CLB5 and *CLB6*, a new pair of B cyclins involved in DNA replication in *Saccharomyces cerevisiae*

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The functions of the Cdc28 protein kinase in DNA replication and mitosis in *Saccharomyces cerevisiae* are thought to be determined by the type of cyclin subunit with which it is associated. G₁-specific cyclins encoded by *CLN1*, *CLN2*, and *CLN3* are required for entry into the cell cycle (Start) and thereby for S phase, whereas G₂-specific B-type cyclins encoded by *CLB1*, *CLB2*, *CLB3*, and *CLB4* are required for mitosis. We describe a new family of B-type cyclin genes, *CLB5* and *CLB6*, whose transcripts appear in late G₁ along with those of *CLN1*, *CLN2*, and many genes required for DNA replication. Deletion of *CLB6* has little or no effect, but deletion of *CLB5* greatly extends S phase, and deleting both genes prevents the timely initiation of DNA replication. Transcription of *CLB5* and *CLB6* is normally dependent on Cln activity, but ectopic *CLB5* expression allows cells to proliferate in the absence of Cln cyclins. Thus, the kinase activity associated with Clb5/6 and not with Cln cyclins may be responsible for S-phase entry. Clb5 also has a function, along with Clb3 and Clb4, in the formation of mitotic spindles. Our observation that *CLB5* is involved in the initiation of both S phase and mitosis suggests that a single primordial B-type cyclin might have been sufficient for regulating the cell cycle of the common ancestor of many, if not all, eukaryotes.

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DNA replication and mitosis occur at discrete stages during the cell cycle of almost all proliferating eukaryotic cells. The mechanisms that determine the timing and order of S phase and mitosis are therefore of fundamental interest. In fungi, where the genetic analysis of cell cycle control is most advanced, a single protein kinase, encoded by *CDC28* in the budding yeast *Saccharomyces cerevisiae* (Pringle and Hartwell 1981; Hartwell 1991) and by *cdc2* in the fission yeast *Schizosaccharomyces pombe* (Nurse and Bisset 1981; Nurse 1990), has a pivotal role in both events. In mammals, these different functions are most likely fulfilled by a family of closely related kinases (Giordano et al. 1989; Fang and Newport 1991; Pines and Hunter 1991). The *cdc2*/Cdc28 kinase is active only when complexed with an unstable cyclin protein (Evans et al. 1983). Many different types of cyclins exist, and it is thought that the successive association of *cdc2*/Cdc28 with different cyclins gives rise to different forms of the kinase, some of which are concerned with S phase and others with mitosis (for review, see Lew and Reed 1992).

The specialization of different cyclins for particular stages of the cell cycle has been well characterized in *S. cerevisiae*. Three so-called G₁ cyclins encoded by *CLN1*,

CLN2, and *CLN3* are concerned with the commitment of cells in late G₁ to entering the cell cycle (known as Start) and thereby, directly or indirectly, with S-phase entry (Sudbery et al. 1980; Cross 1988; Nash et al. 1988; Hadwiger et al. 1989). Deletion of all three *CLN* genes causes cells to arrest in G₁ prior to Start, but deletion of any pair of these genes merely delays Start (Richardson et al. 1989; Cross 1990). Four B-type cyclins encoded by *CLB1*, *CLB2*, *CLB3*, and *CLB4* are concerned with the assembly and maintenance of the mitotic spindle. *CLB1* and *CLB2* encode very similar proteins whose transcripts appear transiently in G₂, whereas *CLB3* and *CLB4* encode a different pair of related proteins whose transcripts appear earlier in the cell cycle, though still later than *CLN1* and *CLN2*. Deletion of *CLB2* alone delays mitosis considerably, whereas mutants lacking *CLB1*, *CLB3*, and *CLB4* enter mitosis with almost normal kinetics. Deleting all four *CLB* genes causes cells to arrest in G₂ with duplicated spindle pole bodies that cannot separate (Surana et al. 1991; Fitch et al. 1992; Richardson et al. 1992).

The notion that different classes of cyclins have cell cycle stage-specific roles may also apply to the mammalian cell cycle. In addition to B-type cyclins, mammals have E-type cyclins that appear in late G₁ well before the onset of S phase (Koff et al. 1991), D-type cyclins that appear in G₁ but are also expressed at other stages (Lew

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et al. 1991; Xiong et al. 1991), and A-type cyclins that appear around S phase and may be necessary for DNA replication (Girard et al. 1991; Pagano et al. 1992). Two themes emerge from the comparison of yeast and mammalian cyclin families. First, the primary sequences of G₁ or S phase-specific cyclins are much less conserved than the B-type class involved in mitosis; for example, E-type cyclins from mammals do not particularly resemble Cln cyclins from yeast, whereas B-type cyclins involved in mitosis in yeast and animals are quite similar. Second, mammals, in addition to having G₁- and G₂-specific cyclins may also have a class (the A-type) specifically concerned with S phase.

Transcriptional controls play an important part in the process by which a G₁ yeast cell becomes committed to the cell cycle and enters S phase. The onset of *CLN1* and *CLN2* transcription may determine the timing of Start (Tyers et al. 1993). *CLN1* and *CLN2* are members of a family of genes, including the *HO* endonuclease gene and the *HCS26* cyclin-like gene, that are regulated by the SBF transcription factor (Breedon and Nasmyth 1987; Nasmyth and Dirick 1991; Ogas et al. 1991). SBF (SCB-binding factor) is composed of a DNA-binding protein encoded by *SWI4* and a regulatory protein encoded by *SWI6* (Andrews and Herskowitz 1989; Taba et al. 1991; Primig et al. 1992). Its activity is dependent on the Cdc28 protein kinase. A positive feedback loop, involving cyclin autoactivation, that is triggered when cells achieve a particular size may be responsible for the sudden activation of *CLN1/2* transcription in late G₁ (Cross and Tinklenberg 1991; Dirick and Nasmyth 1991).

A large number of genes involved in DNA replication in yeast are also activated at Start (for review, see Johnston and Lowndes 1992) by a second Start-dependent transcription factor called MBF (MCB-binding factor; or DSC1), which contains a 120-kD DNA-binding protein (p120) and Swi6 (Dirick et al. 1992; Lowndes et al. 1992). The physiological significance of the transcriptional program governed by MBF is less clear than that conferred by SBF, because most of the genes involved encode stable enzymes whose periodic activation is not essential for S-phase progression, that is, product inherited from the mother cell suffices for several cell cycles (Byers and Sowder 1980). Nevertheless, MBF might also activate genes encoding unstable proteins, whose transcription in late G₁ is required for S phase.

While this study was in progress, Epstein and Cross (1992) described a fifth B-type cyclin (*CLB5*) that was able to rescue the growth defect of a *cln1 cln2 cln3* triple mutant. This gene is expressed in late G₁ and is necessary for normal S-phase transit. Our study confirms these results, provides new information on *CLB5*, and augments our knowledge by the discovery of a novel B-type cyclin gene (*CLB6*). *CLB5* and *CLB6* encode a pair of related cyclins having a specific role in S-phase entry. Deletion of *CLB6* alone has little or no phenotype, deletion of *CLB5* causes a lengthening of S phase, and deletion of both genes delays entry into S phase relative to bud emergence and *CLN2* activation. The transcription of *CLB5* and *CLB6* appears to be activated by MBF in late

G₁ and is normally dependent on *CLN* gene activity. Expression of *CLB5* from the *GAL* promoter bypasses this requirement and allows mutants lacking all three *CLN* genes to enter S phase and proliferate. In the absence of *CLB3* and *CLB4*, *CLB5* also becomes essential for the formation of mitotic spindles. The involvement of a B-type cyclin in S phase as well as mitosis and the fact that this class of cyclins is the most conserved in evolution raise the possibility that the ancestor of fungi and animals may have used a single B-type cyclin to drive the cell cycle oscillation.

Results

A new family of B-type cyclins

In the course of sequencing a suppressor of the *cdc28-1N* mutation (Surana et al. 1991), we discovered that a gene neighboring *CLB2* encodes a novel B-type cyclin. The same gene (called *CLB5*) has been isolated independently by Epstein and Cross (1992) as a result of its ability to suppress the growth defect of a *cln1 cln2 cln3* triple mutant. *CLB5* and *CLB2* are transcribed convergently. Because *CLB1* and *CLB2* encode proteins that are 62% identical and could therefore have arisen by a recent duplication, we sequenced the DNA downstream of *CLB1*. The adjacent gene also encodes a B-type cyclin, which we call *CLB6*.

