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A new pair of B-type cyclins from *Saccharomyces cerevisiae* that function early in the cell cycle

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Two new B-type cyclin genes from *Saccharomyces cerevisiae*, called *CLB5* and *CLB6*, are located in a tail to tail arrangement adjacent to the G_2/M phase promoting cyclins *CLB2* and *CLB1*, respectively. These genomic cyclin arrays are flanked by tRNAs and repeated sequences of Ty elements suggesting an intrachromosomal gene duplication followed by an interchromosomal gene duplication. Based on their deduced protein sequence the *CLB5* and *CLB6* genes form a new pair of B-type cyclins. They are most related to each other and then to the deduced protein sequence of their adjacent genes *CLB1* and *CLB2*. Both genes are periodically expressed, peaking early in the cell cycle. Loss of function mutants are viable, but *clb5*$^-$ mutants exhibit a delay in S phase whereas *clb6*$^-$ mutants show a delay in late G_1 and/or S phase. The clb5 mutant phenotype is somewhat more pronounced in a double null mutant. Both cyclins have the potential to interact with the p34^{CDC28} kinase *in vivo*.
Key words: B-type cyclins/Cdc28 kinase/G_1/S phase/gene duplications/yeast

Introduction

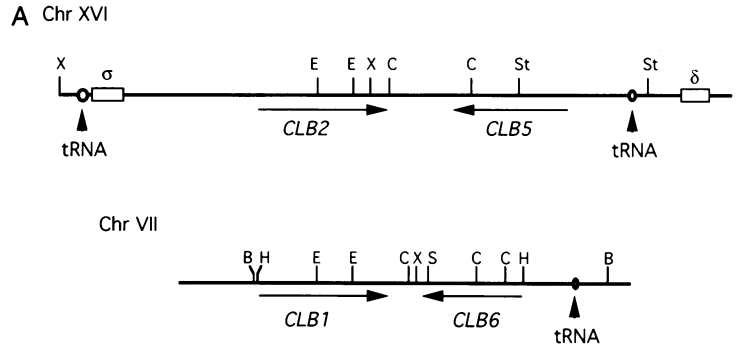
During the past decade much effort has been directed towards the understanding of the molecular basis for the control of cell cycle. The basic mechanisms controlling progression through the eukaryotic cell cycle are highly conserved in different organisms. Many of the key components identified so far show high primary sequence homologies and are able to functionally substitute for their counterparts in heterologous systems *in vivo* (for recent reviews, see Nurse, 1990; Murray, 1992; Reed, 1992).

Genetic analysis in yeast led to the idea that central to the control of cell cycle progression is a serine/threonine protein kinase known as p34^{cdc2}, the gene product of the *cdc2* gene from *Schizosaccharomyces pombe* and the *CDC28* gene for *Saccharomyces cerevisiae*. Different mutations in the *cdc2/CDC28* gene cause arrest at two distinct transition points in the cell cycle. These two, generally assumed to be the predominant control points, are at the G_1/S [in yeast called START (Hartwell, 1993)] and the G_2/M transition borders (Nurse and Bisset, 1981; Piggott *et al.*, 1982; Surana *et al.*, 1991). These findings led to a convincing model, with p34$^{cdc2/CDC28}$ as the key regulator protein mediating passage through these two transition points. In vertebrates and invertebrates, members of a family of highly related p34 proteins, the Cdk proteins, share the multiple control functions of the p34$^{cdc2/CDC28}$ protein. Recently, considerable support has been lent to the idea that in these organisms p34^{cdc2} is required for the G_2 to M phase transition whereas the related Cdk proteins function early in the cell cycle (Reed, 1992; Xiong *et al.*, 1992).

The p34$^{cdc2/CDC28}$ and their related proteins resemble the catalytic subunit of multiprotein kinase complexes transiently formed during the cell division cycle. It is generally assumed that the accumulation of the active form of these kinase complexes, exceeding a critical threshold level at distinct transition points, promotes cell cycle progression. Most likely the downstream events are then obtained by certain phosporylation states of substrates that are critical for cell cycle regulation (Nurse, 1990; Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Murray, 1992).

The kinase activity of p34$^{cdc2/CDC28}$ is dependent on its association with members of a class of proteins called cyclins. Whereas expression of the p34$^{cdc2/CDC28}$ protein is relatively constant throughout the cell cycle, most of the cyclin proteins are very unstable and are only present at a particular point in the cell cycle. This fluctuation ('cycling') in the abundance of the cyclin proteins during meiotic and early mitotic divisions was the basis for their original discovery in marine invertebrates (Evans *et al.*, 1983). At present there are >30 cyclin genes sequenced. Based on amino acid sequence relatedness, by their appearance and their apparent function during the cell cycle they are divided into subclasses of cyclins (Minshull *et al.*, 1990; Hunt, 1991; Lew and Reed, 1992).

The best understood regulatory event in the cell cycle is the G_2/M transition where several post-translational modifications of p34$^{cdc2/CDC28}$, and probably of the cyclins, trigger the switch that mediates progression from G_2 to M phase. In higher eukaryotes A- and B-type cyclins were identified as subunits of the mitotic kinase. The activity of A-type cyclin-associated kinase appears and disappears earlier in the cell cycle than the one of the cyclin B-containing kinases. A and B cyclins contain a mitotic destruction box that serves as signal for a ubiquitin-mediated proteolysis (Glotzer *et al.*, 1991). Whereas B-type genes have been found in every eukaryote analysed, the presence of A-type cyclins has not been reported for yeast. For *S.pombe* the *cdc13* gene was identified as the mitotic B-type cyclin (Solomon *et al.*, 1988). Mutants in *cdc13* stop growth uniformly in G_2. In *S.cerevisiae* four B-type cyclins that act in mitosis (Clb1-4) have been described (Surana *et al.*, 1991; Richardson *et al.*, 1992). These four B-type cyclins exhibit overlapping functions, their transcripts appear in late S phase and they all contain a mitotic destruction box. Single null mutants are viable. The four B cyclins resemble, based on sequence relatedness, two pairs of genes: *CLB1/CLB2* and *CLB3/CLB4*. Whereas loss of function mutants for either



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gcttcactaatctagcgttcatacaacatacaaatcagtgtgttgaaaattattattctctgatattctctccctccttttaggttttaaaATGAATTGT 9
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Fig. 1. Genomic organization of the *CLB* clusters and 5′ sequence of the *CLB6* gene. (**A**) The coding sequences of the genes *CLB2/CLB5* and *CLB1/CLB6* are indicated by arrows. The boxes labelled with σ (sigma) and δ (delta) indicate solo LTRs of Ty elements. Only relevant restriction sites are shown: X, *Xba*I; E, *Eco*RV; C, *Cla*I; St, *Stu*I; B, *Bgl*II; H, *Hin*dIII; S, *Ssp*I. (**B**) Coordinate 1 is the first A in the coding sequence. The MCB boxes (coordinates −437, −402, −371) and the SCB box (coordinate −858) are underlined. The sequence homology with the Ty3-1 flanking sequences are underlined with dots. The corresponding insertion site of the Ty3 element is indicated by an arrow. The $tRNA^{Cys}$ is shown in capital letters.

of these genes are viable, nearly every null mutant combination including *clb2* (except *clb2/clb4*) is lethal.

