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Cyclin-B homologs in *Saccharomyces cerevisiae* function in S phase and in G_2

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We have cloned four cyclin-B homologs from *Saccharomyces cerevisiae*, *CLB1–CLB4*, using the polymerase chain reaction and low stringency hybridization approaches. These genes form two classes based on sequence relatedness: *CLB1* and *CLB2* show highest homology to the *Schizosaccharomyces pombe* cyclin-B homolog *cdc13* involved in the initiation of mitosis, whereas *CLB3* and *CLB4* are more highly related to the *S. pombe* cyclin-B homolog *cig1*, which appears to play a role in G_1 or S phase. *CLB1* and *CLB2* mRNA levels peak late in the cell cycle, whereas *CLB3* and *CLB4* are expressed earlier in the cell cycle but peak later than the G_1-specific cyclin, *CLN1*. Analysis of null mutations suggested that the *CLB* genes exhibit some degree of redundancy, but *clb1,2* and *clb2,3* cells were inviable. Using *clb1,2,3,4* cells rescued by conditional overproduction of *CLB1*, we showed that the *CLB* genes perform an essential role at the G_2/M-phase transition, and also a role in S phase. *CLB* genes also appear to share a role in the assembly and maintenance of the mitotic spindle. Taken together, these analyses suggest that *CLB1* and *CLB2* are crucial for mitotic induction, whereas *CLB3* and *CLB4* might participate additionally in DNA replication and spindle assembly.

[*Key Words*: Yeast; cyclin-B homologs; S-phase; mitosis; Cdc28 kinase]

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The eukaryotic cell cycle is highly conserved in both mechanism and components from yeast to man (for recent reviews, see Cross et al. 1989; Murray and Kirschner 1989a; Lewin 1990; Nurse 1990; Pines and Hunter 1990a; Maller 1991; Reed 1991). Studies carried out in the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* have revealed that a 34-kD serine–threonine protein kinase, known as Cdc28 or cdc2, plays a critical role in at least two key regulatory events. The first is at a point late in G_1 known as START, at which environmental factors, such as nutrient availability and the presence of mating pheromones, act to determine whether a cell will enter the cell cycle (Hartwell et al. 1974; Nurse and Bissett 1981). Second, the Cdc28/cdc2 protein kinase functions at the initiation of mitosis at a point late in G_2 (Nurse and Bissett 1981; Piggott et al. 1982; Reed and Wittenberg 1990; Surana et al. 1991). Studies in *Xenopus* have revealed that Cdc28/cdc2 is an essential component of a factor [M-phase-promoting factor (MPF)] required for the G_2- to M-phase transition (for review, see Hunt 1989; Maller 1991). Furthermore, recent studies in mammalian cells have provided functional evidence that Cdc28/cdc2 is required for the initiation of mitosis (Riabowol et al. 1989; Th'ng et al. 1990), and a newly discovered cdc2-like kinase [cdk2 (Elledge and Spottswood 1991; Ninomiya-Tsuji et al. 1991; Paris et al. 1991; Tsai et al. 1991)] is required for the initiation of S phase (Fang and Newport 1991).

In its active form, the Cdc28/cdc2 protein kinase is a multimeric complex with at least one other protein of 45–60 kD, known as cyclin (for review, see Hunt 1989; Murray and Kirschner 1989a; Maller 1991; Reed 1991; Xiong and Beach 1991; Lew and Reed 1992). Cyclins were initially discovered in the oocytes and embryos of marine invertebrates, as proteins that peaked in abundance just before the onset of mitosis and were then rapidly degraded during mitosis (Evans et al. 1983; Hunt et al. 1992). These "mitotic cyclins" are activators of Cdc28/cdc2 for the G_2- to M-phase transition (for review, see Hunt 1989; Murray and Kirschner 1989a; Nurse 1990; Maller 1991). Mitotic cyclins have been divided into two classes, A-type and B-type, on the basis of sequence relatedness; but they also appear to differ in their kinetics of accumulation, intracellular distribution, and association with different forms of Cdc28/cdc2 (Minshull et al. 1990; Pines and Hunter 1990a,b; Hunt et al. 1992). Cyclin A and B both appear to be capable of promoting entry into mitosis when assayed in the *Xenopus* oocyte system (Swenson et al. 1986; Minshull et al. 1989; Murray and Kirschner 1989b; Murray et al. 1989); however these cyclins do not perform redundant roles in *Drosophila* (Lehner and O'Farrell 1990). In somatic animal cells, cyclin A plays a role at the initiation of S phase (Girard et al. 1991; Pagano et al. 1992).

Another group of cyclins has been defined by three

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genes (*CLN1, CLN2,* and *CLN3*) in the budding yeast *S. cerevisiae* (Sudbery et al. 1980; Cross 1988; Nash et al. 1988; Hadwiger et al. 1989). These "G_1 cyclins" are rate limiting for the START control point in G_1 (Richardson et al. 1989; Cross 1990). The G_1 cyclins associate with Cdc28/cdc2 late in G_1, and this is presumed to result in the activation of the kinase for its essential START function (Wittenberg et al. 1990; Reed 1991). G_1 cyclins may be present in animal cells, although the candidates that have been identified are structurally diverged from the yeast G_1 cyclins (for review, see Xiong and Beach 1991; Lew and Reed 1992).

Genetic analysis of the fission yeast *S. pombe* has identified a cyclin-B homolog, *cdc13*, that is required for entry into mitosis (Booher and Beach 1987; Hagan et al. 1988). Because classical genetic analysis of *S. cerevisiae* had not revealed any cyclin-like genes required for mitosis, we sought to identify these genes by using degenerate oligonucleotides and the polymerase chain reaction (PCR) and by low stringency hybridization. As reported previously, we identified four cyclin-B homologs from *S. cerevisiae*, which we named *SCB1–SCB4* (Ghiara et al. 1991). Surana et al. (1991) have also isolated four cyclin-B homologs from *S. cerevisiae*, named *CLB1–CLB4*, by rescue of a *cdc28-1N* mutant and by PCR. In our last report (Ghiara et al. 1991), we characterized the first of these cyclin-B homologs, *SCB1* (or *CLB1*), and showed that it has properties consistent with a role in the G_2- to M-phase transition. In this report we investigate the function of the other cyclin-B homologs in *S. cerevisiae* and show that these cyclins play a role in the G_2 phase and also the S phase of the cell cycle. This latter role for B-type cyclins may be analagous to the S-phase function inferred for cyclin A in animal cells (Girard et al. 1991; Pagano et al. 1992).

Results

Isolation of four cyclin-B homologs from S. cerevisiae

We (Ghiara et al. 1991) and others (Surana et al. 1991) reported previously the existence of at least four cyclin-B homologs in *S. cerevisiae*. Surana et al. (1991) described the sequence of *CLB1* and *CLB2* and provided partial amino acid sequence for *CLB3* and *CLB4*. Using both PCR and low stringency hybridization approaches (Materials and methods), we had cloned the same four genes. The complete DNA sequences and predicted translation products of *CLB3* and *CLB4* are presented in Figure 1.

As reported previously (Surana et al. 1991), the *CLB* genes are related most closely to *S. pombe* cyclin-B homologs *cdc13* (Booher and Beach 1988; Hagan et al. 1988) and *cig1* (Bueno et al. 1991). Clb1 and Clb2 are closely related to each other and show the highest homology to cdc13 (Table 1). Clb3 and Clb4 are also related to each other, but they show the highest homology with cig1 (Table 1; Fig. 2). *cdc13* has been shown to be required for the G_2- to M-phase transition (Booher and Beach 1987; Hagan et al. 1988), whereas the *cig1* gene appears to play a role in the initiation or execution of S phase (Bueno et al. 1991).

Glotzer et al. (1991) have identified a sequence present at the amino terminus of sea urchin cyclin B that is required for cyclin degradation. This sequence, known as the mitotic destruction box and having the consensus sequence of R-ALGD(NEV)I-N, is highly conserved in cyclin-B and cyclin-A sequences. All four of the Clb proteins have a sequence at the amino terminus that fits this consensus (Table 2). Interestingly, the presumptive destruction box of Clb3 has an arginine at the sixth position. Glotzer et al. (1991) have observed that in all known cyclin-B sequences there is always an asparagine, aspartic acid, or glutamic acid at position six, whereas cyclin-A sequences have a valine residue at this position. Thus, Clb3 does not fit either the cyclin-B or cyclin-A consensus at this position. It has also been noted that cyclin-A and cyclin-B sequences have a lysine-rich region following the destruction box (average of 6 lysine residues in 46 amino acids following the destruction box), which may constitute acceptor sites for ubiquitin conjugation (Glotzer et al. 1991). All four Clb proteins have 4 or 5 lysine residues in a region of 50 amino acids following the destruction box (Fig. 2, data not shown).

CLB3 *and* CLB4 *mRNAs accumulate earlier in the cell cycle than do* CLB1 *and* CLB2 *mRNAs*

We have shown previously that *CLB1* mRNA is periodic during the cell cycle, peaking at a late time consistent with mitotic function, and that this results in a corresponding increase in Clb1 protein (Ghiara et al. 1991). To investigate the accumulation of mRNAs for *CLB2, CLB3,* and *CLB4* during the cell cycle, Northern blot analysis with radiolabeled probes was performed on RNA samples prepared from synchronized cells. Figure 3 shows the results from this analysis compared with *CLB1, CLN1,* and *SWI5* mRNAs. *CLN1* mRNA peaks late in G_1 (Wittenberg et al. 1990), whereas *SWI5* and *CLB1* mRNAs peak late in G_2 (Nasmyth et al. 1987; Ghiara et al. 1991). As for *CLB1*, the mRNAs for the other three *CLB* genes varied in abundance through the cell cycle. However, the cell cycle accumulation of mRNA from the four *CLB* genes was clearly not identical. *CLB2* mRNA accumulation was similar to *SWI5* and *CLB1* mRNAs [consistent with the results of Surana et al. (1991)], peaking late in the cell cycle coincident with the peak in large budded cells. This corresponds to cells late in G_2 or in mitosis. Surprisingly, *CLB3* and *CLB4* mRNAs began to accumulate late in G_1 at the same time as *CLN1* mRNA and reached a peak in abundance at the time when small budded cells were maximal (in S phase, after the peak of *CLN1* mRNA accumulation; Fig. 3). In addition, there appears to be a significant basal level of these species early in G_1. Thus, *CLB1* and *CLB2* mRNAs accumulate at a time that is consistent with a role for these proteins at the G_2/M phase, whereas *CLB3* and *CLB4* mRNAs accumulate earlier in the cell cycle, raising the possibility of a role in S phase.

