Switching transcription on and off during the yeast cell cycle: Cln/Cdc28 kinases activate bound transcription factor SBF (Swi4/Swi6) at Start, whereas Clb/Cdc28 kinases displace it from the promoter in G₂

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When yeast cells reach a critical size in late G₁, they simultaneously start budding, initiate DNA synthesis, and activate transcription of a set of genes that includes G₁ cyclins *CLN1*, *CLN2*, and many DNA synthesis genes. Cell cycle-regulated expression of *CLN1*, *CLN2* genes is attributable to the heteromeric transcription factor complex SBF. SBF is composed of Swi4 and Swi6 and binds to the promoters of *CLN1* and *CLN2*. Different cyclin-Cdc28 complexes have different effects on late G₁-specific transcription. Activation of transcription at the G₁/S boundary requires Cdc28 and one of the G₁ cyclins Cln1–Cln3, whereas repression of SBF-regulated genes in G₂ requires the association of Cdc28 with G₂-specific cyclins Clb1–Clb4. Using *in vivo* genomic footprinting, we show that SBF (Swi4/Swi6) binding to SCB elements (Swi4/Swi6 cell cycle box) in the *CLN2* promoter is cell cycle regulated. SBF binds to the promoter prior to the activation of transcription in late G₁, suggesting that Cln/Cdc28 kinase regulates the ability of previously bound SBF to activate transcription. In contrast, SBF dissociates from the *CLN2* promoter when transcription is repressed during G₂ and M phases, suggesting that Clb1–Clb4 repress SBF activity by inhibiting its DNA-binding activity. Switching transcription on and off by different mechanisms could be important to ensure that Clns are activated only once per cell cycle and could be a conserved feature of cell cycle-regulated transcription.

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The timing of different cell cycle transitions is determined by the sequential activation and inactivation of different cyclin-dependent kinases (CDKs) and their associated regulatory subunits, called cyclins. How are the different waves of CDK activity generated? It is now clear that several mechanisms control cyclin/CDK activities during the cell cycle. These include regulated proteolysis of cyclins, post-translational modifications, specific inhibitors, and the regulated synthesis of CDKs and cyclins (Morgan 1995). The different waves of cyclin/CDK activity are partly generated by transcriptional regulation of cyclin genes.

In the budding yeast *Saccharomyces cerevisiae*, transcription of most cyclin genes is cell cycle regulated. There are at least nine different cyclin subunits that

specify the different functions of the Cdc28 CDK during the cell cycle. G₁ cyclins Cln1, Cln2, and Cln3 are required for entry into the cell cycle in late G₁. B-type cyclins Clb5 and Clb6 promote initiation of S phase, and B-type cyclins Clb1, Clb2, Clb3, and Clb4 control entry into mitosis.

At a point in late G₁, called Start, cells activate a transcriptional program leading to the accumulation of G₁ cyclins *CLN1*, and *CLN2*, and *CLB5*, and *CLB6*, as well as many S-phase proteins (Koch and Nasmyth, 1994). Around the same time, cells initiate DNA replication, start budding, and duplicate their spindle pole bodies. All of these events require the Cdc28 kinase and one of the Cln cyclins. Mutations causing increased stability of Cln proteins or ectopic expression of *CLNs* leads to premature entry into the cell cycle, suggesting that G₁ cyclin activity may be rate limiting for starting the cell cycle (Nash et al. 1988; Tyers et al. 1993; Dirick et al. 1995).

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CLN3 is expressed constitutively throughout the cell cycle (Tyers et al. 1993), whereas *CLN1* and *CLN2* RNAs as well as Cln1- and Cln2-associated kinase activity accumulate transiently at the G_1 -S boundary when cells reach a critical cell size and start a new cell cycle (Wittenberg et al. 1990). The timing of Start is thought to be determined by the transcriptional activation of *CLN1* and *CLN2* (Dirick et al. 1995; Tyers et al. 1993). How they are regulated is therefore an important question if we are to understand the events controlling entry into the cell cycle.

CLN1 and *CLN2* belong to a larger class of late G_1 -specific RNAs whose expression is attributable to a pair of related heteromeric transcription factors called SCB binding factor (SBF) and MCB binding factor (MBF) (Koch and Nasmyth 1994). SBF is a complex containing the Swi4 and Swi6 proteins. It binds to SCB (Swi4–Swi6 cell cycle box, CACGAAA) elements found in the *CLN1* and *CLN2* promoters (Nasmyth and Dirick 1991; Ogas et al. 1991), the *HO* gene (Andrews and Herskowitz 1989; Breeden and Nasmyth 1987a; Nasmyth 1985), and the cyclin-like genes *PCL1* and *PCL2* (Espinoza et al. 1994; Measday et al. 1994). The *CLN2* promoter contains a second, minor, upstream activating sequence (UAS) sequence, which can confer low levels of cell cycle-regulated *CLN2* transcription (Cross et al. 1994; Stuart and Wittenberg 1994). A complex related to SBF, called MBF (also known as DSC-1), contains the Mbp1 and Swi6 proteins (Dirick et al. 1992; Koch et al. 1993; Lowndes et al. 1992) and binds to MCB (*Mlu*I cell cycle box, ACGCGTNA) elements found in the promoters of many DNA synthesis genes, including *CLB5* and *CLB6* (Koch and Nasmyth 1994). Mutants lacking both SBF and MBF arrest the cell cycle in late G_1 , indicating that gene activation is essential for cell cycle entry (Nasmyth and Dirick 1991; Ogas et al. 1991; Koch et al. 1993). Related transcription factors Cdc10, Res1, and Res2 perform equivalent functions in *Schizosaccharomyces pombe* (Tanaka et al. 1992; Caligiuri and Beach 1993; Miyamoto et al. 1994; Zhu et al. 1994). In animal cells, the E2F/DP1 class of transcription factors, though structurally not related to SBF, performs similar functions (La Thangue 1994).

