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The Yeast *CDC16* and *CDC27* Genes Restrict DNA Replication to Once per Cell Cycle

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Summary

***CDC16* and *CDC27* were identified as genes in S. cerevisiae necessary to limit DNA replication to once per cell cycle. A screen for mutants that overreplicated their DNA uncovered new conditional alleles that cause accumulation of up to 8C DNA. DNA overreplication involves all chromosomes and does not require passage through mitosis or another START. It occurs within a single cell cycle and can cause arrest at the *MEC1* checkpoint. Remarkably, Clb2–Cdc28 activity remains elevated in the overreplicating cells. These observations distinguish *CDC16* and *CDC27* from other mutants that accumulate extra DNA after completing an aberrent mitosis, or skipping mitosis altogether, and entering a second, inappropriate G1 and S phase. *CDC16* and *CDC27* may contribute to replication control by targeted proteolysis of an S phase initiator.**

Introduction

A variety of approaches have been taken to understand how DNA replication is limited to once per cell cycle. The basic outlines of these controls were first described by cell fusion and nuclear transplantation experiments, which showed that G2 nuclei are refractory to the positive replication signals in S phase cells (Rao and Johnson, 1970). The molecular mechanisms responsible for this control remain mysterious, although the notion of a "licensing factor" (Blow and Laskey, 1988) has stimulated much interest and received considerable experimental support (Leno et al., 1992; Blow, 1993; Coverley et al., 1993). The cardinal feature of this model is that an essential replication factor, a licensing factor, is destroyed within the nucleus during each S phase and can only be replenished from cytoplasmic stores when the integrity of the nuclear membrane is breached during mitosis. Specific proteins with properties predicted by this model have been identified (reviewed in Tye, 1994, and Huberman, 1995), but it has not yet been shown that their regulation is either necessary or sufficient to limit replication to once per cell cycle.

A different perspective on replication control emerged from experiments in the fission yeast Schizosaccharomyces pombe that focused on the role of mitotic cyclin-dependent kinase (CDK) activity in coordinating S phase with mitosis. A screen for mutants that attained an increased ploidy led to the isolation of mutations in the $cdc2^+$ and $cdc13^+$ proteins (a CDK and a mitotic cyclin, respectively) (Broek et al., 1990; Hayles et al., 1994). An additional study found that overexpression of the $rum1^+$ protein, a CDK inhibitor, also allowed S. pombe cells to undergo sequential, uninterrupted S phases (Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995). In these studies, the cells accumulated extra DNA in whole genomic increments (2C, 4C, 8C, etc.), indicative of repeated S phases with no intervening mitoses. In agreement with these findings, CDK activity is required in Xenopus oogenesis to prevent an inappropriate S phase between meiosis I and meiosis II (Furuno et al., 1994). Moreover, mitotic cyclin B-Cdc2 kinase prevents the assembly of prereplication RPA complexes on DNA (Adachi and Laemmli, 1994). These observations have led to the idea that the inhibitory effect of mitotic CDK activity on DNA replication establishes the obligatory alternation of S phase with mitosis by requiring that mitosis be complete before S phase can begin again. The Saccharomyces cerevisiae *sim1* and *sim2* mutants undergo a single additional round of DNA replication without mitosis, apparently owing to a decrease in CDK activity (Dahmann et al., 1995).

We have studied the control of DNA replication in S. cerevisiae. Our approach involved screening several hundred temperature-sensitive mutants to identify those that possessed excess DNA at the restrictive temperature. Five groups of mutants were classified as "overreplicators," and two of these were shown to contain new alleles of previously characterized CDC genes, *CDC16* and *CDC27*. These mutants overreplicate their DNA despite having continuously elevated mitotic CDK activity, which distinguishes them from other regulators of replication. Although earlier studies have implicated the *CDC16* and *CDC27* genes in predominantly mitosis-specific processes, our work shows that these genes are also essential to limit DNA replication to once per cell cycle.

Results

Yeast Mutants That Accumulate Excess DNA

Individual members of a library containing over one thousand independent temperature-sensitive S. cerevisiae mutants were screened for DNA content after 12 hr of growth at permissive and restrictive temperatures (Hartwell, 1967; Klyce and McLaughlin, 1973). The DNA content of 420 mutants was analyzed by flow cytometry, and 18 were chosen for further study. These appeared to have normal haploid DNA content at the permissive temperature, but accumulated substantially greater than 2C DNA at 36°C. Fluorescence microscopy of propidium iodide– or DAPI-stained cells demonstrated that the increased DNA was nuclear. Direct quantitation and ethidium bromide staining of total DNA confirmed these findings (data not shown). Upon back-crossing to ensure that overreplication segregated as a single mutation, five complementation groups of recessive mutants were assigned. Below we focus on two groups, each containing two different alleles. Both groups of mutants doubled with the same kinetics as wild-type cells when incubated at 25°C; however at 36°C, the mutant cells

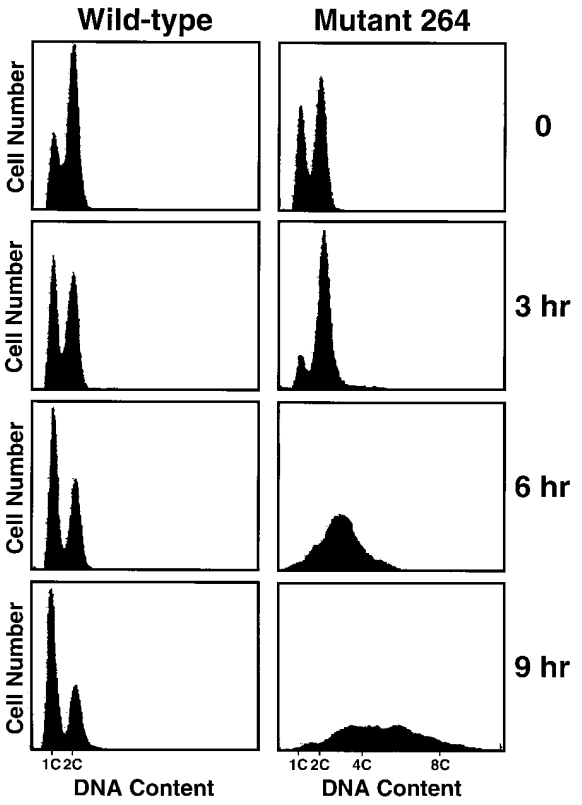


Figure 1. Temperature-Sensitive Yeast Mutants Accumulate up to Four Times the Normal Amount of DNA at 36°C

Exponentially growing wild-type and mutant cells were shifted from 23°C to 36°C and analyzed by flow cutometry. Propidium iodide fluorescence is plotted on the x-axis and is proportional to DNA content. Cell number is shown on the y-axis.

stopped dividing but continued DNA replication. After 12 hr at 36°C, asynchronous mutant populations accrued up to 8C DNA while wild-type cells never contained more than 2C DNA (Figure 1).