Phenotype of null mutations in the CLB *genes*

To investigate the functions of the *CLB* gene products,

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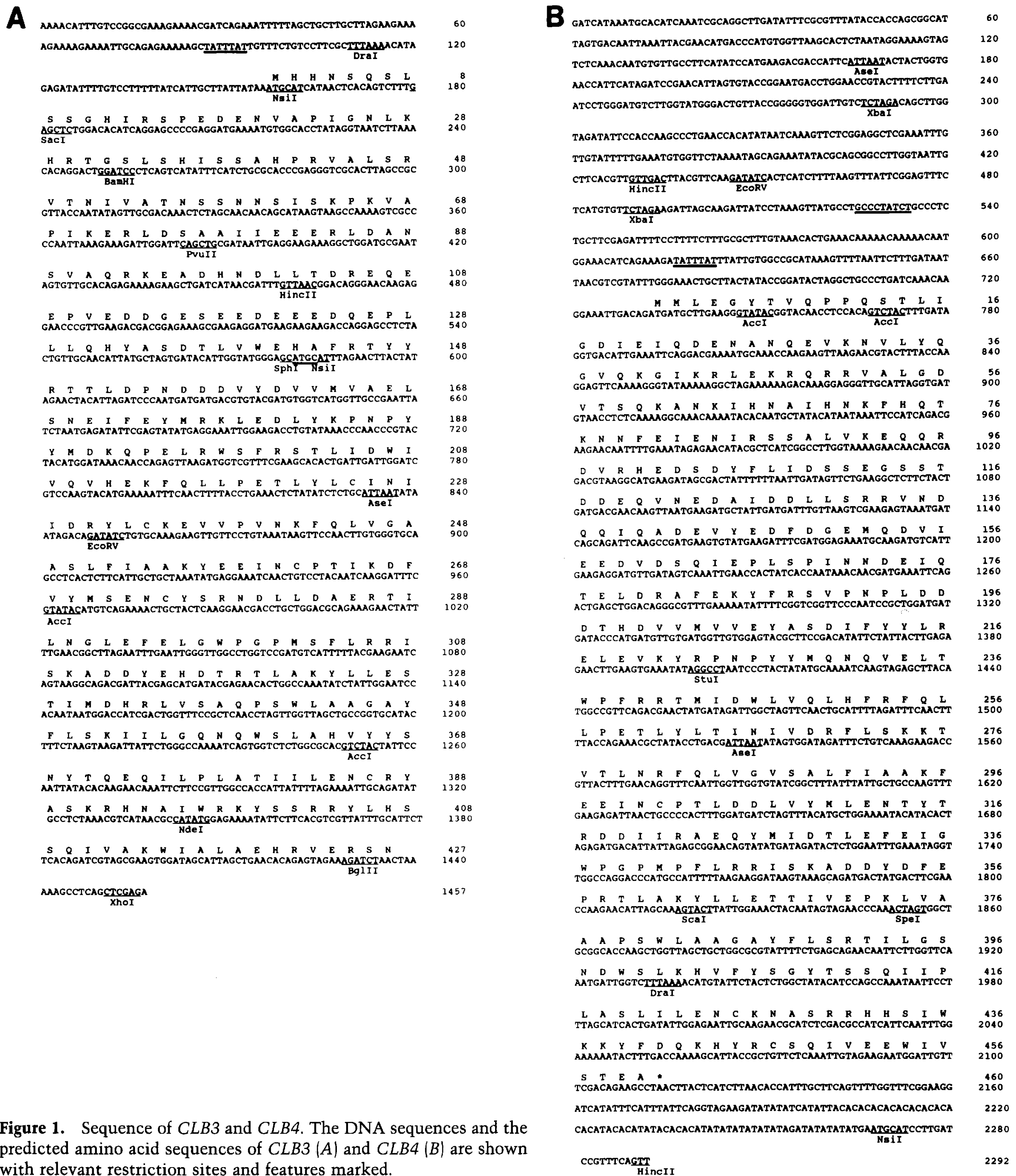

Figure 1. Sequence of *CLB3* and *CLB4.* The DNA sequences and the predicted amino acid sequences of *CLB3* (*A*) and *CLB4* (*B*) are shown with relevant restriction sites and features marked.

null mutations were created in each of these four genes (Materials and methods; Ghiara et al. 1991). Yeast cells containing any single null mutation were viable. *clb2* cells were abnormally elongated (Fig. 4), whereas other single mutations did not display an obvious phenotype. We constructed heterozygous diploid strains, induced sporulation, and analyzed the segregants by tetrad analysis (see Materials and methods) to determine the effect of multiple *clb* null combinations. The null allele combinations *clb1,2* and *clb2,3* were inviable (as were such strains containing additional *clb* mutations), whereas all other combinations were viable (Table 3). Although via-

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Table 1. *Comparison of* S. cerevisiae *Clb proteins to other cyclins*

	Clb proteins (%)			Cyclins (%)[a]									
	Clb2	Clb3	Clb4	cdc13	cig1	HsB1	HsA	HsC	HsD	HsE	Cln1	Cln3	puc1
Clb1	78	43.6	40.7	51.6	39.9	37	30.8	17.2	23.1	24.9	17.9	20.5	20.9
Clb2	—	42.5	39.9	51.6	41.4	38.5	33.3	16.1	23.4	28.2	19	24.9	22.3
Clb3	—	—	62.3	43.5	46.4	36.2	29.7	16.7	23.2	25.7	21.4	19.2	25
Clb4	—	—	—	43.1	45.3	36.2	30.4	17	18.5	21.7	17.4	20.7	22.8

Sequences were aligned using the GAP program (Needleman and Wunsch 1970), with a GAP weight of 3.0 and a GAP length of 0.1. A region of 276 residues at the carboxyl terminus of each cyclin containing the cyclin box was used for the comparisons. Scores indicate percent identical residues within this region.

[a]Sources of cyclin sequences: cdc13 (Booher and Beach 1988); cig1 (Bueno et al. 1991); HsB1 (Pines and Hunter 1989); HsA (Wang et al. 1990); HsC, HsD, HsE (Lew et al. 1991); Cln1 (Hadwiger et al. 1989); Cln3 (Cross 1988); puc1 (Forsburg and Nurse 1991).

ble, *clb3,4* and *clb1,3,4* cells were slightly enlarged (Fig. 4C,D). The fact that *clb1,3,4* cells were viable indicates that Clb2 can perform all essential Clb functions, suggesting that Clb2 is the dominant cyclin-B homolog in the cell during mitotic growth. The fact that mutation of *clb4* did not affect the viability of any other *clb* mutant combination suggests that Clb4 is the cyclin-B homolog of least import in the cell during mitotic growth.

Viable *clb* combinations were analyzed by flow cytometry to determine whether they caused any perturbation in cell cycle distribution. Homozygous diploid strains were used because diploid cells contain twice as much DNA as haploids, and clearer results can therefore be obtained. Flow cytometric analysis of propidium iodide-stained *clb2* cells revealed that there was a dramatic increase in cells with a 4N DNA content, consistent with a delay at the G_2- to M-phase transition (Fig. 5B). In contrast, *clb3,4* or *clb1,3,4* cells did not exhibit any obvious perturbation of the cell cycle distribution in exponentially growing cultures (Fig. 5C,D).

Analysis of viable *clb* deletion strains suggested that *CLB* genes share at least one essential role in the cell cycle, with Clb2 making the greatest contribution and Clb4 the smallest. However, it is not clear from these

Figure 2. Alignment of the *CLB3* and *CLB4* amino acid sequences with *S. pombe cig1*. Identical amino acids between Clb3 and Clb4 are indicated by vertical lines. Conservative substitutions are indicated by two dots. *cig1*, a B-type cyclin form *S. pombe* with G_1- or S-phase functions (Bueno et al. 1991), is also compared.

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Table 2. *Clb destruction box sequences*

			1	2	3	4	5	6	7	8	9
Destruction box consensus	cyclin B		R	X	A	L	G	DNE	I	X	N
	cyclin A							V			
Clb1		35	R	T	I	L	G	N	V	T	N
Clb2		25	R	L	A	L	N	N	V	T	N
Clb3		51	R	V	A	L	S	R	V	T	N
Clb4		43	R	V	A	L	G	D	V	T	S

experiments whether the different *CLB* genes normally perform overlapping or distinct functions: It is possible (for example) that Clb3 and Clb4 normally perform a specialized function in S phase but that in their absence Clb1 and Clb2 can substitute, so that *clb3,4* cells do not display an obvious phenotype.

