CLB5: a novel B cyclin from budding yeast with a role in S phase

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Budding yeast strains have three *CLN* genes, which have limited cyclin homology. At least one of the three is required for cell cycle START. Four B cyclins are known in yeast; two have been shown to function in mitosis. We have discovered a fifth B-cyclin gene, called *CLB5*, which when cloned on a CEN plasmid can rescue strains deleted for all three *CLN* genes. *CLB5* transcript abundance peaks in G_1, coincident with the *CLN2* transcript but earlier than the *CLB2* transcript. *CLB5* deletion does not cause lethality, either alone or in combination with other *CLN* or *CLB* deletions. However, strains deleted for *CLB5* require more time to complete S phase, suggesting that *CLB5* promotes some step in DNA synthesis. *CLB5* is the only yeast cyclin whose deletion lengthens S phase. *CLB5* may also have some role in promoting the G_1/S transition, because *cln1 cln2* strains require both *CLN3* and *CLB5* for viability on glycerol media and *cln1,2,3*$^-$ strains require *CLB5* for rescue by the *Drosophila melanogaster cdc2* gene. In conjunction with *cln1,2,3*$^-$ rescue by *CLB5* overexpression and the coincident transcriptional regulation of *CLB5* and *CLN2*, these observations are suggestive of partial functional redundancy between *CLB5* and *CLN* genes.

[*Key Words*: Cyclin; *CLB5*; S phase; *Saccharomyces cerevisiae*; cell cycle control]

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Progression through the eukaryotic cell cycle is controlled by the protein kinase *cdc2/CDC28*. In budding yeast and fission yeast, this kinase is required for cell cycle START [commitment to cell cycle progression late in G_1 (Pringle and Hartwell 1981)], for mitosis (Hartwell 1991), and for meiosis (Niwa and Yanagida 1988; Shuster and Byers 1989). In higher eukaryotic cells, the involvement of this kinase in mitosis (Hartwell 1991) and meiosis (Labbe et al. 1988) is established. There are also indications that either *cdc2* or a related protein kinase may have a role early in the cell cycle (Blow and Nurse 1990; D'Urso et al. 1990; Pines and Hunter 1990a; Fang and Newport 1991).

cdc2/CDC28 functions only when activated by binding to members of a class of proteins called cyclins. Cyclins of the B-type sequence class are generally associated with activation of *cdc2/CDC28* in mitosis. Four B-type cyclin genes have been reported in *Saccharomyces cerevisiae* (*CLB1*, *CLB2*, *CLB3*, and *CLB4*; Ghiara et al. 1991; Surana et al. 1991). *CLB1*, *CLB2*, and *CLB4* were discovered as high-copy suppressors of a conditional allele of *cdc28* having a G_2/M terminal phenotype (Surana et al. 1991). *CLB1* mutations promoting the stability of the protein lead to M-phase arrest (Ghiara et al. 1991), whereas deletion of *CLB2* leads to delayed entry into M phase (Surana et al. 1991). Simultaneous deletion of *CLB2* and either *CLB1* or *CLB3* arrests cells in G_2 on the basis of tetrad analysis (Surana et al. 1991; D. Lew and S. Reed, pers. comm.). In contrast, the *Schizosaccharomyces pombe cig1*$^+$ gene is a B cyclin that is required for efficient passage of the G_1/S transition (Bueno et al. 1991).

A-type cyclins may also activate *cdc2* in mitosis; however, growing evidence suggests a role for A-type cyclins complexed with Cdc2 or the Cdc2-related kinase Cdk2 earlier in the cell cycle, particularly at DNA replication (Pines and Hunter 1990b; Girard et al. 1991; Tsai et al. 1991). In higher eukaryotes, it is not clear whether cyclin A/kinase complexes are required for S phase throughout its duration or only at the G_1/S transition. In budding yeast, no cyclin-A homologs are known nor is there any genetic evidence that *CDC28* is involved directly in S-phase progression.

In *S. cerevisiae*, START is dependent on the distant cyclin homologs *CLN1*, *CLN2*, and *CLN3* (Richardson et al. 1989), as well as on *CDC28*. The products of the *CLN* genes bind to (Wittenberg et al. 1990; F. Cross and C. Blake, unpubl.) and activate (R. Deshaies, pers. comm.) the *CDC28* protein kinase. The three *CLN* genes are functionally redundant; any one of the three is sufficient for viability (Richardson et al. 1989). Triple *CLN* deficiency causes G_1 arrest, before START (Cross 1990). The genes for human cyclins C, D, and E (Koff et al. 1991; Lew et al. 1991; Matsushime et al. 1991; Xiong et al. 1991) and *Drosophila melanogaster* cyclin C (Lahue et al. 1991; Leopold and O'Farrell 1991) were isolated on the basis of their ability to rescue *cln1,2,3*$^-$ yeast strains. The normal roles of these cyclins are still unclear. Human cyclin A and cyclins B1 and B2, as well as *S. pombe cdc13*$^+$, a mitotic B-type cyclin, also functioned in the

cln1,2,3$^-$ rescue assay (Koff et al. 1991; Lew et al. 1991; Xiong et al. 1991). These rescue experiments all employed a strong yeast promoter and high-copy plasmids, presumably promoting gross overexpression of the foreign cyclin.

Here, we report the discovery of a fifth yeast B cyclin that is capable of rescuing *cln1,2,3*$^-$ lethality when present on a low-copy-number plasmid under the control of its own promoter. Deletion of this gene (*CLB5*) results in a marked slowing of S-phase progression, rather than a delay in the G_1/S or G_2/M transitions. *CLB5* RNA is expressed early in the cell cycle, in contrast to other B-type cyclins (Ghiara et al. 1991; Surana et al. 1991). These observations suggest that *CLB5* is a B-type cyclin functioning early in the cell cycle and is required for efficient DNA replication.

Results

Isolation of a novel B cyclin rescuing the cln1,2,3$^-$ *genotype*

Yeast strains deleted for all three *CLN* genes are inviable but can be maintained if supplied with a plasmid copy of a *CLN* gene. We constructed a library in a CEN (low-copy) plasmid, using genomic DNA from a haploid yeast strain (relevant genotype: *cln1 CLN2 cln3*). The library was screened for plasmids that could rescue a strain with the genotype *cln1 cln2 cln3* [*pGAL1::CLN3/URA3*], under conditions (i.e., glucose media) where *GAL1::CLN3* was not being synthesized. We recovered eight distinct clones of *CLN2* and three distinct clones of a novel gene. Subcloning experiments identified the minimal region of these plasmids required for *cln1,2,3*$^-$ rescue (Fig. 1). This region was sequenced in its entirety (Fig. 2) and was found to contain a single open reading frame of 435 amino acids. The translated sequence was aligned by use of the FASTA homology search program (Pearson and Lipman 1988) to the contents of GenBank (Bilofsky and Burks 1988) and was found to represent a new B cyclin. We named this gene *CLB5*, because four B cyclins (*CLB1, CLB2, CLB3,* and *CLB4*) were already known in budding yeast (Surana et al. 1991). *CLB5* can be further identified as a B cyclin as it contains the conserved FLRR_SK motif (residues 301–307), diagnostic for distinguishing B- and A-type cyclins (O'Farrell and Leopold 1991).