Thus, the question of how *CLN1* and *CLN2* activity is regulated gets down to what regulates SBF activity. Activation of *CLN1* and *CLN2* transcription by SBF in late G_1 requires Cdc28 and Cln cyclins. Any one of the cyclins is capable of activating transcription when ectopically expressed. It has therefore been proposed that the sudden appearance of Cln1 and Cln2 may be important for generating the sharp peak of SBF-dependent transcription in late G_1 (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991). Recent experiments, however, have shown that only *CLN3* is required for activation when wild-type daughter cells reach the critical size (Dirick et al. 1995), indicating that the Cln3/Cdc28 kinase alone is normally responsible for activating late G_1 -specific transcription. It is not known how Cln3 activates late G_1 -specific transcription at Start nor is it known whether activation is caused by changes in Cln3-associated kinase activity during G_1 .

One possible mechanism for induction of *CLN2* expression by SBF would be changes in SBF levels. The abundance of *SWI4* RNAs is cell cycle regulated with a peak in late G_1 (Breeden and Mikesell 1991; Foster et al. 1993). However, several lines of evidence suggest that the accumulation of de novo synthesized Swi4 might not be the main cause for transcriptional activation of *CLN1* and *CLN2* in late G_1 . First, activation of *CLN1* and *CLN2* transcription upon release from *cdc28*-induced G_1 arrest can, at least partly, occur without de novo protein synthesis (Marini and Reed 1992). Second, DNA-binding activity of SBF is detectable in bandshift experiments throughout the cell cycle and does not change much at Start (Taba et al. 1991).

Whereas activation of *CLN1* and *CLN2* transcription depends on Cln3 cyclin, repression in G_2 and M phases requires mitotic cyclins Clb1–Clb4 (Amon et al. 1993). Activation and repression are therefore attributable to different cyclin–Cdc28 complexes and presumably occur by different mechanisms. How Clbs repress *CLN1* and *CLN2* transcription in G_2 and M phases is not known, but the finding that Swi4 is present in immunoprecipitates of Clb2 (Amon et al. 1993) suggested that Swi4 might be a target of Clb1–Clb4/Cdc28 kinases. Consistent with a functional role for this interaction, Mbp1, whose activity is repressed independently of Clb1–Clb4 (Amon et al. 1993), does not coimmunoprecipitate with Clb2 (R. Siegmund, pers. comm.).

To understand how different cyclins have such different effects on *CLN1* and *CLN2* transcription, we analyzed SBF binding in vivo. We find that SBF binds to SCB elements in the *CLN2* promoter in a cell cycle-regulated fashion. SCB elements are occupied in cells arrested prior to Start, as well as in small wild-type daughter cells. In contrast, SCBs are not occupied in G_2 and M-phase cells that contain high Clb kinase activity. Thus, transcriptional activation in late G_1 is mediated by Cln-dependent activation of previously bound SBF, whereas repression in G_2 is mediated by Clb-dependent dissociation of SBF from the promoter. We propose that switching *CLN1* and *CLN2* transcription on and off by different mechanisms could be important to ensure that Clns are activated only once per cell cycle.

Results

In vivo footprinting of the CLN2 promoter

In vitro bandshift experiments have shown that SBF-binding activity is present throughout the cell cycle (Taba et al. 1991), suggesting that SBF might be bound to the promoter all the time. To test this possibility, we analyzed the occupancy of SCB elements in the *CLN2* promoter by in vivo footprinting with dimethylsulfate (DMS). DMS methylates DNA at accessible guanines and, to a lesser extent, at adenosines, providing a chemical probe for protein–DNA interactions. We chose to analyze the *CLN2* promoter because it contains three potential SCB elements in a region shown to be required for high-level *CLN2* expression (Cross et al.

1994; Stuart and Wittenberg 1994) [Fig. 1A]. These sites are recognized by SBF in vitro (Primig et al. 1992), and *SWI4* is required for normal levels of *CLN2* expression (Nasmyth and Dirick 1991; Ogas et al. 1991).

To detect SBF binding we compared initially the DMS methylation pattern around the SCB elements in asynchronously growing wild-type, *swi4*, and *swi4 mbp1* mutant cells (Fig. 1C). Only small differences in DMS reac-

Figure 1. SBF-binding sites in the *CLN2* promoter. (A) Location of putative SCB elements; potential TATA box sequences and mapped transcription start sites (Ogas et al. 1991) are indicated. Sequences between the *NruI* site at -605 and the *SphI* site at -505 are required for high-level *CLN2* transcription [Cross et al. 1994; Stuart and Wittenberg 1994]. (B) Sequences of the *CLN2* promoter between -480 and -679 . Arrowheads mark the 3' end of PCR primers used. Putative SCB elements are boxed (consensus: CACGAAA). Solid and open triangles indicate sites of DMS protection and enhancement, respectively. (C) Genomic footprinting of the *CLN2* promoter. The pattern of DMS modification allowed unambiguous assignment of each band to the sequences shown in B. Sites of DMS protection and enhancement are marked as in B. The assignment of selected guanosine residues to the *CLN2* promoter sequence is indicated. (Lanes 1,5) Wild type (K1107); (lanes 2,6) *cdc28-13* (K3446) arrested in late G_1 by growth at 37°C for 2.5 hr; (lanes 3,7) *swi4::LEU2* (K1939); (lanes 4,8) *swi4 mbp1 GAL-CLN1* (CY130) grown in YEPGal. (Lanes 1–4) Analysis of lower strand (CLN2-1 primer); (lanes 5–8) analysis of upper strand (CLN2-2 primer). (D) Densitometric scanning of genomic footprinting data shown in C (top) lanes 1 and 4; (bottom) lanes 2 and 4. (E) Northern blot analysis of *CLN2*, and *PCL1* RNA levels of the cultures analyzed in C. (cyc) Cycling cells; *CMD1* RNA levels serve as loading control. (F) FACS profile of cultures analyzed in C.

tivity were detected, but densitometric tracing suggested that SCB elements in the *CLN2* promoter are partly protected from methylation in wild-type cells compared to *swi4* and *swi4 mbp1* mutant cells (Fig. 1D). SBF binding might only partly protect SCBs from methylation, in which case DMS would not be appropriate for genomic footprinting. Alternatively, SBF may only be bound in a fraction of the wild-type cells because SBF binding is cell cycle regulated.

