

# Coordinating DNA Replication To Produce One Copy of the Genome Requires Genes That Act in Ubiquitin Metabolism

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**We have developed a genetic screen of the yeast *Saccharomyces cerevisiae* to identify genes that act to coordinate DNA replication so that each part of the genome is copied exactly once per cell cycle. A mutant was recovered in this screen that accumulates aberrantly high DNA contents but does not complete a second round of synthesis. The mutation principally responsible for this phenotype is in the *DOA4* gene, which encodes a ubiquitin hydrolase, one of several yeast genes that encode enzymes that can remove the signalling polypeptide ubiquitin from its covalently linked conjugated forms. *DOA4* is nonessential, and deleting this gene causes uncoordinated replication. Overreplication does not occur in cells with limiting amounts of Cdc7 protein kinase, suggesting that entry into S phase is required for this phenotype. The DNA formed in *doa4* mutants is not highly unusual in the sense that mitotic recombination rates are normal, implying that a high level of repair is not induced. The temperature sensitivity of *doa4* mutations is partially suppressed by extra copies of the polyubiquitin gene *UBI4*, but overreplication still occurs in the presence of this suppressor. Mutations in *DOA4* cause loss of the free ubiquitin pool in cells under heat stress conditions, and extra copies of *UBI4* restore this pool without restoring coordination of replication. We conclude that a ubiquitin-mediated signalling event directly involving the ubiquitin hydrolase encoded by *DOA4* is needed in *S. cerevisiae* to prevent uncoordinated DNA replication.**

In eukaryotic cells, DNA replication is initiated at many sites, often following a complex temporal program (4, 10, 21, 49). As many as 40,000 replicons are used in human cells, and even organisms with much smaller genomes, such as the yeast *Saccharomyces cerevisiae*, must coordinate the use of about 400 initiation sites (43, 51) such that each portion of the genome is copied precisely once per cell cycle.

On the basis of experiments with *Xenopus laevis* eggs, Blow and Laskey have proposed a model to explain how DNA replication is limited to one copy per S phase (2). In their model, a “licensing” factor allows each portion of the genome to serve as a template once in each cell cycle. They propose that the factor has access to the genome only during a limited portion of the cell cycle preceding S phase and is inactivated by replication of the associated DNA sequence. The *MCM2*, *MCM3*, and *MCM5/CDC46* family of genes from *S. cerevisiae* has some of the features expected for such a factor (7, 63): they are needed for normal DNA replication, are localized to the nucleus only during a period preceding S phase, and are degraded after initiation. Recently, partially purified fractions of the *X. laevis* licensing factor have been shown to contain homologs of the yeast Mcm proteins (8, 34, 38), strongly suggesting that this family plays a role in regulating DNA replication.

In the licensing factor model, the license plays two roles: its presence allows initiation, and its destruction prevents reuse of replicated templates. Defects in licensing could therefore be expected either to block initiation or to lead to uncoordinated replication that would produce different numbers of copies of different parts of the genome. The Mcm proteins have been strongly implicated in initiation, but their role in coordination is less clear. Cells with the *mcm3* mutation arrest with a DNA content of 2 C (1 C is the amount of DNA in a haploid cell in

G<sub>1</sub>) (16), implying that this defect still allows coordination of replicon usage since the DNA content of the cells appears to double exactly, even when this gene product is limiting. If the licensing model is correct, then the pathway for coordinating DNA replication would include the licensing factor, a system for activating the license in synchrony with the cell cycle, a mechanism for inactivating the license when replication has occurred, and a replication complex that differentiates between licensed and unlicensed templates. Blocking production or activation of the license would prevent replication, which would be difficult to distinguish from the loss of other components needed for DNA synthesis. However, loss of the ability to inactivate or interpret the license would lead to nonuniform or excessive replication, which would be more diagnostic for errors in the replication coordination pathway.

Mutations in several organisms that allow a normal S phase to occur under inappropriate circumstances (for example, before the completion of mitosis) have been described (12, 22, 26, 39, 41, 56; reviewed in references 27, 37, and 45). Dahmann et al. have recently shown that Clb-Cdk complexes in *S. cerevisiae* play a role in preventing rereplication before mitosis (11). However, DNA replication is normal in these cells in the sense that a single copy of each portion of the genome is produced, leading to a doubling of the genomic DNA content of the cell; only the timing and dependency relationships of other cell cycle events are disturbed. Mutations that cause doubling of the DNA content must retain the ability to coordinate DNA replication normally because they copy each part of the genome once. These cells therefore have abnormal cell cycles but normal coordination within S phase.

To learn about mechanisms that act during replication to ensure that each portion of the genome is copied only once, we have developed a genetic screen of the yeast *S. cerevisiae* for mutants that fail to perform this type of coordination. We assumed that uncoordinated rereplication would be lethal, since it would produce variable copy numbers of different chromosomes and would prevent a subsequent orderly mitosis.

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TABLE 1. Yeast strains used in this study

| Strain     | Genotype   | Background |
|------------|--|------------|
| 3003-19-1  | <i>MAT<math>\alpha</math> dos1-1 trp1 ura3 leu1 his7 ade2 can1 cyh2</i>  | A364a      |
| 3030-19-2  | <i>MAT<math>\alpha</math> trp1 leu2 ura3 dos1-1</i>  | A364a      |
| 3076-14-3  | <i>MAT<math>\alpha</math> dos1-1 leu2 ura3 his3</i>  | A364a      |
| 3091-7-2   | <i>MAT<math>\alpha</math> dos1-1 trp1 his7 ura3 leu2</i>   | A364a      |
| 3113-1-2   | <i>MAT<math>\alpha</math> dos1-1 ade2 ade3 trp1 his3 leu2 (CF:352; LEU2 ade3-2p)</i>   |            |
| 3163-2-3   | <i>MAT<math>\alpha</math> ade2 ade3 leu2 can1 his7 ura3 trp1 doa4-<math>\Delta</math>2(::TRP1) (CF:352; LEU2 ade3-2p)</i>  |            |
| 4053-5-2   | <i>MAT<math>\alpha</math> his7 trp1 leu2 ura3</i>  | A364a      |
| 4053-5-4   | <i>MAT<math>\alpha</math> his7 trp1 leu2 ura3</i>  | A364a      |
| 7236-2-3   | <i>MAT<math>\alpha</math> his3 cyh2 can1 ura3 leu2 trp1</i>  | A364a      |
| 7311-2-4   | <i>MAT<math>\alpha</math> cdc17-1 pep4 prb1 leu2 ura3 his7 can1</i>  | A364a      |
| 7357-8-1   | <i>MAT<math>\alpha</math> ade2 ade3 leu2 can1 his3 ura3 trp1 (CF:352; LEU2 ade3-2p)</i>  |            |
| 7380-2-1   | <i>MAT<math>\alpha</math> trp1 leu2 ura3 can1 his3 his7 cyh2</i>   | A364a      |
| 7496       | <i>MAT<math>\alpha</math> his7 trp1 leu2 ura3 doa4-<math>\Delta</math>2(::TRP1)</i>  | A364a      |
| 7505-2-4   | <i>MAT<math>\alpha</math> his7 trp1 leu2 ura3 doa4-<math>\Delta</math>2(::TRP1)</i>  | A364a      |
| 7537, 7602 | <i>MAT<math>\alpha</math>/MAT<math>\alpha</math> trp1/trp1 leu2/leu2 ura3/ura3 hom3 can1/+,+ his3/+ +/his7 dos1-1/dos1-1</i>   | A364a      |
| 7539       | <i>MAT<math>\alpha</math>/MAT<math>\alpha</math> trp1/trp1 leu2/leu2 ura3/ura3 hom3 can1/+,+ his3/+ +/his7 doa4-<math>\Delta</math>2(::TRP1)/doa4-<math>\Delta</math>2(::TRP1)</i> | A364a      |
| 7553-1-1   | <i>MAT<math>\alpha</math> cdc7 leu2 trp1 ura3 his3</i>   |            |
| 7553-100   | <i>MAT<math>\alpha</math> cdc7 leu2 trp1 ura3 his3 doa4-<math>\Delta</math>4(::URA3)</i>   |            |
| 7601       | <i>MAT<math>\alpha</math>/MAT<math>\alpha</math> trp1/trp1 leu2/leu2 ura3/ura3 hom3 can1/+,+ his3/+ +/his7 doa4-<math>\Delta</math>2(::TRP1)/doa4-<math>\Delta</math>4(::URA3)</i> | A364a      |
| 7862       | <i>MAT<math>\alpha</math>/MAT<math>\alpha</math> trp1/trp1 leu2/leu2 ura3/ura3 hom3 can1/+,+ his3/+ +/his7</i>   | A364a      |

However, lethality that is due to uncoordinated replication would be circumvented by preventing DNA synthesis; the consequences of faulty decision making would then be obviated by the mechanical inability to complete the mistaken course of action. We therefore searched for mutants that were unable to survive a temporary increase in temperature but were rescued by a second mutation that rendered DNA polymerase  $\alpha$  inactive during the high-temperature incubation. In this case, the first mutation causes the cell to attempt to engage in uncoordinated DNA replication, but this does not occur because the malfunctioning replication machinery prevents any DNA synthesis.