CDC16 and *CDC27* Mutants Overreplicate Their DNA

The identity of each overreplication mutant was determined by rescue of the temperature-sensitive phenotype with yeast genomic DNA and confirmed by genetic complementation studies with known yeast mutants. Each was transformed with a low-copy yeast genomic library with a *URA3* selectable marker (Rose et al., 1987). Ura^+ transformants capable of growing at 37°C were selected and analyzed by flow cytometry; all were rescued for both temperature sensitivity and DNA overreplication. Upon loss of the transforming plasmid, all cells reverted to the original thermosensitive phenotype. Transposon insertion was used to identify the relevant open reading frame in the rescuing plasmids (Strathmann et al., 1991). Mutants 264 and 299 were rescued by the wild-type *CDC16* gene, and mutants 22 and 283 were rescued by the wild-type *CDC27* gene. Subsequent subcloning of the *CDC16* and *CDC27* genes from the original plasmids and reintroduction into the mutants confirmed these results. Genetic complementation studies with the original *cdc16-1* and *cdc27-1* alleles supported the identification of *CDC16* and *CDC27* mutants as DNA overreplicators. The two new *CDC16* alleles are referred to as *cdc16-264* and *cdc16-299*, and the new *CDC27* alleles are *cdc27-22* and *cdc27-283*.

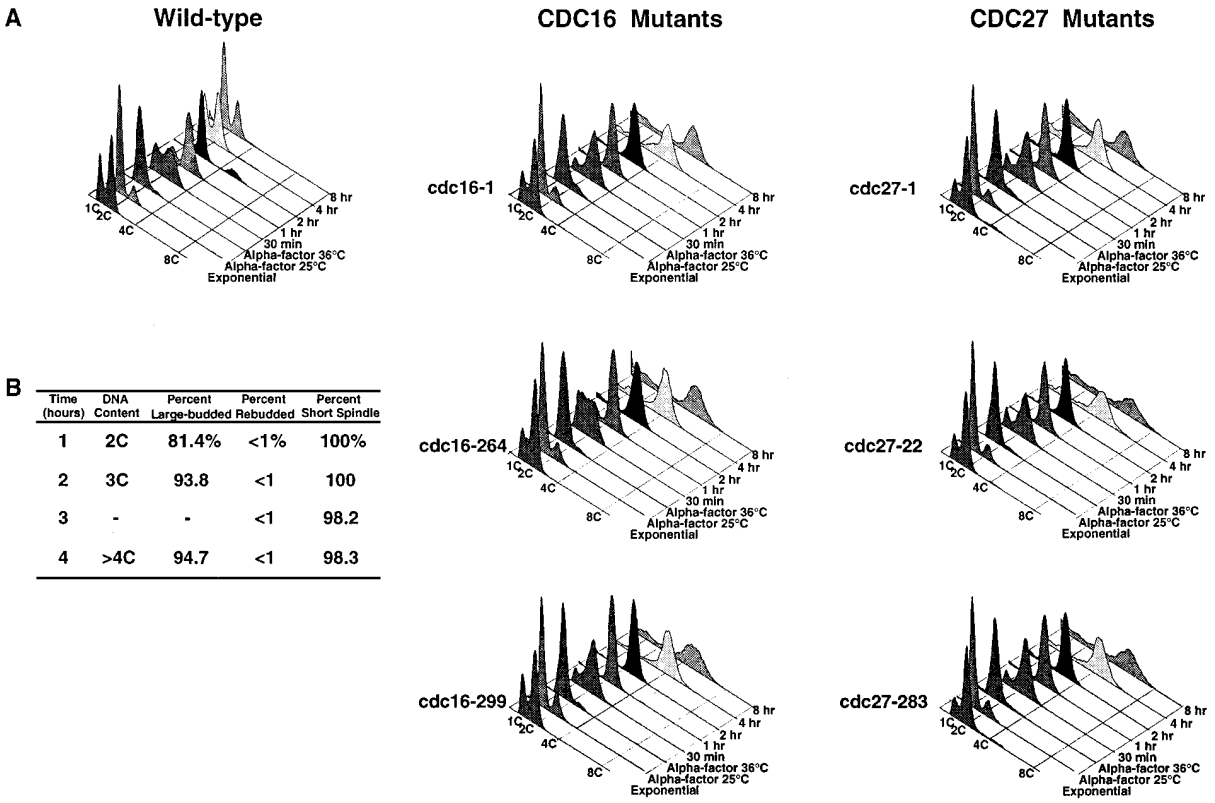
Flow cytometric analyses of synchronous cultures were performed to characterize the nature of DNA overreplication in the mutants and to compare them to the previously described alleles and the wild-type strain (Figure 2A). Cells were synchronized in the G1 phase of the cell cycle with α factor, shifted to 36°C in α factor, and released from the G1 block at 36°C by the addition of pronase. Cells that were α factor arrested contained a uniform 1C DNA. Within 1 hr of release from the G1 block, both wild-type and mutant cells progressed through S phase and accumulated a 2C DNA content. The wild-type cells divided several times during this time course: once between 1 and 2 hr, again between 2 and 4 hr, and at least one more time between 4 and 8 hr. In contrast, the mutants never divided after release from the G1 block. Whereas the wild-type cells oscillated between 1C and 2C with successive cell cycles and eventually lost their synchrony, the mutants continued to accumulate DNA. By 4 hr, the mutant cells contained greater than 2 times the normal amount of DNA (4C content), and at 8 hr, they contained greater than 6C DNA. The wild-type and mutant cells initially followed the same kinetics of budding and spindle formation; however, the mutants remained primarily in a G2-like state as large-budded cells with a short mitotic spindle and a single nucleus (Figure 2B; see below).

The *cdc16-1* and *cdc27-1* cells also accumulated excess DNA after synchronization and subsequent incubation at 36°C. In fact, they exhibited similar patterns of DNA accumulation when compared to the new alleles uncovered in the screen. To rule out the possibility that mitochondrial DNA replication uncoupled from cell cycle control accounted for the overreplication phenotype, *CDC16* and *CDC27* mutants lacking mitochondrial DNA (*rho*^{0} mutants) were also shown to increase DNA content after incubation at 36°C (data not shown).