To distinguish between these possibilities, we analyzed a cell cycle mutant arrested in G_1 . *cdc28-13* mutants were grown at 37°C until they were arrested in G_1 (Fig. 1F) and analyzed for DNA binding of SBF (Fig. 1C) and SBF-dependent transcription (Fig. 1E). As shown in Figure 1C, residues in all SCB elements are almost fully protected against methylation in *cdc28-13* mutants arrested in G_1 but not in *swi4* mutants. In addition, sequences between the SCB elements contain sites of enhanced methylation that are absent in *swi4* mutants. The sites of enhanced methylation may reflect structural changes within this region of the promoter, presumably because of protein–protein interactions between complexes bound to adjacent SCB elements. Sites of protection and enhanced methylation were also detected on the upper strand, but the involved adenosines are methylated only weakly and the data are therefore difficult to interpret. A summary of the DMS protection pattern observed in *cdc28-13* mutants is shown in Figure 1B. The protected sites correspond to the weakly protected sites found in asynchronously growing wild-type cells and are presumably due to the binding of SBF to SCB elements. We conclude that the partial protection observed in asynchronously growing wild-type cells (Fig. 1D) reflects cell cycle changes of SBF binding and that SCB elements are fully occupied in *cdc28* mutants arrested in G_1 .

DMS protection of SCB elements in G_1 is attributable to binding of SBF

To test whether the DMS protection detected in *cdc28* mutants is caused by binding of SBF to SCBs we analyzed the methylation pattern in *cdc28* mutants lacking SBF or MBF. Figure 2 shows that SCBs are fully protected in *cdc28* and *cdc28 mbp1* double mutants arrested in G_1 . The same sites are weakly protected in the *cdc28 swi6* double mutant, which may reflect weak binding of Swi4 in the absence of Swi6. This is consistent with genetic data indicating that Swi4 has residual activity in the absence of Swi6, at least in large cells (Breedon and Nasmyth 1987b; Nasmyth and Dirick 1991). In the *cdc28 swi4* mutant, SCBs are largely accessible to DMS, although a comparison with the methylation pattern in asynchronously growing *swi4 mbp1* mutants (Fig. 1) or *cdc13* mutants arrested in G_2 (see below) suggests minor protection also in the *swi4* mutant. This residual protection might be attributable to binding of MBF in the absence of Swi4, as suggested by the finding that SBF and MBF have partly overlapping functions (Koch et al. 1993).

Two main conclusions can be drawn from these exper-

Figure 2. Methylation protection of SCB elements in the *CLN2* promoter is dependent on SBF. *cdc28* mutants grown in YEPD were arrested in G_1 by growing them at 37°C for 2.5 hr. The lower strand of the *CLN2* promoter was analyzed by DMS footprinting [sites of protection are marked as in Fig. 1]. (Lane 1) *cdc28-13* (K3446); (lane 2) *cdc28-13 swi4::LEU2* (K5133); (lane 3) *cdc28-13 swi6::TRP1*, (K5134); (lane 4) *cdc28-13 mbp1::URA3* (K5135).

iments. First, the methylation protection of SCBs in *cdc28* mutants is dependent on Swi4 and Swi6 and presumably caused by binding of SBF to SCB elements. Consistent with this interpretation, the bases protected in site II correspond to sites where chemical modification interferes with Swi4 binding in vitro (Primig et al. 1992). The second conclusion from these experiments is that SBF can bind to SCB elements in the absence of Cdc28 activity.

Binding of SBF (Swi4/Swi6) to the *CLN2* promoter is cell cycle regulated and is absent in cells with high *Cln* kinase activity

We first addressed at which stages of the cell cycle SBF binds to the *CLN2* promoter using cell cycle mutants to arrest cells at different stages of the cell cycle. As in *cdc28-13* mutants, SCBs are fully occupied by SBF in cells arrested in late G_1 by *Cln* cyclin depletion (Figure 3A, lane 7), that is, prior to the activation of *CLN2* tran-

Figure 3. Binding of SBF to SCB elements is cell cycle regulated. (A) Genomic footprinting of lower strand of the *CLN2* promoter. Cultures of *cdc* mutants and wild-type strains were grown to an OD_{600} of 0.6 in YEPD, shifted to 37°C for 2.5 hr to induce cell cycle arrest, and analyzed for binding of SBF. (Lane 1) Wild-type (K699); (lane 2) *cdc28-13* (K3446); (lane 3) *cdc13* (K2035); (lane 4) *cdc15* (K1993); (lane 5) *clb1-4ts* (K3080); (lane 6) *cdc13* (K2035); (lane 7) *cln1*, *cln2*, *cln3*, *GAL-CLN3* (K3130) arrested in G₁ by growing in YEP/2% raffinose for 3 hr; (lane 8) *cdc4* (K4083) arrested at 37°C for 2.5 hr; (lane 9) *swi6::TRP1* (K1970), grown at 30°C in YEPD. The asynchronously growing *swi6* mutant (used as a control here) shows a protection pattern similar to G₁-arrested *swi6* mutants (see Fig. 2) (B) Northern analysis of cell cycle arrested cultures shown in A. Blots were hybridized to a *CLN2* and a *PCL1* probe. (*CMD1*) Encoding calmodulin, served as a loading control. (C) FACS analysis of cultures analyzed in A.

scription (Fig. 3B). In contrast, no binding was detected in *cdc13* mutants arrested at the end of S phase or in *cdc15* mutants arrested in late anaphase (Fig. 3C). SCBs are also not occupied in cells arrested in G₂ by treatment with the microtubule drug nocodazole (data not shown). SBF-regulated genes are repressed at all of these arrest points (see also Fig. 3B). In summary, SBF is bound to SCB elements in cell cycle mutants arrested in G₁ but not in mutants arrested in G₂ or M phase. SBF binding may therefore be cell cycle regulated.