The second transition point where $p34^{cdc2/CDC28}$ resembles a key regulatory component in yeast is the G_1/S transition (START). So far, however, no essential post-translational modification of the p34 protein itself, affecting G_1/S progression, has been found. Three functional partially overlapping G_1 cyclins (Cln1−3) that participate in the regulation of START have been identified in *S.cerevisiae*. Loss of function mutants of these genes are tolerated, but a deletion of all three genes causes G_1 arrest before START (Richardson *et al.*, 1989). The G_1 cyclins Cln1−3 in *S.cerevisiae* are rate limiting for G_1/S transition (Cross and Tinkelenberg, 1991; Lew *et al.*, 1992). In *S.pombe* a putative homologue to Cln3 is encoded by the *puc1* gene (Forsburg and Nurse, 1991). In the current model for G_1/S transition, $p34^{CDC28}$ is activated by its association with the G_1 cyclins. The transcription of the G_1 cyclins is regulated by the G_1/S-specific transcription factor complex Swi4−Swi6 which in turn is activated by an active $p34^{CDC28}$ kinase complex. This results in a positive feedback loop, producing a certain threshold level of an active G_1/S kinase complex and irreversibly switches to the passage through START (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991). Cross-species complementation screens for *cln* triple mutants from *S.cerevisiae* revealed widely divergent cyclin-like genes from different organisms, that might have a function at the G_1/S transition and in S phase progression (Lew *et al.*, 1991). These cyclins have been classified as C, D and E cyclins. The normal function covered by these cyclins is not clear.

Recently several reports suggest an involvement of a cyclin-dependent kinase for S phase progression. Cyclin A has in addition to its mitotic function an essential role in S phase (Girard *et al.*, 1991; Pagano *et al.*, 1992). In *S.pombe* a B-type cyclin that functions early in the cell cycle with a G_1/S phase role encoded by *cig1* was isolated by a PCR approach (Bueno *et al.*, 1991). A gene isolated as a candidate oncogene called *PRAD1*, now shown to be cyclin D1, has properties to function as an S phase cyclin (Motokura *et al.*, 1991). A possible role for Clb3/Clb4 in S phase has been discussed (Fitch *et al.*, 1992; Richardson *et al.*, 1992).

In eukaryotes, gene redundancy is often observed. In most of the vertebrates and invertebrates, but also in *S.cerevisiae*, cyclins exist as a subgroup of two or more homologue proteins (this is not the case for *cdc13* in *S.pombe*). Studies with *Drosophila* embryos, *Xenopus* egg extracts and *S.cerevisiae* revealed that these related cyclins are partially

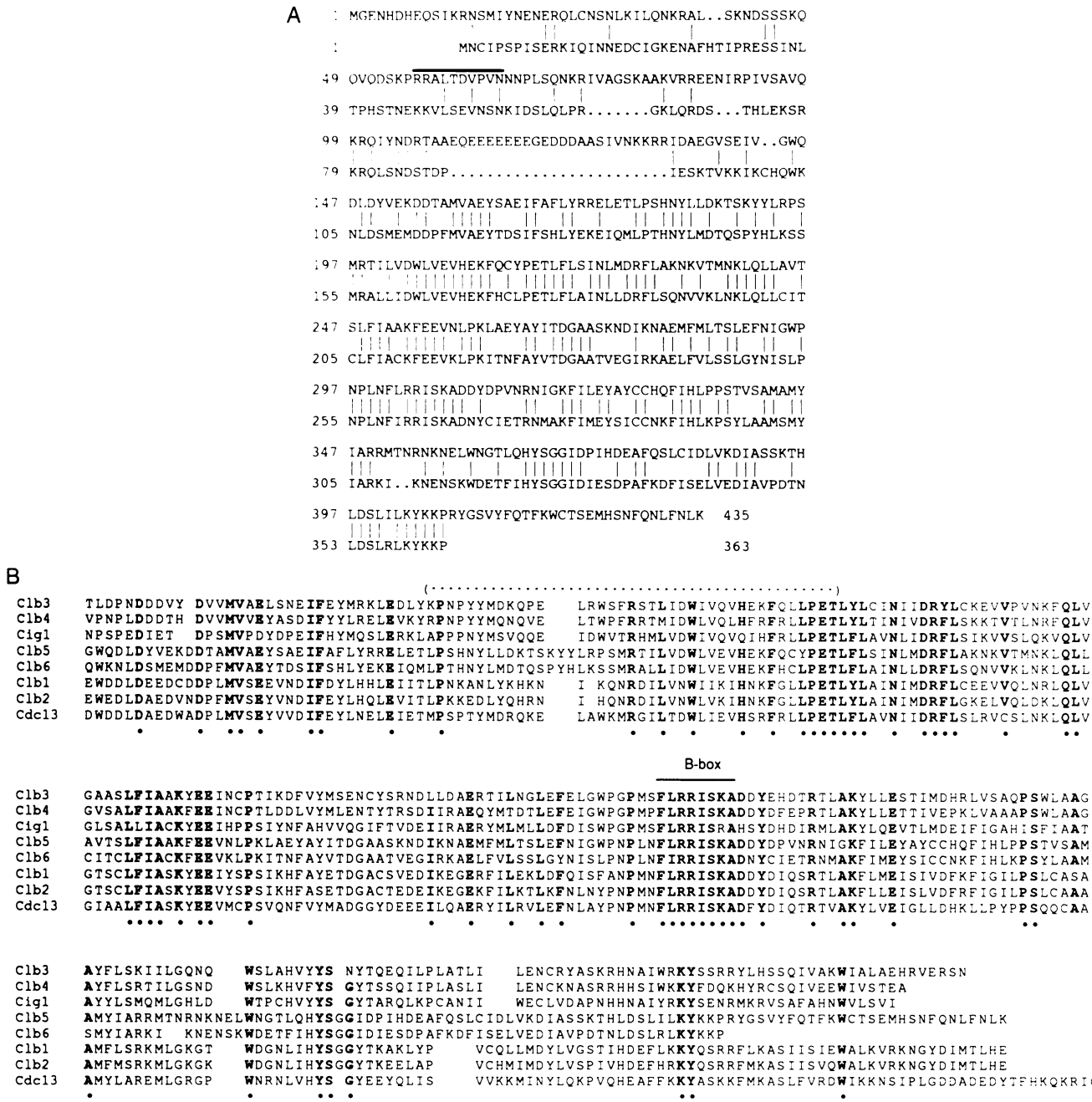


Fig. 2. Clb5 and Clb6 are B-type cyclins and form a pair. (**A**) Alignment from the predicted amino acid sequence of Clb5 (top) and Clb6 (bottom) has been made using the GAP program of the UWGCG package (version 7.2). The mitotic destruction box [R-X-A-L-G-(D/N/E)-I-X-N] in Clb5 is overlined. No obvious destruction box is found in Clb6. The sequence of the *CLB6* gene has been submitted to the EMBL databank (accession number X72890). (**B**) Comparison of the C-terminal parts of the yeast B-type cyclins. All the known B-type cyclins from the yeasts *S.cerevisiae* and *S.pombe* were aligned with the PILEUP program of the UWGCG package (version 7.2) and shown in the order of their relatedness. The alignment includes the proteins Clb3 (residues 151−427), Clb4 (residues 190−460), Cig1 (residues 147−415), Clb5 (residues 144−435), Clb6 (residues 102−363), Clb1 (residues 191−471), Clb2 (residues 211−491) and Cdc13 (residues 185−474) (Booher and Beach, 1987; Bueno *et al.*, 1991; Surana *et al.*, 1991; Richardson *et al.*, 1992). Amino acids identical in at least seven out of the eight proteins are indicated in bold and marked by a dot below the sequence. The B-box and the deletion in Clb5 (.....) are indicated.

redundant and show functional compensation (Minshull *et al.*, 1989; Pines and Hunter, 1989; Nasmyth and Dirick, 1991; Richardson *et al.*, 1992; Surana *et al.*, 1991; Xiong *et al.*, 1992; Knoblich and Lehner, 1993). Because of the redundancy of the cyclin genes in *S.cerevisiae*, recessive mutant screens have failed to detect the cyclin genes in this yeast [this seems also to be the case for G_1 and S phase cyclins in *S.pombe* (Reed, 1992)]. Both the 'G_1 cyclins' and the 'mitotic cyclins' for *S.cerevisiae* were found as dosage suppressors of particular thermosensitive (ts) *cdc28* mutants with the exception of *CLN3* which was isolated as a dominant gain of function mutant (Cross, 1988; Nash *et al.*, 1988).