CLB6 codes for a protein of 380 amino acids (44.1 kD) and shares 49.7% identical residues with Clb5 (435 amino acids, 50.4 kD). Domains homologous to the cyclin box are found in the carboxy-terminal halves of Clb5 and Clb6. They contain 104 (Clb5) and 100 (Clb6) residues in common with the 118 amino acids that are conserved in yeast B-type cyclins (Fig. 1a), including the FLRRISK sequence diagnostic of B-type cyclins (O'Farrell and Leopold 1991). Multiple sequence alignment shows that Clb5 and Clb6 form a new subgroup within the B-type class, as do the Clb1/2 and Clb3/4 cyclins from *S. cerevisiae* (Fig. 1b). Clb5 is distinguished from Clb6 by the presence of a "mitotic destruction box" implicated in proteolysis (Glutzer et al. 1991) and a highly charged 24-amino-acid insertion in its amino-terminal half (Fig. 1a).

Cell cycle control of *CLB5* and *CLB6* transcripts

To assess whether these new cyclins are concerned with early or late aspects of the cell cycle, we analyzed their pattern of expression as cells are released from a G₁ arrest. Cells lacking both *CLN1* and *CLN2* and expressing *CLN3* from the *GAL1-10* promoter proliferate when galactose is present in the medium, arrest uniformly in G₁ prior to Start within 2 hr of its removal, and resume cell division synchronously upon its restoration (Cross 1990). *CLB5* and *CLB6* transcripts are absent in the G₁-arrested cells, reappear within 10 min of galactose addition, and thereafter oscillate with a periodicity that is similar to that of *HO* transcripts (Fig. 2). By this criterion, *CLB5* and *CLB6* regulation resembles that of the

a

Clb5	1	mgenhdhegsikrNsmiynenERqlcnsNlkilqnkrAl...skndSSskq	48
Clb6	1	mNcipsisERkiqinNedcigkenAfhtipresSinl	38
Clb5	49	qvqdsckprrrLtdVpVnnplsQnkRivagskaaKvrReenirpivsavq	98
Clb6	39	tpbstnekkvLseVnsNkidlslQlpR.....gKlqRds...thlektr	78
Clb5	99	KRQiyNDRTaeeqeeeeeegeddddaasivnkrrrIdaegVseIv...gWq	146
Clb6	79	KRQlsNDsTdp.....IepktVkkIkchqWk	104
Clb5	147	dLDyvekDDTaMVAEYSaeIFafLYrrEletLPshNYLlDktSkYyLrpS	196
Clb6	105	nLDsiEmDDpfMVAEYtdsIFshLYekEiqmLPtHNYLmDtqSpYhLksS	154
Clb cons.		-LD-----D--MV-EY--DIF-YL--LE-----P---Y-----	
Clb5	197	MRTiLvDWLVEVHEKfQCyPETLFLsINLmDRFLakNkVtmNKLQLLavT	246
Clb6	155	MRallLiDWLVEVHEKfHCiPETLFLaINLlDRFLsqNvVklNKLQLLciT	204
Clb cons.		-R--LVDW-V-VH--F-LLPETL-L-IN--DRFL---V-LNKLQLVG--	
Clb5	247	sLFIAaKFEEVnLPKlaeyAYiTDGAaskndIknAEmFmLtSLefnIgwP	296
Clb6	205	cLFIAcKFEEVnLPKitnlAYvTDGAatvegIrkaELFvlsSLgyNislP	254
Clb cons.		-LFIA-KYEE---P-I--F-Y--DG-Y---DI--AE-F-L--LEF---WP	
Clb5	297	NPLNFIRRIISKADdYdpvnRNigKFIleYayCChqFIHLpPstvsAMaMY	346
Clb6	255	NPLNFIRRIISKADnYcietRNmaKFImEYsiCCnkFIHLkPSylaAMsMY	304
Clb cons.		-PM-FLIRRIISKAD-YD---R-LAK-LLE---D--FI---PS-AA-A--	
Clb5	347	IARmtnrNkNelWngTlqHYSGGIDpnhDeAFqslcidLVkdIAsskTh	396
Clb6	305	IARki...kNeNskWdeTfiHYSGGIDiesDpAFkdfiseLVeDIAvpdTn	352
Clb cons.		L-R--LG-----W---V-YS-GY-----L-----E-----	
Clb5	397	LDSLILKYKKPryGsVYFqtFkWCtsemhsnfqnlfnlk*	436
Clb6	353	LDSLrLKYKKPkHgmVYFkvFdWCkqkr*	381
Clb cons.		H-----KY-----S-----W-----	

b

Figure 1. Clb5 and Clb6 form a new family of B-type cyclins. (a) Clb5 and Clb6 were aligned using the Gap subroutine of UWGCG. A putative mitotic destruction box of Clb5 is underlined. The consensus sequence (Clb cons.) consists of the common residues in at least six of eight prealigned cyclins from *S. cerevisiae* (Clb1 to Clb6) and *S. pombe* (cdc13 and cig1). Identical residues between Clb5 and Clb6 are in uppercase. (b) Dendrogram of *S. cerevisiae* cyclins clustered by the Pileup program of the UWGCG package (v. 7.2, 1992; Genetics Computer Group 1991). Length of horizontal lines indicates evolutionary distance.

G₁-specific cyclin genes *CLN1* and *CLN2*, appearing only transiently during the cell cycle when cells undergo Start (Cross and Tinkelenberg 1991). A similar cell cycle fluctuation of *CLB5* transcripts is seen in synchronous cultures obtained either by α -factor arrest (Epstein and Cross 1992) or by centrifugal elutriation; *CLB5* RNAs appear shortly before S phase and disappear soon after its completion (see Fig. 6d, below).

CLB5 may be regulated by MBF

The consensus-binding sequences for the Start-specific transcription factors SBF and MBF are CACGAAA (the SCB; Nasmyth 1985b) and ACGCGTNA (the MCB; Johnston and Lowndes 1992), respectively. *CLB5* promoter sequences contain no perfect matches to either sequence but do contain a cluster of four sequences with a single mismatch to the MCB in the interval between -330 and -407 (base pairs upstream of the AUG codon). To determine whether *CLB5* is regulated by SBF or MBF, we analyzed *CLB5* transcript levels in *swi4* and *swi6* single mutants as well as in *swi4 swi6* double mutants (Nasmyth and Dirick 1991). Whereas *CLN1*, *CLN2*, and *HCS26* transcripts, which are regulated by SBF, are greatly reduced in *swi4* and *swi4 swi6* double mutants (Ogas et al. 1991; Moll et al. 1992), there is little change in the level of *CLB5* transcripts (Fig. 3a). Genes regulated by MCBs are still transcribed in *swi6* mutants but are no longer cell cycle regulated (Dirick et al. 1992; Lowndes et al. 1992). Figure 3, b and c, shows that *CLB5* transcripts also have this property. The lack of any depen-

dence of *CLB5* transcription on *SWI4* and the dependence of its regulation on *SWI6* indicate that *CLB5* may be regulated by MBF and not by SBF.

To test whether MBF can bind to the potential MCB sequences within the *CLB5* promoter region, we incubated an end-labeled PCR fragment containing the four MCB-related sequences (DNA between -316 and -422) with a crude yeast extract and analyzed complex formation using a gel-retardation assay. We detect a very slow migrating protein/DNA complex (MBF, Fig. 3d), which is competed by an oligonucleotide containing two MCBs from the *TMP1* promoter (lane 6) but not by an equivalent oligonucleotide in which both MCBs are mutated

Figure 2. Cell cycle-dependent transcription of *CLB5* and *CLB6*. Strain K2762 (*cln1 cln2 cln3::GAL-CLN3*) was arrested in G₁ for 2.5 hr in YEPRaff and released synchronously (at time = 0 min) by the addition of 2% galactose; samples were taken every 10 min, and RNA was isolated. The same blot was hybridized with *CLB5*, *CLB6*, *HO*, *SWI5*, and *CDC15* (as loading control) probes, as indicated.