CLB5 is adjacent to one of the other B cyclins, *CLB2* (Fig. 1). We tested various plasmids containing *CLB5* only, or *CLB2* only, for *cln1,2,3*$^-$ rescue activity and for rescue of a *clb1,2*$^-$ double mutant [see Materials and methods; the double mutant is lethal (Surana et al. 1991)]. We found that the *CLB2*-containing plasmid rescued the *clb1,2*$^-$ lethality but not the *cln1,2,3*$^-$ lethality; the *CLB5*-containing plasmid rescued the *cln1,2,3*$^-$ lethality but not the *clb1,2*$^-$ lethality (Fig. 1). Thus, *CLB5* is qualitatively different from *CLB2*. The failure of *CLB2* to rescue *cln1,2,3*$^-$ lethality, together with the fact that no other B-cyclin genes were recovered from our library, suggests that *CLB5* is unique among yeast B cyclins in its ability to rescue *cln1,2,3*$^-$ lethality when cloned on a CEN plasmid. Lew et al. (1991) were unable to rescue a *cln1,2,3*$^-$ strain by overexpressing integrated *CLB1* from the strong constitutive glyceraldehyde-3-phosphate dehydrogenase promoter, again supporting the inference that *cln1,2,3*$^-$ rescue may be unique to *CLB5*. Although addition of *CLB5* on a CEN plasmid was adequate at *cln* rescue, but not at *clb* rescue, this may simply reflect the time of expression of *CLB5* from its natural promoter, rather than the intrinsic potential of the protein. Overexpression of *CLB5*, or expression from a deregulated promoter, might suffice to rescue *clb1 clb2* mutants. These experiments are in progress.

Northern analysis of CLB5

Four yeast cyclins are known to be subject to cell cycle periodic transcriptional control: *CLN1* and *CLN2* are maximally expressed just before cell cycle START (Wittenberg et al. 1990; Cross and Tinkelenberg 1991), and *CLB1* and *CLB2* are maximally expressed before mitosis (Ghiara et al. 1991; Surana et al. 1991). For these genes, the time of expression is consistent with the time of function; *CLN1* and *CLN2* function to promote START (Richardson et al. 1989), while *CLB1* and *CLB2* regulate entry into mitosis (Ghiara et al. 1991; Surana et al. 1991). Because the time in the cell cycle when a transcript is abundant may provide some insight into the function of the gene (McKinney and Heintz 1991), we examined *CLB5* expression in synchronized cultures. G_1 arrest was induced in a *cln1 cln2 cln3 GAL1::CLN3* strain by incubation in raffinose medium, and cycling was induced by galactose addition, as described (Cross and Tinkelenberg 1991). The defective *cln2* gene in this strain produces a

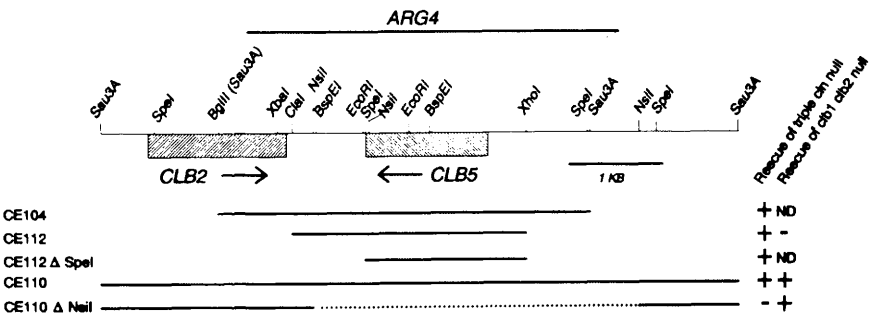

Figure 1. Restriction map and disruption of *CLB5*. CE104 and CE110 were among three overlapping CEN plasmid clones recovered that rescued *cln1,2,3*$^-$ inviability. CE110 contained intact copies of the *CLB2* and *CLB5* genes, but *cln* rescue activity was unique to *CLB5*, whereas *clb1 clb2* rescue was unique to *CLB2*. The region of *CLB5* between the designated *Bsp*EI and *Eco*RI sites was replaced with the yeast *ARG4* gene to generate the *clb5::ARG4* allele. (ND) Not determined.