This screen yielded a temperature-sensitive mutation, which we named *dos1-1*, that causes cells to be sensitive to wild-type DNA polymerase  $\alpha$  and to accumulate aberrant DNA contents when DNA synthesis is permitted. The unique feature of these cells is that they fail to stop replication after the completion of one round of synthesis, and they also fail to complete a second round, indicating that replication no longer affects each sequence equally. The *dos1-1* mutation disrupts *DOA4*, a gene that encodes a ubiquitin hydrolase (47), and disturbs normal metabolism of ubiquitin. Ubiquitin-dependent degradation of cyclins and cyclin-dependent kinase inhibitors, of the S-phase inhibitor Sic1, and of components of the mitotic spindle has been shown to be required to allow cell cycle transitions (19, 28, 30, 31, 33, 42, 46, 62). Since ubiquitin hydrolases act to remove ubiquitin from conjugated forms during either protein degradation or signalling, our results indicate that a ubiquitin-mediated process is also required for normal coordination of template usage during replication and prevention of the aberrant reuse of templates.

## MATERIALS AND METHODS

**Strains, media, and genetic manipulations.** The yeast strains used in this study are described in Table 1. The YM-1, YPD, and defined media used were described previously (24, 52). Cell density was determined by hemacytometer analysis. [*rho*<sup>0</sup>] strains were derived by growing cultures in YM-1 with 20  $\mu$ g of ethidium bromide per ml (61) and then testing clones for the inability to grow on nonfermentable carbon sources and loss of the mitochondrial DNA staining pattern by fluorescence microscopy. For some isolates, a hybridization test for the presence of a mitochondrial genome fragment was also performed (see below). Cells were arrested with  $\alpha$ -factor essentially as previously described (58); cultures were grown in YPD titrated to pH 4.0, concentrated by centrifugation,

and then suspended in the same medium with 5  $\mu$ g of  $\alpha$ -factor per ml. Cultures were incubated for 3 h at room temperature (RT) with aeration, washed twice with distilled H<sub>2</sub>O at 0°C, and then suspended in YPD (pH 4) under the conditions described in the legend to Fig. 7.

**Mutagenesis.** Strain 7311-2-4 (*cdc17-1*) was transformed with YEp24-POL1 (kindly provided by B. Garvik and L. Hartwell, University of Washington, Seattle) and mutagenized with ethylmethane sulfonate as previously described (36). Clones ( $8 \times 10^4$ ) were screened for isolates that failed to survive 6 h of incubation at 38°C. Derivatives lacking the plasmid were then isolated on medium containing 5-fluoroorotic acid (3) and rescreened. Three mutants able to survive the 38°C incubation in the absence but not the presence of the *POL1* plasmid were found; the *dos1-1* mutation is described in this report.

Transposon mTn3-*URA3*-RI is a derivative of mTn3-*URA3* (29) in which the *EcoRI* site was destroyed. This transposon was inserted randomly into a 4,809-bp *HindIII*-*ClaI* fragment containing *DOA4* in pHSS6, and a derivative with the transposon inserted in the intergenic space between *DOA4* and the next gene (as determined by nucleotide sequencing) was obtained. A linear fragment marked with mTn3 was released by using *Bgl*III and *ClaI* and used to transform strain 3003-19-1. This corrects the *dos1-1* mutation only if recombination is distal to the affected site; transformants were screened for temperature sensitivity to determine which had been corrected. A derivative of mTn3-*URA3*-RI was obtained by inserting the 1,576-bp *Sau3A* fragment containing the 2 $\mu$ m replication origin into the *Bam*HI site (54). This mTn3-*URA3*,2 $\mu$ -RI transposon was inserted into pJS108, converting it into a series of high-copy-number yeast vectors which were then screened for the ability to suppress the temperature sensitivity of a *dos1-1* mutation.

**DNA methods.** DNA was extracted (52), digested with endonucleases, separated on agarose gels, transferred to nylon membranes, and probed as previously described (54). *TRP1*, *LEU2*, *HIS3*, and the three rDNA *EcoRI* bands were probed by using YIplac204, YIplac128 (17), pRS303 (59), and total genomic DNA, respectively. Hybridization was quantitated with a PhosphorImager (Molecular Dynamics).

A *dos1-1* strain was transformed with a *CEN*-based genomic library of *Sau3A* partial digestion fragments inserted into the *Bam*HI site of vector p366 (a derivative of Ycp50 in which the *LEU2* gene was substituted for *URA3*; the vector and library were constructed and generously provided by F. Spencer and P. Hieter, Johns Hopkins University, Baltimore, Md.) and plated for colonies at 37°C.

Diphenylamine reactions were carried out as previously described (15) and corrected for the number of cells extracted as determined with a hemacytometer.

*DOA4* was deleted by cloning the 1,006-bp *PstI*-*SphI* and 2,635-bp *EcoRI*-*KpnI* fragments flanking the gene into YIplac204 and YIplac211 (17). Transformation with linear plasmids produced *doa4- $\Delta$ 2(::TRP1)* and *doa4- $\Delta$ 4(::URA3)*, which lack the ability to produce 96% of this gene product. Deletions were confirmed by Southern analysis.

Mitochondrial DNA content was assayed by comparing the signals obtained after probing a total cellular DNA preparation with a mitochondrial genome fragment (the 3,721-bp *EcoRI* fragment from the cytochrome *b* gene [kindly provided by D. Lockshon and W. Fangman, University of Washington]) and a nuclear genome fragment.

**Flow cytometry.** Cells were treated with RNase A, stained with propidium iodide or YOYO-1 iodide (18; a generous gift of Molecular Probes, Eugene,

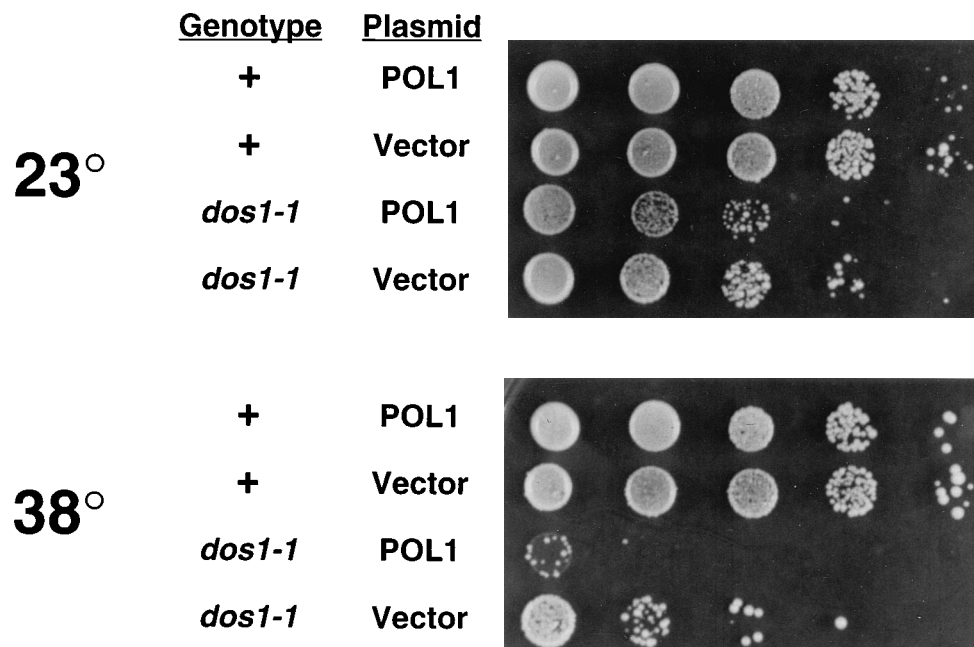


FIG. 1. The *dos1-1* mutation is sensitive to incubation at 38°C only in the presence of a normal copy of the *POL1* gene. Wild-type (7311-2-4) and *dos1-1* mutant (3003-19-1) strains transformed with either vector YEp24 or a vector containing *POL1* were grown to saturation, 10-fold serial dilutions were placed on selective medium and incubated at 23 or 38°C for 6 h, and then both were incubated at 23°C for several days. Both strains contain the *cdc17-1* allele of *POL1*; this mutation blocks DNA replication at 38°C but allows retention of high viability under these conditions (5). 3003-19-1 showed some inviability and sensitivity to the *POL1* plasmid at 23°C, but—unlike the effect obtained with the vector—the lethality induced by the *POL1* plasmid was enhanced about 100-fold by high-temperature incubation.