Figure 3. *CLB5* transcription is regulated by MBF. (a) Steady-state level of *CLB5* transcript is not affected in *swi4* or *swi6* mutants. Northern analysis from exponentially growing wild type (K2832), *swi4*Δ (K2833), *swi6*Δ (K2831) and *swi4*Δ*swi6*Δ (K2392) containing a rescuing *SpADH-CLN2* allele. (b) *SWI6* is required for proper regulation of *CLB5* transcription during the cell cycle. Northern analysis of RNA from congenic strains K2771 (WT) and K2786 (*swi6*) arrested for 2.5 hr in YEPRaff (R150) by Cln depletion and released synchronously by addition of 2% galactose. (*CDC28*), Loading control; (*SWI5*) synchrony control. (c) Quantification of the above using a PhosphorImager

(Molecular Dynamics); (●) K2771 (wild type); (○) K2786 (*swi6::TRP1*). (d) MBF binds to MCBs within the *CLB5* promoter. Gel-retardation assays were performed using whole-cell extracts from wild-type (lanes 2,4,6–10), *swi6* (K1354, lane 11), and *swi4* (R/H1071, lane 12) strains or p120-enriched heparin fraction (lane 5) and a radiolabeled 110-bp PCR fragment containing four MCB motifs from the *CLB5* promoter (lanes 3–12); an oligonucleotide from the *TMP1* promoter was used as control (*TMP1*, lanes 1,2). Competition was observed with a 50-fold molar excess of cold *TMP1* (lane 6) or *CLB5* (lane 8) but not with a mutant *TMP1* oligonucleotide (lane 7; Dirick et al. 1992). Antibody shift experiments were performed with anti-Swi4 (α4, lane 9) and anti-Swi6 (α6, lane 10) antibodies (1 : 20 dilution of sera). (F.P.) Free probe.

(lane 7). The complex is unaltered by adding anti-Swi4 antibodies (lane 9) but is prevented from entering the gel by anti-Swi6 antibodies (lane 10). Extracts from *swi4* mutants (lane 12) still form the complex, but ones from *swi6* mutants cannot (lane 11). Thus, the complex is most likely the result of the binding of MBF, and not SBF, to MCB sequences within the promoter fragment. The above experiments concern *CLB5*, but because the *CLB6* promoter contains two perfect and one imperfect matches to the MCB consensus (between -440 and -370), we expect it to be similarly regulated by MBF.

CLB5 is not essential but important for S-phase progression

Dissection of asci derived from a diploid in which one

CLB5 copy had been replaced by the *URA3* gene yielded four viable spores. *CLB5* is therefore not essential. Strains lacking *CLB5* grow slightly slower than congenic wild-type strains, and their cells are larger (~50%; data not shown). Analysis of the DNA content of individual cells by DNA flow cytometry (FACS) indicates that progression through S phase is retarded in *clb5* mutants, confirming the results of Epstein and Cross (1992). Most wild-type cells have a 2N DNA content, ~25% have a 1N content, and only a minority have intermediate amounts. In *clb5* mutants there are fewer (unbudded) cells with 1N content and many more with intermediate DNA levels, suggesting that many of the cells are delayed in S phase (Fig. 4a). We presume that the decrease in the number of unbudded and G₁ cells in *clb5* mutant cultures is the result of their greater size at division

caused by the delay in S phase. A more rigorous method of assessing S-phase progression is to analyze synchronous cultures prepared by centrifugal elutriation. Wild-type G_1 daughter cells isolated by this means are very small and do not bud nor start DNA replication until after 135 min of incubation but then complete S phase within 30 min (Fig. 4b). *clb5* daughters are born much larger and start budding after only 70 min. DNA replication starts soon afterward but lasts much longer (60 min). A similar lengthening of S phase was seen using cultures synchronized by pheromone (data not shown) or by Cln block-release [Epstein and Cross 1992].

CLB5 and CLB6 are required for initiation of S phase

Deletion of *CLB6* has little or no effect on growth rate, cell size (not shown), or on the distribution of cellular DNA contents in asynchronous cultures (Fig. 4a). Furthermore, G_1 daughter cells of *clb6* mutants isolated by

elutriation resemble wild-type cells in that they enter S phase at the time of bud emergence and complete it within 30–40 min (Fig. 4b). To investigate whether *CLB5* and *CLB6* might have functions that overlap, we analyzed the effect of deleting both genes. *clb5 clb6* double mutant cells are viable but, like *clb5* mutant cells, are large and grow somewhat slower than wild-type cells. Normally, budding and DNA replication are simultaneous processes. It is striking that asynchronous cultures of the double mutant have many more cells with a 1N DNA content even though they have fewer unbudded cells than wild type (Fig. 4a), indicating that deletion of both *CLB5* and *CLB6* delays the onset of S phase relative to bud emergence. This was analyzed directly using a synchronous culture prepared by elutriation. Whereas the onset of S phase, budding, and *CLN2* transcription are roughly simultaneous in wild-type cells, S phase starts 30 min later (at 105 min) than the peak of *CLN2* transcription and budding (75 min) in double mutant cells (Fig. 4b), supporting the above hypothesis. It is also

Figure 4. Phenotype of *clb5*, *clb6* and *clb5 clb6* null mutants. (a) DNA flow cytometric analysis of asynchronous cultures of wild type (K699), *clb6*Δ(K3414), *clb5*Δ(ESY143), and *clb5*Δ *clb6*Δ (K3415) grown to early log-phase in YEPD at 30°C and stained with propidium iodide. Histograms indicate relative DNA content (x-axis) vs. cell number (y-axis). Percent of unbudded cells in these cultures was determined by light microscopy. (b) DNA flow cytometry of cultures synchronized by centrifugal elutriation and grown in YEPD at 30°C. Samples were taken at the indicated time points and processed for DNA content measurement (FACS), Northern analysis, and microscopy. The peak of *CLN2* transcription was determined by Northern analysis and indicated by an arrow aside the budding index.

noticeable that both budding and *CLN2* transcription started earlier in *clb5* and *clb5 clb6* mutants than in wild type. We presume that the delay in S phase leads to daughter cells larger than wild type that consequently need to spend less time growing to the size required for the G₁ cyclin activation. The FACS data on both asynchronous and synchronous cultures indicate that the duration of S phase may be similar in wild type, *clb6* single mutants, and *clb5 clb6* double mutants (~30 min); that is, the lengthening of S phase (to 60 min) that results from deleting *CLB5* is suppressed by deleting *CLB6*. There are two types of explanation for this surprising result: Either *CLB6* is responsible for delaying S-phase progression in *clb5* mutants or the slow progression through S phase in *clb5* mutants can be suppressed by cells starting S phase later in the cell cycle, as will be the case in *clb5 clb6* double mutants.

CLB5 and CLB6 are essential for S phase entry in the absence of CLN1 and CLN2

Deletion of *CLB5* and *CLB6* delays S phase but does not prevent it. Presumably, other cyclins are also able to trigger S phase but only when cells grow larger than normal. There may be additional members of the *CLB5/6* cyclin family that could substitute for *CLB5* and *CLB6*. Alternatively, the function of known cyclin genes could overlap with that of *CLB5/6*; for example, members of the *CLN* family might cooperate with them in starting S phase, and other members of the *CLB* family might help in completing it. We therefore investigated the phenotype of mutating *CLB5* and *CLB6* in combination with other cyclin mutations. We first addressed the phenotype of the *cln1 cln2 clb5* triple mutant. The following were our reasons for choosing this combination. The viability of *cln1 cln2* double mutants depends on the integrity of *SWI6* even though *CLN3* transcription is *SWI6*-independent. It has been suggested that *CLN3* cannot initiate the cell cycle without the help of another factor whose expression is *SWI6*-dependent (Nasmyth and Dirick 1991). *Clb5* and/or *Clb6* could be such a factor because although *SWI6* is not essential for *CLB5/6* transcription, it is necessary for their high-level expression in late G₁ (see Fig. 3b). We therefore constructed a strain deleted for *CLN1*, *CLN2*, and *CLB5* carrying a conditional *GAL-CLB5* allele (see Materials and methods). This strain grows normally in galactose medium, but only very poorly, and not at all at 37°C in glucose medium; cells become large and elongated but do not accumulate at one particular stage of the cell cycle [data not shown]. Similarly, Epstein and Cross (1992) found *cln1 cln2 clb5* mutants to be inviable on glycerol medium. We then deleted *CLB6* in this strain and found that unlike *cln1 cln2* and *clb5 cln6* double mutants, *cln1 cln2 clb5 clb6* cells arrest as large cells with unreplicated DNA upon transfer to glucose medium (Fig. 5b). Many of the quadruple mutant cells fail to bud, whereas others form abnormal buds (Fig. 5a). Thus, the *CLB5/6* gene pair becomes essential for S-phase entry in the absence of *CLN1/2*.