Recently a new cyclin gene for *S.cerevisiae*, *CLB5*, has been reported (Epstein and Cross, 1992). This cyclin has the structural features of a B-type cyclin, but complements a G_1 cyclin (*cln1-3*) deficiency. We have also cloned this *CLB5* gene by the observation that this gene is located downstream of the already known cyclin gene *CLB2*. We

have isolated another, novel cyclin gene, *CLB6*, which forms a subgroup of B-type cyclins together with the *CLB5* cyclin. We show that both genes are transcribed early in the cell cycle at START and that they are involved in G_1/S and/or S phase progression. A possible explanation for the appearance of gene redundancy in cyclin genes for *S.cerevisiae* is discussed.

Results

Duplications of S.cerevisiae B-type cyclins

During sequence analysis of an *S.cerevisiae* B-type cyclin gene, now known as *CLB2* in the literature (Ghiara *et al.*, 1991; Surana *et al.*, 1991; C.Kühne, unpublished results), a new gene coding for a potential B-type cyclin was found in its proximity. These two genes are in a tail to tail arrangement (Figure 1A). The new potential B cyclin shows an open reading frame (ORF) of 435 amino acids with a calculated molecular weight of 50.4 kDa. Disruption of the *CLB5* gene (see below) showed that it did not cover an essential function for growth, reminiscent of all the other cyclins described for *S.cerevisiae* (Richardson *et al.*, 1989, 1992; Surana *et al.*, 1991). This suggests that there might be one or more redundant gene or genes with overlapping functions. Reasoning that the described cyclin pair *CLB1/CLB2* resulted from an interchromosomal gene duplication, it seemed possible that the same array exists at the genomic locus of the redundant B-type cyclin *CLB1*. Thus, an additional B-type cyclin similar to the gene *CLB5* was expected to be present in a tail to tail arrangement with *CLB1*.

Therefore a large genomic fragment containing the *CLB1* gene and flanking sequences was isolated (for details see Materials and methods). As predicted, sequences obtained from downstream of *CLB1* revealed an ORF with strong homologies to B-type cyclins (Figure 1A). This gene is most related to the predicted primary amino acid sequence encoded by the *CLB5* gene and based on this they form a pair. This newly discovered gene (named *CLB6)* and the gene coding for *CLB1* are again in a tail to tail formation and the corresponding stop codons are only 368 bp apart. The DNA sequence of *CLB6* (not shown, EMBL data library: X72890) contains an ORF coding for a 363 amino acid protein with a calculated molecular weight of 40.9 kDa. An alignment of the amino acid sequences of Clb6 and Clb5 is shown in Figure 2A. Whereas the deduced protein sequence for *CLB5* contains a candidate for a mitotic destruction box (overlined in Figure 2A) the Clb6 sequence does not show any obvious candidate sequence for a destruction box. N- and C-terminal extensions and an insertion of a stretch of acidic amino acids in the N-terminal portion of the *CLB5* gene (most obviously, a stretch of seven codons for glutamic acid), account for most of the differences in size of the two related cyclin sequences. It is noteworthy that a similar acidic amino acid stretch is found in the *S.cerevisiae* Clb3 cyclin (Richardson *et al.*, 1992) and in the C-terminal part of the human G_1/S specific cyclin D1 (Lew *et al.*, 1991).

Clb5 and Clb6 form a new subclass of B-type cyclins

Based on given definitions for the classification for B-type cyclin proteins (Minshull *et al.*, 1989), the genes for *CLB5* and *CLB6* fulfil all the requirements for a sequence to be defined as a member of the B-type cyclin family. An alignment done with the PILEUP program of the UWGCG package with all the available cyclin sequences showed that *CLB5* and *CLB6* are more related to each other than to the remaining B-type cyclins. The perfect match with the consensus including the cyclin box and the B-box in an alignment of the conserved C-terminal parts from the known *S.cerevisiae* and *S.pombe* B-type cyclins is shown in Figure 2B.

Table I. Similarities in the 250 C-terminal amino acids of known B-type cyclins from the yeasts *S.cerevisiae* and *S.pombe*

	% Similarity	% Identity						
Clb5	Clb5	Clb6	Clb1	Clb2	Clb3	Clb4	Cdc13	Cig1
Clb5	–	61.4	38.7	41.2	37.9	36.9	38.3	34.1
Clb6	78.2	–	46.2	44.4	35.7	37.3	42.7	35.7
Clb1	58.9	68.2	–	80.1	43.3	40.8	48.7	42.3
Clb2	59.3	67.7	91.2	–	43.8	38.3	50.4	43.1
Clb3	62.1	63.0	68.2	65.8	–	63.3	44.0	48.4
Clb4	57.5	59.7	63.3	62.5	78.0	–	44.6	46.4
Cdc13	61.3	64.0	71.4	73.1	67.0	67.9	–	44.8
Cig1	54.0	55.9	62.3	63.2	66.8	69.6	67.3	–

The percentages of identical (right upper-half) and similar (left lower-half) amino acids were calculated using the GAP program (gap weight = 3.0, gap length weight = 0.1) of the UWGCG package (version 7.2).

For these B-type cyclins the percentage of homology in terms of identity and similarity is outlined in Table I; a comparison given as percent identity and percent similarity for the last 250 amino acids spanning the entire cyclin box of these proteins is shown. The same type of analysis, done with the entire reading frames and the DNA sequences, gave comparable results (data not shown). Based on these comparisons, the pairs Clb1/Clb2, Clb3/Clb4 and Clb5/Clb6 form subgroups of B-type cyclins. Moreover Clb6 is more closely related to Clb1 and Clb2 than is Clb5. This becomes even more pronounced when conservative changes are allowed. On the basis of these comparisons Clb5 is evolutionarily more diverged from the protein encoded by its neighbouring gene *CLB2* than is Clb6. Assuming that the B-type cyclin genes have diverged under similar selection pressure, it is likely that the *CLB1/CLB6* or *CLB2/CLB5* intrachromosomal duplication occurred first in evolution, and that this pair was then interchromosomally duplicated (see below) resulting in the *CLB1/CLB6* or *CLB2/CLB5* clusters.