Oreg.), and sonicated, and their DNA content was determined by flow cytometry as previously described (40).

**Protein electrophoresis.** Two million wild-type cell equivalents (as determined by the absorbance of the cultures) were suspended in sodium dodecyl sulfate sample buffer, boiled, separated on a denaturing 10 to 20% acrylamide gradient gel, transferred to nitrocellulose, and probed with a polyclonal antiserum directed against ubiquitin (a generous gift from Q. Deveraux and M. Rechsteiner, University of Utah, Salt Lake City). This was followed by detection of the antibodies with <sup>125</sup>I-labelled protein A and autoradiography (23).

## RESULTS

**A mutant sensitive to DNA polymerase  $\alpha$  accumulates aberrant levels of DNA.** To identify genes needed to coordinate events during DNA replication, we screened *S. cerevisiae* for mutants that die rapidly at 38°C in the presence of functional DNA polymerase  $\alpha$  but survive if this enzyme is inactive under these conditions (polymerase  $\alpha$  is the product of the *POL1/CDC17* gene; the *cdc17-1* allele exhibits a fast replication stop at 38°C [5, 32]). A strain with these properties was recovered (Fig. 1). The sensitivity to wild-type polymerase  $\alpha$  DNA was found to segregate as a single locus that is linked to temperature sensitivity at 37°C. Mutant cells (now containing a normal genomic *POL1* gene) were assayed by flow cytometry to determine the distribution of DNA contents in populations and were found to contain abnormally large amounts of DNA (Fig. 2 and 3). The mutation was therefore named *dos1-1* (for DNA oversynthesis). The DNA content of *dos1-1* cells was somewhat elevated, even under conditions permissive for growth, and this effect was enhanced by incubation under restrictive conditions (38°C) (Fig. 2 and 3). Upon further backcrossing, temperature sensitivity and polymerase sensitivity comigrated with the elevated DNA content phenotype, but the severity of the temperature-induced change in DNA contents was diminished (Fig. 2), suggesting that a second mutation is present in the original

isolate that enhances the inducibility of overreplication (see below).

To ensure that the signal observed by using flow cytometry after staining with propidium iodide was due to a change in DNA content, DNA was quantitated by using an unrelated dye for flow cytometry (YOYO-1 iodide), the diphenylamine reaction (which is specific for deoxyribose), and hybridization with several specific probes (Fig. 3 and Table 2). Overall, *dos1-1* cells were found to contain an excess of DNA equivalent to 0.1 to 0.3 C under permissive conditions and 0.3 to 1.2 C at 38°C. Since these cells are unusually large, derivatives lacking mitochondrial DNA (*[rho*<sup>0</sup>]) were used in these measurements to ensure that the excess DNA was not due to increased numbers of mitochondrial genomes that can be associated with enlarged cells. Cells with normal mitochondria show a similar elevated DNA content, and a hybridization probe derived from the mitochondrial genome showed that no change in the ratio of mitochondrial DNA to genomic DNA occurred during the 38°C incubation in such cells (see Fig. 6). Hybridization probes for the endogenous yeast plasmid 2 $\mu$ m indicated that this episome does not overreplicate, and the double-stranded RNA genomes of the endogenous killer element (64) were shown to be destroyed by the RNase A treatment used in the flow cytometry protocol (data not shown).

DNA contents between 1 and 2 C are normal for haploid cells in S phase, but single cells should not have a DNA content over 2 C. To determine whether the cells with DNA contents over 2 C carried this DNA in single or multiple nuclei, we examined the nuclear morphology of *dos1-1* mutant cells incubated at 38°C (Table 3). Cells growing at 23°C had nuclear distributions similar to that of wild-type cells, but when incubated at 38°C, *dos1-1* mutant cells were found to have a high proportion of large-budded cells with one nucleus. Overall,

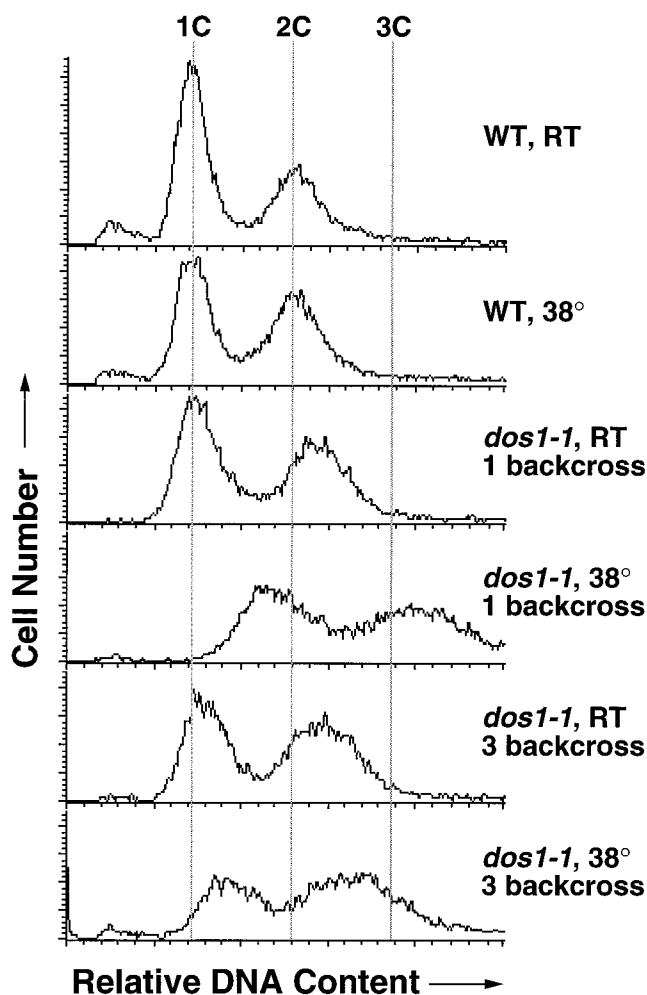


FIG. 2. Cells with the *dos1-1* mutation have elevated DNA contents. Derivatives of strains 4053-5-4 (wild type [WT]), 3003-19-1 (*dos1-1*, one backcross), and 3030-19-2 (*dos1-1*, three backcrosses) lacking mitochondrial DNA (*[rho<sup>0</sup>]* strains) were grown at 23°C (RT) to log phase in YM-1, shifted to 38°C for 6 h, and then processed for flow cytometry as described in Materials and Methods. Vertical lines indicate the position of 1 and 2 C DNA contents inferred from multiple wild-type samples (see Fig. 3A).

95% of the cells had one nucleus (Table 3). About 60% of the population had a DNA content of more than 2 C, and no significant amount of cells with a DNA content of less than 1 C was observed. We conclude that most of the cells with a high DNA content carry that DNA in a single nucleus and that this could not be due to unequal segregation of DNA, which would produce equal numbers of cells with abnormally high and abnormally low DNA contents. Furthermore, since the DNA content did not double to 4 C, DNA synthesis in these cells could not have affected each portion of the genome equally. Adherence to the requirement that replication occur once, and only once, per template per cell cycle has therefore failed in cells with the *dos1-1* mutation incubated at 38°C.