CLB5 expression can trigger S phase in the absence of Cln cyclins

The dependence of *CLB5/6* expression on *Cln* cyclins (see Fig. 2) raises the possibility that the failure of *cln* triple mutants to initiate S phase might be the result of their failure to activate *CLB5* and *CLB6* transcription. To test this notion, we asked whether *CLN*-independent expression of *CLB5* would be sufficient to reverse the lethality of the *cln1 cln2 cln3* triple mutant. We constructed a *cln1 cln2 cln3* strain carrying an unstable *SpADH-CLN2/ADE3* plasmid that confers a uniform red colony color (Kranz and Holm 1990; F. Cvrcková and K. Nasmyth, in prep.) and integrated the *CLB5* gene under the control of the *GAL* promoter. Incubation on galactose medium allowed the loss of the *ADE3* plasmid and caused the formation of white sector colonies. Upon restreaking, cells from the white sectors gave rise to pure white colonies that contain no *CLN* genes (data not shown). We conclude that expression of *CLB5* from the *GAL* promoter bypasses the normal requirement for *CLN* function. The ability of *CLB5* (with its own promoter on a centromeric plasmid) to rescue a *cln1 cln2 cln3* mutant was described previously (Epstein and Cross 1992), although it is not clear to us how this gene was expressed in *Cln*-arrested cells (see Fig. 2).

We then compared the kinetics of S-phase entry in *cln1 cln2 cln3* triple mutants kept alive by *GAL-CLB5* with cells kept alive by *GAL-CLN3* (Fig. 6a). Removal of galactose from cells growing previously in the presence of raffinose and galactose causes G₁ arrest after 3 hr. Restoration of galactose caused both strains to initiate DNA replication within 30 min and to complete it within 75 min. The kinetics of S-phase entry were slightly different. Whereas almost all cells expressing *CLB5* had entered S phase by 30 min, half of the cells expressing *CLN3* were still in G₁ and had not yet entered S phase. *CLB5* may therefore be more effective than *CLN3* in triggering the initiation of DNA replication, at least under these somewhat artificial conditions. We also compared the level of transcripts activated by SBF and MBF in the two cultures to see whether *CLB5* can trigger late G₁-specific transcription in the absence of *CLN* genes. We found that *HCS26* (regulated by SBF) and *RNR1* (regulated by MBF) were rapidly activated following galactose addition in both cultures (Fig. 6b). Whereas *CLB5* caused a more rapid entry into S phase, *CLN3* caused a more rapid transcriptional activation. Both *HCS26* and *RNR1* were activated 10 min earlier by *CLN3* than by *CLB5*. The activation of *CLB5* and *CLB6* transcription by *Cln* cyclins in normal cells could therefore contribute to the full activation of both SBF- and MBF-regulated genes, that is, *CLB5/6* could sustain the G₁ positive feedback loop.

To determine whether *CLB5* expression is also sufficient to induce premature DNA replication in small daughter cells (i.e., another situation in which *CLN1* and *CLN2* transcripts are absent), a wild-type strain containing *GAL-CLB5* was grown in raffinose medium and small unbudded cells were isolated by centrifugal elutri-

Figure 5. *cln1Δ cln2Δ clb5Δ clb6Δ* quadruple mutants arrest in G₁. The quadruple mutant containing *GAL-CLB5* (K3424) was grown to early log phase in YEPRaffGal, filtered, and resuspended in YEPD at 30°C. (a) DIC photomicrographs of wild-type cells (left) or *cln1 cln2 clb5 clb6 GAL-CLB5* cells (right) after 3 hr in YEPD medium. (b) DNA flow cytometry of *cln1 cln2* (K2008, left) and K3424 (right) at different time points after the shift in YEPD.

ation. Half of the cells were incubated further in raffinose, the other half in the presence of both raffinose and galactose. *CLB5* transcripts are rapidly induced in the presence of galactose, but this barely advances *CLN1* transcription and does not cause premature DNA replication (Fig. 6c,d). This result contrasts with the ability of *CLB5* to induce S phase in triple *cln* mutants and suggests that there is an important difference between wild-type early G₁ daughter cells and cells arrested in G₁ as a result of a *Cln* deficiency. Neither population has *CLN1* or *CLN2* transcripts, but daughters probably have *CLN3* transcripts and protein. It seems unlikely, however, that *Cln3* would inhibit S-phase entry. More probably, *CLB5* expression can only trigger S phase once cells have achieved a certain size, which is attained during the G₁ arrest of triple *cln* mutants.

To determine whether the dependence of *CLB5* expression on *Cln* activity might be important for regulating Start, we compared, using a halo assay, the α -factor sensitivity of *MATa bar1* cells expressing *CLN1*, *CLB2*, and *CLB5* from the *GAL* promoter. Neither *CLN1* nor

CLB2 expression caused pheromone resistance, but *CLB5* expression allowed cells to proliferate in the presence of low and high concentrations of α -factor (Fig. 7). Thus, unlike the case for *Cln1* and *Cln2* (Valdivieso et al. 1993), pheromones might not be able to regulate *Clb5* activity at a post-transcriptional level.

CLB5 is required for spindle formation in the absence of CLB3 and CLB4

We then investigated whether *CLB5* might also have overlapping functions with the *CLB3/4* gene pair, whose transcripts appear somewhat after *CLB5/6* but appreciably earlier than *CLB1/2*. Tetrad analysis of a cross between a *clb5* mutant and a *clb3 clb4* double mutant showed that all double mutant combinations are viable but that *clb3 clb4 clb5* triple mutants are dead. The triple mutant spores germinated but arrested as large budded cells. This case of synthetic lethality is not simply the result of combining mutations that each cause sick-

Figure 6. Expression of *CLB5* from the *GAL1-10* promoter rescues a triple *cln1Δ cln2Δ cln3Δ* mutant and triggers entry into S phase. (a) DNA flow cytometry of *cln1Δ cln2Δ cln3Δ* cells rescued either by *GAL-CLN3* (K2762) or *GAL-CLB5* (K3111). Cells were grown to mid-log phase in YEPRaffGal, arrested for 3 hr in YEPRaff; galactose was then added back (time = 0) and samples were taken every 15 min. (b) *CLB5* can activate the G_1 -positive feedback loop in a *cln1Δ, 2Δ, 3Δ* mutant. Northern blot analyses of the above samples were probed with *HCS26* (regulated by SBF), *RNR1* (regulated by MBF), and *CDC15* (as loading control). Activation of both *HCS26* and *RNR1* transcription is triggered by *CLB5* in the absence of G_1 cyclins. (c) *CLB5* is not sufficient for premature entry into S phase in early G_1 cells. Small unbudded *GAL-CLB5* cells (K3072) were isolated by centrifugal elutriation and resuspended either in YEPRaff (left) or YEPRaffGal (right) at 30°C. (d) Northern analysis of the corresponding samples. Ectopic *CLB5* expression in early G_1 cells does not trigger premature *CLN1* transcription.

Figure 7. Cells expressing *CLB5* from the *GAL1-10* promoter are resistant to pheromone-induced G_1 arrest. Filter disks containing 10 μ g (left), 1 μ g (right), or 0.1 μ g (bottom) of α -factor were spotted onto soft agar (0.8% agarose) containing 2.5×10^5 *MATa bar1* cells of the following strains: (1) Wild type (K1534) on YEPD, (2) *GAL-CLB5* (K3072) on YEPD, (3) *GAL-CLB5* (K3072) on YEPGal, (4) *YCp/GAL-CLN1* (ESY51) on YEPGal, (5) *GAL-CLB2* (ESY52) on YEPGal. Halo formation was observed after 24–36 hr incubation at 30°C.

ness. Deletion of *CLB2* causes a strong phenotype, but this mutation is readily combined with a *clb5* deletion (data not shown). In contrast, *clb3 clb4* double mutants have no discernible phenotype, and yet this combination is lethal with a *clb5* deletion.

