9*; filled diamonds, 7373-10-2, Δ*POB1* Δ*RAD9*.

POB1 is identical to *CTF4* (*CHL15*) (10a). Mutations in *CHL15* (chromosome loss) were isolated and characterized in the laboratory of Larionov and independently in the laboratory of Hieter, where the gene was called *CTF4* (chromosome transmission fidelity). In the accompanying paper, Kouprina et al. describe their characterization of the *ctf4* mutants. Of particular relevance to our work, they find that elevated chromosome loss is accompanied by high levels of genetic recombination and that in a minichromosome assay, losses are simple losses (1:0) rather than missegregants (2:0). Mutations known to affect DNA replication display both elevated chromosome loss and genetic recombination, unlike mutations in segregation functions that show only elevated chromosome loss (12). Failure to replicate is expected to cause simple losses rather than missegregation. Kouprina et al. conclude that *CTF4* is needed for producing normal, useful copies of genetic elements.

We have also found that the lack of *POB1* causes (i) an accumulation of cells with a large bud in which the nucleus is found at the neck of the bud, (ii) elevated levels of chromosome loss and genetic recombination, (iii) a shift toward a G_2 DNA content, and (iv) at least a partial requirement for the functional checkpoint provided by the *MEC1* gene.

The accumulation of cells at the large-budded stage with the nucleus at the neck of the bud, together with an overall shift to a G_2 DNA content, seems to indicate that DNA replication is largely completed on a normal schedule in cells lacking *POB1* but that the products of replication are unsuitable for segregation. Perhaps portions of the genome are not replicated at their usual time or the newly replicated DNA is of poor quality. This latter interpretation is consistent with the elevated levels of chromosome loss and recombination observed in Δ*POB1* cells, since attempts to repair mistakes could lead to recombination if successful or to chromosome loss if not.

TABLE 3. Inviable cells in rapidly growing cultures

Strain	Genotype	% Dead cells in log-phase culture	Total observed
7380-6-1	Wild type	3.6	554
7380-3-2	Δ*POB1*	14	207
7380-19-3	*mec1-1*	11	347
7380-6-4	Δ*POB1 mec1-1*	40	245
7373-3-3	Δ*RAD9*	5.2	402
7373-10-2	Δ*RAD9* Δ*POB1*	21	319

Double mutants with *mec1-1* produced a high proportion of inviable cells and a severely distorted morphology. The overall DNA content and distribution of the Δ*POB1 mec1-1* cells appeared to be similar to those of the Δ*POB1* strain (not shown). These observations suggest that cells lacking *POB1* accumulate in G_2 at least partially in response to the *MEC1* checkpoint. In the absence of this checkpoint, the *POB1*-deleted cells often proceed further into the cell cycle than is appropriate, leading to an altered morphology and a high probability of death. Since cells with the *mec1-1* mutation fail to arrest in response to agents that distort DNA synthesis (36a), this result suggests that cells lacking *POB1* form DNA incorrectly.

Double mutants lacking both *POB1* and *RAD9* are more sensitive to UV than is either single mutant. Apparently some of the damage caused by UV, i.e., damage that is normally repaired before the *RAD9* monitoring system acts, remains and must be detected by the *RAD9* checkpoint in *POB1*-deleted cells. Perhaps *POB1* is involved in either detecting or repairing DNA damage. Since *pob1 rad9* double mutants appear somewhat less enlarged than the single *pob1* mutants but have the same overall distribution of morphologies, *RAD9* does not appear to detect the principle signal generated by the loss of *POB1* but does appear to sense some residual fraction of this signal. This view is consistent with the observation that *RAD9* monitors the fitness of the genomic copies in G_2, after the *MEC1*-dependent event (36a).

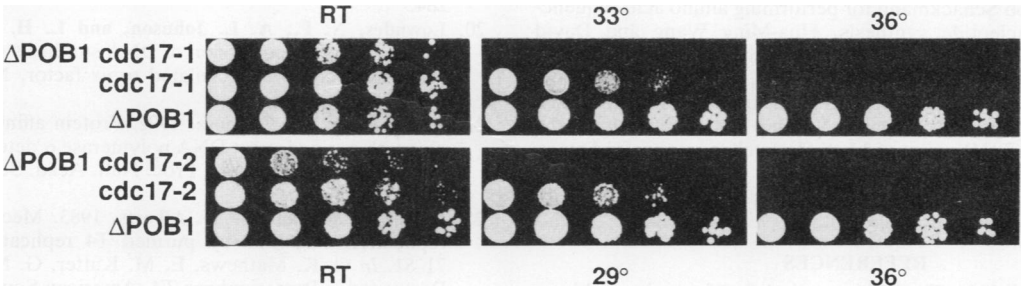

FIG. 9. Temperature sensitivity of Δ*POB1 cdc17* strains. Cells were grown to logarithmic phase, diluted, plated, and grown at the temperature indicated. Each successive spot represents a 10-fold dilution. RT, room temperature (~23°C). From top to bottom, the strains used were 7390-5-2, 7390-5-1, 7390-7-2, 7391-4-2, 7391-1-1, and 7411-10-3.

TABLE 4. Viability of spores derived from diploids lacking the *POB1* gene

No. of strains	Genotype	Viability	% Viable
4	Δ*POB1*/Δ*POB1*	59/152	39
6	Δ*POB1*/+	355/419	85
6	+/+	232/250	93

Cells carrying temperature-sensitive alleles of the essential *POL1* gene can grow at low temperatures but die at high temperatures. We have found that the maximum permissive temperature of these DNA polymerase α mutants is lower in strains lacking *POB1* than in otherwise wild-type strains. This observation is consistent with a physical interaction between POB1 and POL1 proteins in vivo; we might expect the loss of a component of a complex (POB1 protein) to further destabilize the complex already unstable because of the presence of a defective component (polymerase α). Given this interpretation, overproduction of POB1 protein could be expected to alleviate the temperature sensitivity of the *POL1* mutants. To our surprise, we observed just the opposite; when the *POB1* gene was placed under control of the strong, inducible *GAL1* promoter in cells carrying temperature-sensitive mutants of *POL1*, induction led to a lowering of the maximum permissive temperature (not shown). We propose that instead of being driven into normal, stable forms, the excess POB1 protein titrates the available POL1 protein into nonproductive complexes, exacerbating an already tenuous situation. Whatever the interpretation, it is clear that altering the levels of POB1 protein causes a genetic interaction with mutations of *POL1*, consistent with the physical interaction between POB1 and POL1 proteins observed in vitro.

The morphological and genetic characteristics of *POB1*-deleted cells are consistent with the notion that *POB1* is needed to maintain the yeast cell's genome with normal fidelity, allowing it to be included among the group of broadly defined DNA polymerase accessory factors. The similarity to the phenotypes of mutants with defects in other genes known to be active in S phase points toward a role for *POB1* in DNA metabolism. While physical evidence in vitro and genetic evidence in vivo suggest an interaction between POB1 protein and DNA polymerase α, further investigation will be required to unequivocally demonstrate such an interaction in vivo and to elucidate the role played by such a complex in the cell.

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