The *CDC16* and *CDC27* Mutants Arrest with a G2-like Morphology

Microscopic examination of the synchronized cultures demonstrated that the *CDC16* and *CDC27* mutant cells arrested primarily within the first cell cycle after incubation at 37°C (Table 1). The α factor–arrested cells were transferred to prewarmed plates, and microcolonies were observed after two days. In this assay, cells that arrested within the first cycle would be represented by microcolonies of one large-budded cell. Close to 90% of the *CDC16* mutants exhibited a first cycle arrest, whereas the *CDC27* mutants arrested within the first cycle $\sim$80% of the time depending on the particular allele. Analyses of liquid cultures confirmed these findings and showed that there was usually no more than a 10% increase in cell number after release from α factor arrest at 36°C (data not shown).

Fluorescence microscopy of the *CDC16* mutants showed that the cells arrested with a phenotype consistent with an arrest within S phase or G2 (Pringle and



B

Time (hours)	DNA Content	Percent Large-budded	Percent Rebudded	Percent Short Spindle
1	2C	81.4%	<1%	100%
2	3C	93.8	<1	100
3	-	-	<1	98.2
4	>4C	94.7	<1	98.3

Figure 2. *CDC16* and *CDC27* Mutant Cells Overreplicate Their DNA While Remaining in a G2-like Morphology

(A) Wild-type and mutant cultures were synchronized in G1 at 25°C, shifted to 36°C, and released from the G1 arrest at 36°C. Propidium iodide fluorescence is plotted on the x-axis; cell number is shown on the y-axis. (B) Table correlating DNA overreplication in *cdc16-264* cells with cellular morphology.

Hartwell, 1981). DAPI staining of DNA showed that, depending upon the particular *CDC16* allele, 83%–99% of the cells arrested prior to mitosis with a single nucleus usually positioned at the bud neck (Table 1). Anti-tubulin immunofluorescence also demonstrated a premitotic G2-like arrest, since 71%–95% of the cells possessed a short spindle similar to *cdc20*-arrested cells (Figure 2B and Table 1) (Sethi et al., 1991). The *CDC27* alleles were more variable with regard to arrest morphology. The *cdc27-1* cells arrested with one undivided nucleus and a short spindle (96%); however, up to 41% of the *cdc27-22* and 25% of the *cdc27-283* cells contained two well-separated nuclei and an elongated or a postmitotic spindle. Note that the low level of nuclear division in the *CDC16* and *CDC27* mutants can be completely prevented by incubation in the mitotic inhibitor nocodazole, but this does not prevent DNA overreplication (see below). Both groups of mutants budded with kinetics similar to wild-type cells, budding between 30 and 60 min after release from an α factor block. The *cdc16-1* and *cdc16-264* alleles as well as the *cdc27-1* strain rebudded less than 8% of the time; however, the *cdc16-299*, *cdc27-22*, and *cdc27-283* strains possessed additional buds in the cases where nuclear division had occurred.

Overreplication of DNA Does Not Require Passage through Mitosis or START

We tested whether DNA overreplication required the completion of cell cycle events that normally occur after S phase. The same types of synchrony experiments were performed as described above except that various agents were added to specifically block upcoming

Table 1. *CDC16* and *CDC27* Mutants Arrest Primarily in a G2-like Morphology

	Bud Morphology			Nuclei		Spindle Morphology		First Cycle Arrest	Cdc28 Kinase Levels
	Total Budded (%)	1 Bud (%)	2–3 Buds (%)	1 Nucleus (%)	2–4 Nuclei (%)	Short (%)	Other (%)		
cdc16-1	100	94.4	5.6	99.0	0	95.1	4.9	88.0	high
cdc16-264	96.3	88.8	7.5	89.3	10.7	80.8	19.2	94.4	high
cdc16-299	93.0	74.0	19.0	83.2	16.8	71.2	28.8	86.8	high
cdc27-1	96.0	96.0	0	97.1	2.9	96.1	3.9	84.2	high
cdc27-22	94.2	39.8	54.4	58.7	41.3	35.3	64.7	75.0	high
cdc27-283	98.2	61.9	36.3	75.2	24.8	50.0	50.0	85.5	high

stages of the cell cycle. In one experiment, cells were released from α factor–induced G1 synchrony followed by continuous incubation at 36°C in the presence of additional α factor. This caused wild-type cells to arrest in the following G1 period with 1C DNA, but had no effect on DNA overreplication in the *CDC16* and *CDC27* mutants. In a similar experiment, the mitotic inhibitor nocodazole added to α factor–released cells blocked wild-type cells in G2/M with 2C DNA, but the *CDC16* and *CDC27* mutants continued to synthesize DNA. Control experiments showed that overreplication was prevented by the DNA synthesis inhibitor hydroxyurea. Together, these results showed that overreplication can occur without an intervening mitosis or an additional START period, since cells prevented from completing these cell cycle stages accumulated excess DNA.

Density Transfer Experiments Confirm Overreplication

Density transfer experiments directly demonstrated DNA overreplication in the *CDC16* and *CDC27* mutants, and suggest that this occurred within the confines of a single cell cycle. A representative of each mutant class (*cdc16-264* and *cdc27-22*) was pregrown for eight generations at 25°C in minimal media containing [^{13}C]glucose and [^{15}N]ammonium sulfate so that the DNA was uniformly "heavy" in density. The cultures were synchronized according to a similar protocol as that described above and released from G1 arrest at 36°C in medium with no heavy isotopes. A time course of DNA replication is shown in Figure 3. Initially, the DNA resided in a single peak of heavy density. Upon release from the G1 block, a DNA peak of hybrid density accumulated, and by 2 hr most of the DNA had replicated one time. With increased incubation at 36°C, a peak of light density appeared, indicating that DNA sequences had been replicated a second time. The accumulation of DNA first with intermediate and then fully light density suggested that each cycle of DNA replication was semiconservative, and therefore probably reflected execution of a normal S phase. Moreover, light DNA did not appear until most or all of the DNA had become intermediate in density, implying that accumulation of extra DNA may have arisen from sequential, nonoverlapping S phases. During this experiment no nuclear division nor cytokinesis occurred.