Conditional removal of Clb function

The G_2/M delay seen in *clb2* cells (Fig. 5B) suggested that these cyclins play a role in G_2/M phase, which is consistent with previous studies with cyclin B in other systems (Booher and Beach 1987, 1988; Hagan et al. 1988; Murray and Kirschner 1989b). To investigate the

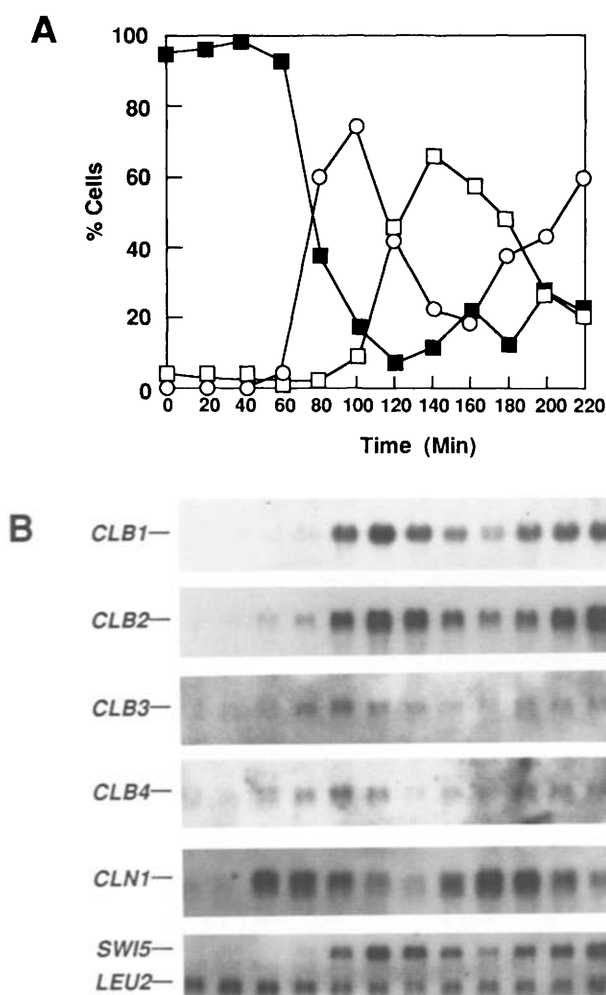

Figure 3. Timing of expression of *CLB* mRNAs during the cell cycle. (*A*) Cells were synchronized by mating pheromone arrest/release as described by Ghiara et al. (1991). (■) Unbudded cells (G_1); (○) cells with small buds (S phase); (□) cells with large buds (G_2/M phase). (*B*) Parallel Northern blot analyses were performed on RNA samples prepared at the indicated times following release from pheromone arrest using probes specific for the Clb1, Clb2, Clb3, Clb4, Cln1, Swi5, and Leu2 mRNAs.

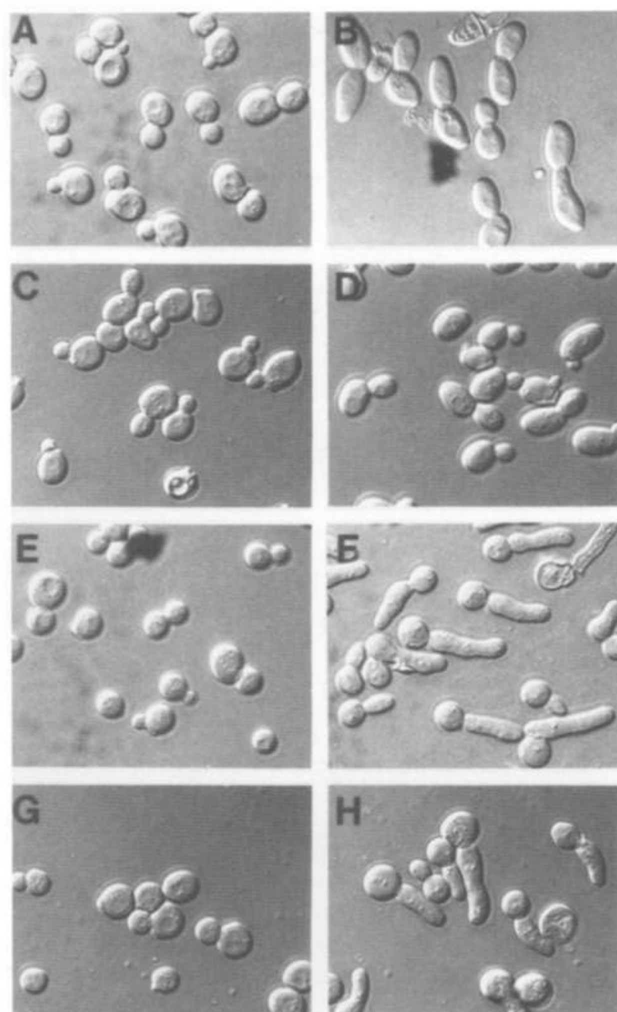

Figure 4. Morphology of *clb* mutant strains. Differential interference contrast microscopy was performed on wild-type (*A*); *clb2* (*B*); *clb3,4* (*C*); *clb1,3,4* (*D*); *clb1,2,3 GAL1::CLB1* growing on YEPGal (*E*); *clb1,2,3 GAL1::CLB1* after addition of glucose for 2 hr (*F*); *clb1,2,3 GAL1::CLB1* growing on YEPGal (G); and *clb1,2,3,4 GAL1::CLB1* after the addition of glucose for 2 hr (*H*).

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Table 3. *Phenotypes of* clb *null mutants*

Genotype	Phenotype
clb1	viable
clb2	viable
clb3	viable
clb4	viable
clb1,2	inviable
clb1,3	viable
clb1,4	viable
clb2,3	inviable
clb2,4	viable
clb3,4	viable
clb1,2,3	inviable
clb1,2,4	inviable
clb1,3,4	viable
clb2,3,4	inviable
clb1,2,3,4	inviable

essential roles of Clb proteins directly, it was necessary to construct a strain in which *CLB* function could be conditionally controlled. To achieve conditional expression of Clb protein, the *GAL1* (galactokinase) promoter, which is induced by galactose but repressed by glucose, was used. A chimeric gene was constructed composed of the *GAL1* promoter fused to the coding region of *CLB1* (Ghiara et al. 1991). This gene was used to rescue cells bearing all of the otherwise lethal *clb* combinations when grown on galactose-based media (for strain constructions, see Materials and methods). The viability of the *clb1,2,3,4 GAL1::CLB1* strain demonstrates that, when overexpressed, Clb1 is able to perform all essential Clb functions.

Homozygous diploid *clb1,2 GAL1::CLB1*, *clb1,2,3 GAL1::CLB1*, and *clb1,2,3,4 GAL1::CLB1* strains were constructed to assess the consequences of removing Clb function incrementally. Cells were grown in galactose liquid medium, and *CLB1* expression was terminated by the addition of glucose to 2%. Samples were taken at various times and cells were fixed for counting, microscopic analysis, and analysis of DNA content by flow cytometry after staining nuclear DNA with propidium iodide (Fig. 6). Consistent with the results of Surana et al. (1991) with germinating spores, the *clb1,2* mutant cells did not arrest on the first cell cycle and the cell number increased slowly through the 3-hr time course (Fig. 6A). Nevertheless, it is clear from the flow cytometric analysis, and the accumulation of budded cells at the 3-hr point, that loss of function of *clb1* and *clb2* causes the majority of cells to delay in G_2 (Fig. 6B,C). On the other hand, within 2 hr after the addition of glucose to the culture, cell proliferation ceased completely for the *clb1,2,3* and *clb1,2,3,4* strains (Fig. 6A). Examination of the DNA content of these cells 2 hr after the addition of glucose showed that while most of the *clb1,2,3* cells arrested in G_2, the *clb1,2,3,4* cells almost all contained less DNA, indicating that the cells had not completed S phase (Fig. 6C; arrows indicate fluorescence corresponding to 2N and 4N DNA content at the beginning of the experiment, representing cells in G_1 or G_2/M, respectively). Because DNA replication is normally completed rapidly once cells bud (~20 min under these growth conditions; data not shown), these data suggest an impairment of some aspect of DNA replication in the *clb1,2,3,4* mutants. It is difficult to assess population DNA content at the 3-hr point, because as arrested mutant cells enlarge, the contributions of autofluorescence and mitochondrial DNA tend to skew populations to higher fluorescence. Hence, the DNA content of mutant populations at 3 hr (and, to a lesser degree, at 2 hr) is likely to be overestimated. These problems prevent a clear conclusion from this experiment regarding whether the *clb1,2,3,4* cells eventually complete DNA replication or remain arrested in S phase. Nonetheless, this experiment suggests that Clb function is required both for mitosis and S phase. The difference between the *clb1,2,3* cells and the *clb1,2,3,4* cells (the contribution of Clb4) suggests that a moderately high level of Clb function is required to perform mitosis successfully, whereas a low level of Clb function is sufficient for DNA replication.

Clb function is required during S phase

In flow cytometric analyses of several of the time points in the experiment described in Figure 6 and similar experiments, it appeared that cells were accumulating in a distinct S-phase peak, rather than arresting at the beginning of, or randomly throughout, S phase (e.g., Fig. 6D, 1

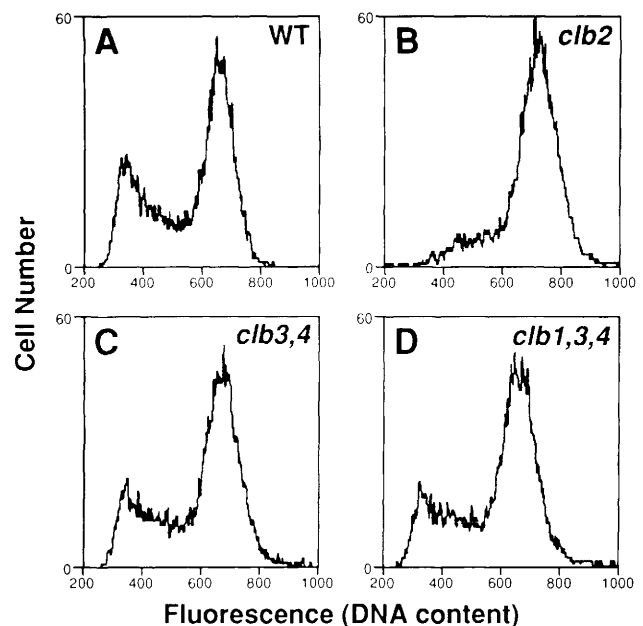

Figure 5. Flow cytometric analysis of viable *clb* disruption mutants. Homozygous diploid strains were used. Fluorescence measures DNA content, as analyzed by staining with propidium iodide. The *left* peak in each distribution corresponds to 2N DNA content (G_1 cells); the *right* peak corresponds to 4N DNA content (G_2/M cells). (*A*) Wild-type; (*B*) *clb2*; (*C*) *clb3,4*; (*D*) *clb1,3,4*.