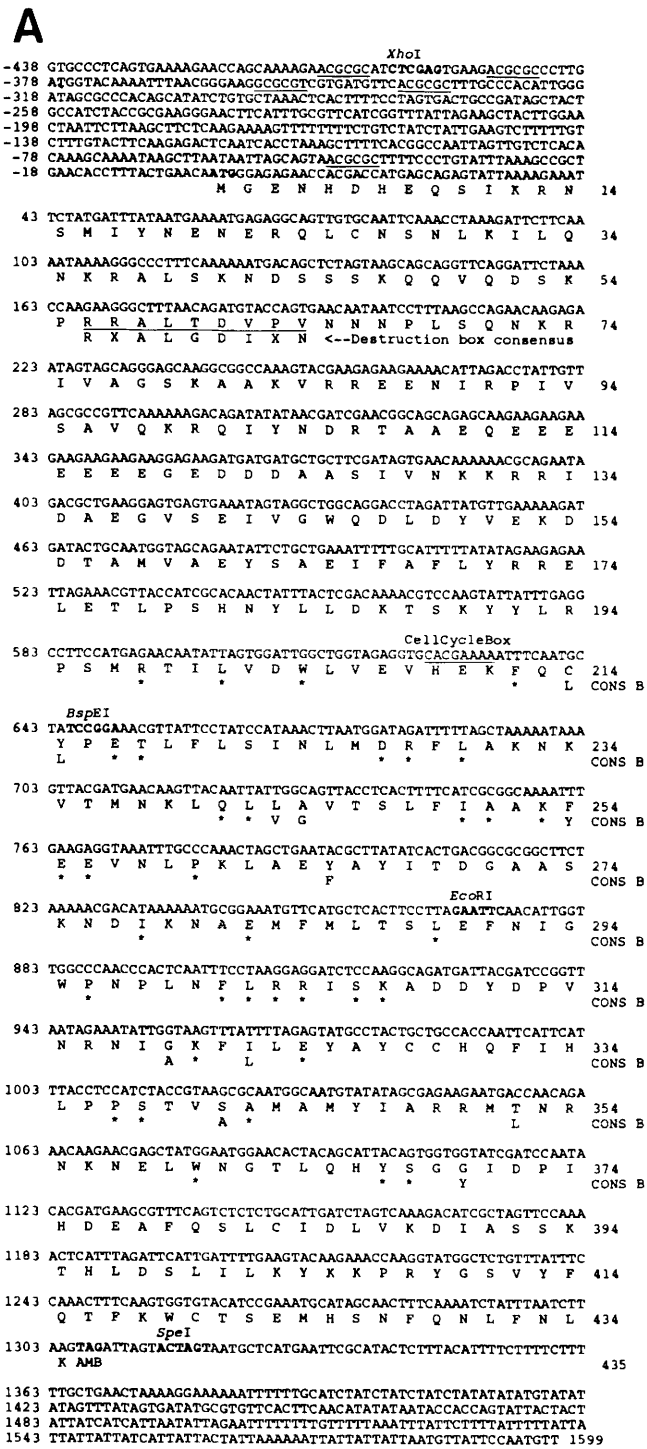

Figure 2. (*A*) *CLB5* is a B cyclin. (*DNA*) The minimal fragment having activity on a CEN plasmid at *cln1,2,3*$^-$ rescue (CE112 ΔSpe; Fig. 1) is the region between the indicated *Xho*I and *Spe*I sites. The DNA between the indicated *Bsp*EI and *Eco*RI sites, which was replaced by the yeast *ARG4* gene, falls near the start of the region of cyclin homology; hence, nearly the entire cyclin box is absent in the *clb5::ARG4* allele. Sequence was determined for both strands, except in the region −438 to −386. (*Potential transcriptional control elements*) Five occurrences of a sequence (ACGCGC and its inverse complement) resembling the MCB are underlined in the 5′ region of the gene. No CCB motifs (CACGAAA, NACGAAA, CNCGAAA, and their inverse complements) are found, with the exception of a single CACGAAAA (underlined), which occurs in the coding region, spanning residues 209–211 of the protein. Previously described CACGAAAA sequences have all been in the 5′-nontranscribed regions (Nasmyth 1985; Nasmyth and Dirick 1991; Ogas et al. 1991). Sequence from −1037 to −438 (data in GenBank, accession no. M91209) showed no further occurrences of CCB or MCB motifs. (*Protein*) In the cyclin homology region (residues 197–370), we consulted O'Farrell and Leopold (1991) to determine amino acids conserved in at least 90% of the 14 B cyclins compared. For those residues, we placed an asterisk under the Clb5 sequence where it matches other B cyclins and placed the B-cyclin consensus amino acid where Clb5 differs. Clb5 matches the consensus at 35 of 46 positions and matches fungal B cyclins at many additional residues, as shown in *B*. A potential destruction box (Glotzer et al. 1991) is underlined, with the consensus given below the line. Consensus destruction boxes begin at residue 42; the Clb5 destruction box begins at residue 41 or 56, depending on the true initiator methionine. (*B*) Alignment of Clb5 with other yeast B cyclins. The Clb5 sequence was aligned to all B cyclins known from the yeasts *S. cerevisiae* and *S. pombe*, by use of the CLUSTAL program within PC/GENE (Higgins and Sharp 1988). CLUSTAL places an asterisk under absolutely conserved residues and a dot under well-conserved residues. The Clb5 sequence is underlined at residues where it fails to conform to sequence conserved in most B cyclins (as determined in the legend to *A*).

properly regulated mRNA (Cross and Tinkelenberg 1991), serving as a control for a gene turned on at START. We found that *CLB5* RNA is expressed exactly in parallel with *cln2* RNA in this protocol (Fig. 3, upper panel). In contrast, *CLB2* RNA comes on later and peaks when the *CLB5* and *cln2* transcripts are near their trough, just preceding nuclear division. This pattern is detectable for two cell cycles in this experiment. The *CLB4* transcript is also cell cycle periodic and peaks at a time intermediate between the *CLB5* and *CLB2* transcripts (data not shown). We have also observed that *CLB5* RNA fluctuates exactly in phase with *CLN2* RNA in an α-factor block–release synchronization protocol with a *CLN1 CLN2 CLN3* strain (data not shown). The fact that *CLB5* expression peaks earlier in the cell cycle than *CLB2* may indicate that *CLB5* principally functions at an earlier cell cycle stage.

To further examine the regulation of the *CLB5* transcript, we repeated the block–release protocol described above, but released in the presence of nocodazole, a microtubule-depolymerizing agent that prevents mitosis (Jacobs et al. 1988). In this protocol, the first bud emergence occurs with normal timing, but mitosis never occurs and cells arrest in G_2 with a single large bud (Jacobs et al. 1988). We found that *cln2* and *CLB5* RNAs peak with timing equivalent to the nocodazole-free treatment and then decline (Fig. 3, bottom panel). The second peak of these RNAs is not observed. *CLB2* RNA also comes up on schedule but, in contrast, remains on at the nocodazole block. These results suggest that the decrease in *CLB2* RNA levels requires nuclear division and that the time in the cell cycle when these gene products function is early for *CLN2* and *CLB5* and late for *CLB2*.

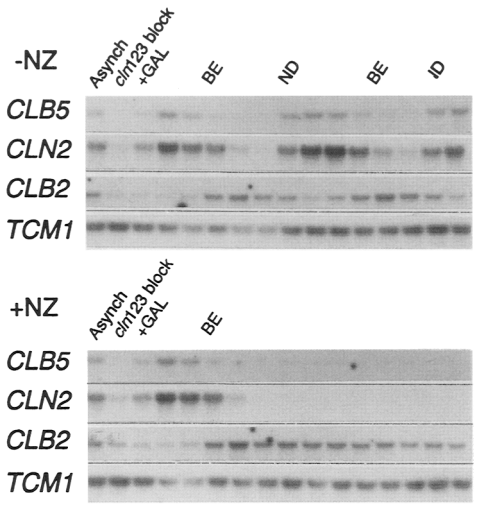

Figure 3. Northern analysis of *CLB5* transcription through the cell cycle. *cln1 cln2 cln3 leu2::LEU2::GAL1::CLN3* strains were grown to log phase in YEP–3% galactose at 30°C and arrested by 150 min growth in YEP–3% raffinose at 30°C. Cultures were released from cell cycle arrest by the addition of galactose to 3%. At 12-min intervals thereafter, samples were taken and RNA was isolated. Replicate blots were hybridized with *CLB5*, *CLN2*, *CLB2*, and *TCM1* (used as a loading control) probes, as indicated. Timing of bud emergence (BE) and nuclear division (ND) was determined by microscopic examination of fixed, sonicated cells, in the latter case stained with DAPI and UV-illuminated. (*Bottom*) Nocodazole was added (15 μg/ml, final concentration) at the time of galactose addition. (Lane *1*) Asynchronous cultures; (lane *2*) cells arrested 150 min at the *cln*$^-$ block; (lane *3*) cells arrested 12 min after galactose addition. Subsequent lanes show additional 12-min intervals.

The initial rise in *cln2* RNA levels in this protocol has been ascribed to *CLN*-dependent positive regulation of *cln2* RNA (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991). The Swi4/Swi6 transcriptional regulators have been proposed as mediators of this regulation, acting through the cell cycle box (CCB) target sequences ACGAAA and C$\underline{C}$GAAA (Nasmyth and Dirick 1991; Ogas et al. 1991). Whereas *CLN1* and *CLN2* have such upstream sites, *CLB5* has none (except in the coding sequence; Fig. 2 legend). The *CLB5* transcript had a similar pattern of cell cycle regulation in both *cln1,2,3*$^-$ *GAL1::CLN3 swi4::URA3* and *SWI4*$^+$ strains (data not shown). The *swi4::URA3* allele abolishes CACGAAAA-binding activity and HO transcription (B.J. Andrews, pers. comm.). Thus, Swi4 is not essential for the periodic transcription of *CLB5*.

Yeast strains have a second class of genes whose transcripts fluctuate in a cell cycle periodic fashion and peak late in G_1. These genes are all involved in DNA replication and include *CDC21* (thymidylate synthase), *CDC9* (DNA ligase), *POL1* (DNA polymerase I), and others (for review, see Andrews and Herskowitz 1990; McKinney and Heintz 1991). All have a sequence motif ACGCGT called the *Mlu*I-containing cell cycle box (MCB) in their 5′-untranscribed region. The MCB is necessary and sufficient for the periodic, late G_1 pattern of expression of these genes (Gordon and Campbell 1991; Lowndes et al. 1991; McIntosh et al. 1991; Marini and Reed 1992). We examined the sequence of *CLB5* for the occurrence of this element and found five ACGCG sites in the 5′-untranslated region of the gene. Four are clustered (three on the sense strand and one on the antisense strand) in the region 407–339 nucleotides upstream of the presumed initiator ATG (Fig. 2); a fifth was found at −47. No complete *Mlu*I sites are present in *CLB5*; however, the sixth nucleotide of the *Mlu*I site may not be critical for MCB activity (McIntosh et al. 1991). The sixth nucleotide of each ACGCG site in *CLB5* is a C. Although we have not demonstrated the role of ACGCGC elements in *CLB5* transcription, it seems possible that MCB activity contributes to the cell cycle regulation of *CLB5* expression.

CLB5 *is required for efficient progression through S phase*

To determine the role of *CLB5* in cell cycle progression, we disrupted the gene, substituting the region between residues Y^{215} and E^{290} with the yeast *ARG4* gene (Fig. 1). The substitution removes most of the cyclin box. Strains carrying the deletion have a 20% increase in cell volume and a 10% increase in doubling time, compared with

isogenic wild-type strains. In addition, the proportion of unbudded cells in an exponential culture was significantly lower in strains lacking *CLB5* (Table 1). These observations suggested that strains lacking *CLB5* are delayed at one or more stages during the budded portion of the cell cycle.