What causes dissociation of SBF from the promoter in G₂ and M phases? Both *cdc13* and *cdc15* mutants arrest with high levels of Clb2-associated Cdc28 kinase activity (Surana et al. 1993; Amon et al. 1994). We therefore tested SBF binding in mutants lacking mitotic Clb/Cdc28 kinase activity. In mutants deleted for Clb1, Clb3, and Clb4, carrying a temperature-sensitive allele of Clb2 (*clb1-4ts* mutants), none of the mitotic Clbs is active. *clb1-4ts* cells arrest in G₂ and fail to repress SBF-dependent transcription (Amon et al. 1993). As shown in

Figure 3, SCBs in the *CLN2* promoter are occupied in the *clb1-4ts* mutant. Occupancy of the promoter was also found in *cdc4* mutants (Fig. 3) that fail to activate Clb kinases and arrest in G_1 with high levels of *CLN1* and *CLN2* RNAs because of a defect in the destruction of the Clb kinase inhibitor $p40^{Stc1}$ (Schwob et al. 1994). Therefore, SBF dissociates from the promoter in *cdc* mutants that arrest with high Clb/Cdc28 kinase activity and fails to dissociate in mutants whose primary defect is the lack of Clb cyclins. Dissociation of SBF from the promoter in G_2 and M phases may therefore be caused by the activity of Clb kinases.

Binding of SBF in G_1 daughter cells

Cell cycle-arrested cells do not represent a "snapshot" of a normal cell cycle because many processes such as cellular growth continue during the arrest. As a consequence, cell cycle-arrested cells become very large and may accumulate proteins to levels not normally present in wild-type cells. We therefore addressed whether SBF binds to the *CLN2* promoter prior to the activation of *CLN2* transcription in normal wild-type cells. This is an important issue because activation of transcription by Cln3/Cdc28 kinase occurs only when cells reach a critical cell size. G_1 daughter cells were isolated using centrifugal elutriation, inoculated in fresh medium at 30°C, and analyzed for SBF binding (Fig. 4A) and *CLN2* transcription (Fig. 4B). We found that SCBs are occupied in two different populations of elutriated wild-type cells which had not yet activated *CLN2* transcription (Fig. 4A, lanes 4,7). Moreover, we observed no difference in the protection pattern between cells below the critical cell size for *CLN2* transcription (Fig. 4B) and cells which had started to accumulate *CLN2* RNAs (cf. Fig. 4A, lane 5,6). Binding of SBF to the promoter therefore occurs in daughter cells prior to the activation of *CLN2* transcription.

We consistently observed weaker DMS protection in small daughter cells than in large *cdc28* or Cln-deficient G_1 cells (Fig. 4). Although the differences in protection are small, sites of enhanced cleavage show clear differences. The differences are not an artifact of the elutriation protocol (see Materials and methods) because a population of elutriated *cln1 cln2* cells, which are much larger than wild-type daughter cells and therefore transcribe *CLN2* RNAs (Dirick et al. 1995) show full protection. The differences between cell cycle-arrested cells and wild-type daughter cells may indicate either that SCB elements are not fully occupied in daughter cells or that in cell cycle mutants SBF/SCB complexes are stabilized because of the accumulation of larger than normal levels of binding activity in large mutant cells. Therefore, it cannot be excluded that an increase of SBF-binding activity during the G_1 period of daughter cells contributes to the sudden appearance of *CLN2* RNAs in late G_1 .

Figure 4. Binding of SBF in elutriated daughter cells. (A) Genomic footprinting of *CLN2* promoter (lower strand). SCBs are marked as in Fig. 1. (Lane 1) Genomic DNA treated with DMS in vitro; (lane 2) *cdc13* (K2035) arrested in G_2 at 37°C; (lane 3) *cln1 cln2 cln3 GAL::CLN3*, (K3130) arrested in G_1 by growth in YEP/2% raffinose for 3 hr; (lane 4) elutriated wild-type cells (K1107); (lanes 5,6) a population of small G_1 daughter cells was inoculated into fresh medium. Samples were taken at 17 fl (lane 5) and at 22 fl (lane 6) and analyzed for SBF binding. (Lane 7) Elutriated wild-type cells (K699); (lane 8) elutriated *cln1 cln2* (K3652) cells. (B) Northern analysis of *CLN2* RNAs from elutriated cells shown in A; The percentage of cells with 2N DNA content as determined by FACS analysis is indicated.

SBF gains access to the CLN2 promoter when cells exit mitosis around the time of cyclin destruction

To follow the appearance of SBF at the *CLN2* promoter as cells progress through the cell cycle, we analyzed SBF binding in cells released from a *cdc15* arrest. *cdc15* mutants arrest at the end of anaphase with high levels of Clb2-associated kinase activity. In the arrested culture, the *CLN2* promoter was not occupied by SBF, and *CLN2* was not expressed (Fig. 5). The mutants were released from the cell cycle block by cooling the culture quickly to 24°C. The cells synchronously recovered from the mitotic block as assayed by the appearance of small budded cells and the activation of *CLN2* transcription (Fig. 5). Binding to SCB elements starts to be detectable 20–30 min after release, shortly before accumulation of *CLN2* transcripts. This is in agreement with our finding that binding of SBF to the *CLN2* promoter precedes *CLN2* transcription in elutriated daughter cells. The kinetics of SBF's binding to the *CLN2* promoter are similar to the kinetics of Clb/Cdc28 kinase inactivation upon release from a *cdc15* block (Surana et al. 1993) and similar to the appearance of *EGT2* RNAs (Fig. 5). *EGT2* expression is dependent on the transcription factor Swi5 and is activated upon Swi5's entry to the nucleus at the end of mitosis when the Clb/Cdc28 kinase is destroyed (T. Schuster, pers. comm.; Koch and Nasmyth 1994). This correlation suggests a causal role for Clb/Cdc28 kinase inactivation in promoting SBF binding to its target sites.