To identify rereplicated templates, genomic DNA was extracted from the same number of *dos1-1* mutant or wild-type cells with and without high-temperature incubation, and then restriction fragments were separated by agarose gel electrophoresis and analyzed by hybridization with specific probes (Fig. 3C). Most of the regions tested overreplicated 50 to 100% in mutant cells but not in wild-type cells, but the *LEU2* gene

TABLE 2. Quantitation of DNA contents with the diphenylamine reaction

| Genotype       | Relative DNA content <sup>a</sup> |      | 38°C/23°C ratio |
|----------------|-----------------------------------|------|-----------------|
|                | 23°C                              | 38°C |                 |
| Wild type      | 1.0                               | 1.0  | 1.0             |
| <i>dos1-1</i>  | 1.10                              | 1.69 | 1.55            |
| <i>doa4-Δ2</i> | 1.08                              | 1.51 | 1.41            |

<sup>a</sup> Two cultures each of 4053-5-4 (wild type), 3091-7-2 (*dos1-1*; four backcrosses), and 7505-2-4 (*doa4-Δ2*) cells were grown in rich medium at 23°C and then either harvested or incubated for 6 h at 38°C before harvesting. DNA content was determined with the diphenylamine reaction (15), and this value was normalized to the number of cells extracted (as determined with a hemacytometer) and the value obtained with the wild-type cultures under the same conditions, which was defined as 1 U. A similar increase in DNA content was noted for a *dos1-1* mutant strain lacking mitochondrial DNA (data not shown).

copy number remained constant under these conditions. While the basis for this exception is not known, *LEU2* therefore provided a convenient internal loading control for this experiment, showing that the increases noted in the mutant cells were not due to a systematic change in the extraction of DNA. To test whether the copy number changed with distance from the replication origins, we performed this same experiment with several probes from the region of chromosome III near *ARS307* (20, 44) but found that none of these sequences appeared to overreplicate (data not shown). Since *LEU2* is also on chromosome III, it is possible that this chromosome, as a whole, does not participate in the overreplication reaction. Anomalous behavior of chromosome III is consistent with the chromosome stability differences noted below.

**Overreplication is caused by a defect in the ubiquitin hydrolase gene *DOA4*.** The temperature sensitivity of the *dos1-1* mutation was complemented by a clone derived from a YCp (low-copy-number) library (Fig. 4). The complementing insert mapped to the left arm of chromosome IV, near *RAD55* and *CDC34* (50). The *dos1-1* mutation was also found to be linked to this region by meiotic mapping (*dos1-1* to *rad55*, 5.7 centimorgans [cM] [62 parental ditype:8 tetratype:0 nonparental ditype]; *dos1-1* to *cdc34*, 9.1 cM [27 parental ditype:6 tetratype:0 nonparental ditype]), suggesting that the cloned DNA represents the wild-type copy of *DOS1*. Complementing subclones contained an open reading frame which had been previously identified as *SSV7* (35) or *DOA4/UBP4* (47) and will subsequently be called *DOA4*. This gene encodes a ubiquitin hydrolase or deubiquitinating enzyme with similarity to the human *tre-2* oncogene (47). The *dos1-1* mutation was recovered by gap repair (53) and found to be a frameshift in codon

TABLE 3. Morphological distribution of cells with the *dos1-1* mutation<sup>a</sup>

| Growth temp (°C) | % of cells with indicated morphology |                      |                      |                     |
|------------------|--------------------------------------|----------------------|----------------------|---------------------|
|                  | No bud, 1 nucleus                    | Small bud, 1 nucleus | Large bud, 1 nucleus | Large bud, 2 nuclei |
| 23               | 49                                   | 18                   | 2                    | 31                  |
| 38               | 26                                   | 6                    | 63                   | 5                   |

<sup>a</sup> Half of a culture of strain 3003-19-1 (*dos1-1*) cells growing at 23°C was collected, the remainder was incubated at 38°C for 6 h, and then both were fixed as for flow cytometry. The cultures were sonicated and then stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) to reveal the nuclei. Gross morphology was determined by phase-contrast microscopy ( $n > 300$ ), and nuclei were examined by fluorescence microscopy ( $n > 100$  large-budded cells). Similar results were obtained with a strain with a deletion of *DOA4* and an *ssv7-1* mutant strain (reference 35 and data not shown).

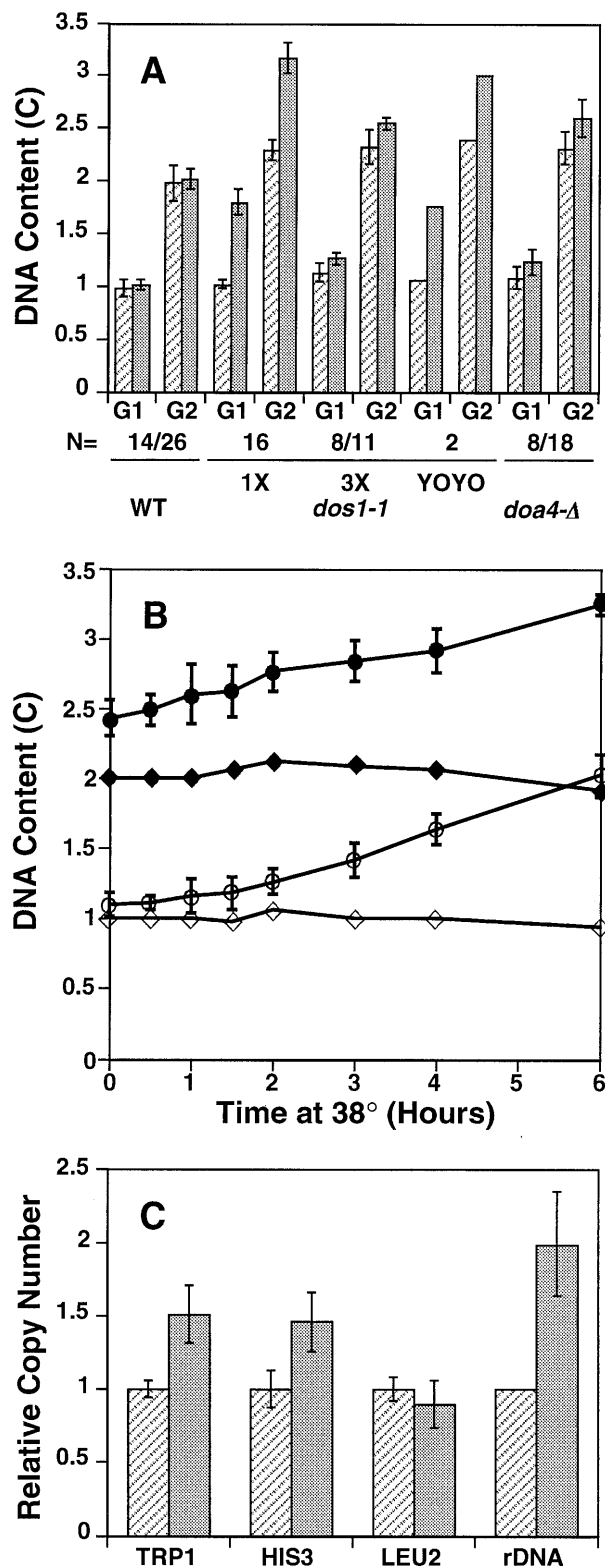


FIG. 3. Quantitation of DNA contents in *dos1-1* and *doa4-Δ* cells. Strains lacking mitochondrial DNA (*rho*<sup>0</sup>) strains were grown in rich medium to log phase and then incubated at 38°C before harvesting and measurement of DNA content by flow cytometry. The relative fluorescence of the two prominent peaks (designated G<sub>1</sub> and G<sub>2</sub> cells) in each distribution was determined and converted to relative DNA content by comparison with the values obtained for wild-type (WT) cultures assayed in parallel, which were defined as 1 and 2 C. (A) The relative DNA contents of multiple samples representing several independent

402 of the *DOA4* gene (AGA→AA), which prevents production of the regions of Doa4 including the hydrolase active sites (Fig. 4). Deletion of *DOA4* resulted in viable cells that were temperature sensitive at 37°C and accumulated excess DNA similarly to multiply backcrossed *dos1-1* strains (Fig. 5).

Deletion of *DOA4* causes phenotypes similar to those caused by the multiply backcrossed *dos1-1* mutation, and *dos1-1* is an alteration in *DOA4*. These observations indicate that *DOA4* is a gene required for promoting coordination of DNA replication. To determine why the singly backcrossed strain has a more severe overreplication phenotype, we deleted the *DOA4* gene from such a strain and used site-directed recombination to correct the *dos1-1* mutation in the same strain. When *DOA4* was deleted from singly backcrossed strain 3003-19-1, the high degree of temperature-inducible overreplication was retained (Fig. 6A and B show similar levels of overreplication for the *dos1-1* parent and its *doa4* deletion derivative, respectively). We then transformed 3003-19-1 with a linear DNA fragment containing wild-type *DOA4* sequences and a selectable marker (which was introduced on a modified Tn3 transposon into the intergenic region adjacent to *DOA4* [29]). Depending on the site of exchange during transplacement, transformants could either retain the mutation or revert to the wild-type sequence. Of 12 isolates tested, 9 remained temperature sensitive and 3 became resistant. The temperature-sensitive isolates had an overreplication phenotype identical to that of the parent strain (Fig. 6C and D show similar levels of overreplication for the *dos1-1* parent and the temperature-sensitive transformant, respectively). In contrast, temperature-resistant isolates failed to overreplicate (Fig. 6E shows that this strain does not produce a significant population of cells with a DNA content of over 2 C). The corrected isolates had normal DNA contents at 23°C and grew at 38°C, but the distribution of DNA contents at 38°C displayed an abnormal delay in S phase (in Fig. 6E, the normal bimodal distribution is replaced by a single broad peak spanning from 1 to 2 C). A wild-type strain transformed with the same marked fragment did not display this phenotype (data not shown). Taken together, these data indicate that loss of *DOA4* alone causes overreplication but that the temperature inducibility of this phenotype can be enhanced by a second mutation present in strain 3003-19-1. This second mutation does not, by itself, cause temperature sensitivity or result in overreplication but does alter progression through S phase at 38°C.