The borders of the cyclin clusters

Intrachromosomal gene duplications often result in gene clusters representing gene families consisting of multiple evolutionarily related copies of one primordial gene (Li and Gradur, 1991; Krumlauf, 1992). It therefore seemed possible that the clusters described for the B-type cyclins from *S.cerevisiae* contain additional members of related cyclins at the same chromosomal locus. Upstream sequences up to −1500 bp (calculated from the AUG initiator codon) for *CLB5* and *CLB6* did not show any significant ORF (data not shown). The same was true for *CLB2* where upstream sequences up to −2500 bp (calculated from the AUG initiator codon) did not show any possible new cyclin candidate (data not shown). Interestingly in the upstream region of *CLB6* and *CLB5* a $tRNA^{Cys}$ gene is located at

positions −678 bp and −652 bp, respectively. Moreover, a $tRNA^{Lys}$ was found upstream of the *CLB2* gene at −1970 bp. The direction for the transcription for the $tRNA^{Cys}$ upstream of the *CLB5* and the *CLB6* gene was found to point towards the genes, whereas the $tRNA^{Lys}$ is located in opposite orientation to the *CLB2* gene (Figure 1).

tRNA upstream regions are known to be hotspots for insertions of retrotransposons found in yeast (Ty elements) (Boeke and Sandmeyer, 1991). Indeed in the upstream region of the *CLB2* gene a long terminal repeat (LTR) from a Ty3 element (sigma element) 16 bp upstream of the observed $tRNA^{Lys}$ was found. Moreover, at the *CLB5* locus 468 bp upstream of the tRNA a Ty1 delta element was found to be inserted. At the *CLB6* locus no sigma or delta element was found in the strain used for isolation of this gene. However, comparison of the flanking sequences from *CLB6* with the EMBL databank revealed homologies to the flanking sequences of the Ty3-1 retrotransposon (Figure 1B) (Hansen and Sandmeyer, 1990). This clearly shows that in some laboratory strains Ty3 elements are inserted in this locus. Most likely it can be assumed that the tRNA and Ty element flankings represent the borders for the two cyclin clusters and were probably involved in the duplications.

The clusters for CLB1/CLB6 and CLB2/CLB5 are located on different chromosomes

For genomic mapping, filters containing most of the *S.cerevisiae* genome in an ordered set of phage clones (kindly provided by L.Riles and M.Olson, Washington University Medical Center, St Louis) and chromosome blots were individually hybridized with *CLB1*, *CLB2*, *CLB5* and *CLB6* specific probes. *CLB2/CLB5* was mapped to the right arm of chromosome XVI, between 720 and 740 kbp. The nearest mapped genes are *aro7* and *sup16* (Mortimer, 1992).

As mapping position for the *CLB1/CLB6* pair, the right arm of chromosome VII between 510 and 525 kbp was determined. The next known gene is *spt6* (Mortimer *et al.*, 1992), mapping between 525 and 550 kbp.

CLB5 and CLB6 mRNA levels peak early in the cell cycle

During the somatic cell cycle the mRNAs from most of the cyclins analysed so far are periodically expressed (Price *et al.*, 1991; Lew and Reed, 1992). In the cases where the appearance and disappearance of the corresponding gene products were analysed, they paralleled the fluctuations of a particular transcript. Information concerning these fluctuations of cyclin mRNA levels can therefore point to a window of the cell cycle in which the respective proteins are present and most probably functional.

The transcripts for *CLB5* and *CLB6* were analysed in Northern blots from synchronized yeast cells. For this, haploid yeast cells were arrested in G_1 at START with the α-factor mating pheromone and after release from the mating factor arrest the RNA was analysed at subsequent time points. As an external parameter for the quality of the synchronization of the cells used for transcript analysis of the *CLB5* and *CLB6* cyclins the budding index was determined by microscopic examination (Figure 3A). In addition the first cycle after release from α-factor arrest was analysed for DNA content with a fluorescence activated cell sorter (FACS) (Figure 3C). Under the conditions employed the transcripts for *CLB5* and *CLB6* were easily detectable 10−20 min after release (Figure 3B and C), similar to the appearance of the RNA for the G_1-specific cyclin *CLN2* and *HO*-endonuclease (data not shown). Consistent with this is that the *CLB5* and *CLB6* transcripts disappear as soon as small buds emerge indicating the G_1 to S transition border (Figure 3A). Moreover, comparison of the DNA content of the cells used for RNA analysis at a particular time point with the fluctuation of the analysed transcripts shows that the RNA for *CLB5* and *CLB6* disappears at or just before DNA synthesis (determined as the peak between 1N and 2N DNA content). Note that the peak of the arrested cells ('α') still shows a 2N peak which is due to strong clumping of the α-factor treated yeast cells (judged by microscopic examination) and explains the discrepancy with the budding index shown in Figure 3A. However, this does not influence the interpretation of the S phase peak. In the presence of α-factor no transcripts from *CLB5* and *CLB6* are detectable. Preliminary results from time course experiments for the *CLB6* transcripts indicate that the mRNA disappears rapidly after the addition of α-factor. These experiments clearly show that the *CLB5* and *CLB6* mRNAs peak early in the cell cycle. This was somewhat surprising because the two cyclins are related to the B-type cyclin family which have been classified as mitotic cyclins. The neighbouring genes *CLB1* and *CLB2* serving as internal controls for the quality of synchronization showed appearance of their RNA later in the cell cycle, at a point where most if not all the DNA is already replicated (Figure 3B and C) (Ghiara *et al.*, 1991; Surana *et al.*, 1991).

For the regulation of a cell cycle-dependent expression in late G_1 or early S phase from *S.cerevisiae* two types of *cis*-acting transcriptional elements were identified so far: the Swi4−Swi6-dependent SCB cell cycle box (CACGAAA or CNCGAAA) and the *Mlu*I containing MCB cell cycle box (ACGCGT or ACGCGC). These *cis*-acting elements are shown to be specifically bound by protein complexes (Dirick *et al.*, 1992; Lowndes *et al.*, 1992; Primig *et al.*, 1992).

The MCB promoter elements are both necessary and sufficient for late G_1-specific transcription for many genes coding for proteins involved in DNA replication in *S.cerevisiae* (Pizzagalli *et al.*, 1988; Andrews and Herskowitz, 1990). The upstream noncoding region of the *CLB5* gene contains multiple but incomplete MCB motifs (ACGCGC) that could be targets for cell cycle-dependent transcriptional regulation (Epstein and Cross, 1992; C.Kühne, unpublished). The upstream region of *CLB6* contains at comparable positions two perfect matches with the MCB box on the coding strand and one inverted incomplete MCB motif on the noncoding strand (Figure 1B). It is likely that these MCB motifs are involved in the regulation of *CLB5* and *CLB6* transcription in late G_1.

The SCB element was found to be responsible for transcriptional control in late G_1 of the HO endonuclease as well as of the *CLN1* and *CLN2* genes. Whereas *CLB5* does not contain an SCB box in the upstream region analysed (not shown) the *CLB6* gene shows a single CACGAAA motif at position −757 (Figure 1B) upstream of the $tRNA^{Cys}$/Ty3 LTR cluster as indicated above. It is not yet clear if this motif is of any relevance for *CLB6* expression and if it is influenced by a Ty3 insertion [found in various laboratory strains (Hansen and Sandmeyer, 1990)].