To directly assess the effect of *clb5* deletion on cell cycle distribution, we performed flow cytometric DNA analysis on exponentially growing cultures of isogenic wild-type and *clb5* strains (Fig. 4, upper panels). Wild-type yeast strains display a characteristic pattern in which over half of the cells are in G_2, a smaller portion are in G_1, and only a small fraction are in S phase. In contrast, *clb5* strains exhibit a significant increase in the proportion of cells in S phase and a diminished fraction of cells in G_1. The increase in the S-phase fraction suggests that *clb5* strains progress more slowly through S phase than *CLB5*$^+$ strains. The reduced G_1 proportion could also be the result of a delay during progression through S phase, because a delay in S phase leads to larger sized daughter cells at the time of cell separation, reducing the requirement for growth (and time) in G_1 in the subsequent cycle (Singer and Johnston 1981; Johnston and Singer 1983; for review, see Cross et al. 1989). The decrease in the G_1 population caused by *clb5* deletion is likely to work by this indirect mechanism, rather than by directly accelerating the G_1/S transition, because *clb5* cells are larger than wild type. Mutations (such as the *DAF1-1* allele of *CLN3*; Cross 1988) that reduce the length of G_1 by accelerating the G_1/S transition are associated with a smaller cell size.

To directly test whether the loss of *CLB5* affects the duration of S phase, we examined the kinetics of S-phase transit in *CLB5* and *clb5* cultures synchronized by *CLN* block–release (as in Fig. 3). We obtained flow cytometric DNA profiles at intervals after releasing cells from the G_1 block. *CLB5* and *clb5* strains both budded and entered S phase simultaneously, ~36 min after release from the G_1 block (Fig. 5A,B). We detected no indication that *clb5* strains entered S phase behind wild-type controls. This suggested that the interval between START and the beginning of S phase was the same in *CLB5* and *clb5* strains. In *CLB5* strains, S phase lasted ~24 min, whereas in *clb5* strains, S phase lasted ~48 min. Synchronized cultures therefore confirm that loss of *CLB5* slows progression through S phase. We can distinguish between a slowed progression through S phase and a delayed beginning of S phase, because the *clb5* mutant culture showed DNA content between 1N and 2N (G1 and G2 DNA content, respectively) for a protracted period compared with the *CLB5* control. In addition, a similar result was obtained by using α-factor to synchronize *CLB5* and *clb5* *CLN1,2,3*$^+$ strains; hence, the *clb5* S-phase delay is not dependent on the *cln*-deficient, *GAL1::CLN3* background (data not shown).

The FACS data indicate that *clb5* strains are delayed during passage through S phase. We wished to confirm this finding by using an alternative method. We used α-factor and hydroxyurea to estimate the proportion of cells in a log phase population that lie between the α-factor and hydroxyurea execution points (Materials and methods). An execution point is defined as the point in the cell cycle when cells have completed the steps that make them sensitive to an agent, such as α-factor or hydroxyurea (Hartwell 1976). Once a cell has passed an execution point, it is insensitive to the corresponding agent for the remainder of the current cell cycle. Because S phase starts shortly after the α-factor execution point (Hereford and Hartwell 1974) and ends at about the hydroxyurea execution point (Hartwell 1976), the interval between these points approximates S phase.

In the *CLB5* wild-type strain, 20% of the cells in an exponential culture were between the α-factor and hydroxyurea execution points (Table 1). In contrast, in the *clb5*-deleted strain, 50% of the cells were in this interval. Given the doubling times of *CLB5* and *clb5* strains, these percentages allow estimation that S phase requires 17 min in a *CLB5* strain and 48 min in a *clb5* strain (Table 1). These results are in reasonable agreement with the length of S phase estimated from the FACS analysis of synchronous cell cycles (i.e., 24 and 48 min for *CLB5* and *clb5*, respectively; Fig. 5).

clb5 *deletion does not prolong* G_2

Because *CLB5* is a B cyclin, and typically B cyclins pro-

Table 1. *The* clb5 *mutation increases the amount of time between the α-factor and the hydroxyurea execution points*

	Percentage of cells before event (minutes after cell division that event occurs)					
	bud emergence	αF execution point	S-phase duration (min)	HU execution point	G_2 + M duration (min)	doubling time (min)
CLB5 CLB2	31% (24)	20% (15)	17	40% (32)	68	100
clb5 CLB2	19% (16)	9% (7)	48	59% (55)	55	110
CLB5 clb2	12% (9)	ND	ND	28% (22)	80	102
clb5 clb2	8% (7)	ND	ND	40% (36)	76	112

The data in Table 2 were analyzed as described in Materials and methods to yield an estimate of the percentage of cells in an asynchronous population that are before a cell cycle event and the time after cell division that the indicated event occurs. S-phase duration was estimated as hydroxyurea (HU) execution point (min) minus α-factor (αF) execution point (min). G_2 + M duration was estimated as doubling time (min) minus HU execution point (min). (ND) Not determined.

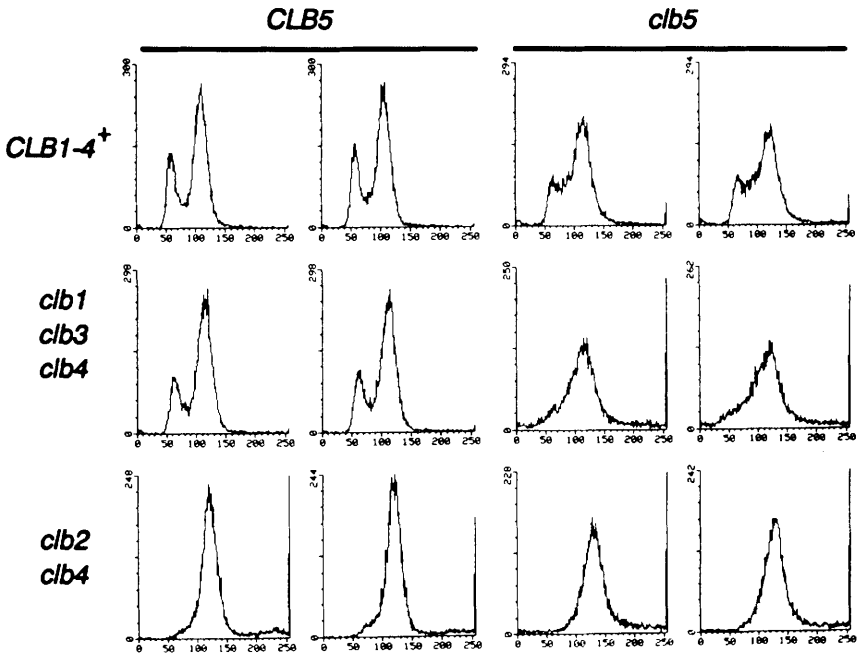

Figure 4. DNA flow cytometry of asynchronous cultures. Log-phase YEPD cultures of the indicated genotypes were sonicated, fixed, and stained with propidium iodide for flow cytometric analysis. For each genotype, two distinct spore clones were used to confirm that phenotypes observed are characteristic of the genotype. Histograms depict relative DNA content (*x* axis) vs. cell number (*y* axis).

mote G_2-to-M-phase progression, we examined whether *CLB5* deletion prolonged G_2. On the basis of FACS analysis of synchronized cells, we estimate that the *clb5* strain entered nuclear division ~12 min after completion of S phase, compared with ~18 min for *CLB5*. [Completion of S phase was inferred at the earliest time when all cells were in a well-defined G_2 peak (Fig. 5A), while the onset of nuclear division was inferred from the rapid increase in percent binucleate cells (Fig. 5C); see legend to Fig. 5.] Hence, there is no indication from these data that *clb5* deletion caused any delay during G_2.