**Overreplication requires normal entrance into S phase.** Initiation of DNA replication in *S. cerevisiae* requires the activity

experiments were compiled. Hatched bars represent samples grown at 23°C, and solid bars are samples incubated at 38°C. N is the number of samples of each type (the number varied for samples grown at 23 and 38°C as shown); the average and standard deviation are plotted. Wild-type (4053-5-4 [*rho*<sup>0</sup>] and 7380-2-1 [*rho*<sup>0</sup>]) and *dos1-1* (1X, one backcross of original isolate, 3003-19-1 [*rho*<sup>0</sup>]; 3X, three backcrosses of original isolate, 3030-19-2 [*rho*<sup>0</sup>]; YOYO, strain 3003-19-1 [*rho*<sup>0</sup>] stained with YOYO-1 iodide instead of propidium iodide) and *doa4-Δ* (7496 [*rho*<sup>0</sup>] and 7505-2-4 [*rho*<sup>0</sup>]) mutant samples are shown. (B) Data were collected and analyzed as for panel A, but in this case samples were taken at the times indicated from four cultures of 3003-19-1 (*rho*<sup>0</sup>) (*dos1-1*; circles) or one culture of 4053-5-4 [*rho*<sup>0</sup>] (wild type; diamonds) cells. The G<sub>1</sub> (open symbols) and G<sub>2</sub> (closed symbols) distributions were plotted with the standard deviation for the *dos1-1* samples. (C) Cultures of 3003-19-1 (*dos1-1*) cells were grown to log phase at room temperature (hatched bars), and then half of each culture was shifted to 38°C for 8 h (shaded bars). DNA was extracted from equal numbers of cells as determined by hemacytometer analysis, digested with *EcoRI*, electrophoresed through 0.8% agarose, and then transferred to a solid membrane. The relative hybridization signal obtained with the probes indicated was determined from several lanes with a PhosphorImager. The signal from cells grown at 23°C was defined as 1; wild-type cultures treated the same way did not show any signal increase when incubated at 38°C (data not shown).

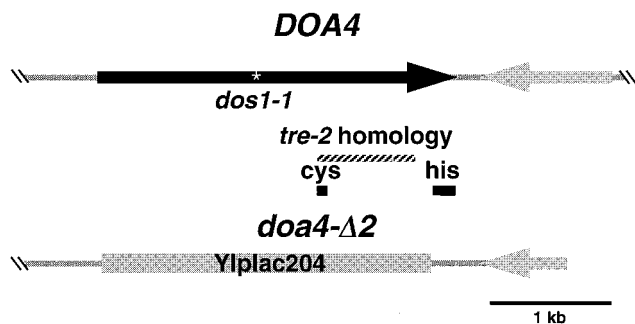


FIG. 4. The *DOA4* region. Plasmids that complemented the temperature sensitivity of *dos1-1* were found to contain the *DOA4* gene by searching the GenBank database with the partial nucleotide sequence (48). Deletions marked with YIplac204 (*TRP1* [17]) were constructed and inserted into *S. cerevisiae* by transplacement (53) with the homologous regions shown. The location of *dos1-1* is shown; this mutation truncates the Doa4 product before synthesis of the regions of similarity to other ubiquitin hydrolases (cys is the active-site cysteine, and his is a histidine-containing region common to this family of enzymes), including the human *tre-2* oncogene (47).

of several genes, including the protein kinase encoded by *CDC7* (reviewed in reference 55). To determine whether cells without *DOA4* accumulate excess DNA while attempting to perform S phase or whether overreplication can occur in other parts of the cell cycle, we constructed isogenic strains containing a temperature-sensitive allele of *cdc7* and either the normal *DOA4* gene or a *doa4* deletion. When growing under permissive conditions, the *cdc7* strain displayed a  $G_2$  delay but the relative DNA contents were normal (Fig. 7A). In comparison, the *cdc7 doa4* double mutant had the slightly elevated  $G_2$  DNA contents characteristic of *doa4* cells (compare Fig. 7A with Fig. 5). When treated with the pheromone  $\alpha$ -factor and then placed at a restrictive temperature, both strains arrested with similar 1 C DNA contents (Fig. 7B). This 1 C arrest was even tighter in cells in which the high-temperature arrest was coupled with continuation of the pheromone block (Fig. 7C). It proved necessary for observing reliable arrests in this experiment to use cells containing normal mitochondrial genomes, so the arrested populations appear at a higher DNA content than the 1 C level inferred from growing populations (the ratio of mitochondrial to genomic copy numbers for the *doa4* strain was actually lower than for the *DOA4* strain under all conditions [Fig. 7]). However, in each case, the double mutants arrested with the same DNA contents as or slightly lower DNA contents than the *cdc7* single mutants, indicating that the double mutants do not progress any further into S phase than do *cdc7*

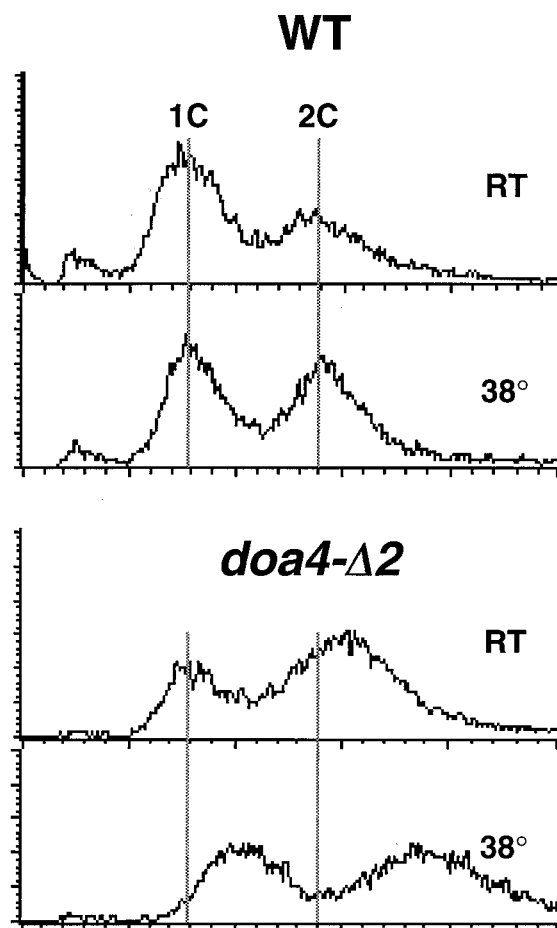


FIG. 5. Deletion of *DOA4* causes overreplication. Strains 4053-5-4 ( $[rho^0]$  wild type [WT]) and 7496 ( $[rho^0]$  *doa4-Δ2*) lacking mitochondrial DNA were grown in YM-1 to log phase and either collected or incubated at 38°C for 6 h. DNA contents were then determined as described in the legend to Fig. 2.

mutants. Cells released from the  $\alpha$ -factor block resumed the original patterns of DNA distribution (compare Fig. 7A and D). This shows that *doa4* cells blocked from performing S phase accumulate with normal 1 C DNA contents and suggests that they are able to acquire abnormal DNA contents only if they can enter S phase.