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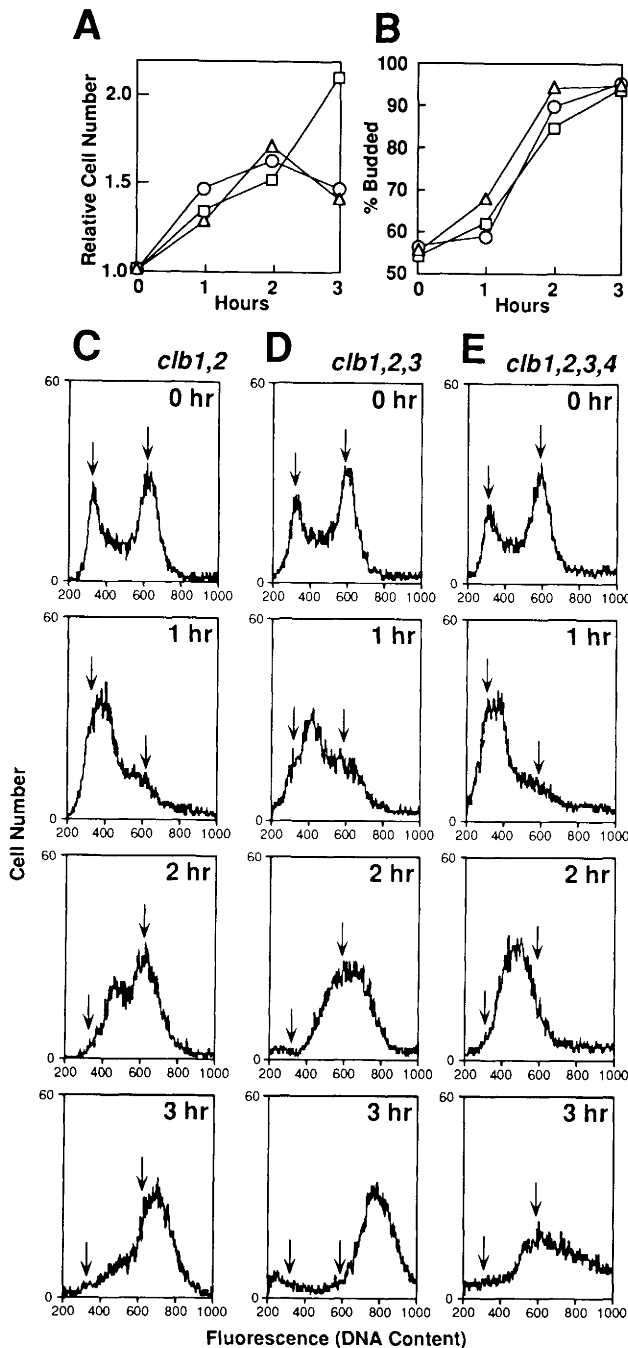

Figure 6. Flow cytometric analysis of lethal *clb* disruption mutants upon loss of the rescuing Clb1. (*A*) Analysis of cell number increase after termination of Clb synthesis by addition of glucose. Samples were analyzed at 0, 1, 2, and 3 hr. Strains were homozygous diploids as follows: (□) *clb1,2 GAL1::CLB1*; (○) *clb1,2,3 GAL1::CLB1*; (△) *clb1,2,3,4 GAL1::CLB1*. (*B*) Analysis of the percentage of budded cells after termination of Clb synthesis. Symbols are as in *A*. (*C–E*) Flow cytometric analysis of mutants after termination of Clb synthesis. (*C*) *clb1,2 GAL1::CLB1*; (*D*) *clb1,2,3 GAL1::CLB1*; (*E*) *clb1,2,3,4 GAL1::CLB1*. Arrows indicate the positions of the 2N (G_1 cells, *left*) and 4N (G_2/M cells, *right*) peaks for reference. Times are indicated beginning from the time of glucose addition.

hr, or Fig. 6E, 2 hr). This suggested that cells lacking *Clb* proteins might be able to begin DNA replication but then might encounter a restriction at some specific point in S phase. To obtain more precise data germane to this issue, we selected a synchronous population of *clb1,2,3,4* cells in G_1 and observed their progress through the cell cycle (Fig. 7B). We made the assumption, by analogy with results in *Xenopus*, that Clb degradation would be greatest during mitosis. Thus, G_1 cells formed during incubation of *clb1,2,3,4 GAL1::CLB1* cells in glucose medium should be devoid of Clb function, because the residual Clb1 synthesized in the previous cell cycle would have been degraded when the cells went through mitosis. Operationally, homozygous diploid *clb1,2,3,4 GAL1::CLB1* cells were incubated in glucose medium for 1 hr (Fig. 7A), and the G_1 cells formed during this incubation were isolated by centrifugal elutriation (Materials and methods). These cells were incubated in glucose medium for another 2 hr, during which the DNA content was monitored by flow cytometry (Fig. 7B). Essentially 100% of the cells budded during this period (Fig. 7C). It is clear from this experiment that despite the presumed complete absence of Clb proteins, these cells were all able to enter S phase. However, the cells stopped increasing their DNA content within a short window, about one-third of the way through S phase. The gradual drift of the S-phase peak to increasing fluorescence with time probably reflects increasing autofluorescence as the cells enlarge, although we cannot rule out the possibility that the cells are slowly and synchronously replicating more of their DNA. This experiment suggests that Clb function becomes critical about one-third of the way through S phase.

Cells without Clb proteins arrest before the hydroxyurea execution point

We sought to use an alternative method to test the idea of an S-phase role for Clb proteins. Hydroxyurea, an inhibitor of the enzyme ribonucleotide reductase that maintains DNA precursor pools, arrests yeast cells that have not completed DNA replication in S phase and prevents cell division (Slater 1973). Cells that have completed S phase divide in the presence of hydroxyurea and become arrested on the subsequent cell cycle. To determine the extent to which they had completed DNA replication, the conditionally lethal *clb1,2*, *clb1,2,3*, and *clb1,2,3,4* mutant cells were transferred to glucose medium for 2 hr and then tested for sensitivity to hydroxyurea. Cells were plated on medium containing hydroxyurea, with and without galactose, to allow resumption of Clb synthesis. The percentages of cells arrested immediately versus those that arrested after one division were tabulated (Table 4; for details, see Materials and methods). For the *clb1,2* mutant, 95% of the budded cells were able to undergo a single division in the presence of hydroxyurea, indicating that they had completed DNA replication during the 2-hr incubation in glucose, consistent with a G_2 arrest. For the *clb1,2,3* mutant, however, 26% of the budded cells were unable to divide,

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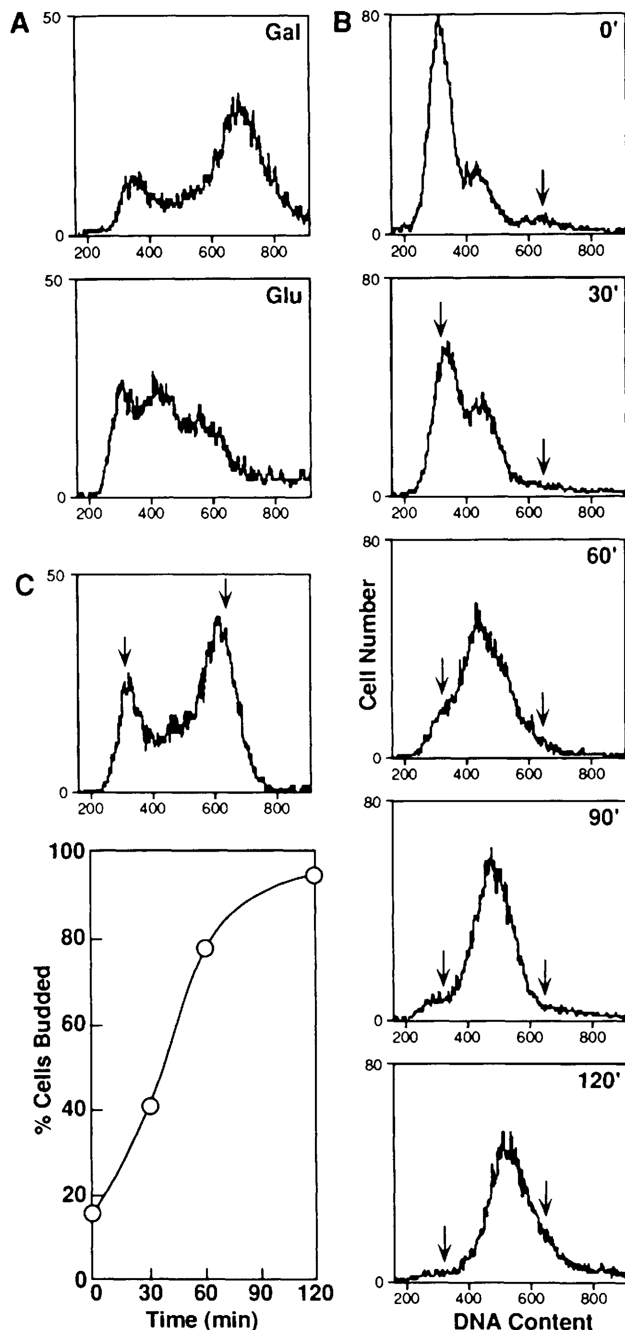

Figure 7. Flow cytometric analysis of synchronized *clb1,2,3,4 GAL1::CLB1* cells. (*A*) Flow cytometry of homozygous diploid *clb1,2,3,4 GAL1::CLB1* cells growing on galactose (Gal) or 1 hr after addition of 2% glucose to terminate Clb1 synthesis (Glu). These cells show three peaks of DNA content corresponding to cells in G_1, G_2, and S phase. (*B*) Small cells were isolated from the population treated with glucose (*A*) by centrifugal elutriation. These cells were predominantly in G_1 (*B*, 0'). The cells were inoculated into medium containing glucose and incubated at 30°C for the indicated times before analysis of DNA content (vertical strip, 0–2 hr). Arrows indicate the DNA content of G_1 (*left*) and G_2 (*right*) cells. (*C*) As a control for the positions of the G_1 and G_2 DNA contents for elutriated cells (which are smaller than the starting asynchronous culture), cells were elutriated and returned to galactose medium for 1 hr (*C*, upper panel). The arrows indicating 2N and 4N positions were derived from this control culture. The lower panel indicates the percent of cells budded for the same cells and time points shown in *B*.

suggesting that they had not yet exited from S phase. The most dramatic outcome was with the *clb1,2,3,4* mutant, where 92.5% of the cells were restrained from dividing by hydroxyurea. Thus, the bulk of these cells have an unreplicated or incompletely replicated DNA content. These data strongly support an S-phase role for Clb proteins.