A similar conclusion was reached from the determination of hydroxyurea execution points in exponential cultures. Assuming that G_2 starts at the hydroxyurea execution point, the length of G_2 + M (including cytokinesis) can be estimated from the data in Table 1 to be 68 min for *CLB5* and 55 min for *clb5* (see Table 1 footnote). Here, again, G_2 is possibly shorter, and certainly no longer, in strains lacking *CLB5*. Note that the G_2 + M estimate is substantially greater than the FACS-based G_2 estimate, because it includes the interval between nuclear division and cell separation. In contrast, a *clb2* strain was estimated to require 80 min for the completion of G_2 + M (Table 1). This increase in the proportion of the cell cycle after the hydroxyurea-sensitive step is presumably the result of G_2 delay (Surana et al. 1991). This is consistent with the view that *CLB2* has an important role in G_2, rather than in S phase; the major role of *CLB5* appears to be in S phase and not in G_2.

Interactions between clb5 *and other B-cyclin mutations*

We reasoned that if other B cyclins acted in concert with *CLB5* to promote S-phase transit, then strains bearing combinations of B-cyclin mutations might either have a more pronounced S-phase delay than *clb5* single mutants or be altogether unable to transit S phase and, consequently, inviable. We used tetrad analysis to generate all viable combinations of B-cyclin null mutations. The simultaneous deletions *clb1 clb2* and *clb2 clb3* are inviable, whereas genotypes *clb1 clb3 clb4* and *clb2 clb4* are viable (S. Reed and D. Lew, pers. comm.). We found that deletion of *CLB5* did not cause lethality in conjunction with any viable set of B-cyclin deletions.

We therefore determined the cell cycle distributions of viable combinations of B-cyclin null mutations. We found that exponential cultures of *clb1,3,4*$^-$ strains have a similar FACS profile to wild-type strains (Fig. 4; cf. top left and central left panels); however, their G_1 peak is slightly depressed. The deletion of these B cyclins does not cause a detectable S-phase delay; some delay in G_2 may be inferred from the diminution of the G_1 peak. In contrast, *clb1,3,4,5*$^-$ strains display a FACS profile suggestive of a combination of S-phase and G_2 delays (Fig. 4, central right panels). These data do not suggest that quadruply deleted strains are significantly more delayed in S phase than *clb5* single mutants, although some effect of *clb5* on G_2 delay, or of *clb1,3,4*$^-$ on S-phase delay, cannot be ruled out. Exponential cultures of *clb3,4,5*$^-$ strains appear similar by FACS to *clb1,3,4,5*$^-$ strains, indicating that the presence of *CLB1* does little to overcome the delays attributable to the simultaneous deletion of *CLB3*, *CLB4*, and *CLB5* (data not shown).

Deletion of *CLB2* alone (Surana et al. 1991), or *CLB2* and *CLB4*, leads to accumulation of cells in G_2 in a log-phase culture (Fig. 4, bottom left panels). The FACS profile of *clb2,4,5*$^-$ strains is also consistent with the accumulation of cells in G_2, but some S-phase delay attributable to the loss of *CLB5* function may still be apparent,

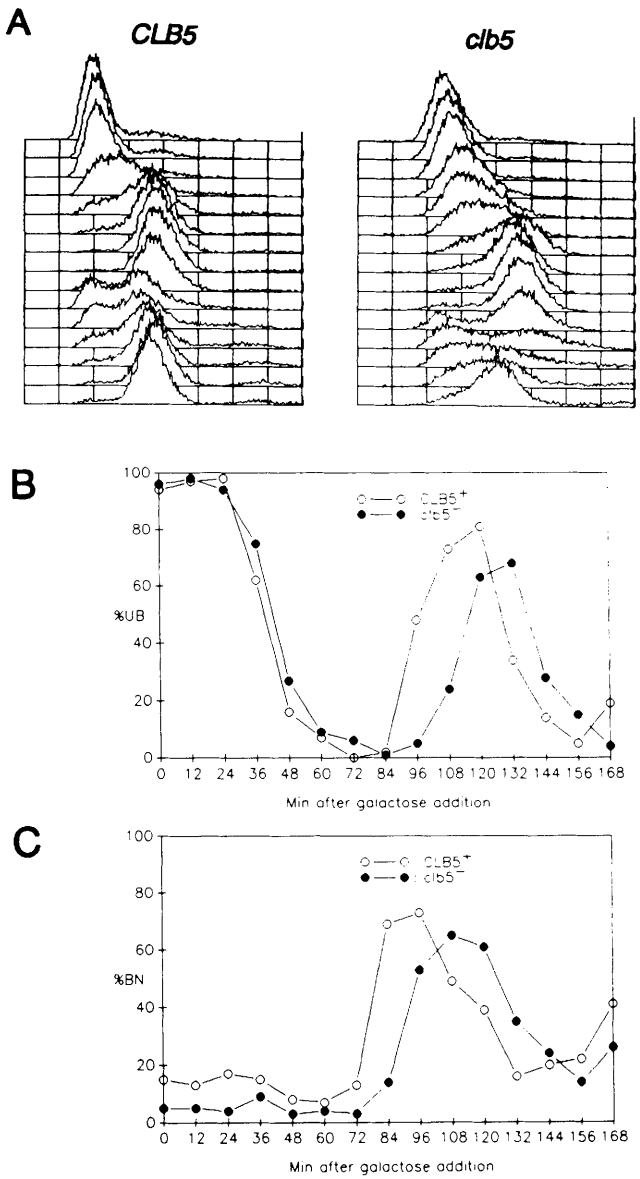

Figure 5. DNA flow cytometry of synchronized cultures following release from arrest in G_1. *cln1,2,3*$^-$-deficient strains were synchronized as in Fig. 3. Strains were either *CLB5* or *clb5::ARG4*, as indicated. Initial samples were taken after 150-min arrest, and subsequent samples were taken at 12-min intervals following the addition of galactose. (*A*) DNA content histograms. The top curve represents the arrested culture; subsequent curves are arranged below. S phase is complete 60 min after release in *CLB5* cells but not until 84 min after release in *clb5* cells. (*B*) Percent unbudded cells. (*C*) Percent binucleate cells. Nuclear division occurs ~78 min after release in *CLB5* cells and ~96 min after release in *clb5* cells.

because the G_2 peak in the latter mutant is shorter and broader (Fig. 4, bottom right panels). To further examine the possible interaction of *CLB5* and *CLB2*, we determined where in the cell cycle the HU execution point occurs in double mutant strains (Table 1, line 4). We found that in a *clb2* background, the loss of *CLB5* increases the interval between bud emergence and the HU execution point from 13 to 29 min, whereas the interval between the HU execution point and cell division remains virtually unchanged (80 vs. 76 min). With bud emergence as a rough morphological marker of START (Pringle and Hartwell 1981), this result is consistent with our conclusion from DNA flow cytometry (Fig. 4, bottom) that loss of *CLB5* prolongs S phase in a *clb2* background while having no incremental effect on the G_2 delay attributable to the loss of *CLB2*.