TABLE 4. Chromosome loss and recombination in *doa4* mutants<sup>a</sup>

| Genotype      | Chromosome III disomic fragment |                      |                           | Chromosome V diploids |                                |                              |                    |                                |                              |
|---------------|---------------------------------|----------------------|---------------------------|-----------------------|--------------------------------|------------------------------|--------------------|--------------------------------|------------------------------|
|               | No. of events/<br>no. of cells  | Loss rate            | Mutant/wild-type<br>ratio | 23°C                  |                                |                              | 36°C               |                                |                              |
|               |                                 |                      |                           | Loss ( $10^{-5}$ )    | Recombination<br>( $10^{-5}$ ) | Mutant/wild-type<br>ratio(s) | Loss ( $10^{-5}$ ) | Recombination<br>( $10^{-5}$ ) | Mutant/wild-type<br>ratio(s) |
| Wild type     | 10/67,400                       | $1.5 \times 10^{-4}$ | 1                         | 0.42                  | 0.92                           | 1                            | 1.2                | 3.7                            | 1                            |
| <i>dos1-1</i> | 7/1,260                         | $5.6 \times 10^{-3}$ | 37                        | 0.47                  | 1.2                            | 1.1, 1.3                     | 0.54               | 6.1                            | 0.45, 1.6                    |
| <i>doa4-Δ</i> | 10/1,308                        | $7.6 \times 10^{-3}$ | 51                        | 0.59                  | 0.88                           | 1.4, 0.96                    | 0.96               | 6.3                            | 0.8, 1.7                     |

<sup>a</sup> Wild-type (7357-8-1), *dos1-1* (3113-1-2), and *doa4-Δ* (3163-2-3) strains containing a fragment of chromosome III containing the *ADE3* gene were grown at RT under selection for the fragment and then placed on rich medium. The fragment causes colonies to develop a red pigment (57). Half-sectored colonies indicate that the fragment was lost in the first division after plating, allowing the rate of loss per mitosis to be determined (57). Diploids that were wild type (7862) or homozygous for the *dos1-1* (7537 and 7602) or *doa4-Δ* (7539 and 7601) mutation and heterozygous for the *can1* and *hom3* mutations were used to determine the rates of loss and recombination on chromosome V by using fluctuation analysis (25). Five to seven colonies grown at RT or at 36°C (the maximal permissive temperature for both *dos1-1* and *doa4-Δ* mutant strains) were used.

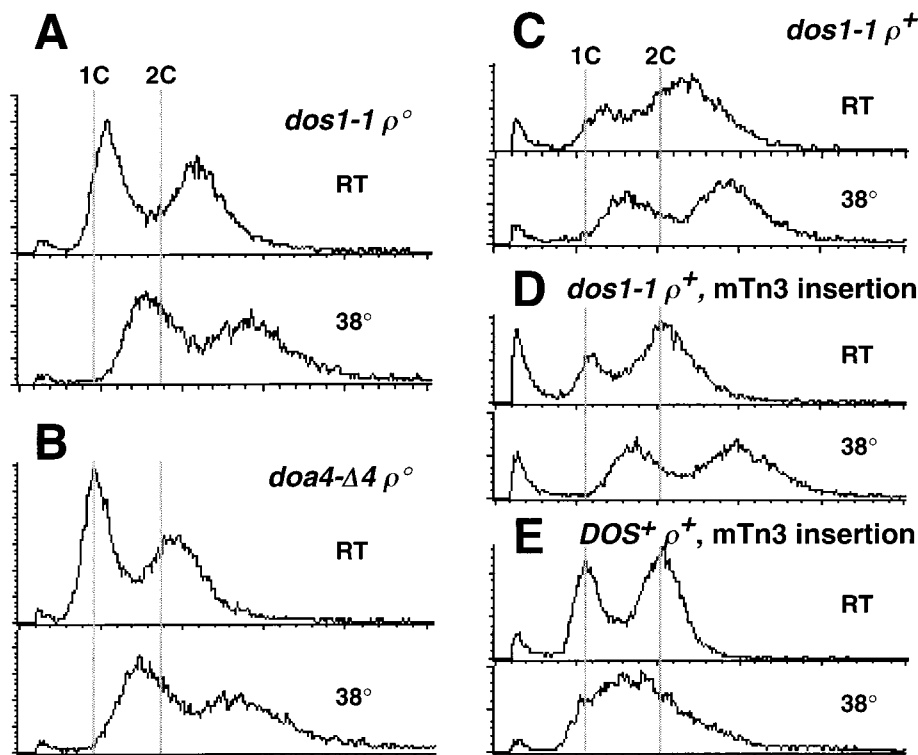


FIG. 6. Deletion of *DOA4* in a *dos1-1* mutant strain produces a similar phenotype, and introduction of normal *DOA4* sequences reverses the overreplication phenotype. *DOA4* was deleted from strain 3003-19-1 ( $[rho^0] dos1-1$ ), and the DNA contents of both strains were assayed as described in the legend to Fig. 2. (A) 3003-19-1  $[rho^0]$ . (B) 3003-19-1  $[rho^0]$  transformed with a construct to delete *DOA4* and introduce *URA3*. 3003-19-1  $[rho^+]$  (C) was transformed with a linear fragment containing an mTn3-*URA3* transposon in the intergenic region adjacent to the wild-type *DOA4* sequence including the position of the *dos1-1* mutation (29). Of 12 *URA3*<sup>+</sup> clones, 3 were temperature resistant; the remainder presumably recombined closer to the mTn3 marker than the location of the *dos1-1* mutation and therefore did not correct the defect. Temperature-sensitive (D) and -resistant (E) clones were assayed for DNA contents as described in the legend to Fig. 2. These strains retain mitochondrial genomes and were therefore tested for mitochondrial DNA content. RT and 38°C samples were found to have similar ratios of mitochondrial DNA to genomic DNA.

**Cells deficient in *DOA4* do not display elevated levels of recombination typical of induction of repair.** Strains experiencing high levels of DNA damage repair often display elevated frequencies of recombination and chromosome loss (14, 25). To see if the uncoordinated DNA replication occurring in *doa4* mutants causes these defects, we tested the stability of two types of test element. A large, multiply marked telocentric fragment representing about one-half of chromosome III was introduced into strains with and without *doa4* mutations. Since these strains contain a complete copy of this chromosome, the fragment is dispensable and changes in its rate of loss can be determined as a function of different mutations (57). As shown in Table 4, the stability of this element was strongly diminished by *doa4* mutations, even in cells growing under permissive conditions (a similar effect was seen with a centromeric plasmid [data not shown]). A second assay was used to measure the rates of both loss and recombination with an intact chromosome V. In this case, diploids heterozygous for *can1* and *hom3* mutations and homozygous for either normal or mutant copies of *DOA4* were assayed for production of cells resistant to canavanine and then the rates of both loss and recombination were determined by fluctuation analysis (25). No significant change in either rate was observed, even in cells that had been grown at the maximal permissive temperature of 36°C, a condition that should maximize the stress caused by limiting *Doa4* function.

Several aspects of the two assays are different, but we do not know which differences are responsible for the variance in loss rates observed. However, the lack of participation by chromo-

some III markers in overreplication suggests that this chromosome is anomalous. We concluded that at least one intact chromosome is lost and experiences recombination at normal frequencies in *doa4* mutants, suggesting that the accumulation of excess DNA is not due to a highly error-prone process inducing widespread DNA damage.

**Overreplication is linked to a defect in ubiquitin metabolism.** During our attempts to obtain plasmids that complement the temperature sensitivity of the *dos1-1* mutation, we obtained a second set of clones from both low- and high-copy-number genomic libraries. These clones had overlapping inserts, provided only partial complementation, and mapped to chromosome XII. They therefore represented an unlinked suppressor of the *dos1-1* mutation. These isolates were found to contain the structural gene encoding polyubiquitin, *UBI4*. Subcloning and transposon mutagenesis showed that the suppressor was, in fact, the *UBI4* gene (Fig. 8). A plasmid with the *UBI4* gene was mutagenized with a derivative of transposon Tn3 containing a selectable marker and a high-copy-number yeast replication origin, such that a series of derivatives were obtained that were all capable of high-copy-number transformation of yeast cells but differed in the location of the transposon. Only those isolates in which the transposon was inserted outside the first repeat of the polyubiquitin gene retained suppression activity. Therefore, the ability to produce ubiquitin is essential for the suppression. This result suggested that the defects observed in *doa4* cells might be due to depletion of ubiquitin pools in these cells. Analysis of free ubiquitin levels by immunostaining