One potential caveat concerning the result with the *clb1,2,3,4* mutant pertains to a loss of viability after prolonged incubation in glucose. We observed that whereas *clb1,2* and *clb1,2,3* mutants recovered rapidly in galactose after 2 hr in glucose, the *clb1,2,3,4* mutant recovered much more slowly. In fact 40% of arrested cells in this experiment were incapable of recovery (these were statistically removed from the analysis). If the remaining 60% were impaired from recovery by the presence of hydroxyurea, the data for this mutant might be skewed in favor of S-phase arrest. To control for this possibility, we tested whether *clb1,2,3,4* cells that had been incubated in glucose medium for 2 hr suffered any additional loss of viability upon exposure to hydroxyurea in galactose medium. Cells were transferred from glucose to galactose/hydroxyurea liquid medium, incubated for 3 hr at 30°C, and plated on galactose medium without hydroxyurea to assess their viability. The results indicated that the galactose/hydroxyurea incubation had only a small effect on the viability of the cells (data not shown), which would not alter the conclusion that the great majority of *clb1,2,3,4* cells had arrested in S phase.

Morphological analysis of clb *mutants*

Microscopic examination of arrested *clb1,2,3* cells by Nomarski optics (Fig. 4F), or after staining nuclei with DAPI and spindles with an anti-tubulin antibody (Fig. 8A,B), revealed that the arrested cells had large elongated buds, an unlobed nucleus, and a short spindle extending across the nucleus, consistent with arrest in G_2 (Reed

Table 4. *Arrest of Clb-deficient strains in S phase*

Genotype	Adjusted hydroxyurea sensitive[a]	Adjusted hydroxyurea insensitive[b]	Percent in S phase
clb1,2	24	463	5.0
clb1,2,3	123	352	26.0
clb1,2,3,4	295	24	92.5

[a]Total number of cells/600 that did not divide on hydroxyurea minus the number of cells/600 that did not recover after 2 hr in glucose minus the number of cells/600 that were unbudded (prior to S phase) when plated.
[b]Total number of cells/600 that divided on hydroxurea minus the number of clumps of cells/600 units plated (that would be confused for divided cells).

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and Wittenberg 1990; Ghiara et al. 1991). These data indicate that *CLB* function is required for the G_2- to M-phase transition.

A similar analysis of the conditional *clb1,2,3,4* mutant yielded a strikingly different result. The arrested cells were examined microscopically using Nomarski optics (Fig. 4H), or after staining with DAPI (to visualize nuclei) and with anti-tubulin antibodies (to visualize spindles). As shown in Figure 8, C and D, all cells arrest with elongated buds and an unlobed nucleus (as do the *clb1,2,3* cells), but these cells contained no discernible spindle, although fine microtubule fibers were apparent extending from single-microtuble organizing centers. From these observations we conclude that cyclin-B function at some level is required for the assembly or maintenance of an intranuclear spindle. However, we cannot determine whether the spindle pole body has duplicated, an event that normally takes place in late G_1 and is controlled at START (Byers and Goetsch 1975).

Because virtually all *clb1,2,3,4* cells contained unipolar structures, one might infer that cells already containing spindles and duplicated spindle pole bodies might regress to this state once Clb function is eliminated. To test this idea directly, *clb1,2,3,4 GAL1::CLB1* cells in galactose medium were arrested in S phase by treatment with hydroxyurea (Slater 1973). Under these conditions, cells contain short G_2-like intranuclear spindles (Fig. 9A,B). After 3.5 hr, cells were resuspended in glucose medium without hydroxyurea to terminate Clb1 synthesis and to release the S-phase block. After 3 hr, most spindles had regressed to unipolar structures (Fig. 9C,D). Flow cytometric analysis indicated that by this time a significant amount of DNA replication had occurred (data not shown). Thus, a low but significant level of Clb function is required for the maintanence of an intranuclear spindle, even after the spindle pole bodies have been duplicated.

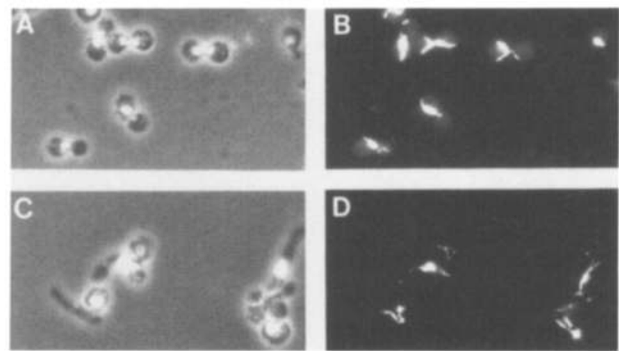

Figure 9. Spindle regression in the absence of Clb function. (*A,B*) *clb1,2,3,4 GAL1::CLB1* in YEP Gal arrested using hydroxyurea (0.2 M for 3.5 hr). (*C,D*) Same cells after termination of Clb1 synthesis by addition of glucose and release from S-phase block by removal of hydroxyurea for 3 hr. (*A,C*) DAPI staining; (*B,D*) anti-tubulin staining.

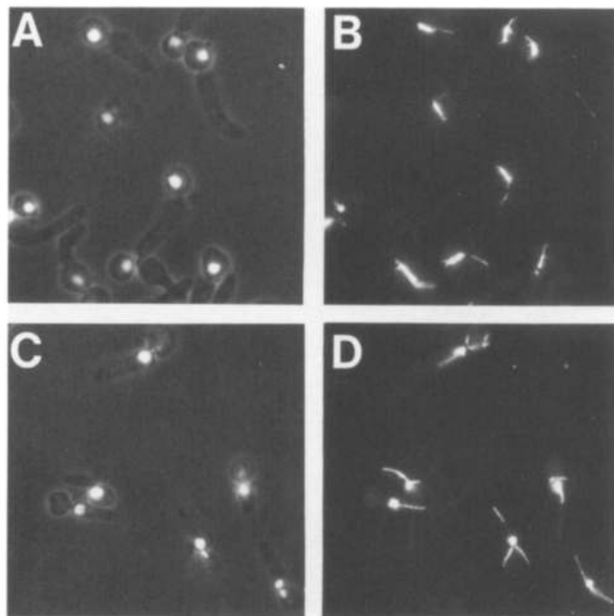

Figure 8. Nuclear and spindle morphology upon loss of Clb function. (*A,B*) Homozygous diploid *clb1,2,3 GAL1::CLB1* 2 hr after addition of glucose; (*C,D*) homozygous diploid *clb1,2,3,4 GAL1::CLB1* 2 hr after addition of glucose. (*A,C*) Staining of nuclear DNA using DAPI and a combination of fluorescent and phase-contrast optics; (*B,D*) immunofluorescent staining of same field using anti-tubulin antibodies.

Discussion

Clb proteins are essential for mitosis

B-type cyclins are thought to promote entry into mitosis in a wide variety of organisms through their activation of Cdc28/cdc2 protein kinases. Our results are fully consistent with the idea that Clb proteins play a similar role in *S. cerevisiae*. *clb2* mutants were delayed in the G_2 phase of the cell cycle, and *clb1,2* or *clb1,2,3* mutants became arrested in G_2 (Figs. 5 and 6; Surana et al. 1991). These arrested cells displayed a short intranuclear microtubule spindle characteristic of G_2 cells, before the spindle elongation that occurs during mitosis (Fig. 8). In addition, Clb proteins bind to and activate the histone H1 kinase activity of Cdc28, with maximal activation in mitosis (Ghiara et al. 1991; N. Grandin and S.I. Reed, in prep.). Finally, overexpression of a truncated allele of Clb1 causes an inability to exit from mitosis (Ghiara et al. 1991), suggesting that high Clb/Cdc28 kinase activity maintains the mitotic state. Thus, activation of Clb/Cdc28 kinase at the G_2/M transition is very likely to be the trigger for mitosis in *S. cerevisiae*, as it is in other organisms.

Clb proteins are required for completion of DNA replication

clb1,2,3,4 mutants underwent arrest with an S-phase DNA content upon loss of the rescuing Clb1 (Fig. 6).

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Furthermore, when the arrested cells were returned to growth on media that reinduced Clb1, it could be seen that they were still sensitive to hydroxyurea (Table 4). Both of these observations suggest that elimination of Clb function prevents completion of DNA replication. Analysis of synchronized cells lacking Clb proteins showed that these cells all entered S phase and arrested uniformly after replicating approximately one-third of their nuclear DNA (Fig. 7). This surprising result suggests that Clb function may not be required for the initiation or elongation phases of DNA replication but, rather, for a distinct function that only becomes essential for DNA replication once a significant part of the genome has been replicated. Possible functions might include initiation of DNA replication at late origins or activation of topoisomerase required to relieve DNA supercoiling generated by replication. In this regard, it is interesting to note that an additional B-type cyclin, Clb5, has recently been identified in *S. cerevisiae* based on its ability to function as a G_1 cyclin (Epstein and Cross 1992; S.I. Reed, unpubl.). Although the functional relationship of Clb5 to the other Clb proteins is not yet clear, it appears that Clb5 is required for efficient progression through S phase (Epstein and Cross 1992). It is possible that Clb5 and the other Clb proteins perform overlapping functions in S phase and that the DNA replication seen in the *clb1,2,3,4* mutant was the result of Clb5 activity. However, different Clb proteins might perform distinct specialized roles in S phase. Recently, cyclin A has been shown to be required for initiation of, or progression through, S phase in mammalian somatic cells (Girard et al. 1991; Pagano et al. 1992). Although Clb3, Clb4, and Clb5 do not share strong structural homology with cyclin A, they may perform analogous functions in yeast.