Is CLB5 *functionally redundant with the* CLN *genes?*

Because we discovered *CLB5* on the basis of its ability to rescue *cln1,2,3*$^-$ yeast when overexpressed and then found that its transcript is present at times in the cell cycle coinciding with those of *CLN2* (and *CLN1*), we were curious to determine whether it could be demonstrated to have *CLN*-like functions. We tested all combinations of *cln* deletions with the *CLB5* deletion and found that *CLB5* was not essential in strains bearing any viable combination of *cln* deletions (Fig. 6, left panels). However, when cells were tested on YEP–glycerol media, *cln1 cln2 clb5* strains were nearly inviable, whereas *cln1 cln2 CLB5* strains were fully viable (Fig. 6, right panels). This observation is suggestive of partial functional redundancy between *CLB5* and *CLN* genes: *cln1,2*$^-$ strains rely on both *CLN3* and *CLB5* for growth on glycerol media. Deletion of *CLB2* in the *cln1,2*$^-$

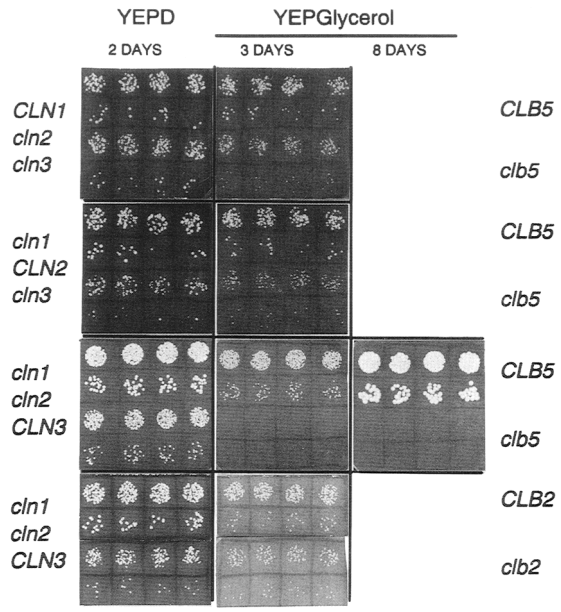

Figure 6. *cln1 cln2 clb5* strains are nearly inviable on glycerol media. Strains of the indicated genotypes were serially diluted and plated at two densities on YEP media supplemented with either 2% glucose (YEPD) or 3% glycerol. Photographs were taken after growth for the indicated number of days. *cln1 cln2 clb5* strains formed detectable colonies on YEP–glycerol after ~1 week of growth, with a plating efficiency of ~5% (glycerol/glucose).

background did not impair viability on glycerol (Fig. 6, bottom panels). Diploids made by mating *cln1 cln2 clb5* strains to a ρ^0 (mitochondrial deficient) *cln3* strain were viable on glycerol media; hence, the glycerol lethality of *cln1 cln2 clb5* strains is not the result of a defect in their mitochondrial DNA.

Leopold and O'Farrell (1991) reported that overexpression of the *D. melanogaster cdc2* gene could rescue *cln1,2,3*$^-$ yeast strains. They speculated that the *Drosophila Cdc2* protein was interacting with an unidentified cyclin. We have found that rescue of *cln1,2,3*$^-$ yeast by *Drosophila cdc2* is dependent on *CLB5*. *cln1,2,3*$^-$ *clb5 GAL1::CLN3* yeast strains transformed with the *Drosophila cdc2* plasmid are inviable on glucose, yet isogenic *CLB5* transformants are viable (data not shown). This result further supports the idea that *CLB5* function may overlap with *CLN* gene functions.

Discussion

CLB5 *functions to promote S phase*

We report the discovery of a fifth B cyclin in budding yeast. Although this gene is not essential, its deletion significantly depresses the rate at which cells replicate chromosomal DNA. DNA flow cytometry of both exponential and synchronized cultures shows that *clb5* strains are slowed during progression through S phase. Also, we have shown that the proportion of the cell cycle falling between the α-factor and hydroxyurea execution points expands in *clb5* strains. It is plausible to infer that the α-factor execution point approximates the start of S phase (Hereford and Hartwell 1974) and the hydroxyurea execution point marks the completion of S phase, in both *clb5* and wild-type strains (Hartwell 1976). Hence, both experimental approaches indicate that *CLB5* functions to promote S-phase transit. *CLB5* is the only yeast cyclin whose deletion causes prolonged S phase. Deletion of other B cyclins either prolongs G_2 (*CLB2*) or has little effect on cell cycle distribution (*CLB1*, *CLB3*, and *CLB4*; Fig. 4), whereas deletion of any two *CLN* genes does not cause an S-phase delay detectable by DNA flow cytometry (data not shown). Deletion of the B-cyclin *cig1*$^+$ in *S. pombe* causes a delay during G_1 or in the G_1/S transition but does not affect the rate of S-phase progression (Bueno et al. 1991).

The loss of *CLB5* seems to affect the overall rate of progression through S phase. In contrast, other known cyclins promote transitions between discrete phases of the cell cycle, such as the START event (*CLN*s; Richardson et al. 1989), the G_2/M transition (Murray and Kirschner 1989; Nurse 1990; Hartwell 1991), and meiotic induction (Westendorf et al. 1989). However, *CLB5* may not act throughout S phase. An effect on the rate of S-phase progression could be caused by a defect in initiation (e.g., owing to the to use of a smaller number of origins of replication). Moreover, we note the possibility that *CLB5* affects the efficiency of S-phase transit by acting at a time other than S phase, for example, during G_1, or G_2 of the preceding cycle.

We considered that slow S-phase transit in *clb5* strains might result from defective regulation of the genes involved in DNA replication and expressed under the control of the DSC1-binding activity and the MCB (Lowndes et al. 1991; for review, see McKinney and Heintz 1991). In the synchronous time course shown in Figure 5, we examined the regulation of *CDC21* (an MCB gene). *CDC21* RNA accumulation peaked with similar levels and kinetics in *CLB5* and *clb5* strains (data not shown). We also did not detect a significant defect in histone H2A transcription in *clb5* strains. H2A is an S-phase-transcribed gene not under MCB control (for review, see Osley 1991). In light of these results, it seems unlikely that the basis for the prolonged S phase of *clb5* strains is a defect in the transcriptional control of gene products involved in DNA synthesis. Consistent with this conclusion is the observation that the *clb5* deletion fails to affect DSC1 DNA-binding activity (L. Johnston, pers. comm.).

In human cells there is some evidence for the involvement of a cyclin-dependent kinase in DNA synthesis (Blow and Nurse 1990; D'Urso et al. 1990; Furakawa et al. 1990; Fang and Newport 1991). In higher eukaryotes it appears that *cdc2* has multiple homologs [called cdks (Tsai et al. 1991)]. Distinct kinases may be involved in S-phase and M-phase promotion (Fang and Newport 1991). Multiple cyclin/cdk complexes are present during S phase, and at least three of them (cyclin A/cdc2, cyclin A/cdk2, and cyclin E/cdk2) are catalytically active at this time (Pines and Hunter 1990b; J. Roberts, pers. comm). No A-type or E-type cyclins are known in budding yeast. Perhaps *CLB5* plays a role in yeast similar to the role played by cyclin A or cyclin E in human cells. In addition, it remains to be tested whether *CLB5* activates Cdc28 kinase.

Does CLB5 *function elsewhere in the cell cycle?*

Although *CLB5* was discovered on the basis of *cln1,2,3*$^-$ rescue, we cannot be sure whether *CLB5* functions in concert with *CLN*s in their role in START. The fact that the *CLB5* transcript appears and disappears in synchrony with the *CLN2* transcript, together with the fact that extra copies of the *CLB5* gene under the control of the natural promoter rescue *cln1,2,3*$^-$ strains, suggests that the Clb5 protein may be present early enough to have a role in START. The fact that strains lacking *CLN1* and *CLN2* require *CLB5* for viability on glycerol suggests that *CLB5*, like *CLN3*, can promote *CLN*-dependent events. A similar conclusion is implied by the fact that *cln1,2,3*$^-$ strains require *CLB5* for rescue by the *D. melanogaster cdc2* gene.

Does *CLB5* have an additional role in promoting the G_2/M-phase transition? The data presented (Figs. 4 and 5; Table 1) do not suggest any delay during G_2 in *clb5* mutants. However, the presence of a potential mitotic destruction box (Glotzer et al. 1991) in Clb5 suggests that the protein may persist until M phase, consistent with a possible role in G_2. Finally, *CLB5* appears to be required for the normal progression through meiosis (C.

Epstein and F. Cross, unpubl.), although we have not determined at which meiotic stage *CLB5* becomes essential.