Analysis of Overreplication by Pulsed-Field Electrophoresis

Chromosomes from wild-type and mutant cells grown at 36°C were analyzed by pulsed-field electrophoresis (Figure 4) (Carle and Olson, 1985). Wild-type yeast cells treated with the DNA synthesis inhibitor hydroxyurea as well as mutants such as *cdc46-1* that are incapable of proceeding through S phase give rise to abnormal chromosomal electrophoretic patterns, presumably because they possess replication-specific DNA structures (Hennessey et al., 1991). Instead of being well resolved through the agarose matrix, the chromosomes of these cells are retained in the wells. Chromosomes from wild-type cells entered the gel normally at the α factor block in G1, remained in the well at the 30 min time point when

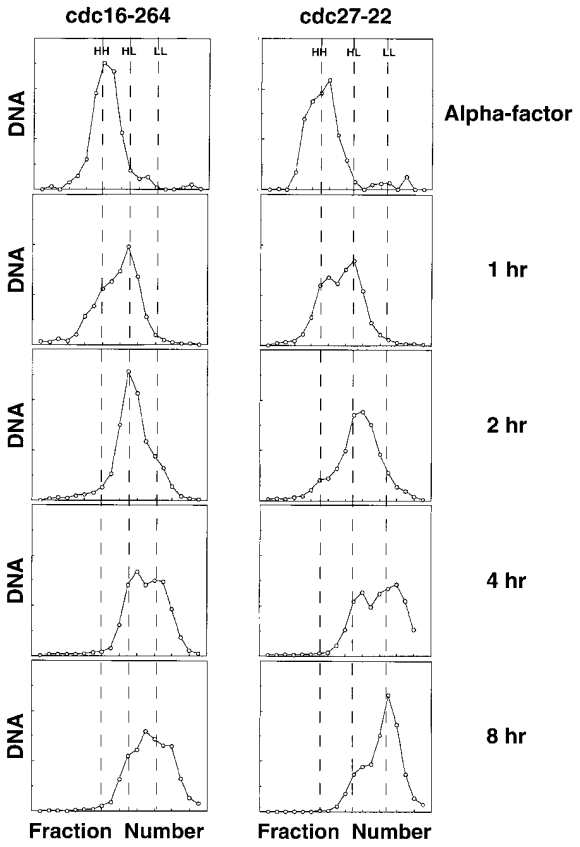


Figure 3. Density Transfer Analysis Shows That *CDC16* and *CDC27* Mutant Cells Replicate Their DNA More Than Once Per Cell Cycle

cdc16-264 and *cdc27-22* cells were pregrown in ^{13}C/^{15}N–labeled minimal media at 25°C, synchronized with α factor, shifted to 36°C, and released from G1 arrest at 36°C in YPD media. Shown on the x-axis are fraction numbers with density decreasing from left to right. The positions of "heavy-heavy" (HH), "heavy-light" (HL), and "light-light" (LL) DNA are shown above to indicate unreplicated, once-replicated, and twice-replicated DNA sequences. The y-axis represents DNA amount as measured by Southern blot analysis.

DNA synthesis had begun, and were once again well resolved after 1 hr, when the DNA sequences had been replicated one time. With regard to these time points, the *cdc16-264* cells had chromosomal patterns identical to the wild-type. However, after continued incubation at 36°C, the electrophoretic mobility of the wild-type chromosomes were well resolved, since the cultures lost their synchrony (data not shown) while the mutant chromosomes once again became retarded and possessed a pattern resembling that of chromosomes in the midst of DNA synthesis (Figure 4). Mutant cells blocked in S phase or G2/M at 25°C by treatment with hydroxyurea or nocodazole showed similar patterns to wild-type controls shown (data not shown). These findings illustrate the fact that all mutant chromosomes were subject to overreplication at 36°C, since there were no biases with regard to electrophoretic mobility. One can also rule out that excess DNA accumulation was the result of chromosome nondisjunction, amplification of only one or a few DNA sequences, or rampant mitochondrial DNA synthesis. Similar results were obtained for the other *CDC16* and *CDC27* alleles (data not shown).

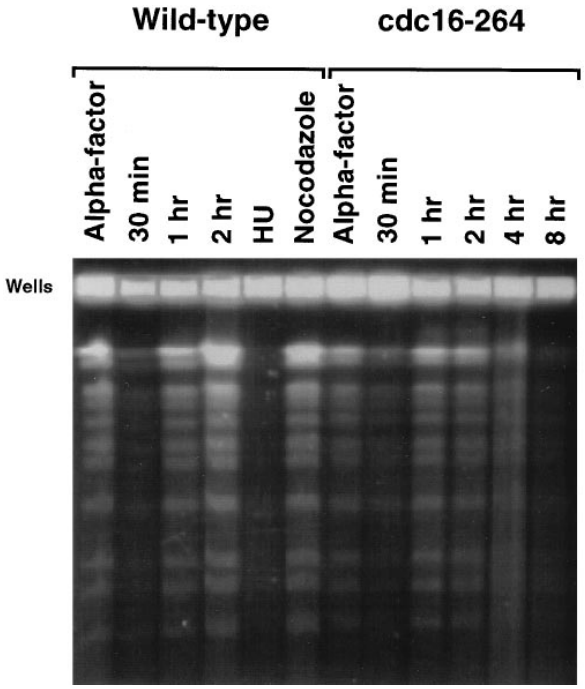


Figure 4. Pulsed-Field Electrophoresis Demonstrates That All Chromosomes Are Abnormally Replicated

Whole chromosomes of synchronized wild-type and *cdc16-264* cells were subjected to pulsed-field electrophoresis. The 30 min time points correspond to cells in the midst of DNA synthesis (see Figure 2). Wild-type cells were treated with hydroxyurea (HU) as a positive control for cells blocked in DNA synthesis.

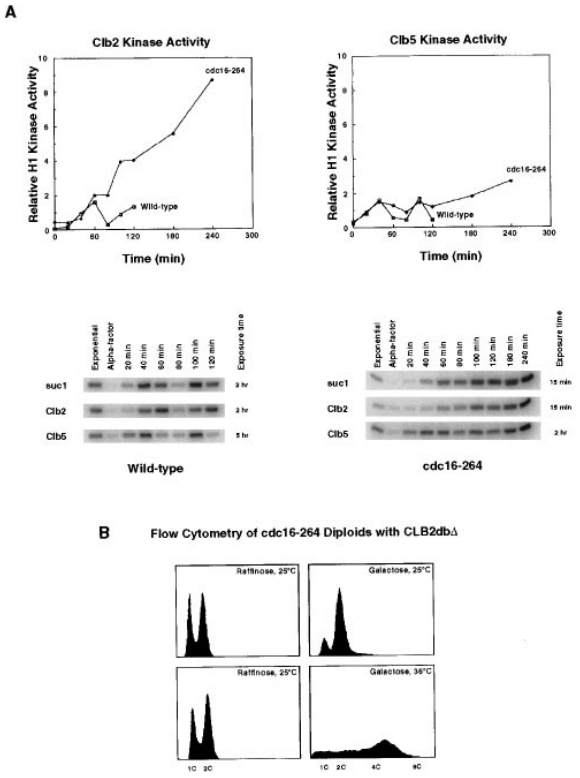


Figure 5. *CDC16* Cells Overreplicate Their DNA despite Continually Elevated Clb-Cdc28 Kinase Activity

(A) Cdc28 kinase levels of synchronous wild-type and *cdc16-264* mutant cultures were assayed by histone H1 phosphorylation. Identical amounts of total protein were used, but exposure times were varied to display amounts in the linear range. The graphs above directly compare relative amounts of kinase activity. Total Cdc28 kinase activity measurements utilized suc1-beads; Clb2- and Clb5-associated kinase activity used cyclin-specific immunoprecipitation.