To characterize the phenotype of *clb3 clb4 clb5* triple mutant cells, we constructed a triple mutant strain kept alive by *CLB5* expression from the *GAL* promoter. These cells proliferate somewhat slower than *clb3 clb4* cells and accumulate in G_2 , either because S phase is accelerated or because mitosis is delayed by *CLB5* overexpression. There is a transient increase in the fraction of G_1 cells with the shift to glucose medium. Nevertheless, all cells eventually arrest with a G_2 DNA content (see Fig. 8c, left panel). To better analyze the phenotype of these cells, we prepared a population of mainly unbudded G_1 cells by centrifugal elutriation of a culture recently shifted to glucose medium. All cells budded and replicated their DNA (Fig. 8b) after 210 min continued incubation in glucose medium, but they failed to make mitotic spindles (Fig. 8a). The G_2 arrest of *clb3 clb4 clb5* triple mutants is therefore similar to that of *clb1 clb2 clb3 clb4* quadruple mutants, which arrest with dupli-

Figure 8. *clb3Δ clb4Δ clb5Δ* triple mutants arrest in G_2 without mitotic spindle. Strain K3131 (containing *GAL-CLB5*) was grown to mid-log phase in YEPRaff + 0.5% Gal at 23°C, incubated for 1 hr in YEPD to enrich the G_1 population that was then isolated by centrifugal elutriation in YEPRaff. The elutriated cells (80% unbudded) were resuspended in YEPD at 30°C, and samples were taken every 30 min. (a) In situ immunofluorescence of K3131 cells growing in YEPRaffGal (Gal) or after 4.5 hr in YEPD (Gluc.); (left) DIC photomicrographs, (middle) DAPI staining, (right) antitubulin staining. (b) DNA flow cytometry of elutriated K3131 cells at various time points after shift in YEPD. (c) Deletion of *CLB6* in a *clb3Δ clb4Δ clb5Δ* background delays entry into S phase. DNA flow cytometry of asynchronous K3131 (left) and *clb3Δ clb4Δ clb5Δ clb6Δ GAL-CLB5* (K3418) cultures. Cells were grown to early log phase in YEPRaff + 0.5% Gal at 30°C, filtered, and then incubated in YEPD at 30°C.

cated but unseparated spindle pole bodies (Fitch et al. 1992), though the state of the spindle pole body in the triple mutant remains uncharacterized. The mitotic defect of the *clb3 clb4 clb5* triple mutant is unlikely to be the result of partial inactivation of *CLB2* during the process of disrupting *CLB5*, because its growth defect is

completely reversed by a centromeric plasmid carrying *CLB5* but is not affected by a multicopy plasmid carrying *CLB2* (data not shown). Furthermore, the *clb3 clb4 clb5 GAL-CLB5* strain still produces almost wild-type levels of Clb2-associated kinase activity (data not shown). We conclude that *CLB3* and *CLB4*, whose transcripts appear

in S phase, share with *CLB5* a function that is essential for mitosis.

These data do not, however, exclude the possibility that *CLB3* and *CLB4* also have a minor role in S phase. We find that many of the arrested *clb3 clb4 clb5 clb6* cells have unreplicated DNA (Fig. 8c, right), suggesting that DNA replication is less efficient than in the *clb5 clb6* mutants (see Fig. 4b). Thus, the initiation of DNA replication in *clb5 clb6* mutants not only depends on *CLN1* and *CLN2* but also on *CLB3* and *CLB4*.

Discussion

A new family of B-type cyclins in S. cerevisiae

Previous analyses suggested that Cln cyclins (Cln1–Cln3) regulate Start and entry into S phase, whereas B-type Clb cyclins (Clb1–Clb4) are responsible for the formation and function of the mitotic spindle. The discovery of a new pair of B-type cyclins, Clb5 and Clb6, shows this picture to have been an oversimplification. Our data suggest that Clb5 and Clb6 have functions that overlap with both Cln and Clb1–Clb4 cyclins. Clb5, for example, has roles in the initiation of both DNA replication and mitosis.

We discovered *CLB5* and *CLB6* as a result of their chromosomal locations, downstream of *CLB2* and *CLB1*, respectively. Linkage of related genes is rare in yeast and may therefore serve some function in this case. This cannot be common transcriptional control because the genes are transcribed convergently and at very different stages of the cell cycle. Linkage disequilibrium could help to maintain the compatibility of cyclins that must cooperate in the formation of the mitotic spindle. The proteins encoded by *CLB5* and *CLB6* form a closely related pair (49.7% identity over the entire sequence) as do those encoded by *CLB1* and *CLB2* (62% identity) (see Fig. 1b). Hence, the *CLB1/6* and *CLB2/5* loci probably arose by gene duplication. However, they no longer perform equivalent functions. The *CLB2/5* locus seems more important for vegetatively growing cells, in that mutations in either gene have marked phenotypes, whereas deletion of *CLB1* or *CLB6* has little effect. The *CLB1/6* locus may be more important for meiosis (Grandin and Reed 1993).

CLB5 and CLB6 are important for entry into S phase

Transcripts from *CLB5* and *CLB6* are tightly cell cycle regulated. Their accumulation in late G₁ (around the time that cells undergo Start) and decline in G₂ resembles the fluctuation of *CLN1* and *CLN2* transcripts and suggests a function early in the cell cycle. The most marked phenotype of *clb5* mutants is an extended S phase. DNA replication seems to start punctually (i.e., relative to other Start-related events such as budding and *CLN2* transcription, though a slight delay is not ruled out), but it then lasts more than twice as long as in wild-

type cells. This phenotype, which in a somewhat less severe form has also been described by Epstein and Cross (1992), could be a result of either slower chain elongation or a defect in the firing of certain (e.g., late) replication origins. Deleting *CLB6* alone has little effect on the timing or duration of S phase, but deleting both *CLB5* and *CLB6* causes the onset of S phase to be delayed by at least 30 min. It is possible, therefore, that both single and double mutant phenotypes are the result of defective initiation events, in which case, we suppose that either Clb5 or Clb6 is sufficient for the punctual firing of early origins but that only Clb5 suffices for the efficient firing of late origins. This hypothesis should be testable by analyzing the utilization of individual origins in *clb5* and *clb6* mutants. It is not clear what eventually triggers DNA replication in the absence of Clb5 and Clb6. It is possible that cells wait for the accumulation of Clb3/4 or a yet undiscovered cyclin. It is unlikely that Cln1/2 play a role because their RNAs have declined when DNA replication occurs in the *clb5 clb6* mutant (see Fig. 4b).

All seven previously characterized cyclins in yeast have been shown to interact with the Cdc28 kinase (Wittenberg et al. 1990; Grandin and Reed 1993; Tyers et al. 1993). It is thought that their main function is to activate the kinase and maybe to determine its substrate specificity. Overexpression of *CLB5* partially rescues *cdc28-13* mutants (data not shown), suggesting that Clb5 might be an activator of the Cdc28 protein kinase. Previous work has implicated vertebrate cyclin A and the cdk2 kinase in S phase (Fang and Newport 1991; Girard et al. 1991). Clb5 and cyclin A might therefore perform similar functions, even though these two cyclins do not particularly resemble each other. Potential targets for Clb5/6-associated kinases include the replication factor Rpa, which shows S phase-specific phosphorylation (Din et al. 1990) and constituents of the Orc complex that binds replication origins in yeast (Bell and Stillman 1992).

Regulation and function of G₁ cyclins

Preparations for all aspects of the cell division process occur soon after cells undergo Start (Pringle and Hartwell 1981): Cells enter S phase; they duplicate their spindle pole bodies, the first step in forming a mitotic spindle; and they lay down proteins required for cytokinesis in the vicinity of the future bud site (Kim et al. 1991). It was thought that only Cln cyclins (Cln1–Cln3) were necessary for these early cell cycle events and, moreover, that the three Cln cyclins had quasiequivalent roles (Richardson et al. 1989). Clb5 and Clb6 must now be included in this picture. We suggest that Cln3, Cln1/2, and Clb5/6 have three distinct functions at the beginning of the yeast cell cycle (see Fig. 9).