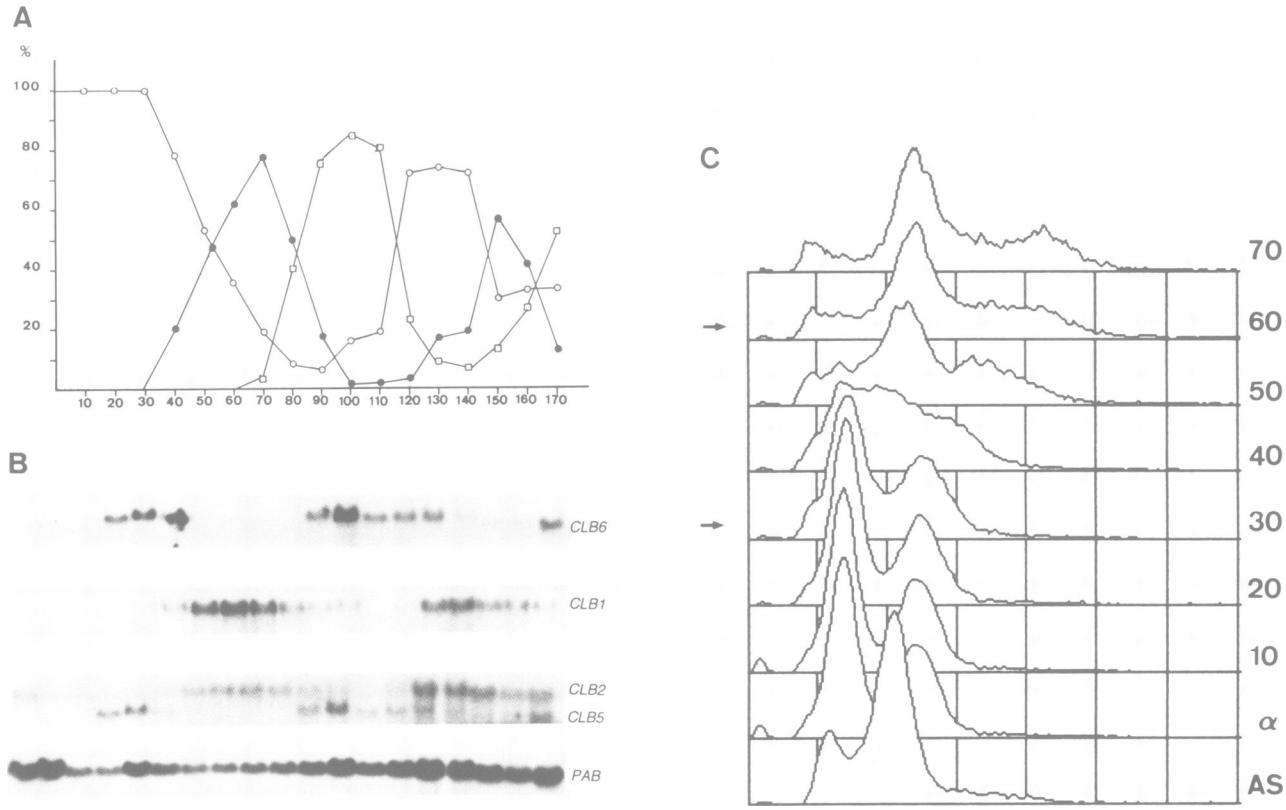


Fig. 3. The mRNAs of *CLB5* and *CLB6* appear early in the cell cycle. Cultures were synchronized by α-factor treatment. Samples for determination of the budding index, RNA preparation and FACS analysis were taken at 10 min intervals. (**A**) For the budding index fixed cells were manually counted under the microscope after release from α-factor arrest at the time points indicated in minutes. Open circles, unbudded cells; closed circles, small buds; open squares, large buds. (**B**) Parallel Northern blots were hybridized with the specific probes indicated at the right. The *PAB1* gene [poly(A) binding protein] was used as a control for the amounts of loaded RNA. The first sample on the left in each row represents RNA preparation from asynchronous cultures prior to synchronization. The second sample in each row represents the RNA preparation from cells at the release point (0 min). (**C**) Analysis of cell number versus DNA content in asynchronous cultures (AS), α-factor arrested cells (α) and cells at 10 min intervals after release in a FACScan. Only the results from the first cell cycle are shown. The arrows indicate the samples in which the *CLB1/CLB2* (upper) and *CLB5/CLB6* (lower) mRNAs are most strongly expressed.

CLB5 and CLB6 are involved in G_1-S progression

As shown above, the abundance of the *CLB5* and *CLB6* transcripts was maximal at the G_1 to S phase transition suggesting a function as G_1 or S phase cyclins. To obtain more information on the time and mode of function of the newly discovered cyclins during the cell cycle, loss of function mutations in these genes were analysed. A gene replacement resulting in a deletion of the entire cyclin box of the *CLB5* gene was not lethal to the cells. However, fluorescence-activated cell sorting (FACS) analysis for DNA content from proliferating *clb5*$^-$ cells showed, compared with otherwise isogenic wild type cells, a greater portion of cells with an intermediate DNA content as judged from the number of cells between the 1N and 2N peaks (Figure 4A). This indicates that clb5$^-$ cells are delayed in S phase. Similar results were obtained by Epstein and Cross (1992). Microscopic examination of *clb5*$^-$ cells revealed that most of the cells were enlarged in size and had elongated daughter cells (Figure 4C). This was further analysed by measuring the relative cell size by forward angle light scattering (FSC) in a flow cytometer (Lew *et al.*, 1992). In the case of the *clb5* mutant population the overall cell size distribution was shifted towards larger cells (Figure 4B). In contrast to the results obtained with *CLB5*, a deletion of most of the *CLB6* gene revealed a higher portion of unbudded cells as judged by microscopic examination. No obvious morphological change for *clb6* mutants was observed (Figure 4B and C). Analysis of the DNA distribution of proliferating *clb6*$^-$ cells, showed a slight but reproducible increase of cells with a 1N and of cells with an intermediate DNA content relative to comparable wild type cells. The increase in the S phase population was not as strongly pronounced as in the case of the *clb5* mutants. The described analysis of the deletion mutants does not distinguish if the observed time points for the delays in late G_1 and S phase represent the time point of function or if this is an indication for an event that appears to occur earlier in the cell cycle.

Based on sequence relatedness, the appearance of the RNA and the observed phenotype of *clb5* and *clb6* mutants, these cyclins form a pair and resemble a new subclass of B-type cyclins. To test for overlapping functions double mutants were analysed for these genes. Therefore a heterozygote diploid for *CLB5/clb5* was transformed with the *CLB6* disruption allele. Transformants that were heterozygote for both genes were sporulated and analysed for progeny carrying both selectable markers indicative of deleted *clb5* and *clb6* alleles. Haploid cells carrying both markers were obtained, indicating that a simultaneous deletion of both of these genes is not lethal. However, cells deleted for *clb5* and *clb6* showed a strong increase in cells with an intermediate DNA content compared with the strains carrying single mutations (Figure 4A). Moreover