(B) Flow cytometry of *cdc16-264*/*cdc16-264* diploids containing galactose-inducible *CLB2db*Δ were grown for 12 hr in yeast extract–peptone plus raffinose at 25°C (left graphs). Galactose was added to 2% and the cultures were incubated at 25°C (top) or 36°C (bottom) for 12 hr.

CDC16 and *CDC27* Mutants Arrest with Elevated Cdc28 Kinase Levels

At the restrictive temperature, *CDC16* and *CDC27* mutants contain high Cdc28 kinase levels, consistent with an arrest in S phase or G2. Cellular extracts were prepared from α factor–synchronized cells at 36°C, and total Cdc28 kinase activity was measured by assaying the histone H1 kinase activity of protein immobilized on $p13^{suc1}$ beads (Brizuela et al., 1987). As shown in Figure 5A, the kinase levels of *cdc16-264* cells increased with similar kinetics to the wild-type cells; however, these levels continued to increase with increased time at 36°C. Cdc28 levels of wild-type cells were periodic with successive cell cycles, the troughs coinciding with cell division. At later time points when synchrony was lost in the wild-type cells (180 and 240 min), kinase levels were comparable to the exponential time point shown (data not shown). Similar findings were obtained from experiments in which wild-type and mutant cells were transformed with an HA-tagged version of Cdc28, and Cdc28 kinase activity was assayed by immunoprecipitation with anti-HA antibodies (data not shown). Such elevated Cdc28 kinase levels of the mutants are comparable to those seen in nocodazole-treated cells blocked in mitosis (data not shown). Further analysis showed that Clb5- and Clb2-associated kinase levels remained high in the *cdc16-264* mutant throughout the time course, demonstrating that neither S phase nor M phase kinases oscillated. Elevated kinase activity was also observed with the other *CDC16* and *CDC27* alleles (data not shown). The finding that most of the mutants do not rebud at 36°C is consistent with the presence of high Cdc28 kinase levels.

Although unlikely, it remained a formal possibility that Cdc28 kinase activity oscillated at the single cell level but was not detected because of the asynchrony of the cell population. To firmly establish that DNA overreplication did not require a decrease in Cdc28 kinase activity, a *cdc16-264* diploid strain was constructed containing a galactose-inducible form of the Clb2 protein that lacked the amino acids required for ubiquitin-mediated degradation (*GAL-CLB2db*Δ) (Surana et al., 1993). Asynchronous cultures were incubated at 25 or 36°C in the presence of galactose, and DNA content was assayed by flow cytometry (Figure 5B). When grown at 25°C, cells arrested with a G2 content of DNA, as would be predicted for cells unable to complete mitosis. At 36°C expression of the stable Clb2 protein had no effect on

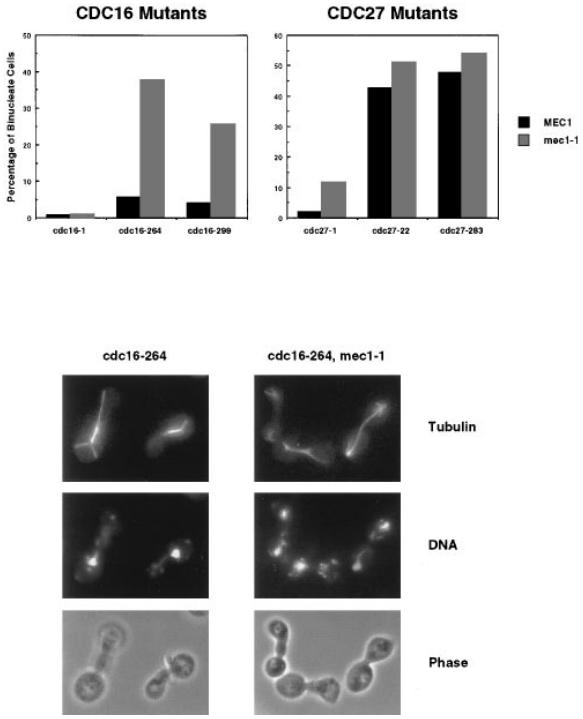


Figure 6. The Terminal Arrest Phenotype of *CDC16-264* and *CDC16-299* Cells Is *MEC1* Dependent

CDC16 and *CDC27* mutants, and double mutants containing the *mec1-1* mutation, were synchronized in G1, shifted to 36°C, and released at 36°C. Cultures were fixed 4 hr after G1 release. Mitotic spindles are visualized by anti-tubulin immunofluorescence; nuclei, by DAPI staining. Graphs shown above compare percentage of cells containing two nuclei.

DNA overreplication and the cells accumulated up to an 8C DNA content. Wild-type cells containing the *GAL-CLB2db*Δ construct did not show DNA overreplication under the same conditions (data not shown).

Overreplicating Mutants Are Blocked at the MEC1 Checkpoint

Genetic studies of the overreplication mutants suggest that the cells are actively prevented from continuing in the cell cycle due to "checkpoints" that can sense ongoing or aberrant DNA replication. The *CDC16* and *CDC27* alleles were introduced into a known checkpoint-deficient strain, *mec1-1*. The *MEC1* gene is required for preventing mitosis in cells that are blocked in S phase or contain DNA damage (Weinert et al., 1994). Flow cytometry of the *CDC16* and *CDC27* mutants showed that excess DNA still accumulated when the cells were deficient for the *MEC1* checkpoint; further, microcolony studies demonstrated that the double mutants did not undergo an additional cell division (data not shown). Interestingly, fluorescence microscopy revealed that a large proportion of the *cdc16-264 mec1-1* and *cdc16-299 mec1-1* cells arrested with two nuclei and an extended mitotic spindle (Figure 6). In contrast, the *cdc16-1* allele still showed a premitotic arrest, consistent with a previous report (Weinert et al., 1994). Viability studies demonstrated that *cdc16-264 mec1-1 and cdc16-299 mec1-1* mutants possessed decreased survival rates after incubation at the nonpermissive temperature; however, the *cdc16-1 mec1-1* cells were unaffected (data not shown). The *cdc27-1 mec1-1* cells showed a 5-fold increase in the number of binucleate cells; however, it was difficult to determine whether the *cdc27-22* and *cdc27-283* alleles were affected by the *mec1-1* checkpoint mutation, since a relatively large percentage of these cells normally arrest with two nuclei.