Transcription driven by a LexA–Swi4 fusion is cdc28 dependent

Our footprinting analysis indicates that activation of *CLN2* transcription is not caused by changes in the ability of SBF to bind to the promoter but by changes in its ability to activate transcription. If this is the case, then a version of SBF that is constitutively targeted to its promoter should still exhibit Cdc28-dependent activation of transcription. We tested this hypothesis by replacing Swi4's DNA-binding domain with that of the bacterial repressor LexA. A fusion gene in which the first 115 amino acids of Swi4 are replaced with the DNA-binding domain of LexA (Golemis and Brent 1992) was integrated at the *URA3* locus under the control of the constitutive *ADH1* promoter. Transcriptional activity of the fusion protein was monitored using a *LEU2* gene under the control of several LexA operators. Transcription of the reporter gene was dependent on the presence of the Swi4 sequences (data not shown) and strongly reduced in *swi6* mutants (Fig. 6). *cdc28* mutants expressing the fusion protein were arrested in G₁ by shifting the cells to the restrictive temperature and released into nocodazole-containing medium at 25°C, which arrests cells in the subsequent M phase. Figure 6 shows that the LexA–Swi4 fusion protein is transcriptionally inactive during the *cdc28* arrest. After release from the cell cycle block, LexA–Swi4 dependent gene expression is reactivated, although the reactivation is slower than the activation of a cognate SBF target gene *PCL1*, which is only transiently

Figure 5. SBF gains access to SCBs as cells exit mitosis upon release from *cdc15* arrest. (Top) Genomic footprinting of SCBs in the *CLN2* promoter; (bottom) Northern analysis of *CLN2*, *CLB2*, and *EGT2* RNAs. RNAs were quantitated relative to the level of *CMD1* RNA (internal loading control). A 10-liter culture of *cdc15-2* cells (K1993) was grown at 25°C to a density of 0.6 OD₆₀₀ in YEPD, harvested by filtration, and resuspended in 8 liters YEPD at 37°C. After arresting cells for 2.5 hr, the culture was released from the cell cycle block by cooling the culture quickly to 23°C with the addition of frozen YEPD medium. For each time point, 1.3 liter of culture was harvested and analyzed further. In lanes 7 and 8 of the footprinting analysis the methylation pattern around site I was inconclusive, but the pattern at site II indicates an inhibition of SBF binding as cells switch off transcription.

expressed upon release from the arrest. As cells become arrested in nocodazole, expression of the reporter gene decreases only slowly and is not completely inactivated, suggesting that the fusion protein may be partly resistant to transcriptional repression in G₂ phase. SBF normally dissociates from the promoter in nocodazole arrest. The

Figure 6. LexA–Swi4-mediated gene expression is *cdc28* dependent. Strain K4538 (*cdc28-13*, *LexAop-LEU2*), harboring the LexA–Swi4 fusion was grown in YEPD medium at 25°C to early log phase. The culture was shifted to 37°C for 3 hr and subsequently released into medium containing nocodazole at 25°C. Samples were taken at the indicated time and analyzed for expression of the *LEU2* reporter gene under the control of LexA operators. (Right) LexA–Swi4 dependent transcription analyzed in (K4538) and congenic *swi6* mutant cells grown at 25°C in YEPD.

failure to repress transcription mediated by the fusion protein could therefore be a consequence of constitutive LexA binding throughout the cell cycle. The behavior of the LexA–Swi4 fusion in G_2 -arrested cells is thus consistent with the notion that repression of SBF-dependent transcription in G_2 involves SBF's removal from the promoter. The main conclusion from the behavior of the LexA–Swi4 fusion is that transcription remains Cdc28-dependent, supporting the idea that Cln/Cdc28 kinase activates previously bound SBF at Start. However, it is clear that the fusion protein does not reproduce fully the pattern of SBF-dependent gene activation, because the kinetics of RNA accumulation upon cell cycle release from the *cdc28* block are quite different.

Discussion

Transcriptional activation of *CLN1* and *CLN2* attributable to SBF is thought to be the rate limiting step for cell cycle entry in yeast (Tyers et al. 1993; Dirick et al. 1995). How their transcription is normally activated in late G_1 when cells reach a certain size and how their expression is subsequently repressed in G_2 and M phases are therefore key questions. In this paper we investigated whether changes in SBF's binding to the promoter might have a role. Using genomic footprinting, we have shown that SBF binds to the *CLN2* promoter in a cell cycle-regulated fashion. SCB elements are protected in G_1 but not in G_2 or M-phase cells. Clb1-4/Cdc28 kinase activity is required to remove SBF from the promoter in G_2 , suggesting that Clb1–Clb4 may repress *CLN1* and *CLN2* transcription by inhibiting the DNA-binding activity of SBF.

How is *CLN2* transcription activated in late G_1 ?

Several lines of evidence indicate that activation of *CLN2* transcription in late G_1 is not caused by changes

in SBF binding. We found that SCB elements are fully occupied in *cdc28* mutants and in mutants arrested in G_1 because of G_1 cyclin depletion, which fail to activate SBF-dependent transcription. Furthermore, the methylation pattern in mutant cells that arrest in G_1 prior to activation of SBF-dependent transcription (*cln1 cln2 cln3* and *cdc28*) was identical to the pattern in mutants that arrest with fully activated *CLN* transcription (*cdc4* and *clb1–clb4ts*). This suggests that there are no changes in SBF binding as cells activate *CLN2* transcription. Importantly, we found that SCBs were almost fully occupied in small daughter cells before the activation of late G_1 -specific transcription. We also did not detect changes in SBF binding as daughter cells started to accumulate *CLN2* RNAs. Hence, changes in SBF levels attributable to the accumulation of *SWI4* RNAs in late G_1 (Breedon and Mikesell 1991) are unlikely to be important for activation of *CLN2* transcription even in daughter cells. The conclusion that transcriptional activation is not attributable to changes in SBF binding is also supported by our finding that a protein fusion where Swi4's DNA-binding domain is replaced by the DNA-binding domain of the bacterial repressor LexA confers *cdc28*-dependent transcription.