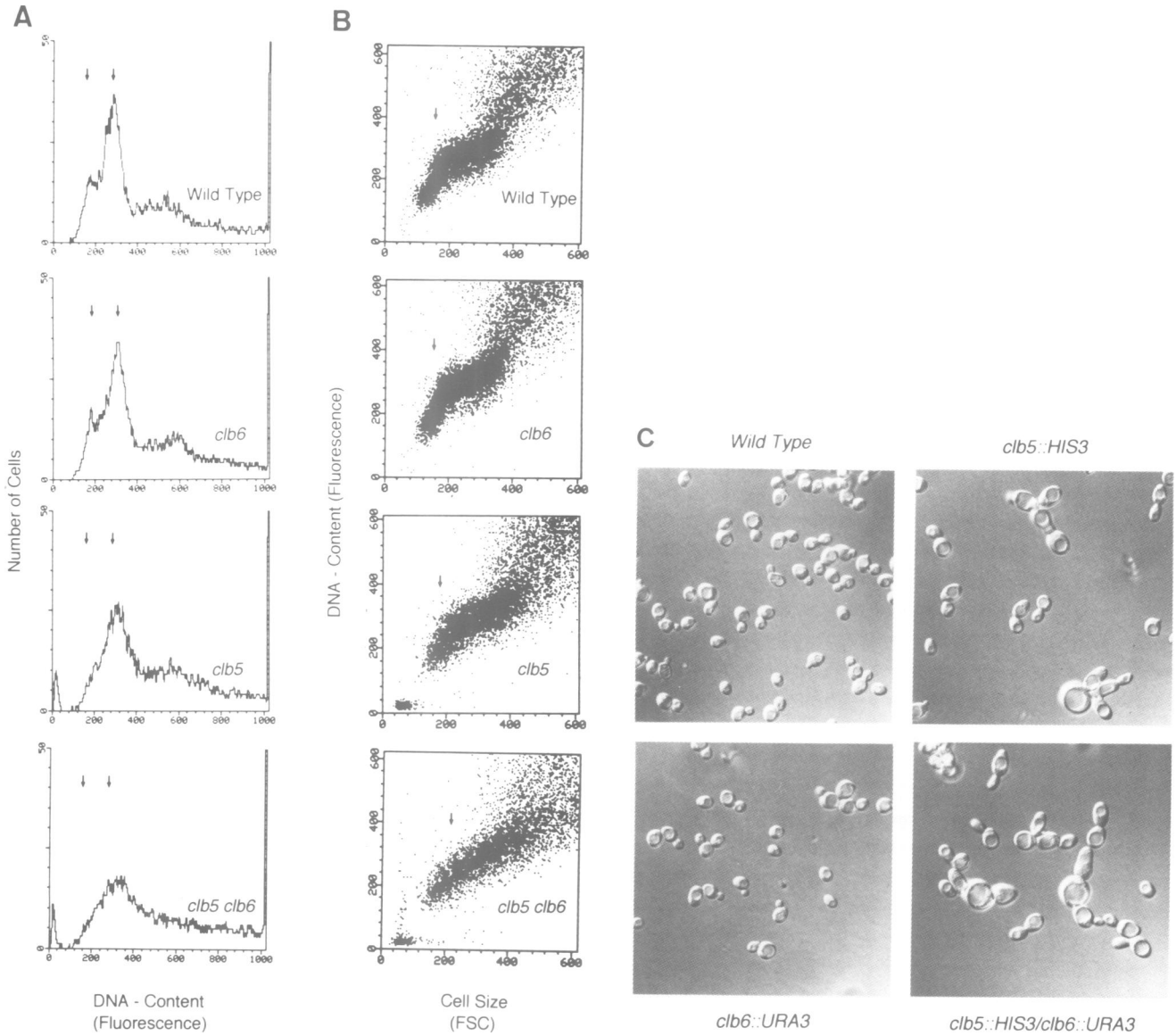
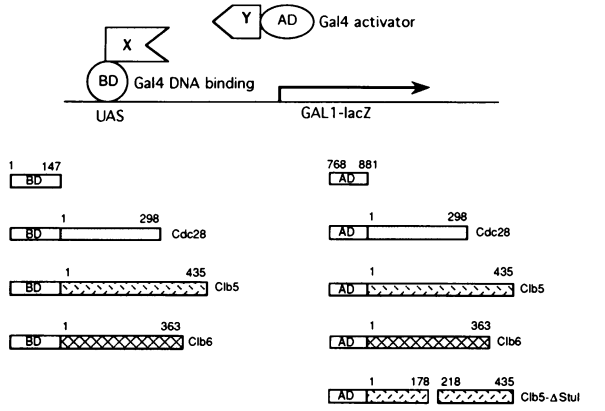


Fig. 4. Distribution in the cell cycle and morphological appearance of *clb5* and *clb6* mutant cells. Cells were exponentially grown in YPD and fixed in 70% ethanol for cell sorting analysis. (**A**) DNA flow cytometry of proliferating cultures from wild type, *clb6::URA3*, *clb5::HIS3* and *clb5::HIS3/clb6::URA3* strains. The two arrows indicate the positions for 1N (left) and 2N (right) DNA contents. (**B**) Dot plot of DNA content (propidium iodide fluorescence) versus cell size (FSC, forward angle light scattering) from proliferating cells. The strains used are as in panel A. The arrows indicate the smallest cells in G_2. (**C**) Differential interference contrast micrographs of exponentially growing wild type, *clb5::HIS3*, *clb6::URA3* and *clb5::HIS3/clb6::URA3* cells.

microscopic inspection and forward angle light scattering showed that the population of the double mutants shifted to a bigger cell size than the single *clb5* mutant (histogram not shown). A dot plot of the cell size (FSC) against the DNA content shows an increase in overall cell size (Figure 4B). Judged by this plot, the minimal budding size determined according to Lew *et al.* (1992) seems not to be affected. The enlarged cell size of the smallest cells in the 2N population seems to be rather due to an overall increase in cell size in the *clb5* and *clb5/clb6* mutants (Figure 4B). These phenotypic observations of double minus mutants are indicative of overlapping but also distinct functions of the cyclins Clb5 and Clb6 early in the cell cycle.

Clb5 and Clb6 interact with p34^{CDC28} in vivo

For Clb1 and Cln2 an interaction with the p34^{CDC28} kinase and the formation of an active kinase complex has been shown (Ghiara *et al.*, 1991; Reed, 1992). It is generally assumed that every specific stage in the cell cycle has its very particular form of p34^{CDC28} kinase complex. At least some of these complexes might be very unstable and difficult to study biochemically. A possible interaction between p34^{CDC28} and the newly discovered cyclins Clb5 and Clb6 was analysed with the 'Gal4 two hybrid system' which allows the detection of protein−protein interactions *in vivo* (Fields and Song, 1989). The basis for this system is the fact that the yeast transcription factor Gal4 carries two distinct domains (Keegan *et al.*, 1986) that can be separated but must be assembled together to form a functional activator. The N-terminal domain binds to specific upstream activating DNA sequences (UAS) and the C-terminal part is necessary to activate transcription. Therefore, a hybrid protein formed by the Gal4 DNA-binding domain with a protein of interest (X) can reconstitute transcriptional activation when this



Gal4 DNA binding-domain-X hybrid	Gal4 activation-domain-Y hybrid	Colony color *	units of β-galactosidase
pGBT9	pGAD424	White	< 0.1
Cdc28	Clb5	Blue	9.4
Cdc28	Clb6	Blue	11.5
Cdc28	Clb5-ΔStul	White	< 0.1
Clb5	Cdc28	Blue	8.7
Clb6	Cdc28	Blue	9.3
Clb5	pGAD424	White	< 0.1
Clb6	pGAD424	White	< 0.1
pGBT9	Clb5	White	< 0.1
pGBT9	Clb6	White	< 0.1

Fig. 5. *In vivo* Clb5, Clb6 and p34^{CDC28} protein−protein interactions. The fusions with the DNA binding and the activation domains are shown. Amino acid are indicated by numbers above the constructs. The reporter strain carrying the plasmid constructs indicated in the first and second columns were grown on selective SD plates. The colour of the colony and the enzyme activities are given in the third and fourth columns. The β-galactosidase units in the ONPG assay were calculated according to Guarente (1983). *, Incubation for colour development was for <1 day. BD, Gal4 binding domain; AD, Gal4 activation domain.

protein interacts with a second protein (Y) fused to the Gal4 transcription activating domain. This activation is detected by a galactokinase promoter−*lacZ* reporter gene (*GAL1*−*lacZ*) (for illustration see Figure 5). This method should enable examination of transient interactions occurring only in a short period in the cell cycle. The fact that a stable protein is obtained from the gene product of the used reporter gene allows the accumulation of a weak signal over time (Chien *et al.*, 1991).