Clb proteins play a role in the assembly and maintenance of the intranuclear spindle

In *S. cerevisiae*, the spindle pole bodies (the major microtubule-organizing centers in these cells) are duplicated near the G_1/S transition (Byers and Goetsch 1975). During S phase, the duplicated spindle pole bodies separate and a short intranuclear spindle forms between them, which is maintained through G_2 and elongates during mitosis (Byers and Goetsch 1975). Most *S. cerevisiae* S-phase and G_2-arresting *cdc* mutations confer a terminal phenotype that consists, in part, of a short, thick, intranuclear spindle (Byers and Goetsch 1975; Ghiara et al. 1991). It was therefore rather surprising that the *clb1,2,3,4* mutants deprived of the rescuing Clb1 arrested uniformly without a discernible intranuclear spindle (Fig. 8). Moreover, the loss of Clb1 in this strain resulted in the disappearance of spindles that had already formed during a hydroxyurea arrest (Fig. 9). Thus, Clb proteins are required for the maintenance (and possibly assembly) of the short G_2 spindle, as well as for the elongation of this spindle during mitosis (see above).

The finding that both DNA replication and spindle assembly were defective in *clb1,2,3,4* mutants raised the possibility that one of these phenotypes was indirectly caused by the other (i.e., that DNA replication was blocked because of the inability to assemble a spindle or that spindle assembly was blocked because of the inability to complete DNA replication). We consider this possibility unlikely because previous studies have shown that DNA replication proceeds unhindered even when microtubule structures are disrupted by nocodazole or β-tubulin mutations (Huffaker et al. 1988; Jacobs et al. 1988). Similarly, spindle assembly proceeds normally even when DNA replication is blocked by hydroxyurea or various *cdc* mutations (Byers 1981; Pringle and Hartwell 1981). Thus, we consider it more likely that Clb proteins directly regulate both DNA replication and spindle assembly.

Another surprising finding was that *clb1,2,3,4* mutants suffered a dramatic loss of viability 3–4 hr after withdrawal of the rescuing Clb1. In contrast, *clb1,2,3* mutants did not show a significant loss of viability during this period. This difference suggests that the loss of viability was a result of the defects observed uniquely with the *clb1,2,3,4* mutant, namely impaired DNA replication and spindle assembly. Possibly, the unipolar microtubule structure of this mutant corresponds to an irreversible state, blocking further cell cycle progress. Alternatively, the defect in DNA replication may lead to lesions that cannot be repaired efficiently.

Which Clb protein does what?

The genetic data reported here demonstrate that Clb proteins perform several critical functions during the cell cycle (above), with Clb2 making the strongest contribution to these functions and Clb4 the weakest. Our observations with different lethal *clb* mutant combinations suggest that a low level of Clb function is required for DNA replication and a high level of Clb function is required for chromosome segregation. Similarly, a low level of Clb function suffices for assembly and maintenance of a short interphase spindle while a higher level is required for spindle elongation in mitosis. All four Clb proteins are able to provide the DNA replication and spindle assembly functions, and at least Clb1, Clb2, and Clb3 can contribute to the mitotic functions. However, these data do not reveal whether different Clb proteins are normally responsible for the separate functions (but can substitute for each other when necessary) or whether all Clb proteins participate redundantly in all functions.

Although not revealed by the genetic experiments, there are strong reasons to believe that Clb proteins fall into two functional classes, with Clb3 and Clb4 performing the S-phase and early spindle assembly roles and Clb1 and Clb2 performing the mitotic roles. First, sequence analysis shows that Clb3/Clb4 and Clb1/Clb2 form pairs that are related much more closely to each other than they are to members of the other pair. Furthermore, Clb3 and Clb4 are related most closely to the *S. pombe* cyclin cig1 (Fig. 2), which appears to play a role in G_1 or S phase (Bueno et al. 1991), whereas Clb1 and Clb2 show highest homology to the *S. pombe* cyclin cdc13 involved in the initiation of mitosis. Second,

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mRNAs for Clb3 and Clb4 are expressed [and corresponding kinases are activated (N. Grandin and S.I. Reed, in prep.)] earlier in the cell cycle than those for Clb1 and Clb2 (Fig. 3). Thus, we propose that in the normal cell cycle Clb3 and Clb4 function in S phase to promote DNA replication and spindle assembly, while Clb1 and Clb2 (possibly aided by Clb3 and Clb4) are mainly responsible for mitotic induction. This model, however, requires a certain plasticity of Clb function, such that Clb1 and/or Clb2 can fulfill early cell cycle functions if Clb3 and Clb4 are not present.

Substrates for the Clb/Cdc28 protein kinase

Given the broad range of precedents for cyclin B in other eukaryotic cells and our previous results with Clb1 (Ghiara et al. 1991), it is very likely that Clb proteins exert their effects through activation of the Cdc28 protein kinase and consequent phosphorylation of appropriate substrates. The Clb functions identified in this report provide clues to the possible identity of these substrates. The DNA replication function may reflect a need to phosphorylate proteins that form part of the replication complexes, whereas spindle-associated motor proteins are attractive candidates for substrates involved in spindle elongation. The role uncovered here in spindle maintenance might also reflect a requirement to phosphorylate kinesin-like motors shown recently to be crucial for spindle pole body separation (Hoyt et al. 1992; Roof et al. 1992). Alternatively, perhaps nucleation of microtubules on the nuclear side of the spindle pole body requires phosphorylation of spindle pole body components by the Clb/Cdc28 kinase. Consistent with this idea, the collapse of already assembled spindles in the absence of Clb function is reminiscent of the return of spindle pole bodies to a side-by-side configuration in cells treated with microtubule-depolymerizing drugs such as benomyl and nocodazole (Jacobs et al. 1988).

Materials and methods

Cloning the CLB2, CLB3, *and* CLB4 *genes*

The *S. cerevisiae* cyclin-B homolog *CLB1* (*SCB1*) was isolated by using PCR and degenerate oligonucleotides designed to the AA(S)KYEE and AKYL(F)M(VI)E motifs (Ghiara et al. 1991). To isolate other cyclin-B homologs from *S. cerevisiae* using the PCR technique (Saiki et al. 1988) the following degenerate oligonucleotides were used: 5′ primer, 5′-CCGGATCCMRNYTNCARYTNGTNGG-3′ corresponding to the amino acid sequence KLQLVG; 3′ primer, 5′-CGAATTCYTCNAYNARRTAYTTNGC-3′ corresponding to the amino acid sequence AKYL(F)M(VI)E (Y = C + T, R = A + G, M = C + A, N = A + G + C + T). The *Bam*HI and *Eco*RI restriction sites used for cloning are underlined. The PCR products were digested with *Bam*HI and *Eco*RI restriction enzymes, gel purified, and cloned into the *Bam*HI and *Eco*RI sites of pT7T319U (Pharmacia).

Using PCR and the KLQLVG and AKYL(F)M(VI)E primers, a second cyclin-B homolog was isolated from *S. cerevisiae*. The derived amino acid sequence of this clone was identical to the partial amino acid sequence of the *CLB3* gene, as presented by Surana et al. (1991). To isolate the complete genomic clone, a radiolabeled probe made from the PCR clone of *CLB3* was used to screen a *S. cerevisiae* genomic library in the vector λ Dash (obtained from Merl Hoekstra, Salk Institute, San Diego, CA). DNA sequence analysis revealed an open reading frame of 1278 bp, predicted to encode a protein of 426 amino acids (Fig. 1A).

The *CLB2* gene was obtained by screening the λ Dash *S. cerevisiae* genomic library under low stringency conditions (as described below) with a *CLB1* probe (0.5-kb *Eco*RV fragment) containing the cyclin box region. The sequence identified had several discrepancies relative to that reported by Surana et al. (1991): At position −108, they have TATAACCCC, whereas we have TATAAAAAC, which fits the consensus sequence for a TATA box; and at position 1510, they have TGA, whereas we have TAA.

The *CLB4* gene was isolated by screening the λ Dash *S. cerevisiae* genomic library under low stringency conditions (as described below) with a *CLB3* probe specific for the cyclin box (the *CLB3* PCR clone). The derived amino acid sequence of this gene was similar to the partial amino acid sequence of *CLB4* presented by Surana et al. (1991).

Radiolabeled probes were made using the random primer DNA labeling kit (Boehringer Mannheim) according to the manufacturer's instructions. For Southern blot analysis, DNA was transferred to nitrocellulose filters (Schleicher & Schuell).

Construction of clb *null mutations*

The *CLB2* null allele (*clb2::LEU2*) was constructed by utilizing a 2.8-kb *Eco*RI subclone derived from a phage clone of *CLB2*. This clone enabled the 0.38-kb *Eco*RV–*Bgl*II fragment from the cyclin box region to be replaced with a 2.5-kb *Pvu*II–*Bam*HI fragment containing *LEU2* (derived from a clone of *LEU2* in pUC19) The construct was then excised with *Nsi*I and used to transform BF264-15DUa. Transformants were selected on minimal plates supplemented with amino acids but lacking leucine.

The *CLB3* null allele (*clb3::TRP1*) was constructed as follows. A 1.5-kb *Bgl*II–*Hin*cII fragment containing the *TRP1* gene (from the plasmid YRp7) was used to replace the 0.58-kb *Bgl*II–*Eco*RV fragment containing the cyclin box region of *CLB3*. The target for insertion was a 2.7-kb *Cla*I–*Sal*I clone of *CLB3* in pT7T3 19U. The resulting disruption construct was then excised with *Sal*I and *Cla*I and used to transform BF264-15DUa. Transformants were selected on minimal plates supplemented with amino acids but lacking tryptophan.