Unlike other cyclins, Cln3 protein does not fluctuate in abundance during the cell cycle. The observation that *cln1 cln3* double mutants are viable without the Swi6 transcription factor, whereas *cln1 cln2 swi6* mutants are dead, suggests that Cln3 cannot alone support entry into the cell cycle but needs additional genes whose expression is dependent on *SWI6*. It has therefore been pro-

Figure 9. Transcriptional activation and the role of yeast cyclins. (Top) *CLB5* and *CLB6* transcripts parallel those from *CLN1/2* in late G_1 , preceding those from *CLB3/4* (S/ G_2) and *CLB1/2* (G_2 /M). (Bottom) Model for the activation and functions of budding yeast cyclins. *CLN3* may be the initial trigger for the G_1 -positive feedback loop involving *CLN1* and *CLN2*. *CLB5* and *CLB6* are activated by *CLN1/2* or, in their absence, by *CLN3*. *CLB5,6* can also activate late G_1 -specific transcription but only when cells have reached a critical size (dotted arrow). *CLNs* have two major functions: Initiate the budding process and turn on *CLB5/6* transcription. *CLB5* and *CLB6* are directly involved in DNA replication. In the absence of *CLB5/6*, DNA replication is delayed and triggered by *CLNs* or unknown cyclins; conversely, in the absence of *CLN1/2*, budding is promoted by *CLB5/6* or unknown cyclins (dashed arrows with question mark). *CLB3, CLB4*, and *CLB5* are required for the assembly of a short intranuclear, preanaphase spindle. *CLB1*, and *CLB2* are involved in spindle maintenance and later events of mitosis.

posed (Tyers et al. 1993) that the primary function of Cdc28 kinase activity associated with Cln3 is to trigger the transcription in late G_1 of other cyclin genes, such as *CLN1/2* and *CLB5/6*. Loss of Cln3 function is not lethal but causes Start and presumably also *CLN1/2* and *CLB5/6* transcription to be delayed (Cross 1988; Nash et al. 1988). Our observation that *cln1 cln2 clb5 clb6* quadruple mutant cells cannot enter S phase or bud properly despite having an active *CLN3* gene confirms the fact that Cln3 is not sufficient (as regards cyclin function) for Start and is consistent with a function in the activation of *CLN1/2* or *CLB5/6*.

How might *CLN3* then help to trigger transcription of the *CLN1/2* and *CLB5/6* gene pairs? Two different transcription factors, SBF and MBF, have been implicated in late G_1 -specific transcription in yeast. SBF is required to activate *CLN1*, *CLN2*, and the distantly related *HCS26* gene (Ogas et al. 1991). The lack of *SWI4* dependence, the deregulation in *swi6* mutants, and the binding of MBF to MCB-like elements in the *CLB5* promoter all point to MBF and not SBF being involved in *CLB5* regulation. The *CLB6* promoter contains several matches to the MCB element, suggesting that it too is regulated by MBF. Many other DNA replication genes are regulated by MBF, but *CLB5* and *CLB6* are the first examples of genes whose transcription in late G_1 is important for S-phase entry. Most replication genes encode stable enzymes that can be inherited from the previous cell cycle (Byers and Sowder 1980). All genes regulated by SBF and MBF depend on Cdc28 and on Cln activity.

The activation in late G_1 of *CLN1* and *CLN2* has different functions than that of *CLB5* and *CLB6*. Both budding and entry into S phase are delayed in *cln1 cln2* double mutants (Hadwiger et al. 1989), whereas only S phase

seems delayed in *clb5 clb6* double mutants (Fig. 4b). We therefore propose that *CLN1/2* activation has two main functions: to promote budding and to activate genes regulated by MBF (e.g., *CLB5* and *CLB6*). Both functions might be facilitated by Cln3 (Fig. 9). Activation of *CLB5* and *CLB6*, on the other hand, is neither needed for *CLN1/2* transcription nor for budding but is particularly important for S-phase entry. We have not yet analyzed the timing of spindle pole body duplication in *clb5 clb6* mutants, and it is therefore not possible to say whether the *CLN1/2* or *CLB5/6* genes are more important for this process.

Despite their specialization, it is likely that the *CLN1/2* and *CLB5/6* gene pairs can partly substitute for each other. In *cln1 cln2* double mutants, *CLB5* and *CLB6* become important for budding, and in *clb5 clb6* double mutants, *CLN1* and *CLN2* are necessary for S-phase entry. According to this scheme, *cln1 cln2 cln3* triple mutants are inviable because Cln1/2 proteins are missing and the *CLB5/6* genes are not transcribed. As predicted by this hypothesis, expression of *CLB5* independently of Cln function, that is, from the *GAL* promoter, suppresses the lethality of the triple *cln* mutant. Several truncated forms of human A- or B-type cyclins can also suppress the triple mutant, but the full-length *S. cerevisiae CLB1* does not (Lew et al. 1991), suggesting that *CLB5* has some propensity for this task. Under these circumstances, *CLB5* not only promotes budding but also the activation of genes regulated by SBF and MBF. Clb5 (at least when sufficiently expressed) can therefore perform all functions normally performed by Cln cyclins. The increase in *CLB5* transcription in late G_1 might therefore contribute to the dynamics of gene activation at Start.

CLB5 and *CLB6* activation is necessary for punctual S-phase entry, but it is not sufficient. Expression of *CLB5* from the *GAL* promoter neither advances the onset of S phase nor triggers premature transcription of *CLN1* in small daughter cells isolated by centrifugal elutriation. It is likely that none of the genes regulated by MBF are prematurely activated either. This may be the reason why premature S-phase entry does not occur, that is, the initiation of DNA replication may require the activation by MBF of genes other than *CLB5* and *CLB6*. It is mysterious that *CLB5* expression triggers S phase and the SBF/MBF transcriptional program in *cln1 cln2 cln3* triple mutant cells arrested in late G₁ but not in small daughter cells. Cell size could be the crucial difference between these two situations, in which case, there must exist factors required for the Start-promoting activity of *CLB5* (other than Cln1-3) that are limiting in small cells. Because of a lack of such factors, *CLB5* expression in small daughter cells might not in fact immediately produce Cdc28 kinase activity, and this might be another reason why S phase is not initiated prematurely.

G₂ cyclins and the formation of mitotic spindles

Clb5's possession of sequences characteristic of the destruction boxes found in mitotic B-type cyclins (Glötzer et al. 1991) raises the possibility that Clb5 protein made in late G₁ might not be degraded before mitosis and might therefore have a function in G₂ as well. We found, accordingly, that deletion of *CLB5* causes *clb3 clb4* mutant cells to arrest in G₂ without mitotic spindles. This finding suggests that Clb1/2, Clb3/4, and Clb5 represent three classes of B-type cyclins involved in spindle function in yeast. Transcription of *CLB5* in late G₁ and S, *CLB3/4* in S and G₂ (Fitch et al. 1992; Richardson et al. 1992), and *CLB1/2* in G₂ and M (Ghiara et al. 1991; Surana et al. 1991) is consistent with these three cyclin types being important at different stages of spindle morphogenesis and function.

Determining the exact role of each cyclin in wild-type cells has been complicated by their overlapping functions. For example, mitotic spindles still form in mutants lacking cyclins from each class; *clb5* mutants and *clb3 clb4* double mutants are viable, and *clb1 clb2* double mutants, although blocked in mitosis, still form short (preanaphase) spindles (Surana et al. 1991). Formation of spindles in *clb3 clb4* mutants depends on both Clb5 and Clb1/2, unlike in wild type where neither set of genes is needed. Electron microscopy has shown that spindle pole bodies duplicate but do not separate in *clb1 clb2 clb3 clb4* quadruple mutants. Light microscopic analysis suggests that the phenotype of *clb3 clb4 clb5* triple mutants might be similar. We therefore propose that the formation of a bipolar spindle following spindle pole body duplication normally involves Clb3, Clb4, and Clb5. In the absence of Clb3 and Clb4, Clb1 and Clb2 cooperate with Clb5 to perform the same function, explaining why *clb3 clb4* mutants are viable. The phenotype of *clb3 clb4 clb5* mutants disproves the suggestion, based on earlier analyses of *clb1-clb4* mutant combina-

tions (Fitch et al. 1992; Richardson et al. 1992), that Clb2 might be sufficient for spindle assembly. *CLB2* does not seem sufficient even when overexpressed (from a multicopy plasmid; data not shown). Cdc28 kinase activity associated with Clb3, Clb4, and Clb5 could act directly in spindle assembly, for example, by phosphorylating kinesin-like motors (Hagan and Yanagida 1990; Hoyt et al. 1992). Alternatively, such kinases could act indirectly, for example, by activating forms of the Cdc28 kinase associated with Clb1 and Clb2. Whether *CLB1* and *CLB2* transcripts are absent at the G₂ arrest of *clb3 clb4 clb5* triple mutants is not known, but Clb2-associated H1 kinase activity is lowered 10- to 20-fold compared with nocodazole-arrested wild-type cells (data not shown). The failure to make Clb1/2 kinase could therefore be partly responsible for the defect in mitotic spindle assembly.