The key question that remains is how transcription is only activated when cells reach a certain size. Interestingly, no changes in the levels of Cln3 RNA or protein have been detected when daughter cells reach the critical cell size (Tyers et al. 1993), yet the timing of *CLN1* and *CLN2* transcription is strongly dependent on the levels of Cln3 protein (Cross and Blake 1993; Tyers et al. 1993). It has been speculated that activation of transcription might be caused by the accumulation of a critical level of Cln3-associated kinase activity (Tyers et al. 1993; Dirick et al. 1995). Our data suggest that Cln3 kinase alters the ability of previously bound SBF to activate transcription. If Cln3/Cdc28 has to interact directly with this complex, then the nuclear concentration of Cln3/Cdc28 kinase might become very important as it is now titrated against a constant level of DNA-bound substrate.

How is *CLN1/CLN2* transcription repressed during G_2 and M phases?

We have shown that SBF fails to bind to the *CLN2* promoter in G_2 and M-phase-arrested mutant cells despite being present in those cells (Taba et al. 1991). Thus, SBF proteins must be present in a form unable to bind to SCB elements during G_2 and M phases. The intracellular localization of Swi6 protein changes during the cell cycle. Most of the protein is nuclear during G_1 and S phase, whereas it is predominantly cytoplasmic in M-phase-arrested cells (Taba et al. 1991). Could the cytoplasmic localization of Swi6 explain transcriptional repression? This is unlikely, because <50% of the cells in a cycling culture have SBF bound to the promoter (Fig. 1D), yet most cells in a cycling wild-type culture show nuclear staining of Swi6 (Taba et al. 1991). Thus, Swi6's exclusion from the nucleus may contribute to repression of

SBF regulated genes during M phase, but it is unlikely to be responsible for repression during G₂.

What causes dissociation of SBF from the promoter during G₂ and M phases? We found that Clb1–Clb4/Cdc28 kinases are required to remove SBF from the promoter in G₂. Furthermore, inactivating Clb kinases by overexpression of the Clb kinase inhibitor p40^{SIC1} is sufficient to cause reactivation of SBF-dependent transcription in nocodazole-arrested cells (Dahmann et al. 1995). Inhibition of SBF binding may therefore be a direct effect of Clb1–Clb4/Cdc28 kinases.

How do Clb1–Clb4 kinases inhibit SBF binding? The finding that Swi4 is present in immunoprecipitates of Clb2 (Amon et al. 1993), suggests that Clb2 acts directly on Swi4. DNA binding of SBF might be inhibited by phosphorylation or by association of Clb2/Cdc28 with Swi4. Clb2 binds to the ankyrin repeat domain of Swi4 located in the center of the protein (R. Siegmund, pers. comm.). Swi4 is a substrate for Cdc28 kinase in vivo (M. Neuberg, pers. comm.), suggesting a role for phosphorylation in the regulation of Swi4 activity. So far, however, mutations in Cdc28-dependent phosphorylation sites of Swi4 did not alter cell cycle regulation of Swi4-dependent RNAs (M. Neuberg, pers. comm.). Although this does not rule out that Clb/Cdc28 kinase acts by phosphorylation, it suggests that the physical association of Clb2 with Swi4's ankyrin repeats might also be important.

A model for the regulation of SBF-dependent transcription

The finding that ectopic activation of Cln/Cdc28 kinase in G₂ cells does not reactivate SBF-dependent gene expression (Amon et al. 1993) predicted that Clbs do not just reverse activation by inhibiting Cln/kinase activity but that repression must occur because of an indepen-

dent mechanism that is dominant over activation. Our finding that Clbs inhibit SBF binding shows this to be the case. Three different changes in Cdc28 activity contribute to the cyclical transcription of SBF-regulated genes like *CLN2* (Fig. 7): In G₁, SBF binds to the promoter but fails to activate transcription. When cells reach a critical cell size, Cln3/Cdc28 kinase acts on previously bound SBF to activate transcription of *CLNs*. In G₂, Clb1–Clb4/Cdc28 kinases become active and repress *CLN2* transcription by removing SBF from the promoter. The third step of the SBF cycle occurs upon exit from mitosis when Clbs are rapidly inactivated by proteolysis and SBF rebinds to the promoter, albeit in an inactive form. Reactivation of *CLN2* transcription therefore cannot occur until repression is relieved by destruction of Clb kinases at the end of the cell cycle.

It has been shown that in *swi4* mutants *CLN2* expression is strongly reduced; however, this reduced expression remains cell cycle regulated (Cross et al. 1994; Stuart and Wittenberg 1994). Our in vivo footprinting analysis showed that most, if not all, binding to SCB elements in wild-type cells is attributable to SBF. In the absence of SBF, the related factor MBF has been proposed to substitute partly for SBF (Koch et al. 1993; Stuart and Wittenberg 1994). We have not analyzed DNA binding of MBF in vivo, but it is conceivable that it may be regulated similarly.

Why is the mechanism of repression different from the mechanism of transcriptional activation?