The *CLB4* null allele (*clb4::HIS2*) was constructed by the replacement of a 1.8-kb *Eco*RV–*Nsi*I fragment containing the *CLB4* gene with a 1.9-kb *Pst*I–*Eco*RI fragment containing *HIS2* (derived from a clone of *HIS2* in pUC118). This insertion was carried out in the 3.8-kb *Eco*RI clone of *CLB4*. The construct was excised with *Ase*I and *Eco*RI and used to transform YS102. Transformants were selected on minimal plates supplemented with amino acids but lacking histidine.

In all cases, transformants were screened for the gene disruption by Southern blot analysis of genomic DNA.

Yeast strains, genetic procedures, media, and growth conditions

All yeast strains used in this study were derivatives of BF264-15DU: *MAT***a** *ade1 his2 leu2-3,112 trp1-1*^{a} *ura3Dns* (Richardson et al. 1989). The relevant genotypes of strains used in this study are shown in Table 5.

Standard genetic procedures for yeast were used (Sherman et al. 1982). Yeast transformations were carried out by the alkali

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Table 5. *Strain list*

Strain	Relevant genotype
YH134	*MAT***a** *clb1::URA3*
YH135	*MAT***a** *clb2::LEU2*
YS101	*MAT***a** *clb3::TRP1*
YS102	*MAT***a** *clb1::URA3 clb3::TRP1*
YS104	*MAT***a** *clb1::URA3 clb3::TRP1 clb4::HIS2*
YS105	*MAT***a** *GAL1::CLB1(LEU2) clb1::URA3 clb3::TRP1 clb4::HIS2*
YS106	*MATα clb2::LEU2*
YS107	*MAT***a** *GAL1::CLB1(LEU2) clb1::URA3 clb2::LEU2 clb3::TRP1*
YS108	*MAT***a** *GAL1::CLB1(LEU2) clb1::URA3 clb2::LEU2 clb3::TRP1 clb4::HIS2*
YS112	*MAT***a** *clb4::HIS2*
YS109	*MAT***a** *GAL1::CLB1(LEU2) clb1::URA3 clb2::LEU2*
YS114	*MAT***a** *clb1::URA3 clb4::HIS2*
YS115	*MAT***a** *clb2::LEU2 clb4::HIS2*
YS118	*MAT***a** *clb3::TRP1 clb4::HIS2*
YS201	*MAT***a***/α clb1::URA3/CLB1 clb3::TRP1/CLB3*
YS202	*MAT***a***/α clb1::URA3/CLB1 clb3::TRP1/CLB3 clb2::LEU2/CLB2*
YS203	*MAT***a***/α GAL1::CLB1(LEU2) clb1::URA3/CLB1 clb3::TRP1/CLB3 clb2::LEU2/CLB2 clb4::HIS2/CLB4*
DLY005	*MAT***a***/α*
DLY373	*MAT***a***/α clb2::LEU2/clb2::LEU2*
DLY378	*MAT***a***/α clb3::TRP1/clb3::TRP1 clb4::HIS2/clb4::HIS2*
DLY379	*MAT***a***/α clb1::URA3/clb1::URA3 clb3::TRP1/clb3::TRP1 clb4::HIS2/clb4::HIS2*
DLY380	*MAT***a***/α GAL1::CLB1(LEU2) clb1::URA3/clb1::URA3 clb2::LEU2/clb2::LEU2*
DLY382	*MAT***a***/α GAL1::CLB1(LEU2) clb1::URA3/clb1::URA3 clb2::LEU2/clb2::LEU2 clb3::TRP1/clb3::TRP1*
DLY384	*MAT***a***/α GAL1::CLB1(LEU2) clb1::URA3/clb1::URA3 clb2::LEU2/clb2::LEU2 clb3::TRP1/clb3::TRP1 clb4::HIS2/clb4::HIS2*

cation method (Ito et al. 1983). Genomic sequence replacements were performed as described by Rothstein (1983).

Yeast cultures were grown at 30°C in YEP (1% yeast extract, 2% Bacto-peptone, 0.005% adenine, 0.005% uracil) supplemented with glucose (2%) or galactose (2%). Conditional Clb1 synthesis was terminated (*GAL1:CLB1* strains) by adding 50% glucose to cultures growing in YEP/galactose medium to a final concentration of 2%. Cultures were arrested in S phase (Fig. 8) by growth in YEP/galactose supplemented with 0.2 M hydroxyurea for 3.5 hr and released from the block by resuspension in YEP/glucose.

Hydroxyurea execution point experiments (Table 4) were performed by growing *GAL1::CLB1 clb1,2*, *GAL1::CLB1 clb1,2,3*, and *GAL1::CLB1 clb1,2,3,4* mutant strains in glucose-supplemented medium for 2 hr, after which cells were subjected to a short pulse of sonic disruption and plated on each of three types of solid medium: (1) minimal synthetic medium containing 2% glucose, (2) YEP/galactose, and (3) YEP/galactose supplemented with 0.4 M hydroxyurea. The first plate, which was immediately stored at 4°C, served as a record of the composition of the plated cell population. The second plate served as viability control for the nutritional shift from glucose to galactose and was maintained at 30°C for 12 hr. The third plate, kept at 30°C for 20 hr, was used to determine whether individual cells could undergo a round of division in the presence of the replication inhibitor hydroxyurea. Cells were observed directly on the agar surface, using a low power objective and a stage modified to accommodate 100-mm petri dishes. The plating control (minimal plate) was scored by assigning cells to three catagories: unbudded cells, singly budded cells, and units composed of multiple buds or cells. The viability control was scored by assigning cells to two catagories: single cells and microcolonies. Single cells were assumed to be inviable. The experimental plate was scored by assigning cells to two catagories: undivided (singly budded) cells and divided (two side-by-side budded) cells. To calculate the percentage of S-phase (hydroxyurea sensitive) cells, the number of inviable cells (viability control) and unbudded cells (plating control) was subtracted from the number of cells that did not divide on hydroxyurea to give an adjusted hydroxyurea-sensitive cell number. The number of multiple cells (plating control) was subtracted from the number of cells that divided on hydroxyurea to give an adjusted hydroxyurea-insensitive cell number. The percentage of S-phase cells was then calculated by dividing the adjusted hydroxyurea-sensitive cell number by the sum of the adjusted hydroxyurea-sensitive and -insensitive cell numbers. The calculations were based on scoring 600 independent structures (cells, pairs of cells, or microcolonies) on each plate.

Photomicroscopy, immunofluorescence staining, cell counting, flow cytometry, and cell synchrony

Yeast cells were photographed live, using differential interference contrast (Nomarski) optics with a 100× objective. Fluorescent photomicroscopy on fixed and stained cells was performed using a Zeiss Axiophot photomicroscope with a 100× objective. DAPI and anti-tubulin antibody staining was performed as described by Ghiara et al. (1991).

Cell numbers and budding percentages were determined using a hemacytometer and a Leitz SM-Lux phase-contrast microscope with a 40× objective.

Cell cultures were analyzed for DNA content using flow cytometry by the method of Hutter and Eipel (1979). Yeast cells were fixed in 70% ethanol, stained with propidium iodide, and analyzed for fluorescence using a Becton-Dickinson FACScan analyzer (Lew et al. 1992).

Cell synchrony using mating pheromone was as described by

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Ghiara et al. (1991). Centrifugal elutriation was performed as described by Lew et al. (1992).

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Note added in proof

Sequence data described in this paper have been submitted to the GenBank data library.

References

Booher, R. and D. Beach. 1987. Interaction between *cdc13*+ and *cdc2*+ in the control of mitosis in fission yeast; dissociation of the G1 and G2 roles of the *cdc2*+ protein kinase. *EMBO J.* **6:** 3441–3447.

———. 1988. Involvement of cdc13+ in mitotic control in *Schizosaccharomyces pombe*: Possible interaction of the gene product with microtubeules. *EMBO J.* **7:** 2321–2327.

Bueno, A., H. Richardson, S.I. Reed, and P. Russell. 1991. A fission yeast B-type cyclin functioning early in the cell cycle. *Cell* **66:** 149–159.

Byers, B. 1981. Cytology of the yeast life cycle. In *The molecular biology of the yeast* Saccharomyces: Life cycle and inheritance (ed. J.N. Strathern, E.W. Jones, and J.R. Broach), pp. 59–97. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Byers, B. and L. Goetsch. 1975. Behavior of spindles and spindle plaques in the cell cycle and conjugation of *Saccharomyces cerevisiae*. *J. Bacteriol.* **124:** 511–523.

Cross, F. 1988. *DAF1*, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **8:** 4675–4684.

———. 1990. Cell cycle arrest caused by *CLN* gene deficiency in *Saccharomyces cerevisiae* resembles START-arrest and is independent of the mating-pheromone signalling pathway. *Mol. Cell. Biol.* **10:** 6482–6490.

Cross, F., J. Roberts, and H. Weintraub. 1989. Simple and complex cell cycles. *Annu. Rev. Cell Biol.* **5:** 341–395.

Elledge, S.J. and M.R. Spottswood. 1991. A new human p34 protein kinase, CDK2, identified by complementation of a *cdc28* mutation in *Saccharomyces cerevisiae*, is a homolog of *Xenopus* Eg1. *EMBO J.* **10:** 2653–2659.

Epstein, C.B. and F.R. Cross. 1992. *CLB5*: A novel B cyclin from budding yeast with a role in S phase. *Genes & Dev.* **6:** 1695–1706.

Evans, T., E.T. Rosenthal, J. Youngblom, D. Distel, and T. Hunt. 1983. Cyclin: A protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* **33:** 389–396.

Fang, F. and J.W. Newport. 1991. Evidence that the G1-S and G2-M transitions are controlled by different cdc2 proteins in higher eukaryotes. *Cell* **66:** 731–742.

Forsburg, S.L. and P. Nurse. 1991. Identification of a G1-type cyclin *puc*+ in the fission yeast *Schizosaccharomyces pombe*. *Nature* **351:** 245–248.