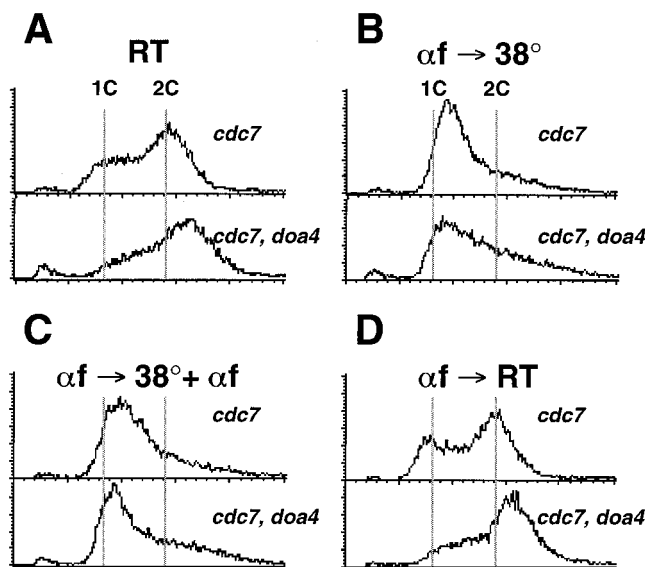


FIG. 7. The *cdc7* mutation prevents overreplication due to the *doa4* mutation. The *DOA4* gene was deleted from strain 7553-1-1 (*cdc7*) to produce the otherwise isogenic strain 7553-100 (*cdc7 doa4-Δ2*). The strains were grown in rich medium to log phase at RT (A), treated for 3 h with 5  $\mu$ g of  $\alpha$ -factor ( $\alpha$ f; Sigma) per ml, washed, transferred to rich medium, and incubated with (C) or without (B)  $\alpha$ -factor at 38°C for 3 h or released to rich medium and incubated without  $\alpha$ -factor at RT for 4 h (D; see Materials and Methods). Total DNA contents were assayed as described in the legend to Fig. 2, and mitochondrial DNA was assayed as described in Materials and Methods. Strain 7553-100 had a lower ratio of mitochondrial DNA to genomic DNA than did strain 7553-1-1 under all conditions (50 to 60%), and the ratio did not change significantly under the conditions shown, indicating that the DNA content changes observed were not caused by changes in the level of mitochondrial DNA.

showed that ubiquitin pools are, in fact, depleted under heat stress conditions when *DOA4* is mutated (Fig. 9).

Since restoration of free ubiquitin levels partially restored the temperature resistance of *doa4* cells, we tested the DNA contents of these cells (Fig. 10). Addition of the *UBI4* gene to *dos1-1* cells on a high-copy-number vector did not correct the overreplication phenotype, indicating that loss of the free ubiquitin pool is not responsible for the loss of replication coordination.

## DISCUSSION

We have found that the *DOA4* gene in *S. cerevisiae* is needed both for normal coordination of DNA replication and for ubiquitin metabolism. Mutations in *DOA4* cause cells with single nuclei to acquire genomic DNA contents of between 2 and 3.2 C. DNA replication in these cells failed to stop after copying the genome once and also failed to complete a full second round of synthesis. Mutations in *DOA4* therefore cause a failure of the coordination of DNA replication such that each region of the genome is no longer copied precisely once per cell cycle. A second, uncharacterized mutation can enhance the extent of the overreplication produced, but loss of coordination is due to disruption of *DOA4*. Some ubiquitin-mediated process is thereby implicated in limiting DNA replication to one round per cell cycle.

The *dos1-1* mutation was isolated in a screen for mutations sensitive to functional DNA polymerase  $\alpha$ . We assumed that excess or uncoordinated replication would be lethal and that this effect would be abolished in the absence of DNA synthesis. While *dos1-1* cells do die rapidly if normal polymerase  $\alpha$  is

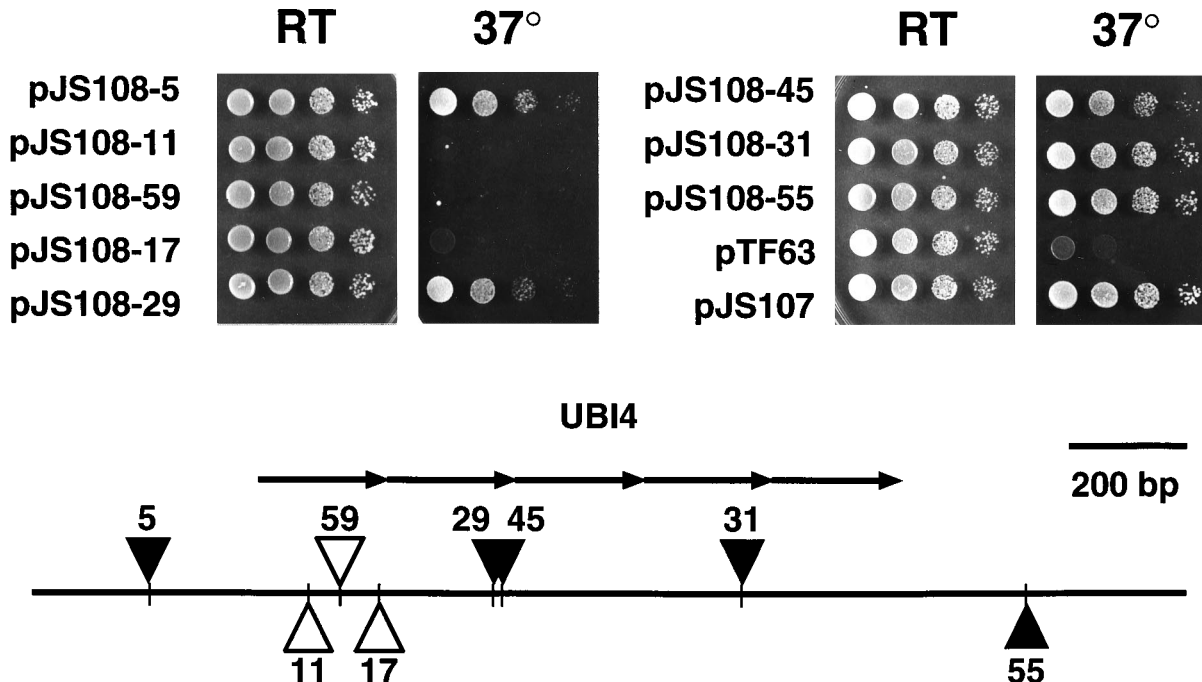


FIG. 8. The *UBI4* gene is a suppressor of *dos1-1*. A 2.8-kbp *Bam*HI-*Sal*I fragment containing the *UBI4* gene (and 276 bp of the bacterial tetracycline resistance gene) was subcloned into pTF63 (40) to produce high-copy-number yeast plasmid pJS107 and into pHSS6 (29) to produce Tn3-compatible target plasmid pJS108. A derivative of transposon Tn3 (mTn3-*URA3* [29]) containing a 2  $\mu$ m origin of replication was introduced into pJS108 to produce derivatives that contain a selectable marker and a high-copy-number yeast replication origin. Clones with the transposon in the positions shown (as determined by nucleotide sequencing) were introduced into strain 3003-19-1 (*dos1-1*). After selective growth, 10-fold serial dilutions were placed on selective medium and incubated at RT or 37°C. *UBI4* encodes five fused tandem repeats of the ubiquitin monomer (abutting arrows). Transposon insertions that retain suppressor activity are represented by closed triangles, while those lacking activity are represented by open triangles. Symbols above and below the line differ in the orientation of the transposon. Loss of complementation was observed only in constructs in which the transposon disrupts the first ubiquitin repeat, blocking the ability to produce any monoubiquitin. Similar suppression was obtained with a strain with a deletion of *DOA4* (data not shown).



