CLN3, not positive feedback, determines the timing of CLN2 transcription in cycling cells

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Transcriptional activation of the budding yeast *CLN1* and *CLN2* genes during the late G₁ phase of the cell cycle has been attributed to a positive feedback loop, wherein the transcription of both genes is stimulated by the accumulation of their protein products. We demonstrate that in cycling cells *CLN2* does not play a role in determining the timing of its own transcriptional activation. First, we show that *CLN3* alone is sufficient to maximally activate *CLN2* transcription. Cells that lack functional *CLN1* and *CLN2* genes activate the *CLN2* promoter with the same kinetics and at the same size as cells in which all three *CLN* genes are functional. In addition, *CLN2* transcription is activated with similar kinetics in cells that have *CLN2* as their only functional *CLN* gene and in *CLN*-deficient cells. Promoter analysis shows that *CLN3*-dependent activation of *CLN2* transcription is directed primarily through the previously identified UAS1 region although another *cis*-acting region, UAS2, also can contribute to *CLN2* activation under some conditions. The ability to activate transcription of *CLN2* is not a unique property of *CLN3* because ectopically expressed *CLN2* can both activate the endogenous *CLN2* promoter and induce Start. We propose that failure of the endogenous *CLN2* gene to contribute significantly to activation of its own transcription results from its relative effectiveness at inducing Start, cell cycle progression and, subsequently, inactivation of *CLN2* expression.

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During the course of a eukaryotic cell cycle a series of rigorously orchestrated events allow cellular components to be duplicated successfully and segregated faithfully by cell division. In the budding yeast *Saccharomyces cerevisiae* regulation over cell cycle progression is exerted predominantly during G₁ phase at a point referred to as Start (for review, see Pringle and Hartwell 1981). At Start cells choose between entry into a quiescent stationary state (if deprived of a carbon source), conjugation (in the presence of cells of the opposite mating type), progression into the meiotic pathway (if diploid cells are deprived of a nitrogen source), or initiation of a new mitotic cell cycle (Hartwell et al. 1974).

Major cell cycle transitions in eukaryotic cells require the activity of one or more cyclin-dependent protein kinases (CDKs) (for review, see Reed 1992). Activation of this family of kinases is governed in part by their association with regulatory subunits known as cyclins. *S. cerevisiae* utilizes a single CDK encoded by *CDC28* for passage through both Start and mitosis (Reed 1992). The Cdc28-associated cyclin regulatory subunits can be divided into three classes, three G₁ cyclins or *CLNs*, which are essential for Start (Richardson et al. 1989; Cross

1990); two B-type cyclins, *CLB5* and *CLB6*, which are important for entry into and progression through S phase (Schwob and Nasmyth 1993); and four B-type cyclins, *CLB1-CLB4*, which are important for the G₂/M transition as well as for the maintenance of S phase (Surana et al. 1991; Richardson et al. 1992).

The *CLN* genes are functionally redundant with regard to their ability to support cell proliferation (Cross 1990; Richardson et al. 1992). Although inactivation of all three *CLN* genes results in G₁ arrest, any one of the three is sufficient to allow cells to proliferate. Despite their functional redundancy, there are fundamental differences between *CLNs*. At the level of primary structure the *CLN1* and *CLN2* proteins share a high degree of identity, whereas *CLN3* is considerably divergent (Nash et al. 1988; Hadwiger et al. 1989). Moreover, expression of *CLN1* and *CLN2* is regulated coordinately. The genes are periodically transcribed with a peak of mRNA accumulation occurring during late G₁, a pattern that is reflected in the accumulation of the respective proteins and protein kinase activities (Wittenberg et al. 1990; Tyers et al. 1993). In contrast, *CLN3* is expressed throughout the cell cycle and displays less fluctuation in mRNA, protein, and associated kinase activity (Wittenberg et al. 1990; Tyers et al. 1992).

The G₁ cyclins are rate-limiting activators of Start.

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Mutant Cln2 and Cln3 proteins that have increased stability accelerate the execution of Start and as a consequence, shorten G₁ phase and decrease the mean cell size (Sudbery et al. 1980; Cross 1988; Nash et al. 1988; Hadwiger et al. 1989). Similarly, overexpression of any of the three G₁ cyclins can accelerate the timing of Start and reduce the minimal cell size required for budding (Lew et al. 1992). Thus, the regulation of *CLN* gene expression is a critical determinant of the kinetics of Start execution.