Ghiara, J.B., H.E. Richardson, K. Sugimoto, M. Henze, D.J. Lew, C. Wittenberg, and S.I. Reed. 1991. A cyclin B homolog in *S. cerevisiae*: Chronic activation of the Cdc28 protein kinase by cyclin prevents exit from mitosis. *Cell* **65:** 163–174.

Girard, F., U. Strausfeld, A. Fernandez, and N.J.C. Lamb. 1991. Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. *Cell* **67:** 1169–1179.

Glotzer, M., A.W. Murray, and M.W. Kirschner. 1991. Cyclin is degraded by the ubiquitin pathway. *Nature* **349:** 132–138.

Hadwiger, J.A., C. Wittenberg, H.E. Richardson, M. de Barros Lopes, and S.I. Reed. 1989). A novel family of cyclin homologs that control G1 in yeast. *Proc. Natl. Acad. Sci.* **86:** 6255–6259.

Hagan, I.M., J. Hayles, and P. Nurse. 1988. Cloning and sequencing of the cyclin related *cdc13*+ gene and a cytological study of its role in fission yeast mitosis. *J. Cell Sci.* **91:** 587–595.

Hartwell, L.H., J. Culotti, J.R. Pringle, and B.J. Reid. 1974. Genetic control of the cell division cycle in yeast. *Science* **183:** 46–51.

Hoyt, M.A., L. He, K.K. Loo, and W.S. Saunders. 1992. Two *Saccharomyces cerevisiae* kinesin-related gene products required for mitotic spindle assembly. *J. Cell Biol.* **118:** 109–120.

Huffaker, T.C., J.H. Thomas, and D. Botstein. 1988. Diverse effects of β-tubulin mutations on microtubule formation and function. *J. Cell Biol.* **106:** 1997–2010.

Hunt, T. 1989. Maturation promoting factor, cyclin and the control of M-phase. *Curr. Opin. Cell Biol.* **1:** 268–274.

Hunt, T., F.C. Luca, and J.V. Ruderman. 1992. The requirements for protein synthesis and degradation, and the control of destruction of cyclins A and B in the meiotic and mitotic cell cycles of the clam embryo. *J. Cell Biol.* **116:** 707–724.

Hutter, K.-J. and H.E. Eipel. 1979. DNA determination of yeast by flow cytometry. *J. Gen. Microbiol.* **113:** 369–375.

Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153:** 163–168.

Jacobs, C.W., A.E.M. Adams, P.J. Szaniszlo, and J.R. Pringle. 1988. Functions of microtubules in the *Saccharomyces cerevisiae* cell cycle. *J. Cell Biol.* **107:** 1409–1426.

Lehner, C.F. and P.H. O'Farrell. 1990. The roles of *Drosophila* cyclins A and B in mitotic control. *Cell* **61:** 535–547.

Lew, D.J. and S.I. Reed. 1992. A proliferation of cyclins. *Trends Cell Biol.* **2:** 77–81.

Lew, D.J., V. Dulic, and S.I. Reed. 1991. Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. *Cell* **66:** 1197–1206.

Lew, D.J., N.M. Marini, and S.I. Reed. 1992. Different G1 cyclins control the timing of cell cycle commitment in mother and daughter cells of the budding yeast *S. cerevisiae*. *Cell* **69:** 317–327.

Lewin, B. 1990. Driving the cell cycle: M phase kinase, its partners, and substrates. *Cell* **61:** 535–547.

Maller, J. 1991. Mitotic control. *Curr. Opin. Cell Biol.* **3:** 269–275.

Minshull, J., J.J. Blow, and T. Hunt. 1989. Translation of cyclin mRNA is necessary for extracts of activated *Xenopus* eggs to enter mitosis. *Cell* **56:** 947–956.

Minshull, J., R. Golsteyn, C.S. Hill, and T. Hunt. 1990. The A-

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and B-type cyclin-associated *cdc2* kinases in *Xenopus* turn on and off at different times in the cell cycle. *EMBO J.* **9:** 2865–2875.

Murray, A.W. and M.W. Kirschner. 1989a. Dominoes and clocks: The union of two views of the cell cycle. *Science* **246:** 614–621.

———. 1989b. Cyclin synthesis drives the early embryonic cell cycle. *Nature* **339:** 275–280.

Murray, A.W., M.J. Solomon, and M.W. Kirschner. 1989. The role of cyclin synthesis in the control of maturation promoting factor activity. *Nature* **339:** 280–286.

Nash, R., G. Tokiwa, S. Anand, K. Erickson, and A.B. Futcher. 1988. The *WHI1*$^{+}$ gene of *Saccharomyces cerevisiae* tethers cell division to cell size and is a cyclin homolog. *EMBO J.* **7:** 4335–4346.

Nasmyth, K., A. Seddon, and G. Ammerer. 1987. Cell cycle regulation of *SWI5* is required for mother-cell-specific *HO* transcription in yeast. *Cell* **49:** 549–558.

Needleman, S.B. and C.D. Wunsch. 1970. A general method applicable to the search for similarities in the amino acid sequence of two proteins. *J. Mol. Biol.* **48:** 443–453.

Ninomiya-Tsuji, J., S. Nomoto, H. Yasuda, S.I. Reed, and K. Matsumoto. 1991. Cloning of a human cDNA encoding a CDC2-related kinase by complementation of a budding yeast *cdc28* mutation. *Proc. Natl. Acad. Sci.* **88:** 9006–9010.

Nurse, P. 1990. Universal control mechanism regulating onset of M-phase. *Nature* **344:** 503–508.

Nurse, P. and Y. Bissett. 1981. Gene required in G1 for commitment to cell cycle and in G2 for control of mitosis in fission yeast. *Nature* **292:** 448–460.

Pagano, M., R. Pepperkok, F. Verde, W. Ansorje, and G. Draetta. 1992. Cyclin A is required at two points in the human cell cycle. *EMBO J.* **11:** 961–971.

Paris, J., R. Le Guellec, A. Couturier, K. Le Guellec, F. Omilli, J. Camonis, S. MacNeill, and M. Philippe. 1991. Cloning by differential screening of a *Xenopus* cDNA coding for a protein highly homologous to cdc2. *Proc Natl. Acad. Sci.* **88:** 1039–1043.

Piggott, J.R., R. Rai, and B.L.A. Carter. 1982. A bifunctional gene product involved in two phases of the yeast cell cycle. *Nature* **298:** 391–393.

Pines, J. and T. Hunter. 1989. Isolation of a human cyclin cDNA: Evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34^{cdc2}. *Cell* **58:** 833–846.

———. 1990a. p34^{cdc2}: The S and M kinase? *New Biol.* **2:** 389–401.

———. 1990b. Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. *Nature* **346:** 760–763.

Pringle, J.R. and L.H. Hartwell. 1981. The *Saccharomyces cerevisiae* cell cycle. In *The molecular biology of the yeast* Saccharomyces: Life cycle and inheritance (ed. J.N. Strathern, E.W. Jones, and J.R. Broach), pp. 97–142. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Reed, S.I. 1991. G1-specific cyclins: In search of an S-phase-promoting factor. *Trends Genet.* **7:** 95–99.

Reed, S.I. and C. Wittenberg. 1990. A mitotic role for the Cdc28 protein kinase of *S. cerevisiae*. *Proc Natl. Acad. Sci.* **87:** 5697–5701.

Richardson, H.E., C. Wittenberg, F. Cross, and S.I. Reed. 1989. An essential G1 function for cyclin-like proteins in yeast. *Cell* **59:** 1127–1133.

Riabowol, K., G. Draetta, L. Brizuela, D. Vandre, and D. Beach. 1989. The cdc2 kinase is a nuclear protein that is essential for mitosis in mammalian cells. *Cell* **57:** 393–401.

Roof, D.M., P.B. Meluh, and M.D. Rose. 1992. Kinesin-related proteins required for assembly of the mitotic spindle. *J. Cell Biol.* **118:** 95–108.

Rothstein, R.J. 1983. One-step gene disruption in yeast. *Methods Enzymol.* **101:** 202–211.

Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239:** 487–491.

Sherman, F., G. Fink, and J.B. Hicks. 1982. *Methods in yeast genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Slater, M.L. 1973. Effect of reversible inhibition of deoxyribonucleic acid synthesis on the yeast cell cycle. *J. Bacteriol.* **113:** 263–270.

Sudbery, P., A.R. Goodey, and B.L.A. Carter. 1980. Genes which control cell proliferation in the yeast *Saccharomyces cerevisiae. Nature* **288:** 401–404.

Surana, U., H. Robitsch, C. Price, T. Schuster, I. Fitch, A.B. Futcher, and K. Nasmyth. 1991. The role of *CDC28* and cyclins during mitosis in the budding yeast *S. cerevisiae*. *Cell* **65:** 145–161.

Swenson, K.I., K.M. Farrell, and J.V. Ruderman. 1986. The clam embryo protein cyclin A induces entry into M-phase and the resumption of meiosis in *Xenopus* oocytes. *Cell* **47:** 861–870.

Th'ng, J.P.H., P.S. Wright, J. Hamaguchi, M.G. Lee, C.J. Norbury, P. Nurse, E.M. Bradbury. 1990. The FT210 cell line is a mouse G2 phase mutant with a temperature-sensitive *CDC2* gene product. *Cell* **63:** 313–324.

Tsai, L.-H., E. Harlow, and M. Meyerson. 1991. Isolation of the human *cdk2* gene that encodes the cyclin A- and adenovirus E1A-associated p33 kinase. *Nature* **353:** 174–177.

Wang, J., X. Chenivesse, B. Henglein, and C. Brechot. 1990. Hepatitis B virus integration in a cyclin A gene in a hepatocellular carcinoma. *Nature* **343:** 555–557.

Wittenberg, C., K. Sugimoto, and S.I. Reed. 1990. G1-specific cyclins of *S. cerevisiae*: Cell cycle periodicity, regulation by mating pheromone, and association with the p34^{CDC28} protein kinase. *Cell* **62:** 225–237.

Xiong, Y. and D. Beach. 1991. Population explosion in the cyclin family. *Curr. Biol.* **1:** 362–364.

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