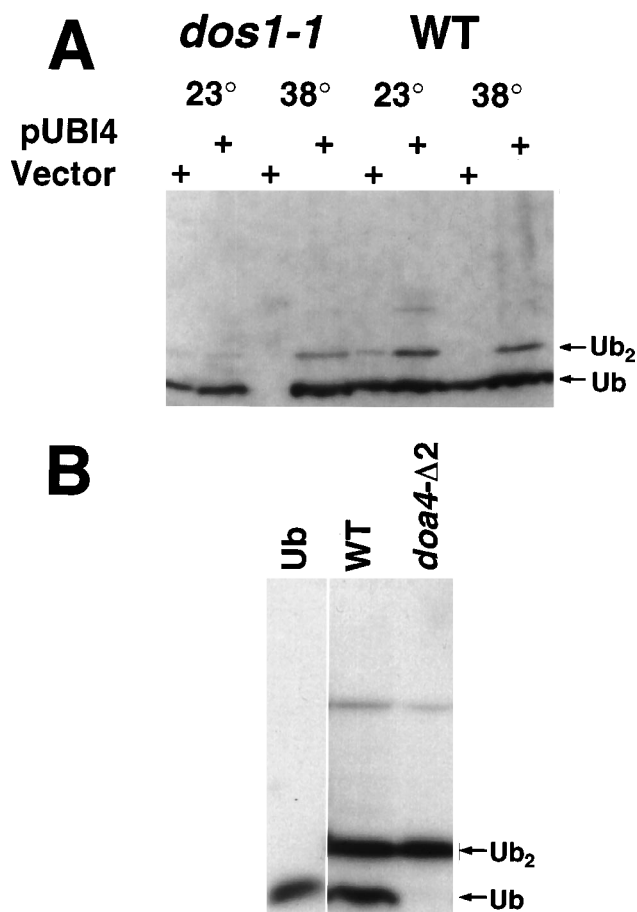


FIG. 9. Mutations in *DOA4* cause loss of free ubiquitin (Ub) pools under heat stress conditions. (A) Strains 3076-14-3 (*dos1-1*) and 7236-2-3 (wild type [WT]) were transformed with pTF63 (vector) or pJS107 (pUBI4), grown in selective medium at 23°C, and then either collected or shifted for 6 h to 38°C and then collected. Proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were immunostained with antiubiquitin antibodies. An identical gel stained with Coomassie blue dye indicated that uniform amounts of protein were loaded onto each lane. The identity of the dimeric ubiquitin band was inferred from the migration of this species relative to that of molecular weight standards. (B) Strains 4053-5-2 (WT) and 7496 (*doa4-Δ2*) were grown to log phase in YM-1 at 23°C, incubated at 38°C for 6 h, and then processed as described for panel A.

available, we have found that death does not correlate strongly with excess replication. In fact, the *UBI4* gene suppresses the temperature sensitivity of *dos1-1* cells even though it allows overreplication to proceed. Under these conditions, the cells grow slowly and exhibit an aberrant morphology but remain viable. The errors that occur in *dos1-1* cells can therefore eventually be detected and corrected, suggesting that the excess DNA does not take on a highly unusual form. Consistent with this interpretation, we have found that cells with *doa4* mutations display normal levels of recombination and so do not appear to experience unusually high levels of repair, since this is associated with induction of recombination.

The lethality induced by *doa4* mutations varies with the number of copies of the *POL1* gene in the cell. In the presence of the high-copy-number *POL1* plasmid used in our initial screen, the viability of *dos1-1* cells decreased about 100-fold during 6 h of incubation at 38°C (Fig. 1). With a low-copy-number *POL1* plasmid or a single genomic copy of *POL1*, viability is almost normal after the same treatment (a *dos1-1*

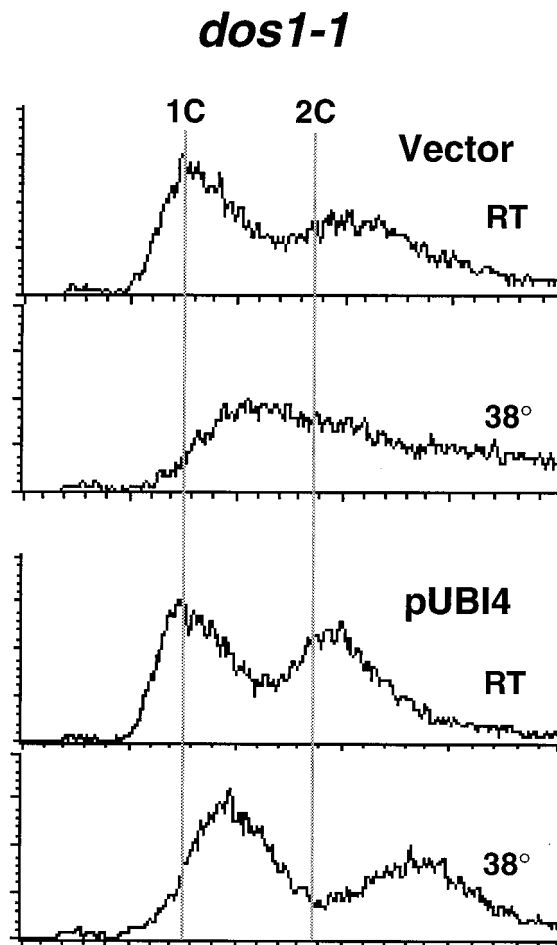


FIG. 10. Elevated levels of *UBI4* do not reverse the overreplication phenotype of *dos1-1*. Strain 3003-19-1 [*rho*<sup>0</sup>] (*dos1-1*) was transformed with pTF63 (vector) and pJS107 (pUBI4), grown to log phase in selective medium, shifted to 38°C, and assayed by flow cytometry as described in the legend to Fig. 2.

*POL1* culture retained 92% viability after 6 h of incubation at 38°C) (60). Both types of cells exhibit similar levels of overreplication, so it appears that death is caused by a combination of elevated *POL1* and excess replication. Coverley and Laskey have proposed a model for initiation of replication in eukaryotes in which multiple polymerase  $\alpha$ -mediated events compete for eventual elongation by a replication holoenzyme (10). Perhaps elevated levels of polymerase  $\alpha$  stress a mechanism of this sort such that too many or inappropriate initiation events occur in *doa4* mutants.

If overreplication is due to repeated refiring at origins, then the copy number of sequences near origins should be elevated relative to that of sequences far from origins. We were unable to test this model explicitly, since information regarding the pattern of genomic origin usage over large regions has been obtained only for chromosome III (20, 49), which apparently does not participate in overreplication. However, the *TRP1* region is associated with an origin and shows only the average 1.5-fold increase in copy number, suggesting that overreplication is not confined strongly to origin regions.

Cells lacking *DOA4* have nearly normal 1 C DNA contents when grown under permissive conditions but have elevated DNA contents in G<sub>2</sub>. In the presence of a mutation in *CDC7*, which encodes a protein kinase needed for entry into S phase,

both *DOA4*-proficient and -deficient cells arrest with 1 C DNA contents at the restrictive temperature, while growing or released cells show elevated DNA contents only if they lack *DOA4*. These results suggest that *doa4* cells enter S phase with normal DNA contents but fail to complete S phase normally, instead producing more than one copy of some genomic sequences. This situation is somehow detected and corrected, leading to normal mitosis after a G<sub>2</sub> delay and regeneration of normal 1 C DNA-containing G<sub>1</sub> cells.

Ubiquitin hydrolases can remove ubiquitin from ubiquitin-conjugated proteins, and they can also convert ubiquitin polymers to monomers (9, 13, 28). *S. cerevisiae* has many ubiquitin hydrolases, but the effects caused by *doa4* mutations are far more pronounced than the phenotypes caused by other hydrolase mutations (1, 13, 31), indicating that these enzymes are not functionally redundant. Loss of Doa4 causes slower degradation of several proteins (6, 47), possibly because the failure to remove ubiquitin from conjugates blocks the release of substrates from the 26S protease complex, thereby slowing general ubiquitin-dependent proteolysis (reviewed in reference 28).

The loss of a ubiquitin hydrolase could affect DNA replication in at least two ways. First, a general slowing of proteolysis due to inhibition of the proteasome could cause persistence of a factor that promotes DNA replication. Second, if the activity of a factor that promotes or limits replication is controlled by reversible modification with ubiquitin, the absence of a hydrolase could alter the state of this regulation. Interpreted in terms of the licensing factor model (2), in the first case, cells would be deficient in destroying the licensing factor during DNA replication, or in the second case, cells would be unable to switch the license to an "off" conformation in response to a passing replication complex.

A plasmid carrying the *UBI4* gene that encodes polyubiquitin suppresses many of the defects found in cells lacking the Doa4 protein. The *UBI4* plasmid restores the free ubiquitin pools that are lost under heat stress conditions in these cells and might rescue them in this way. In this view, a variety of processes using the same pool of free ubiquitin are blocked by the decimation of this pool in *doa4* cells, leading to the observed set of pleiotropic defects. Restoration of the pool by increased production of the Ubi4 protein reverses some of these effects but does not block overreplication at high temperatures (Fig. 10). We therefore suggest that overreplication is a direct consequence of the loss of *DOA4*, while the other phenotypes are indirect effects of the loss of the free ubiquitin pool.

These results link DNA replication control with a gene needed for normal ubiquitin metabolism. Elucidation of substrates acted upon by Doa4 and further characterization of the conditions that convert overreplication of DNA from a survivable to a lethal event will produce a clearer picture of the mechanisms that restrict DNA replication to a single round per cell cycle and coordinate the use of many replicons in eukaryotic cells.

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