Cyclical transcription of *CLN2* could, in principle, be generated by periodic accumulation of Cln3 or Clb kinases only. But does Cln3 kinase activity cycle? There are two main reasons to believe that it does not. First, no changes in RNA and/or protein levels have been detected during the cell cycle. Second, Cln3 activity is thought to

Figure 7. A model for SBF-mediated gene expression. Before Start, SBF (Swi4/Swi6) is bound to the promoter, but fails to activate transcription. At Start, Cln3/Cdc28 acts on SBF (shaded) to activate transcription. In G₂ and M phases, active Clb1–Clb4/Cdc28 kinases repress transcription, by inhibiting SBFs DNA binding activity. Destruction of Clbs upon exit from mitosis allows reassociation of SBF to the promoter.

be dependent on cell size, that is, the level of protein synthesis. It has been proposed that Cln3 kinase activity accumulates during early G_1 relative to its presumptive nuclear targets and once it reaches a threshold level, activates transcription (Tyers et al. 1993; Dirick et al. 1995). If this were the case, Cln3 kinase, once having reached this threshold, will persist throughout the remaining cycle, until it is reduced when a small daughter cell is born by asymmetrical cell division. This may be one of the reasons why repression in G_2 occurs by a mechanism other than activation.

It can be imagined that the cyclical activity of Clbs, because of their periodic activation and destruction, would be sufficient to drive the SBF cycle. In this case, there would be two states: In the absence of Clbs, SBF binds to the promoter and activates transcription. When Clbs are active, SBF dissociates from the promoter and *CLN1* and *CLN2* transcription is off. This would provide a simple means of alternating between the activity of Clns and Clbs. However, the immediate reactivation of Clns after exit from mitosis would start the cell cycle in small daughter cells at the same time as in large mother cells. Hence, entry into the cell cycle could not be coordinated with cellular growth. Delaying polarized growth and DNA replication until a critical cell size is reached, by awaiting Cln3 activation, is therefore important for size homeostasis, whereas the removal of SBF from the promoter in G_2 is required to repress transcription independent of the state of Cln3 kinase activity.

The significance of dual controls for the cell cycle

A key aspect of the proposed model for the regulation of *CLN* transcription is that each of the three transitions is attributable to a different mechanism, thereby generating a linear sequence of irreversible events. This contrasts to most systems of inducible gene expression (Hill and Treisman 1995) where reversibility is not only the rule but may in fact be essential for efficient adaptation to changing external conditions. Why does the cell cycle use irreversible systems? Irreversible controls over *CLN* expression will help to prevent the unscheduled reactivation of Cln kinase during G_2 and M phase, which would interfere with cytokinesis (Lew and Reed 1993).

The controls that we have described are not dissimilar to the mechanisms that prevent rereplication of DNA in G_2 . B-type cyclins promote DNA replication in S phase, presumably by activating preformed replication complexes, and they inhibit rereplication in G_2 (Dahmann et al. 1995; Hayles et al. 1994) by preventing the reassembly of prereplication complexes after DNA synthesis (Dahmann et al. 1995). This ensures that even if the signal for activation of replication were to persist during G_2 , rereplication would still not recur until Clbs are inactivated. This is similar to the effect of Clbs on *CLN2* transcription where they prevent the assembly of transcription complexes even if Cln3 kinase activity persists during G_2 . In both of these systems reactivation in the next cycle requires the previous destruction of B-type cyclins at the end of mitosis. The efficiency of this kind

of dual control is particularly evident in the control of replication, as it is thought that activation and repression of replication are controlled by the same Clb kinase activities (Dahmann et al. 1995). The existence of different mechanisms for activation and inactivation of cell cycle events may be a common theme to ensure the faithful succession of different cell cycle steps, that is, its processive nature.

The opposing effects of different cyclin/CDK complexes on late G_1 -specific transcription is reminiscent of the situation in mammalian cells where different cyclin/CDK complexes regulate the activity of the E2F/DP1 class of transcription factors (Krek et al. 1994; La Thangue 1994). Cyclin D/CDK4 is thought to activate the factors by releasing them from inhibitory complexes with Rb, whereas binding of cyclinA/CDK2 to E2F may reduce the ability of E2F/DP1 heteromers to bind to their target sequences (Krek et al. 1994). However, in contrast to yeast, there is little or no *in vivo* evidence to evaluate the physiological importance of the proposed mechanisms.

Materials and methods

In vivo footprinting

Genomic footprinting was performed using DMS. Methylated sites were detected by primer extension with *Taq* polymerase (Primig et al. 1991). Cultures (600–1000 ml) of *cdc* mutants were grown in YEPD at 25°C to an OD₆₀₀ of 0.6. Cultures were shifted to 37°C until cells were uniformly arrested. Cells were harvested rapidly by filtration and resuspended in YEPD to give a final volume of 2–4 ml. After adding 2.5 μ l of DMS/ml of cells, cells were incubated for 4 min (25°C). Reactions were stopped by adding 30 ml of ice-cold TNE β buffer (10 mM Tris-Cl at pH 8.0, 1 mM EDTA, 40 mM NaCl, 100 mM β -mercaptoethanol). After centrifugation, cells were washed once in 30 ml of SCE β buffer (182 grams/liter of sorbitol, 29.4 grams/liter of tri-Na-citrate/2H₂O, 2.92 grams/liter of EDTA at pH 7, 100 mM β -mercaptoethanol), and resuspended in 2 ml of SCE β buffer containing 5 mg/ml of Zymolyase 20T. After incubating at 37°C for 45 min, cells were lysed with 2 ml of lysis buffer (2% SDS, 0.1 M Tris-Cl at pH 9.0, 50 mM EDTA) and incubated at 65°C for 5 min. After adding 2 ml of 5 M potassium acetate, samples were put on ice for at least 1 hr. Debris was removed by centrifugation at 13,000 rpm for 40 min at 4°C. DNA was precipitated with 1 volume of ethanol and 0.5 M NH₄OAc at pH 5.5, washed twice with 50% ethanol, and resuspended in 4 ml of TE. DNA precipitation was repeated several times with 0.3 M NaOAc and 1 volume of ETOH, each time reducing the volume. DNA was digested with RNase A (DNase free) for 1 hr at 37°C, precipitated as above, and resuspended in 0.3 ml of TE. For each primer extension reaction, 50 μ l of DNA was digested with 25 units of *Hae*III and 1 μ g of RNase A for 4 hr, extracted with phenol/chloroform, and precipitated. Approximately 1 ng of 5'-end-labeled oligonucleotide primer (1 $\times$ 10⁶ cpm), 10 μ l of 10 $\times$ PCR buffer [166 mM (NH₄)₂SO₄, 670 mM Tris-HCl at pH 8.8, 67 mM MgCl₂, 100 mM β -mercaptoethanol, 1 mg/ml of BSA], 2 μ l of 100 mM MgCl₂, and H₂O were added to the DNA to give a final volume of 96 μ l. The sample was heated to 95°C for 5 min, and after adding 3 μ l of 10 mM dNTPs and 8 units of native *Taq* polymerase (Perkin-Elmer), 40 cycles of primer extension were performed (1 min at 94°C, 2 min at 65°C, and 3 min at 72°C) followed by a 10 min extension at 72°C. PCR products were

