**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 G₂, 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 G₁/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 G₁ (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. 1991; 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 **cdc2** having a G₂/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 G₂ 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 G₁/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 G₁/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 homologos 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 G₁ arrest, before START [Cross 1990]. The genes for human cyclins C, D, and E [Koff et al. 1991; Lew et al. 1991; Matsushima 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 G1/S or G2/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 Lipman 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. G1 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

\begin{figure}
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\caption{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 BspEI and EcoRI sites was replaced with the yeast ARG4 gene to generate the clb5::ARG4 allele. [ND] Not determined.}
\end{figure}
begins at residue 41 or 56, depending on the true initiator methionine. (BI Alignment of Clb5 with other yeast B cyclins. The Clb5 conserved in at least 90% of the 14 B cyclins compared. For those residues, we placed an asterisk under the Clb5 sequence where it fails to conform to sequence conserved in most B cyclins (as determined by use of the CLUSTAL program within GENES & DEVELOPMENT 1697. The Clb5 sequence is underlined at residues where it corresponds to sequence conserved in most B cyclins (as determined by use of the CLUSTAL program within GENES & DEVELOPMENT 1697. The Clb5 sequence is underlined at residues where it fails to conform to sequence conserved in most B cyclins (as determined by use of the CLUSTAL program within GENES & DEVELOPMENT 1697.

Figure 2. [A] CLB5 is a B cyclin. [DNA] The minimal fragment having activity on a CEN plasmid at clin-1.23 rescue [CE12] (spe) is the region between the indicated XhoI and EcoRI sites. The DNA between the indicated BspEI and EcoRI 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 (CCGCCG and its inverse complement) resembling the MCB are underlined in the S’ region of the gene. No CCB motifs [CAC-GAAA, 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 CACGAAA sequences have all been in the S’ nontranscribed regions [Nasmyth 1985; Nasmyth and Dirick 1991; Ogas et al. 1991]. Sequence from – 1037 to – 386 (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 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 G₂ 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.

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 CCGAAA [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 G₁. These genes are all involved in DNA replication and include CDC21 (thymidylate synthase), CDC29 (DNA ligase), POL1 (DNA polymerase II), and others [for review, see Andrews and Herskowitz 1990, McKinney and Heintz 1991]. All have a sequence motif AGCGG called the Mül-containing cell cycle box (MCB) in their S'-untranslated region. The MCB is necessary and sufficient for the periodic, late G₁ 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 AGCGG 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 Mül sites are present in CLB5, however, the sixth nucleotide of the Mül site may not be critical for MCB activity [McIntosh et al. 1991]. The sixth nucleotide of each AGCGG site in CLB5 is a C. Although we have not demonstrated the role of AGCGG elements in CLB5 transcription, it seems possible that MCB activity contributes to the cell cycle regulation of CLB5 expression.

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] 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.

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 Y215 and E290 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 G1, a smaller portion are in G2, 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 G1. The increase in the S-phase fraction suggests that clb5 strains progress more slowly through S phase than CLB5+ strains. The reduced G1 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 G1 in the subsequent cycle (Singh and Johnston 1981; Johnston and Singer 1983; for review, see Cross et al. 1989). The decrease in the G1 population caused by clb5 deletion is likely to work by this indirect mechanism, rather than by directly accelerating the G1/S transition, because clb5 cells are larger than wild type. Mutations such as the DAFI-1 allele of CLN3; Cross 1988) that reduce the length of G1 by accelerating the G1/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 G1 block. CLB5 and clb5 strains both budded and entered S phase simultaneously, ~36 min after release from the G1 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).

### 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 | α-factor execution point | S-phase duration | HU execution point | G2 + M duration | doubling time |
|----------------|------------------------|------------------|------------------|----------------|---------------|
| 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]. G2 + M duration was estimated as doubling time [min] minus HU execution point [min]. (ND) Not determined.
mote G2-to-M-phase progression, we examined whether CLB5 deletion prolonged G2. 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 G2 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 G2.

A similar conclusion was reached from the determination of hydroxyurea execution points in exponential cultures. Assuming that G1 starts at the hydroxyurea execution point, the length of G2 + 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, G2 is possibly shorter, and certainly no longer, in strains lacking CLB5. Note that the G2 + M estimate is substantially greater than the FACS-based G2 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 G2 + M (Table 1). This increase in the proportion of the cell cycle after the hydroxyurea-sensitive step is presumably the result of G2 delay (Surana et al. 1991). This is consistent with the view that CLB2 has an important role in G2, rather than in S phase; the major role of CLB5 appears to be in S phase and not in G2.