All possible combinations of fusions with the Gal4 DNA binding domain and the Gal4 activator domain with the entire ORFs of the *CDC28*, *CLB5* and *CLB6* genes were constructed. These hybrid genes were then used to test for protein−protein interaction in a haploid yeast strain with the appropriate genetic background (*GAL1*−*lacZ*). Interaction was detected by testing the various combinations for β-galactosidase activity by the appearance of blue colonies on X-gal containing plates and quantitated by the ONPG assay (Figure 5). All combinations of the *CDC28* gene and one of the cyclin genes expressed in this system resulted in the production of β-galactosidase activity. The observed *lacZ* expression was independent of the fusion type (activation or binding domain) of *CDC28* and *CLB5* or *CLB6* (Figure 5). No activity was detected if only one of the two plasmids carried a fusion with one of these genes. To confirm the specificity of the interaction of the cyclins with the p34^{CDC28} kinase in the two hybrid system, an in-frame deletion was made in *CLB5* (Leu178 to Thr218; Figure 2B). The deleted region is highly conserved among cyclins and was shown to be essential for the *in vitro* interaction of A-type cyclins with its kinases (Lees and Harlow, 1993). This construct did not give any measurable β-galactosidase activity. Based on these criteria Clb5 and Clb6 interact with p34^{CDC28} *in vivo*. This finding is supported by the observation that overexpression of a N-terminal truncated version of the Clb5 protein is lethal at permissive temperature for a strain carrying the ts *cdc28-4* mutation, whereas the truncated protein is tolerated in an isogenic wild type strain (C.Kühne, unpublished results).

Discussion

Gene duplications are generally assumed to be a major driving force for evolution of life (Li and Gradur, 1991). In this communication an example of gene duplications leading to members of a gene family that is central to the control of the progression of the cell division cycle, the cyclins, is described. In *S.cerevisiae* the genes for the mitotic cyclins Clb1 and Clb2 together with the B-type cyclin homologue Clb5 (recently described by Epstein and Cross, 1992) and a yet previously undescribed cyclin Clb6, are arranged as inverted repeats forming the arrays *CLB1/CLB6* and *CLB2/CLB5*. Based on their sequence the cyclins in these clusters form the subgroups *CLB1/CLB2* and *CLB5/CLB6*. Surprisingly, the function of the group *CLB5/CLB6* is quite distinct from their respective neighbouring genes *CLB1/CLB2* and shows some novel features for B-type cyclins.

First, these genes are transcribed early in the cell cycle at the G_1 to S transition border. The upstream sequences of *CLB5/CLB6* contain multiple MCB elements that are likely to be *cis*-acting elements for cell cycle-regulated expression of these genes in late G_1 similar to many proteins involved in DNA replication. This early appearance of a cyclin transcript in *S.cerevisiae* is so far only reported for the 'G_1 cyclins' *CLN1* and *CLN2*. However, the *CLN1* and *CLN2* expression in late G_1 is regulated by an SCB element.

Second, the FACS analysis shows that *clb5::HIS3* mutants are delayed in S phase progression and *clb6::URA3* mutants exhibit a delay in late G_1 and/or early S phase, demonstrating a function early in the cell cycle. This is consistent with the fluctuation pattern of the corresponding transcripts.

The function of Clb5 partially overlaps with the Clb6 function

Although double minus mutants are not lethal, they show an enhancement of the single mutant phenotypes resulting in enlarged cells that are strongly delayed in S phase. Clearly, *clb5* mutations affect S phase progression more than do *clb6* mutations. The enhancement of the phenotype for *clb5* in a *clb6*$^-$ background argues for a partially overlapping function. Since Clb5/Clb6 are not essential for viability of cells, which is also observed for various combinations of other *clb* or *cln* mutants, it is likely that there are other genes that compensate for Clb5/Clb6 deficiency in *S.cerevisiae*. Southern blot analysis under lowered stringency and results obtained during the isolation of the *CLB1/CLB6* genomic locus suggest that there is at least one other undescribed B-type cyclin homologue in *S.cerevisiae*.

Clb5 and Clb6 interact with p34^{CDC28}

In *S.cerevisiae* an association of the p34^{CDC28} kinase activity in G_2 and early M phase with the B-type cyclin Clb1 was shown (Ghiara *et al.*, 1991). Since Clb1, Clb2, Clb3 and Clb4 have overlapping functions it is likely that they all interact with the p34^{CDC28} kinase. Although we have not shown a Clb5- or Clb6-dependent kinase activity, we could demonstrate an *in vivo* protein−protein interaction of these cyclins with p34^{CDC28}. It is quite possible that Clb5 and Clb6 are involved in the maintenance of the kinase activity in early S phase suggested by the results of Surana *et al.* (1991).

In higher eukaryotes several kinases can interact with different types of cyclins at particular points in the cell cycle. In yeast, however, so far only one cyclin-dependent kinase is known. The interaction of several cyclins with a particular member of the kinase family (p34^{CDC28}) should define conserved structural features of these cyclins. This could explain that in yeast B-type cyclins are found to function at different points in the cell cycle. The fact that the early appearing Clb5 and Clb6 cyclins interact with p34^{CDC28} is consistent with this explanation. In higher eukaryotes it is possible that one of the described cyclins for G_1 and S phase functions analogous to Clb5 and Clb6 and that the highly diverged primary amino acid sequences evolved ectopically with the different members of the p34 kinase family associated with these proteins. Alternatively, the functions of the early B-type cyclins might be specific for budding yeast. In higher eukaryotes analogous functions, if they exist at all, might occur later in the cell cycle. Finally, it might be that the corresponding B-type cyclins have not yet been found.

Redundancy of S.cerevisiae B-type cyclins

B-type cyclins known from *S.cerevisiae* form pairs with partially overlapping functions. From the sequence relatedness of the two pairs of cyclins reported here, it is most likely that the intrachromosomal duplications occurred prior to the interchromosomal event. In fact the sequences show that the genes present in an intrachromosomal cluster are significantly more diverged from each other than is the case for the corresponding genes on the other chromosome. This is consistent with their apparent function. *CLB1* and *CLB2* form a pair exhibiting overlapping essential functions with mutants in *CLB2* having a stronger effect. For the pair *CLB5/CLB6* mutations in the *CLB5* gene have a much stronger phenotype. It is interesting to note that *CLB5* lies downstream of the stronger mitotic allele *CLB2*. Because *CLB5* is more diverged from its neighbour *CLB2* than is *CLB6* from *CLB2* and *CLB1*, we favour the idea that the *CLB2/CLB5* cluster is the ancestor of the *CLB1/CLB6* cluster. The tRNAs and the Ty flanking sequences could have served as targets for a DNA mediated interchromosomal gene duplication event. The presence of Ty sequences prompts us to speculate that the interchromosomal gene duplication could have taken place by retroevolution of an ancestral cluster by reverse transcription from a pre-mRNA spanning both genes. The potential for retroevolution of a heterologous gene between two LTRs by Ty reverse transcriptase is well documented (Derr *et al.*, 1991; Derr and Strathern, 1993). The complete answer about the existence of similar clusters in other cyclins and for other genes representing a gene family with subgroups of genes will be obtained, at least for *S.cerevisiae*, as soon as other loci of cyclin genes have been analysed or the genome sequencing project will be completed.

Materials and methods

Yeast strains, media and genetic methods

All yeast strains used in this study were, unless otherwise described, derivatives of the wild type strain CW04 (*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3*) (Banroques *et al.*, 1986). The *bar1* strain RH448 (*MATa leu2 his4 lys2 ura3 bar1*) was obtained from H.Riezman. The Y526 strain for the 'Gal4 two hybrid' system was obtained from S.Fields. Standard genetic procedures and media for yeast were used (Sherman *et al.*, 1986). Yeast transformations were carried out by the LiCl method (Ito *et al.*, 1983). The genomic one-step transplacements were performed as described (Rothstein, 1983).