Table 1. Strain list

Strain	Genotype
K1970*	<i>MATa swi6::TRP1</i>
K3294*	<i>MATa mbp1::URA3</i>
K1939*	<i>MATa swi4::LEU2</i>
CY130*	<i>MATa swi4::URA3 mbp1::URA3 YCPGAL-CLN1/LEU2</i>
K5133	<i>MATa swi4::LEU2 cdc28-13</i>
K5134	<i>MATa swi6::TRP1 cdc28-13</i>
K5135	<i>MATa mbp1::URA3 cdc28-13</i>
K2035	<i>MATa cdc13-1</i>
K3130	<i>MATa cln1::hisG cln2del cln3::GAL-CLN3/URA3</i>
K3080	<i>MATa clb1del clb3::TRP1 clb4::HIS3 clb2-VI</i>
K3446	<i>MATa cdc28-13</i>
K3445	<i>MATa cdc28-13</i>
K4083	<i>MATa cdc4</i>
K1993	<i>MATa cdc15-2</i>
K1994	<i>MATa cdc15-2</i>
K3652	<i>MATa cln1 cln2</i>
K4538	<i>MATa cdc28-13 leu2::lexAop-LEU2</i>

Strains were derivatives of W303 (K699): *MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 Gal⁺psi⁺ssd1-d*. Strains marked with an asterisk are derived from K1107: *MATa HLMa HMRa ho-LacZ ura3 ade2-1 can1-100 met⁻ his3 leu2-3,112, trp1-1 SSD1-v*. The *lexAop-LEU2* gene (three LexA binding sites), which replaces the chromosomal *LEU2* gene, was introduced by genetic crosses with EGY48 (Gyuris et al. 1993).

purified by addition of 10 μ l of CTAB solution (5% hexadecyltrimethyl-ammoniumbromide in ethanol) and precipitation on ice for 20 min. DNA was collected by centrifugation (30 min, 14,000 rpm at 4°C), resuspended in 0.2 ml of 0.5 M $\text{NH}_4\text{OAc}/0.3$ M NaOAc, and precipitated again with 660 μ l of ethanol. After a second ethanol precipitation, samples were resuspended in 5 μ l of 100 mM NaOH, 1 mM EDTA, and 5 μ l of gel loading buffer. An aliquot of 2 μ l was analyzed on 8% sequencing gels. PCR primers used for the analysis of the *CLN2* promoter: CLN2-1, (–691) 5'-TTAATAATGATACTGAGGTTCAAAAGTGCC-3' was used to analyze the lower strand; CLN2-2, (–466) 5'-CAG-GCTACGCAATGTGCTCTTCGCTAGGT-3' was used to probe the upper strand.

Genetic techniques and plasmids

Standard techniques were used for culturing yeast and genetic crosses. If not stated otherwise, cells were grown in YEPD (Rose et al. 1990). The *lexA-SWI4* fusion gene was constructed by inserting *SWI4* sequences starting from amino acid 116 (*Bam*HI site) to a *Sal*I site located 3' of the coding region of *SWI4* into the *Bam*HI–*Sal*I sites of the LexA fusion vector pEG202, which expresses the fusion from the *S. cerevisiae ADH1* promoter (Golemis and Brent 1992). An *Sph*I fragment harboring the fusion gene was subsequently transferred to the *Sph*I site of the integrating vector YIplac211 (Gietz and Sugino 1988). The resulting plasmid (pCK102) was integrated at the *URA3* locus.

Cell cycle synchronization

Synchronizations of cultures by release from cell cycle arrest were performed as described (Koch et al. 1993; Schwob and Nasmyth 1993). For elutriation, cells were grown in YEP/2% raffinose medium to an OD_{600} of 2.0 (Schwob and Nasmyth 1993). Because of the large amount of cells required for each footprinting sample, the amount of cells recovered from the elutriator was only sufficient for a single footprint and tended to be contaminated with G_2 cells. Therefore, to harvest large

amounts of small cells (from ~12 liters of culture), cells were collected, cooled to 4°C and elutriated in two batches. Populations of the same cell size (Dirick et al. 1995) were then combined. After elutriation, cells were resuspended into fresh medium at 30°C and analyzed for DNA binding after 20–30 min (to avoid any perturbation due to the long exposure to 4°C). Analysis of DNA content by FACS and RNA analysis were done as described (Epstein and Cross 1992; Schwob and Nasmyth 1993). Quantitation of RNA levels was performed using a PhosphorImager (Molecular Dynamics). *CMD1* (calmodulin) RNA levels that do not fluctuate during the cell cycle served as internal loading control for all Northern blots.

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Switching transcription on and off during the yeast cell cycle: Cln/Cdc28 kinases activate bound transcription factor SBF (Swi4/Swi6) at start, whereas Clb/Cdc28 kinases displace it from the promoter in G2.

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