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 clb3 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 G2 peak is slightly depressed. The deletion of these B cyclins does not cause a detectable S-phase delay; some delay in G2 may be inferred from the diminution of the G1 peak. In contrast, clb1,3,4,5− strains display a FACS profile suggestive of a combination of S-phase and G2 delays (Fig. 4, central right panels). These data do not suggest that quadrupliy deleted strains are significantly more delayed in S phase than clb5 single mutants, although some effect of clb5 on G2 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 G2 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 G2, but some S-phase delay attributable to the loss of CLB5 function may still be apparent,
A B cyclin with a role in yeast S phase

Figure 5. DNA flow cytometry of synchronized cultures following release from arrest in G1. 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 G2 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 G2 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−
background did not impair viability on glycerol [Fig. 6, bottom panels]. Diploids made by mating cln1 cln2 clb5 strains to a ρ0 [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 G1 [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 G1, or in the G1/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 [CLNs; Richardson et al. 1989], the G2/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 G1, or G2 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 CLNs 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 G2/M-phase transition? The data presented [Figs. 4 and 5; Table 1] do not suggest any delay during G2 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 G2. 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 pmle12Pst-ARG4-BglII[F(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 Spel site at 504 and the SpeI 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 (Ghara 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 swit4::URA3 allele consisted of a duplication of an internal BamHI fragment of SW14, 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 0.2 M. Nocodazole [Sigma] was used in liquid media at 15 μg/ml, from a dimethylsulfoxide (DMSO) stock at 50 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 [pGAL1::CLN3/P3]. 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). 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 Hieter 1989). pCE110 and pCE112 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. pCE101 ΔNLS, lacking the CLB5 gene, was derived from pCE110.

To make a null allele of CLB5, we partially digested CE111 ΔNLS with EcoRI and ligated it to EcoRI-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 EcoRI site at residues 290-291 of CLB5, with the orientation 5′ CLB5 → camR → ARG4 → 3′ CLB5. This was partially digested with BspEI, and a fragment lacking the BspE1 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, CB5-ARG4ΔBspEI, was digested with XhoI and SpeI and transformed into arg4 strains. Integration was demon-
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strated to be at the CLB5 locus by Southern blotting. pclb5::
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. Dou-
ble-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.

cbl1 clb2 rescue by pCEN/CLB2

Diploid YCE997 (clb1::URA3/CLB1 clb2::LEU2/CLB2) was
transformed with pCE110, pCE112, and pCE110 ΔNsiI (Fig. 1).
From each transformation, a colony was picked, cultured in
Yed-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 ΔNsiI], 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 Tip + spores (i.e., 8.25) should
be Ura + Leu +, given the independent assortment of
CLB1 and CLB2. The finding that none is significant [X² = 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 an α-factor-
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 (MATa barl
CLB5) or YCE1029-10B (MATa barl clb5::ARG4) in YEPD me-
dium 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 cate-
gories were considered to be past the execution point. Similarly
for α-factor, ideally all cells should be one or two cells per mi-
crocolony; we observed ~10–15% of cells in the three, four, or
five-cell categories. These were considered to be past the α-fac-
tor 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 (hydrox-
yurea execution point) or two (α-factor execution point) sepa-
rates experiments, and the data were pooled (Table 2). To calcu-
late 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)/ln2, 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 divi-
sion to the execution point in minutes was calculated by mul-
tiplying 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::GALI::CLN3 cells
were synchronized by incubating in YEP-3% raffinose for 150
tables.

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

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

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.

αF α-factor; (HU) hydroxyurea.

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m. 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 EcoRI fragment; CLB2, the 767-bp BglII–Cul fragment; CLB4, the 500-bp SpeI–SspI 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 - cells deficient for cells were synchronized exactly as in Northern blots, or by treating a bar1 MATa strain with 10^{-7} M α-factor (Sigma) for 120 min.

Doubling times were determined from log-phase cultures. OD_{600} readings were taken at intervals, log transformed, and regressed versus time. Electronic cell volumes were determined as described (Cross 1988).

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