Cloning and sequencing

The *CLB5* containing region was isolated during a chromosomal walk designed for a gene lying upstream of the *CLB2/CLB5* locus (data not shown). A *Sau*3A−*Xba*I fragment containing *CLB5* and upstream regions was cloned into the *Bam*HI−*Xba*I sites of pTZ18R and pTZ19R (Pharmacia). For *CLB2* a 4 kb *Xba*I fragment (Figure 1) was cloned into pTZ18R in both orientations. For cloning of the downstream region of the *CLB1* locus a yeast genomic library in YEp13 (Nasmyth and Reed, 1980) was screened by colony hybridization. As a hybridization probe, a fragment obtained by PCR amplification from genomic yeast DNA with appropriate primers spanning exactly the entire reading reading frame of *CLB1* was used. The *CLB6* gene was isolated from a YEp13 clone containing the *CLB1* gene. A 6.2 kb fragment was subcloned in both orientations using *Xba*I (cutting in the insert and in the 2μ vector from YEp13) into pTZ18R. Subsequently the clones were trimmed to smaller sizes by nested deletion with *Exo*III and mung bean nuclease (Stratagene protocol). DNA sequence analysis with the T7 polymerase sequencing kit (Pharmacia) was performed on subclones in the vector pTZ18R (Pharmacia) or by using specific oligonucleotides. The subclones were obtained from nested deletions.

'Gal4 two hybrid' system

The *CDC28* gene was isolated by PCR amplification from genomic DNA using specific oligonucleotides designed from the ATG codon with a *Bam*HI extension and an oligonucleotide covering the Stop codon with a *Sal*I extension. The functionality of this isolate was verified by its ability to give a galactose-dependent rescue of the conditional ts *cdc28-4* allele when placed under the heterologous GAL1 promoter (data not shown). The *CDC28* gene was also cloned into the *Bam*HI/*Sal*I sites of the vectors pGBT9 and pGAD424.

The *CLB5* gene was cloned into the *Bam*HI/*Sal*I sites of the vectors pGBT9 and pGAD424 by using an insert obtained by PCR amplification of the entire reading frame with a specific oligonucleotide around the ATG and the universal primer oligonucleotide in the pTZ18 vector. The *CLB6* gene was cloned into the *Bam*HI/*Sal*I sites of the vectors pGBT9 and pGAD424 (Fields and Song, 1989) similar to *CLB5*. The internal deletion in *CLB5* in the pGAD424 plasmid was obtained by *Exo*III/Mung Bean digestion at the single *Stu*I site (+582 bp from ATG) and religation. Resulting plasmids were sequenced to confirm the reading frame.

For measurement of β-galactosidase activity three independent clones from each of the constructions were analysed. All three replicates of each plasmid tested gave essentially the same results. Plasmids were transformed individually into strain Y526. Transformed cells were streaked on selective plates and after 2 days the cells were transferred onto nitrocellulose filters which were then incubated on X-gal (5'-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg/ml)-containing SD plates. Alternatively the colonies from the transformation plates were directly transferred to X-gal-containing plates by the filter replica method. For quantification of β-galactosidase activities from cells grown on selective plates the *o*-nitrophenyl-β-D-galactoside (ONPG) method was used (Guarente, 1983).

Cell cycle synchronization by α-factor treatment

Mating pheromone α-factor arrest experiments were carried out by growing 1 l of the haploid bar1$^-$ strain RH448 to a density of A_{600} = 0.4 in YPD at 25°C followed by a treatment with 100 ng/ml of synthetic α-factor peptide (Sigma T-6901) for 150 min at 25°C. Cells were collected by brief centrifugation, washed twice in conditioned YPD medium and once in fresh YPD medium and then incubated for further 170 min in conditioned medium 1:1 diluted with fresh YPD. Conditioned YPD medium was obtained by growing the BAR1$^+$ strain CW04 to a density of A_{600} = 0.4 and then

filtered through a 0.45 μm sterile filter. The filtrate was taken as conditioned medium. For RNA preparation 30 ml samples were taken at intervals of 10 min and immediately filtered through a 1.2 μm filter. The cells were resuspended in ice-cold double-distilled water, centrifuged and immediately frozen in dry ice–ethanol. The pellets were stored at −70°C prior to RNA preparation. In addition samples were fixed in 70% ethanol at each time point for FACS analysis and in 3.7% formaldehyde for determination of the budding index.

RNA purification and Northern analysis

Total yeast RNA was prepared by the hot phenol method according to Domdey *et al.* (1984) with minor modifications. Briefly, 300 μl 50 mM sodium acetate (pH 5.3), 10 mM EDTA, 30 μl 10% SDS and 500 μl of phenol saturated with the same buffer was added to the frozen pellet and the tubes were placed at 60°C for 10 min with occasional mixing. Then the tubes were placed on dry ice–ethanol and the frozen extractions were centrifuged for 15 min in a microcentrifuge. The phenol phase was discarded and new acid-saturated phenol was added for a second round of heating and freezing. After centrifugation the supernatant was re-extracted in phenol–chloroform and the supernatant was precipitated with ethanol in the presence of LiCl (final concentration 0.8 M). Total RNA was separated on 1% agarose gels containing formaldehyde and transferred to nylon membranes by the capillary method. The hybridization was performed as described by Sambrook *et al.* (1989). Specific hybridization probes were radiolabelled with [α-^{32}P]dATP according to standard methods. All the DNA hybridization probes were gel-purified PCR fragments obtained by amplification of the DNA encoding the entire reading frame of the genes used with custom-synthesized DNA oligonucleotide primers.

Construction of CLB5 and CLB6 loss of function mutations

For construction of synthetic null alleles of the *CLB5* and *CLB6* genes large fragments were replaced with the auxotrophic markers *HIS3* and *URA3*, respectively. The *CLB5* null allele (*clb5::HIS3*) was constructed starting from a clone obtained during sequence analysis spanning from coordinate +423 (from the ATG) to the *Xba*I site in pTZ18R. In this construction a 1.1 kb *Stu*I–*Cla*I fragment containing the cyclin box region was replaced with a 1.3 kb *Bam*HI–*Xho*I fragment encoding the *HIS3* gene. This construct was then excised with *Eco*RI and *Xba*I and used to transform an isogenic diploid derivative of CW04.

For the construction of a *CLB6* null allele (*clb6::URA3*) the vector pTZ18R containing the 2.14 kb *Bgl*II–*Xba*I fragment of the *CLB6* locus was used. A 0.55 kb *Cla*I–*Ssp*I fragment containing the cyclin box information was replaced with a 1.1 kb *Bgl*II fragment containing the *URA3* gene (Bonneaud *et al.*, 1991). This construct was then excised with *Hin*dIII and used to transform an isogenic diploid derivative of CW04. For construction of *clb5/clb6* double mutants a heterozygote diploid containing a *clb5::HIS3* allele was transformed with the *clb6::URA3* allele. The obtained diploid strains were sporulated for 7 days on sporulation plates and dissected onto YPD plates and incubated at 30°C. The spore clones were analysed for their respective prototrophies.

Flow cytometry and cell counting

For analysis of DNA content using flow cytometry the cells were fixed in 70% ethanol, stained with propidium iodide as described (Nash *et al.*, 1988) and analysed for fluorescence using a Becton-Dickinson FACScan analyser with the Lysis II software.

To determinate the percentages of budding cells for the estimation of the quality of the synchronization, the same number of cells fixed in 3.7% formaldehyde were examined for the absence of buds, small buds and large buds using a haemocytometer and a Zeiss microscope with a 40 × objective. Photographs of unfixed cells were taken in a Zeiss Axiophot microscope using a 100× Plan-Apochromat objective.

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