Mutation of *CLB5* causes less severe phenotypes in another strain background (Epstein and Cross 1992) than in the W303 strain of our studies. The extension of S phase is less extreme, and *clb3 clb4 clb5* triple mutants are viable. One explanation is the existence of polymorphic loci affecting cyclin function, which could even encode yet undiscovered cyclin genes.

The phylogeny of cyclins

It is becoming increasingly apparent that the diverse functions of Cdc28 during the yeast cell cycle are performed by forms of the kinase that are distinguished by their cyclin subunits. Entry into the cell cycle at Start involves the Cln cyclins. S-phase entry needs Clb5 or Clb6. Bipolar spindle formation involves Clb3, Clb4, and Clb5. Mitosis needs Clb1 and Clb2. Much of the order and timing of cell cycle events involves the progressive activation of Cdc28 kinase activities associated with different cyclins, whose periodicity during the cycle is determined by both transcriptional and post-transcriptional controls (for review, see Nasmyth 1993).

In considering how the diversity of cyclin function might have evolved, it is striking that mitotic B-type cyclins are the most conserved class. G₁ cyclins from yeast are no more similar to G₁ cyclins from vertebrates than either class is to mitotic cyclins (Koff et al. 1991). The conservation of mitotic cyclins presumably reflects common ancestry and suggests that they might be the most ancient members of the cyclin family. We speculate that duplications of a primordial B-type cyclin gene followed by functional specialization led to the current diversity. This would explain why B-type cyclins are involved in regulating both DNA replication and mitosis in yeast. In many cases, specialization is not complete because there is much functional redundancy, at least under our experimental conditions, between different types of yeast cyclins. Those concerned with adjacent phases of the cell cycle have retained overlapping functions (e.g., Cln/Clb5 Clb6, Clb5/Clb3 Clb4, Clb3 Clb4/Clb1 Clb2), but types that are expressed at widely separated stages have not (e.g., Cln/Clb1 Clb2).

The apparent overlap between Clb5 and Cln cyclin

function is so extensive that cells lacking all three Cln cyclins can proliferate rapidly when *CLB5* is expressed from the *GAL* promoter. Why, then, do cells bother to have Cln cyclins? The answer may be that gene multiplication expands the regulatory and functional repertoire of a cell. Cyclin types are not only distinguished by the range of cell cycle events that they affect but also by the different forms of regulation imposed on them. Pheromonal control, for example, may be exerted directly on Cln cyclin activity via the Far1 protein and not directly on other cyclin types such as Clb5 (Chang and Herskowitz 1990; Valdivieso et al. 1993). This could explain why expression of *CLB5* from the *GAL* promoter allows cells to escape pheromone-induced G_1 arrest. In normal cells, pheromones prevent *CLB5/6* activation and entry into S phase because their transcription is dependent on Cln cyclins. It may be that much of the apparent redundancy of cyclin function seen in yeast stems from our ignorance of how these genes and their products are regulated in the wild.

Materials and Methods

Strains and media

Yeast strains used in this study were derivatives of the standard wild-type strains W303 and K1107 (Table 1). Cells were grown in YEP medium (1% yeast extract, 2% Bacto-peptone, 50 mg/l adenine) supplemented as indicated with 2% glucose (YEPD), raffinose [Raff], or galactose [Gal] unless otherwise stated. Plates contain 2% agar. Yeast transformation, transplacement, and DNA analysis were performed as described by Nasmyth (1985a). Standard molecular biology techniques were performed according to Sambrook et al. (1989). α -Factor was a generous gift of H. Riezman (Biocenter, Basel, Switzerland). The monoclonal anti-tubulin antibody (YOL1/34) was obtained from J. Kilmartin (Medical Research Council, Cambridge, UK).

Cloning and genetic manipulations of *CLB5* and *CLB6*

An open reading frame with homology to B-type cyclins was found by sequencing a previously isolated multicopy suppressor (*sup1A*) of the *cdc28-1N* mutation (Surana et al. 1991). The *sup1A* plasmid contained a large insert (6.1 kb) carrying the carboxy-terminal half of the new gene named *CLB5* but also the full-length *CLB2* gene that is responsible for the *cdc28-1N* suppression. The whole *CLB5* gene was then cloned by screening a YEp13 genomic library with a 0.5-kb *EcoRI* fragment containing the cyclin box of *CLB5*. Two positive clones having large overlapping inserts were obtained, from which a 3.0-kb *BstXI*-*XbaI* fragment was subcloned into pBluescript and fully sequenced on both strands following unidirectional exonuclease III treatment (Genbank accession number X70435). The independent cloning of the same gene was recently reported (Epstein and Cross 1992).

A gene related to *CLB5* was found at the equivalent position adjacent to *CLB1* and was named *CLB6*. *CLB6* was cloned by screening a genomic library in YCplac111 (a generous gift from F. Cvrcková, IMP) with a 0.75-kb *XbaI*-*EcoRV* fragment containing the carboxyl terminus of *CLB1*. Eleven positive clones were isolated, and a 3.8-kb *EcoRI* fragment was subcloned in pBluescript KS(+). The region between the *BglIII* and *SphI* sites was fully sequenced on both strands using both subcloning and specific primers (Genbank accession number X70436).

For driving *CLB5* by the *GAL1-10* promoter, the 0.7-kb

EcoRI-*BamHI* of *GAL1-10* was cloned in front of the 2.2-kb *HindIII* fragment of *CLB5* in the Ylplac204 (*TRP1*) and Ylplac211 (*URA3*) plasmids (Gietz and Sugino 1988). The resulting plasmids were linearized with *EcoRV* and used for yeast transformation. The *GAL-CLB2* (Fitch et al. 1992), *GAL-CLN1* (Cross and Tinkelenberg 1991), and *GAL-CLN3* (Xiong et al. 1991) constructs have been described elsewhere.

Disruption of *CLB5* and *CLB6*

The *CLB5* and *CLB6* loci were mutated by one-step gene disruption with the *URA3* and *LEU2* genes, respectively. The 0.7-kb *StuI*-*EcoRI* carboxy-terminal fragment containing the cyclin box of *CLB5* was replaced with either a 1.1-kb *URA3* or a 3.8-kb *hisG-URA3-hisG* (Alani et al. 1987) marker. For *clb6::LEU2*, the 2.0-kb *XbaI*-*PstI* fragment of the *LEU2* gene (pJJ282; Jones and Prakash 1991) was substituted for the 1.1-kb *MluI*-*NsiI* fragment of *CLB6*. The wild-type diploid K842 was transformed with the *HindIII*-digested *clb5::URA3* plasmid (C2254) and subsequently with the *XbaI*-*BglIII*-digested *clb6::LEU2* plasmid (C2476) to generate K3353. After sporulation and dissection, the genotype of the individual spores was determined by replica-plating on selective plates at 30°C.

RNA analysis

RNA isolation was performed according to Cross and Tinkelenberg (1991), and Northern analysis as described by Price et al. (1991). Probes were prepared by random priming of the 0.5-kb *EcoRI* fragment of *CLB5* and 1.0-kb *Clal* fragment of *CLB6*.

Cell synchronization

For centrifugal elutriation, cells were inoculated to $A_{600} = 0.05$ and grown overnight in 1–2 liters of YEP Raff (+/- Gal) at 23°C until mid-log phase ($A_{600} = 2-4$). Cells were harvested by filtration, resuspended in 200 ml of cold YEP Raff, and sonicated for 30 sec with a mid-size tip on a Kontes Micro Ultrasonic Cell Disrupter set at 50% output. Cells were kept on ice, loaded onto a Beckman J6M/E centrifuge (JE 5.0 rotor) at 4°C, 4000 rpm, with the pump (Masterflex) set at 0.8 until the chamber was 80% full, and left to equilibrate for 15 min by washing with cold YEP Raff medium. The pump speed was then gradually increased by 0.02 increments until small daughter cells were elutriated (usually between 1.12 and 1.30 pump speed). Cells were collected (0.3–1.5 l) until a sufficient amount was reached or the apparition of budded cells; they were then centrifuged and resuspended in their respective prewarmed media.

For synchronization by Cln depletion, cells were pregrown in YEP Raff Gal, washed by filtration with 3 volumes of YEP Raff, arrested for 2.5–3 hr in this medium, and then released by the addition of 2% Gal.