CDC16 mutant alleles were also tested in another genetic background containing a defective checkpoint gene. The *RAD9* gene product is essential for arresting cells in the G2 phase of the cell cycle in response to DNA damage (Weinert and Hartwell, 1988). Double mutants containing either *cdc16-1*, *cdc16-264*, or *cdc16-264* and *rad9*Δ behaved similarly to the *cdc16 mec1* mutants. The *cdc16-1 rad9*Δ cells arrested with a single nucleus (100%) while the *cdc16-264 rad9*Δ and *cdc16-299 rad9*Δ strains frequently arrested with divided nuclei (25% after a 4 hr release from α factor arrest). Thus, at least for the *cdc16-264* and *cdc16-299*, these strains seemed to be actively blocked from progressing further in the cell cycle rather than being incapable of performing subsequent functions such as nuclear division.

Discussion

In searching for S. cerevisiae mutants that were defective in controlling DNA synthesis, we uncovered new alleles of two *CDC* genes, *CDC16* and *CDC27*. These new alleles, in addition to the originally described mutants, accumulated between 2C and 8C DNA contents when incubated at the nonpermissive temperature. Density transfer experiments showed that the increased DNA content was accomplished by semiconservative DNA synthesis, and pulsed-field gel electrophoresis showed that all chromosomes had overreplicated.

By multiple criteria DNA overreplication in *CDC16* and *CDC27* mutants occurred within the confines of a single cell cycle. The morphology of overreplicating cells indicated that they were blocked in a G2-like state. They arrested as large-budded cells with a single undivided nucleus and a short mitotic spindle that never elongated or broke down. Moreover, the mitotic inhibitor nocodazole had no effect on terminal morphology of the mutants, nor did it prevent DNA overreplication. Biochemical measurements also indicated that the overreplicating cells were arrested in a G2-like state because they contained high levels of the mitotic Clb2-Cdc28 kinase. This was further supported by experiments showing that expression of a dominant Clb2 mutant that prevents completion of mitosis did not prevent overreplication in the *cdc16-264* mutant. Finally, genetic experiments showed that cell cycle progression in the *CDC16* and *CDC27* mutants was constrained by the *MEC1* and *RAD9* G2 checkpoints, since double mutants progressed further in the cell cycle and completed nuclear division. Thus, DNA overreplication occurred while cells were arrested at the *MEC1* and *RAD9* checkpoint, which presumably recognized the presence of aberrantly replicated or damaged DNA and caused the cell cycle to stop prior to the completion of mitosis.

DNA overreplication also does not require passage through a second START. Readdition of α factor did not prevent overreplication, nor did *CDC16* or *CDC27* mutants bud again before beginning to accumulate more than 2C DNA. Our major conclusion is that mutations in *CDC16* and *CDC27* eliminate the strict requirement that S phase alternate with mitosis, and allow the overreplication of chromosomal DNA within a single cell cycle.

The alterations in cell cycle control observed in *CDC16* and *CDC27* mutants contrasts with the phenotypes of other yeast mutants that accumulate extra DNA. One type of mutant with increased DNA content exhibits continued cell cycle progression despite missegregation of chromosomes. In *ESP1* (McGrew et al., 1992) and *MPS1* (Winey et al., 1991) mutants, chromosomes do not disjoin correctly because of defects in spindle pole body segregation and duplication. Nevertheless, *ESP1* and *MPS1* mutants exit mitosis and start a new cell cycle as evidenced by the emergence of a new bud and, at least in the *ESP1* mutants, breakdown of the mitotic spindle and decline in mitotic Cdc28 kinase activity (Surana et al., 1993). These cells reenter S phase, and acquire greater than diploid amounts of DNA. Mutations in *ESP1*-related genes *cut1*$^+$ in S. pombe (Hirano et al., 1986; Creanor and Mitchison, 1990; Uzawa et al., 1990), and *BimB* in Aspergillus nidulans (May et al., 1992) have similar phenotypes. A second class of mutants increase their ploidy by skipping mitosis altogether. These mutants have inactive mitotic CDKs, and reveal the essential role of mitotic CDK activity in establishing the obligate alternation of S and M phases. The phenotype of *CDC16* and *CDC27* mutants described here differs from both of these others, because DNA overreplication in *CDC16* and *CDC27* mutants occurs without beginning a new cell cycle.

Functions of *CDC16* and *CDC27*

We infer from our results that one essential function of *CDC16* and *CDC27* is to restrict DNA replication to once per cell cycle. Recent insights into the biochemical functions of these proteins provide important clues as to how they might participate in the control of DNA replication.

CDC16, *CDC23*, and *CDC27* are members of the TPR (tetratricopeptide repeat) protein family (Sikorski et al., 1990). Homologs of *CDC16* and *CDC27* have been found in a variety of eukaryotic organisms ranging from fungi to humans (Hirano et al., 1988; O'Donnell et al., 1991; Tugendreich et al., 1993, 1995). The Cdc16, Cdc23, and Cdc27 proteins associate with each other, both in vivo and in vitro, forming part of a 20S complex involved in ubiquitin-mediated proteolysis (Lamb et al., 1994; King et al., 1995). These proteins do not appear to be generally required for protein destruction, since many unstable proteins (including some degraded by the ubiquitin pathway) remain unstable in *CDC16* and *CDC23* mutants (Irniger et al., 1995). Rather, it is thought that the Cdc16-Cdc23-Cdc27 complex targets specific proteins for proteolysis, perhaps by acting as a cell cycle–regulated E3 ubiquitin-ligase. This protein complex has been referred to as the "anaphase-promoting complex" because of its essential role in degrading mitosis-specific targets and allowing progression through the metaphase to anaphase transition (King et al., 1995).

The only specific protein substrates of the 20S complex containing Cdc16-Cdc23-Cdc27 known so far are the B-type mitotic cyclins. Biochemical experiments in Xenopus egg extracts have directly shown that this protein complex catalyzes cyclin B ubiquitination during mitosis (Glotzer et al., 1991; Hershko et al., 1994; King et al., 1995). Moreover, specific mutations in either *CDC16* or *CDC23* result in stabilization of cyclin B and cause anaphase arrest (Irniger et al., 1995). *CDC16*, *CDC23*, and *CDC27* have a distinct function that is required for sister chromatid separation at the metaphase to anaphase transition (Holloway et al., 1993; Irniger et al., 1995; Tugendreich et al., 1995). This also appears to involve ubiquitin-dependent protein turnover, because competitive inhibition of the cyclin B–proteolysis pathway with an N-terminal peptide of cyclin B prevents sister chromatid separation (Holloway et al., 1993).