Materials and methods

Strains and media

All strains were isogenic with BF264-15D (*trp1-1a leu2-3,112 ura3 ade1 his2*). A nonreverting null allele of *ARG4* was installed in the BF264-15D background with pmlc12Pst*ARG4*Bgl-Fill[Bam*URA3*] (Sun et al. 1989). The *cln1* and *cln3* deletions were as described (Cross and Tinkelenberg 1991), whereas the *cln2* deletion was made by a similar procedure to the *cln2* deletion in that reference, except that it extended between the *Sph*I site at −504 and the *Spe*I site at amino acid 162 of the *CLN2*-coding sequence. This new *cln2* allele was demonstrated to be null for *CLN* function as described (Cross and Tinkelenberg 1991). Strains bearing null alleles of *CLB1* (Ghiara et al. 1991), *CLB2*, *CLB3*, and *CLB4* (unpubl.) were kindly supplied by S. Reed and D. Lew (Scripps Research Inst., La Jolla, CA; unpubl.). The *swi4::URA3* allele consisted of a duplication of an internal *Bam*HI fragment of *SWI4*, with the *URA3* between the duplicated sequences. This construct was provided by B. Andrews (University of Toronto, Canada) and was introduced into the BF264-15D background by J. McKinney (Rockefeller University, New York). The structures of all disruptions were confirmed by Southern blotting. Hydroxyurea (Sigma) was used in solid media at 0.2 M. Nocodazole (Sigma) was used in liquid media at 15 μg/ml, from a dimethylsulfoxide (DMSO) stock at 10 mg/ml. Standard techniques were employed for strain constructions (Sherman et al. 1989).

Construction of CEN plasmid library

We cloned *CLB5* fortuitously while attempting to clone an unlinked dominant mutation, the *CLN* bypass mutation (*CBM8*), whose phenotypes include cell division and colony formation in a *cln1,2,3*$^-$ background. A full description of these mutations is in preparation. Briefly, *CBM8* was isolated as a spontaneously occurring mutation, which conferred viability on a strain whose genotype was *cln1 cln2 cln3* [p*GAL1::CLN3/URA3*]. After the original isolation and backcross of *CBM8*, it was maintained in the presence of *GAL1::CLN3*, avoiding selection of further modifiers of viability of the *cln1,2,3*$^-$ condition.

We sought to clone *CBM8* by making a library in a yeast CEN plasmid, from genomic DNA isolated from strain YCE933-1C (*cln1 CLN2 cln3 CBM8*$^{4th\ Backcross}$). We then isolated plasmids from this library that conferred viability on *cln1,2,3*$^-$ strains. We included *CLN2* in the library as a positive control. We made our library in a novel yeast cloning vector, pCE101, which was made from pRS314 (Sikorski and Heiter 1989) by substituting a pBR322 ORI for the existing pUC ORI.

Yeast YCE933-1C was inoculated into 1 liter of YEPD and cultured to an OD of 1.15. Yeast DNA was then isolated essentially as described in Holm et al. (1986). DNA was 1/16 underdigested with *Sau*3A, phenol extracted, RNase treated, and precipitated with polyethylene glycol (PEG) to eliminate residual RNA. The pellet was recovered and run over a 10–30% glycerol gradient for 5 hr at 40,000 rpm in an SW40 rotor. The gradient was fractionated, and a fraction was retained having a modal DNA fragment length of ~12 kb.

pCE101 was cut with *Bam*HI, treated with calf intestinal phosphatase, and combined, at a concentration of 3 μg/ml, with the glycerol gradient-fractionated yeast DNA. The mixture was ligated overnight at 15°C with T4 DNA ligase and transformed into *Escherichia coli* strain DH5α. Bacterial colonies were rinsed off of the Luria broth (LB)/ampicillin plates and pooled from 50 independent transformations and platings of the ligated DNA, cultured for 2 hr with an added 100 ml of LB (50 μg/ml of ampicillin), and plasmid DNA was prepared by the alkaline lysis method.

Isolation of CLB5 *gene from library*

Library DNA was transformed into yeast strain YCE960-3C (*cln1 cln2 cln3* [p*GAL1::CLN3/URA3*], and transformants were selected on YcGal-trp. Once colonies had grown up, replicas were taken to YEPD agar. Secondary YEPD replicas were taken from the primary replicas after 24 hr of growth. These were allowed to grow for 2–3 days, although [p*CLN2/TRP1*] clones formed robust patches after 1 day of growth. Candidate clones were picked from the secondary YEPD replicas, and the colony was purified on YcGal-trp-ura. Growth on YEPD was demonstrated to be dependent on inheritance of a *TRP1/CEN* plasmid based on cosegregation, following plasmid loss during nonselective growth. Plasmids were recovered from 1.5-ml overnight YEPD cultures, as described (Ausubel et al. 1987).

Overall, from two independent libraries made from the same glycerol gradient fraction, we recovered eight distinct clones of *CLN2* (a total of 42 times), three distinct clones of *CLB5* (a total of 3 times), and a single clone bearing *MPK1*, found previously to suppress *cln1,2,3*$^-$ lethality in a high-copy plasmid (O. Fields and J. Thorner; B. Futcher; both pers. comm.). Clones of *CLN2* were identified on the basis of hybridization to a radiolabeled *CLN2* probe. The *CLB5* and *MPK1* genes were subcloned into the integrating vector RS304, yielding pCE105 and pCE117, respectively. These were each linearized at a unique site in the insert and transformed into diploid YCE957 (*cln1/cln1 cln2/cln2 cln3/cln3 CBM8* [p*GAL1::CLN3/URA3*]. Transformants were recovered on YcGal-trp, sporulated, and dissected. Southern blots confirmed that the RS304 derivatives had integrated by homologous recombination at the *CLB5* and *MPK1* loci, as expected. *CBM8* was not linked to either *CLB5* or *MPK1*; hence, our *CLB5* and *MPK1* clones represent wild-type DNA.

Plasmids, subcloning, and sequencing

pCE104 was one of the *CLB5* plasmids isolated from our CEN plasmid library. pCE111 and pCE112 were made by subcloning the pCE104 entire insert and *Xho*I–*Cla*I fragments, respectively, into pRS314 (Sikorski and Hieter 1989). pCE111 Δ*Cla*I and pCE112 Δ*Spe*I were derived from their respective parent plasmids by limit digestion and recircularization. pCE110 was another of the *CLB5* plasmids isolated from the library. Restriction mapping, sequencing, and hybridization experiments revealed that it contained the *CLB2* gene, as well as the *CLB5* gene. pCE110 Δ*Nsi*I, lacking the *CLB5* gene, was derived from pCE110.

To make a null allele of *CLB5*, we partially digested CE111 Δ*Cla*I with *Eco*RI and ligated it to *Eco*RI-digested pMLC28Pst6-*ARG4* (Sun et al. 1989). The ligation mixture was transformed into *E. coli*, and transformants were selected on LB, 50 μg/ml of ampicillin, and 34 μg/ml of chloramphenicol. Several of these transformants were restriction mapped, and one was selected in which the *ARG4* plasmid had been cloned into the *Eco*RI site at residues 290/291 of *CLB5*, with the orientation 5′*CLB5* → camR → *ARG4* → 3′*CLB5*. This was partially digested with *Bsp*EI, and a fragment lacking the *Bsp*EI fragment spanning residues 216–291 of *CLB5*, plus part of the camR gene, was gel purified and recircularized with T4 DNA ligase. The resulting plasmid, *clb5::ARG4* Δ*Bsp*EI, was digested with *Xho*I and *Spe*I and transformed into *arg4* strains. Integration was demon-

strated to be at the *CLB5* locus by Southern blotting. p*clb5::ARG4/CEN/TRP1* was also transformed into *cln1,2,3*$^-$ yeast as an intact CEN plasmid and shown to have no activity at *cln1,2,3*$^-$ rescue.