Construction of conditional lethal *clb5* strains

cln1 cln2 double mutants are unviable in the K699 background because it is *ssd1* (F. Cvrcková and K. Nasmyth, in prep.). For this reason, we used the K1107 strain background (where *cln1 cln2* cells rely on *CLN3* for growth) to investigate the role of *CLB5/6* at Start. First, a *cln1Δ* strain (K1971) was transformed with the *clb5::hisGURA3* disruption plasmid (C2255) cut with *SalI* × *XbaI* to generate K3224. Second, a *cln1Δ cln2::URA3* (K2008) was transformed at the *trp1* locus with the *GAL-CLB5/TRP1* integrative plasmid (C2233) linearized with *EcoRV* × *XbaI* to produce K3293. Then K3224 was crossed to K3293 and sporulated, and 30 tetrads were dissected. Several (9)

Table 1. Strain list

Strain	Genotype	Source
K699	<i>MATa</i>	W303-1a
K700	<i>MATα</i>	W303-1b
K842	<i>MATa/MATα</i>	K699 × K700
K1354**	<i>MATa, pep4-3 prb1-1122 prc1-407 swi6::TRP1</i>	K1268 Tfn (a)
K1393	<i>MATα, cdc28-13</i>	S. Reed
K1534	<i>MATa, bar1Δ</i>	K699 Tfn
K2063	<i>MATα, cln1::hisGURA3</i>	K700 Tfn (a)
K2392*	<i>MATa, swi4::LEU2 swi6::TRP1 URA3::Ylplac211-SpADH-CLN2(2×)</i>	(a)
K2638	<i>MATα, cln1::hisG</i>	K2063 FoA R
K2652	<i>MATa, clb1::URA3 clb3::TRP1 clb4::HIS3</i>	(d)
K2685	<i>MATa, cln1::hisG cln2Δ YCpADE3/ADH-CLN2</i>	K2664 × K2676 9c (b)
K2696	<i>MATα, cln1::hisG cln3::GAL-CLN3</i>	K2638 Tfn
K2739	<i>MATa, cln1::hisG cln2Δ cln3::LEU2 YCpADE3/ADH-CLN2</i>	K2726 Tfn (b)
K2762	<i>MATα, cln1::hisG cln2Δ cln3::GAL-CLN3</i>	K2696 × K2685 11b
K2771*	<i>MATa, cln1::hisG cln2Δ cln3::LEU2 YCpGAL-CLN1</i>	K2342 8a (a)
K2786*	<i>MATa, cln1::hisG cln2Δ cln3::LEU2 YCpGAL-CLN1 swi6::TRP1</i>	K2771 Tfn
K2831*	<i>MATα, swi6::TRP1 URA3::SpADH-CLN2(2×)</i>	K1950 × K2392 (a)
K2832*	<i>MATa, URA3::SpADH-CLN2(2×)</i>	K1950 × K2392 1c (a)
K2833*	<i>MATa, swi4::LEU2 URA3::SpADH-CLN2(2×)</i>	K1950 × K2392 8d (a)
K2884	<i>MATa, cln1::hisG cln2Δ hcs26::LEU2 orfD::TRP1 YCpADE3/ADH-CLN2</i>	K2726 Tfn (b)
K3072	<i>MATa, bar1Δ TRP1::GAL-CLB5(2×)</i>	K1534 Tfn
K3080	<i>MATa, clb1Δ clb2-ts clb3::TRP1 clb4::HIS3</i>	(c)
K3093	<i>MATa, clb3::TRP1 clb4::HIS3</i>	K2652 × K700 8d
K3098	<i>MATα, clb5::hisGURA3</i>	K700 Tfn
K3103	<i>MATα, clb2-ts clb3::TRP1</i>	K3080 × K700 11b
K3107	<i>MATa, cln1::hisG cln2Δ cln3::LEU2 YCpADE3/ADH-CLN2 TRP1::GAL-CLB5</i>	K2739 Tfn
K3111	<i>MATa, cln1::hisG cln2Δ cln3::LEU2 TRP1::GAL-CLB5</i>	K3107 white
K3114	<i>MATα, clb2-ts clb3::TRP1 clb5::URA3</i>	K3103 Tfn
K3122	<i>MATa, clb2-ts clb3::TRP1 clb4::HIS3</i>	K3080 × K3114 5a
K3128	<i>MATα, clb5::hisGURA3 TRP1::GAL-CLB5</i>	K3098 × K3072 7a
K3131	<i>MATα, clb3::TRP1 clb4::HIS3 clb5::hisGURA3 TRP1::GAL-CLB5</i>	K3093 × K3128 4d
K3224*	<i>MATa, cln1::hisG clb5::hisGURA3</i>	K1971 Tfn (a)
K3293*	<i>MATα, cln1::hisG cln2::URA3 TRP1::GAL-CLB5</i>	K2008 Tfn (a)
K3296*	<i>MATα, cln1::hisG cln2::URA3 clb5::hisGURA3 TRP1::GAL-CLB5</i>	K3224 × K3293 4d
K3353	<i>MATa/MATα, clb5::URA3/CLB5 clb6::LEU2/CLB6</i>	K842 Tfn
K3414	<i>MATα, clb6::LEU2</i>	K3353 7c
K3415	<i>MATa, clb5::URA3 clb6::LEU2</i>	K3353 7d
K3418	<i>MATα, clb3::TRP1 clb4::HIS3 clb5::hisGURA3 TRP1::GAL-CLB5 clb6::LEU2</i>	K3131 Tfn
K3424*	<i>MATα, cln1::hisG cln2::URA3 clb5::hisGURA3 TRP1::GAL-CLB5 clb6::LEU2</i>	K3296 Tfn
ESY51	<i>MATa, bar1Δ YCpGAL-CLN1</i>	K1534 Tfn
ESY52	<i>MATa, bar1Δ URA3::GAL-CLB2</i>	K1534 Tfn
ESY61	<i>MATα, cdc28-13 TRP1::GAL-CLB5</i>	K1393 Tfn
ESY108	<i>MATa, clb2-ts clb3::TRP1 clb4::HIS3 URA3::GAL-CLB5</i>	K3122 Tfn
ESY143	<i>MATa, clb5::URA3</i>	K3353 7b

Unless marked with an asterisk, all strains were isogenic derivatives of (or at least backcrossed four times to) K699, whose full genotype is *MATa, HMLα, HMBa, ho ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 ssd1*. Strains marked with a single asterisk (*) are isogenic derivatives of K1107 whose full genotype is *MATa, HMLa, HMRa, ho-βgal ade2-1 trp1-1 can1-100 met leu2-3,112 his3 ura3*; those marked with two asterisks (**) are isogenic to K1268 whose full genotype is *MATa, ade2-1 trp1 leu2 his3 ura3 pep4-3 prb1 prc1*. Tfn under Source indicates that the strain was constructed by DNA transformation, usually transplacement. The loci concerned were always checked by Southern blot analysis.

(a) Nasmyth and Dirick (1991).

(b) F. Cvrcková and K. Nasmyth (in prep).

(c) A. Amon, M. Tyers, B. Futcher, and K. Nasmyth (in prep).

(d) Fitch et al. (1992).

Ura⁺ Trp⁺ glucose-sensitive segregants were obtained. All were determined, by Southern analysis, to carry both the *cln2::URA3* and *clb5::hisGURA3* alleles. One of them (K3296) was transformed with *clb6::LEU2* (C2476-cut *Clal*) and selected on galactose plates lacking leucine (K3424).

We were not successful in transforming directly *clb5::URA3* in a *clb1 clb3 clb4* haploid strain (K2776). To test whether *clb3 clb4* and *clb5* are synthetic lethals, we crossed K3093 (*clb3::TRP1 clb4::HIS3*) with K3096 (*clb5::URA3*) and dissected 20 tetrads. Whereas all double combinations were viable,

a *clb3 clb4 clb5* triple mutant could never be obtained, even though 12 such mutants were expected from the segregation pattern. In most cases, the spores germinated and died in the budded portion of their first cell cycle. To construct the *clb3 clb4 clb5 GAL-CLB5* strain, K3093 [*clb3::TRP1 clb4::HIS3*] was crossed to K3128 [*clb5::hisGURA3 TRP1::GAL-CLB5*], and $\text{Trp}^+ \text{His}^+ \text{Ura}^+$ glucose-sensitive segregants were obtained and checked by Southern analysis. One of them (K3131) was used for further analysis.

Other techniques

Conditions for gel-retardation experiments were as described previously (Dirick et al. 1992). Flow cytometry DNA quantitation was determined according to Epstein and Cross (1992) on a Becton-Dickinson FACScan. In situ immunofluorescence and photomicroscopy were performed according to Nasmyth et al. (1990).

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