These results have led to the idea that *CDC16* and *CDC27* regulation of DNA replication might also involve cell cycle–dependent protein destruction. We have no direct evidence that this is the case; however, at least some of the mutations in *CDC16*, *CDC23*, and *CDC27* that appear to effect their ability to target proteins for destruction also cause DNA overreplication. It is intriguing to consider the relationship between these results and the licensing factor model of replication control (Blow and Laskey, 1988). Perhaps the Cdc16 and Cdc27 proteins participate in destroying licensing factor during S phase, and the *CDC16* and *CDC27* mutants, being unable to revoke the licensing signal, cause reinitiation of DNA replication without mitosis. Potential targets include the Mcm and Cdc6 proteins, which have been postulated to be directly involved in promoting DNA replication at origins of replication (Hogan and Koshland, 1992; Tye, 1994; Huberman, 1995; Liang et al., 1995; Piatti et al., 1995). Support for the role of *CDC6* in controlling DNA replication in budding yeast comes from the finding in S. pombe that very high overexpression of the p65^{cdc18} protein (an S. pombe Cdc6 homolog) causes repeated rounds of DNA replication and accumulation of up to 8C DNA (Nishitani and Nurse, 1995).

Coordinating DNA Synthesis with the Cell Cycle

In the absence of *CDC16* or *CDC27* function, DNA synthesis becomes uncoupled from cell cycle progression. Previous experiments have shown that the temporal ordering of S phase and mitosis is determined by the inhibitory effect of mitotic CDK activity on DNA replication. Thus, it is both remarkable and unexpected that *CDC16* and *CDC27* mutations allow cells to continue DNA synthesis despite high levels of Clb2-Cdc28 kinase. This apparent paradox might be resolved if Cdc16 and Cdc27 proteins function downstream of mitotic CDK activity to enforce their inhibitory effect on DNA replication. In cells compromised for *CDC16* or *CDC27* function, mitotic CDK's would be unable to prevent DNA replication, and the cell cycle would behave as though no mitotic CDK activity were present. Experimental precedent indicates that this will result in reentry into S

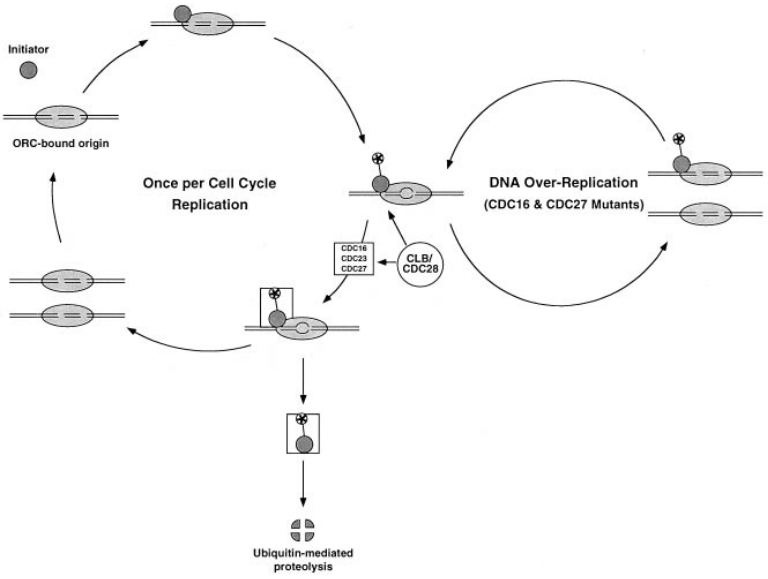


Figure 7. *CDC16-CDC23-CDC27* Restrict DNA Replication to Once per Cell Cycle

A molecular model describing the role of the Cdc16-Cdc23-Cdc27 complex in regulating DNA replication is shown. A putative DNA replication initiator protein interacts with the ORC-bound origin of replication. Activation of this initiator protein may be *CDC28*-dependent. Once initiation has occurred, this protein is inactivated by the Cdc16-Cdc23-Cdc27 complex. According to this model, inactivation of the initiator protein is controlled by the concerted action of the Clb-Cdc28 kinase and the Cdc16-Cdc23-Cdc27 complex. Cdc28 may phosphorylate the initiator protein and thereby render it subject to inactivation by Cdc16-Cdc23-Cdc27. Alternatively, the specific inhibitory activity of this complex may be induced by *CDC28*-dependent phosphorylation. In the *CDC16* and *CDC27* overreplicator mutants, the initiator protein, while marked for degradation, remains in its active state and DNA replication can continue unabated.

phase from G2, and accumulation of extra DNA (Broek, et al., 1990; Hayles et al., 1994).

Based upon this work and the work of others, one hypothesis is that activation of the Cdc16-Cdc23-Cdc27 complex leads to the turnover of specific targets including an essential and limiting replication factor, a protein that controls sister chromatid cohesion, and cyclin B. One specific molecular mechanism is shown in Figure 7. We propose that a key replication protein is phosphorylated by Clb-Cdc28 kinases, signaling its usage and targeting it for *CDC16-CDC23-CDC27*–mediated proteolysis. An alternative is that Clb-Cdc28 kinase activates the Cdc16-Cdc23-Cdc27 complex allowing it to recognize and destroy specific targets. In either case, the loss of *CDC16* or *CDC27* function allows this replication factor to remain active to promote additional rounds of DNA replication. A very parsimonious idea would be that phosphorylation both activates the initiator and targets it for destruction.

This model makes no specific assumptions about the timing of Cdc16-Cdc23-Cdc27 action in regulating DNA replication except that it must occur after S phase has begun. In this regard, it is now evident that *CDC16* and *CDC27* participate in the control of multiple, temporally distinct cell cycle events. Destruction of cyclin B in anaphase and separation of sister chromatids at the metaphase-to-anaphase transition both require *CDC16* and *CDC27*. Our results show that these proteins are also required prior to metaphase to prevent overreplication of chromosomal DNA. Thus, as demonstrated by double mutant studies with known checkpoint genes, *cdc16-264* and *cdc16-299* mutants are capable of completing sister chromatid separation but are prevented from doing so by a prior failure to cease DNA synthesis. These observations show that the functions of *CDC16*, *CDC27*, and perhaps their associated proteins in the anaphase-promoting complex extend beyond anaphase and are probably required at several points in the cell cycle to promote transitions from one phase to the next.

Experimental Procedures

Strains and Plasmids

The genotypes of the S. cerevisiae strains used in this study are all congenic with strain A364a except for the diploids, which are derived from both A364a and W303. All of the haploid strains used were MATa. The collection of temperature-sensitive mutants used for the original flow cytometric search is described in Hartwell, 1967, and Klyce and McLaughlin, 1973, and was obtained from C. McLaughlin (University of California, Irvine). The original *cdc16-1* and *cdc27-1* strains were obtained from L. Hartwell (University of Washington) and were backcrossed to the A364a parent strain. The *mec1-1* and *rad9*Δ strains (A364a background) were obtained from T. Weinert (University of Arizona) and were used to create the double mutants. The diploid strain containing homozygous *cdc16-264* mutations and the *CLB2db*Δ construct was made by integrating the *GAL-CLB2db*Δ plasmid (plasmid 2434 from K. Nasmyth, Research Institute of Molecular Pathology, Vienna; Surana et al., 1993) into haploid Gal^+ *cdc16-264* cells (mixed A364a and W303 strain background) and subsequently mating the transformants to a *cdc16-264* strain of the opposite mating type (A364a background).