Dideoxy sequencing with Sequenase (U.S. Biochemical) was performed according to the manufacturer's instructions. Double-stranded DNA was sequenced on both strands, by use of exonuclease III deletion derivatives of pCE111 and pCE112, restriction enzyme deletion derivatives, and custom synthesized primers.

clb1 clb2 *rescue by pCEN/*CLB2

Diploid YCE997 (*clb1::URA3/CLB1 clb2::LEU2/CLB2*) was transformed with pCE110, pCE112, and pCE110 Δ*Nsi*I (Fig. 1). From each transformation, a colony was picked, cultured in YcD–trp liquid, sporulated, and dissected. Replicas were taken from the dissection agar to score *clb1::URA3*, *clb2::LEU2*, and the *TRP1*/CEN plasmid. From 997[pCE110] haploid progeny strains, 5 Ura$^+$ Leu$^+$ Trp$^+$ colonies were found among 22 Trp$^+$ spores (23%). From 997[pCE110 Δ*Nsi*I], 2 Ura$^+$ Leu$^+$ Trp$^+$ colonies were found among 7 Trp$^+$ spores (29%). From 997[pCE112], no Ura$^+$ Leu$^+$ Trp$^+$ colonies were found among 33 Trp$^+$ spores, although 44% of the viable progeny were Trp$^-$, 37% were Ura$^+$, and 28% were Leu$^+$. If pCE112 rescued *clb1 clb2* mutants, then one-fourth of Trp$^+$ spores (i.e., 8.25) should be Ura$^+$ Leu$^+$, given the independent assortment of *CLB1* and *CLB2*. The finding that none is significant ($X^2 = 11.00$, $P < 0.005$).

Determination of α-factor and hydroxyurea execution points

The arrest morphology owing to α-factor treatment is a large unbudded cell (Pringle and Hartwell 1981). Therefore, a cell past the α-factor execution point at the time of plating on α-factor-containing solid medium will divide, and each of the new cells will arrest without budding, resulting in an adjacent pair of large unbudded cells. A cell before the α-factor execution point will arrest as a single, large unbudded cell.

The arrest morphology owing to hydroxyurea treatment is a large budded cell (Pringle and Hartwell 1981). Therefore, a cell past the hydroxyurea execution point at the time of plating on hydroxyurea-containing solid medium will divide, and each of the new cells will bud and arrest, resulting in an adjacent pair of large budded cells. A cell before the hydroxyurea execution point will arrest as a single large budded cell.

Exponentially growing cultures of YCE1029-12B (*MAT***a** *bar1 CLB5*) or YCE1029-10B (*MAT***a** *bar1 clb5::ARG4*) in YEPD medium were sonicated and plated on YEPD, YEPD + 10^{-8} M α-factor, or YEPD + 0.2 M hydroxyurea (Hartwell 1976). After 4-hr (3.5 hr in one experiment) incubation at 30°C, the plates were examined microscopically. By the end of the incubation on the control YEPD plate, >90% of the cells had divided and budded again at least once, so we did not consider viability to be a significant concern in the analysis. Although ideally all cells on the hydroxyurea plate should be either two or four cells plus buds per microcolony (Hartwell 1976), ~10% of the cells were found in the one, three, and five cells plus buds per microcolony categories. The one-cell category was considered to be before the hydroxyurea execution point; the three- and five-cell categories were considered to be past the execution point. Similarly for α-factor, ideally all cells should be one or two cells per microcolony; we observed ~10–15% of cells in the three-, four-, or five-cell categories. These were considered to be past the α-factor execution point in the analysis. The percentage of budded cells was determined at the time of plating. A total of 100 cells each were scored for two independent cultures in three (hydroxyurea execution point) or two (α-factor execution point) separate experiments, and the data were pooled (Table 2). To calculate the proportion of the total cell cycle time at which various events occurred from the percentage of cells in the population past those events, we employed the age-distribution function (Mitchison 1971) $Ex(F) = 1 - \ln(2 - F)/\ln 2$, where F is the fraction of cells before the execution point, and $Ex(F)$ is the point in the cell cycle at which the execution point occurs (in units of cell cycle times, from 0 to 1). The time from cell division to the execution point in minutes was calculated by multiplying $Ex(F)$ by the doubling time of the strain. The hydroxyurea execution point was determined similarly for a *clb2::LEU2* strain.

Northern blotting

Northern blotting was performed as described in Cross and Tinkelenberg (1991). *cln1,2,3*$^-$ *leu2::LEU2::GAL1::CLN3* cells were synchronized by incubating in YEP–3% raffinose for 150

Table 2. *Cell division and budding upon plating wild-type and* clb5 *yeast on solid media containing inhibitors*

Strain	Time (hr)	Medium[a]	Cells + buds per microcolony (S.E.M.)					Number of experiments forming mean
			1	2	3	4	>4	
CLB5 CLB2	0		31 (3)	67 (3)	2 (1)	2 (0)	0 (0)	6
	4	αF	20 (3)	65 (4)	6 (2)	7 (2)	2 (2)	4
	4	HU	3 (0)	33 (2)	4 (2)	58 (3)	2 (1)	6
clb5 CLB2	0		19 (2)	76 (2)	3 (1)	2 (1)	0 (0)	6
	4	αF	8 (0)	79 (2)	6 (0)	6 (2)	1 (0)	4
	4	HU	2 (1)	56 (3)	2 (1)	36 (2)	5 (1)	6
CLB5 clb2	0		12 (2)	86 (3)	1 (1)	1 (1)	0 (0)	4
	4	HU	0 (0)	25 (2)	3 (1)	70 (1)	2 (1)	4
clb5 clb2	0		8 (3)	89 (2)	2 (1)	2 (2)	0 (0)	4
	4	HU	1 (2)	35 (7)	4 (1)	57 (6)	3 (1)	4

The number of cells + buds formed by each cell plated was determined by microscopic examination of cells plated on appropriate media (see Materials and methods). For each condition in each experiment, 100 cells were scored. The means and standard errors (S.E.M.) of the data are shown, and the number of replicate experiments used to calculate the mean is indicated.
[a](αF) α-Factor; (HU) hydroxyurea.

min. Galactose was added to 3% to start the cycle. Nocodazole was used at 15 μg/ml. DNA fragments used as probes were as follows: *CLB5*, the 462-bp *Eco*RI fragment; *CLB2*, the 767-bp *Bgl*II–*Cla*I fragment; *CLB4*, the 500-bp *Spe*I–*Ssp*I fragment, from a clone kindly supplied by B. Futcher (Cold Spring Harbor Lab, New York); *CLN2* and *TCM1*, as described (Cross and Tinkelenberg 1991).

Determination of cell cycle parameters

Flow cytometric DNA quantitation was determined as follows: Cells were stained with propidium iodide and prepared for FACS analysis by use of a Becton Dickinson FACScan, as described (Lew et al. 1992). In most experiments, 10,000 cells were examined. Events were either live-gated to have a DNA fluorescence above background or gated after acquisition on the basis of forward and side scatter, to exclude cell debris. In the former case (Fig. 5), all 10,000 events are plotted in the DNA content histograms; in the latter (Fig. 4), up to ~20% of the events are discarded. The profiles were affected very little by the gating method chosen. When the latter method was used, the *y* axes were adjusted to compensate for the fact that different numbers of events are represented on different histograms. A constant proportion of the cell population corresponds to a constant area in the histogram, regardless of the gating method used. For FACS analysis of synchronized cells, *cln1,2,3*$^-$- deficient cells were synchronized exactly as in Northern blots, or by treating a *bar1 MAT***a** strain with 10^{-7} M α-factor (Sigma) for 120 min.

Doubling times were determined from log-phase cultures. OD_{660} readings were taken at intervals, log transformed, and regressed versus time. Electronic cell volumes were determined as described (Cross 1988).

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