Bacterial strains pox38 and JGM were used for random transposon insertion (Strathmann et al., 1991). The yeast genomic library used for mutant rescue is described in Rose et al., 1987.

Media and Growth Conditions

Media recipes were obtained from Ausubel et al., 1987. Cells were grown in yeast extract–peptone–dextrose (YPD) media unless otherwise indicated. Yeast extract–peptone plus 2% raffinose was used for pregrowth prior to galactose induction; 2% each of raffinose and galactose were utilized for inductions. Permissive temperatures ranged from 23°C–25°C. Nonpermissive temperatures ranged from 35°C–37°C. α factor was added at 5 μg/ml and was later removed upon the addition of 10 μg/ml pronase E (Sigma).

Flow Cytometry

Yeast samples were prepared for flow cytometric analysis adapted from Lew et al., 1992. Cultures were fixed in 70% ethanol, followed by a 4 hr digestion with RNase A. Cells were stained with propidium iodide overnight at 4°C. Samples were diluted and sonicated for 10 s. Fluorescence was measured using a Becton Dickinson FACScan and analyzed using Reproman or CELLQuest software.

Cloning and Complementation

The yeast genomic library was transformed into each *ura3* mutant using standard techniques (Ausubel et al., 1987). Ura^+ transformants

were selected at 37°C. Rescuing plasmids were subjected to random transposon insertions (Strathmann et al., 1991). Transposon-tagged sequences were reintroduced into the mutant alleles, and Ura^+ transformants that could no longer rescue growth at 37°C were identified. Rescuing genes were identified by sequencing from within the ends of the transposon and continuing outward into the yeast genomic DNA. Complementing genes were identical to the wild-type sequences contained in the GenBank and EMBL databases.

Cell Synchrony and Time Course Assays

Logarithmically growing cells were incubated in the presence of 5 μg/ml α factor for 2 hr at 25°C. Cultures were shifted to 36°C 2 hr in α factor. Pronase E was added to 10 μg/ml, and the cultures continued incubation at 36°C. For the α factor "add-back" experiment, cells were released from α factor arrest at 36°C by transfer to YPD alone. After 30 min, α factor was added to 5 μg/ml and incubation at 36°C was continued. For the hydroxyurea experiment, 0.15 M hydroxyurea was added to the cultures at α factor release at 36°C. Cultures utilized for the nocodazole experiment were pulsed at 36°C in α factor for 4 hr and released into 15 μg/ml nocodazole at 25°C.

Density Transfer

Cells were pregrown for at least 8 generations in yeast minimal medium containing [^{13}C]glucose and [^{15}N]ammonium sulfate (Wellinger et al., 1993). After arrest in α factor and incubation at 36°C for 2 hr, cultures were switched to YPD at the nonpermissive temperature. Total DNA was extracted, digested with SnaBI restriction enzyme and RNase A, and centrifuged through a CsCl gradient as described (McCarroll and Fangman, 1988). Fractions were collected, adsorbed to Hybond N membranes, and hybridized to radioactively labeled total yeast DNA. Note that the majority of the signal was likely due to hybridization to the rDNA repeats that are represented in high copy in the genome. DNA content was quantitated by Phosphorimager. Peaks were determined by comparison to DNA from cells grown in "heavy" or "light" media. Experimental samples were directly compared to known densities of control peaks. Densities were aligned accordingly in Figure 3.

Pulsed-Field Analysis of Yeast Chromosomes

Samples were fixed in 70% ethanol for at least 30 min at room temperature until all were collected. Yeast DNA was prepared essentially as described by Carle and Olson, 1985. Samples were electrophoresed in 0.5X Tris–borate–EDTA for 27 hr at 14°C with a field strength of 6 V/cm and a switching time of 73.6 s. The gel was stained for 30 min with ethidium bromide and destained in water overnight.

Immunofluorescence

Anti-tubulin immunofluorescence and DNA staining by DAPI were performed as a modification of the protocols outlined in Pringle et al., 1989. Fixed cells were incubated for 1–2 hr at 25°C with a 1:40 dilution of anti-tubulin monoclonal antibody YOL1/34 followed by incubation with fluorescein isothiocyanate–conjugated anti-rat secondary antibody (1:100) for 1–2 hr. Slides were mounted in medium containing DAPI.

Preparation of Cell Extracts

Total yeast protein extracts were prepared in TNN (50 mM Tris–Cl [pH 7.5], 5 mM EDTA, 250 mM NaCl, 0.1% NP40) plus protease inhibitors and 50 mM sodium fluoride. Cells were lysed by vortexing with glass beads. Protein concentrations were determined by Bradford assay (Bio-Rad).

H1 Kinase Assays

Cdc28 kinase activity was assayed using histone H1 as a substrate. For total Cdc28 kinase activity, 10 μg of total protein was incubated with 20 μl of 2 mg/ml suc1 beads (Brizuela et al., 1987) for 2 hr at 4°C. For immunoprecipitations using anti-Clb2 and anti-Clb5 antisera (courtesy of C. Dahmann and K. Nasmyth, Research Institute of Molecular Pathology, Vienna, Austria), 5 and 10 μg of total protein were incubated with a 1:50 dilution of antibody. Incubations were performed overnight at 4°C. Protein A beads were added and incubation continued for 2 hr at 4°C. Beads were washed 3 times with TNN and once with kinase buffer (50 mM Tris–Cl [pH 7.5], 10 mM magnesium chloride, 1 mM dithiothreitol) (Booher et al., 1993). Kinase assays included 50 μM ATP, 0.5 μCi [γ-^{32}P]ATP and 2 μg histone H1 (Boehringer Mannheim). Kinase reactions proceeded for 10 min at 37°C. Phosphorylated histone H1 was quantitated by Phosphorimager.

***CLB2db*Δ Experiment**

Logarithmically growing *cdc16-264* homozygous diploid cells containing an integrated copy of a galactose-inducible *CLB2db*Δ construct were transferred to yeast extract–peptone plus 2% raffinose and incubated at 25°C for 12 hr. The cultures were divided and galactose was added to 2%. Cells were incubated at 25°C or 36°C.

Microcolony Assays

Cells were synchronized by α factor incubation and shifted to 36°C for 2 hr in α factor. Cells were sonicated briefly and spread onto prewarmed plates (37°C). Microcolonies were allowed to form at 37°C for 2–3 days.

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