CLN1 and *CLN2* mRNA are present at very low levels in small daughter cells early in G₁ phase. However, a dramatic increase in the rate of *CLN1* and *CLN2* transcript accumulation occurs as cells reach a critical size (Dirick and Nasmyth 1991; Price et al. 1991; Lew et al. 1992). Because the low level of expression of these genes that occurs early in G₁ is independent of functional *CDC28*, and because maximal expression is dependent on *CDC28* and G₁ cyclins, it has been proposed that *CLN1* and *CLN2* are regulated via a positive feedback loop (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991). That hypothesis holds that accumulation of a low level of Cln/Cdc28 kinase activates *CLN1* and *CLN2* transcription and that, once activated, these genes generate additional stimulatory protein kinase activity and, as a consequence, induce the rapid and irreversible execution of Start. It is thought that gradual accumulation of Cln-associated Cdc28 kinase activity is associated with cell growth and that activation of *CLN1* and *CLN2* expression occurs once a threshold level is achieved (Nasmyth 1993). Because *CLN3* is expressed throughout the cell cycle it is believed to be an important source of Cln/Cdc28 kinase during early G₁ phase (Tyers et al. 1992). However, it is also presumed that once activated, the *CLN1* and *CLN2* genes contribute to activation of their own expression (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991; Ogas et al. 1991). The weak kinase activity associated with Cln3 has led to the proposal that Cln3 does not participate directly in driving cell cycle progression but, instead, that its primary role is to induce expression of *CLN1*, *CLN2*, and other genes that then can directly perform Start (Tyers et al. 1993). Consistent with this hypothesis, two other cyclin-like genes (*PCL1* and *PCL2*) have been shown to be essential when *CLN1* and *CLN2* are inactivated (Measday et al. 1994). However, there is no evidence that expression of either of these cyclins, which associate with Pho85 (another member of the CDK family), is sufficient to induce Start in the absence of *CLNs*.

Peak expression of the *CLN1* and *CLN2* genes is dependent on transcription factors encoded by *SWI4* and *SWI6* (Nasmyth and Dirick 1991; Ogas et al. 1991), which were originally identified as factors essential for G₁-specific expression of the *HO* endonuclease gene (Breedon and Nasmyth 1985, 1987; Andrews and Herskowitz 1989). Together, they form components of a transcription factor known as SCB-binding factor (SBF), which binds to and transcriptionally activates promoters containing Swi-dependent cell cycle boxes (SCBs) (Andrews and Herskowitz 1989; Nasmyth and Dirick 1991; Ogas et al. 1991). This *cis*-acting regulatory motif has

been identified in the upstream regions of the *HO*, *CLN1*, *CLN2*, *PCL1*, and *PCL2* genes (Nasmyth 1985; Frohlich et al. 1991; Ogas et al. 1991). *SWI6* also interacts with the DNA-binding protein *MBP1* (Koch et al. 1993) to form a transcription factor complex, known as MCB-binding factor (MBF) or DNA synthesis control (DSC1) (Lowndes et al. 1991; Dirick et al. 1992; Koch et al. 1993). This transcription factor binds to *cis*-acting regulatory sequences known as MluI cell cycle box (MCB) elements (Dirick et al. 1992; Lowndes et al. 1992), which occur upstream of many genes whose products are involved in DNA synthesis as well as in two B-type cyclin genes, *CLB5* and *CLB6* (Breedon 1988; Andrews and Herskowitz 1990; Epstein and Cross 1992; Johnston and Lowndes 1992; Kuhne and Linder 1993; Schwob and Nasmyth 1993). Analysis of the *CLN1* and *CLN2* promoter sequences reveals the presence of both MCB and SCB elements (Ogas et al. 1991), and at least in the case of *CLN2*, both types of elements appear to be functional and to contribute to peak levels of *CLN2* transcription (Cross et al. 1994; Stuart and Wittenberg 1994). Both SBF and MBF activate transcription during late G₁ and are stimulated by activation of the Cln/Cdc28 protein kinase. As a result, they are proposed to be the targets of the Cln/Cdc28-dependent feedback loop (Taba et al. 1991; Dirick et al. 1992). Consistent with the kinetics of *CLN1* and *CLN2* transcription, SBF- and MBF-regulated genes are repressed as the cells enter S phase (for review, see Koch and Nasmyth 1994). Whereas inactivation of SBF, and consequently *CLN1* and *CLN2* expression, depends upon the activity of a B-type cyclin-associated Cdc28 kinase, the mechanism of MBF inactivation has not been characterized (Amon et al. 1993).

To elucidate the mechanisms governing the accumulation of G₁ cyclins and to determine how their activity controls Start, we previously performed a detailed analysis of the *CLN2* promoter. Those studies identified two distinct regions that are competent to confer G₁-specific expression to *CLN2*, a region containing SCB and MCB elements, which we refer to as UAS1, and a second region devoid of previously characterized motifs, which we refer to as UAS2 (Stuart and Wittenberg 1994). Here we describe experiments designed to establish the importance of these promoter elements for transcriptional activation of *CLN2* both early and late in G₁ phase. We find that the two *CLN2* promoter regions contribute differentially to the expression of *CLN2* during G₁ phase. Both are important for *CLN2* mRNA accumulation in the absence of functional *CLN* genes. However, UAS1 is of primary importance in cells having functional *CLN3*. Thus, whereas UAS1 is important for achieving peak levels of *CLN2* transcription, UAS2 is likely to participate in an as yet unidentified, *CLN3*-independent aspect of *CLN2* regulation.

Because the *CLN2* promoter is subject to distinct forms of regulation, depending on the status of other *CLN* genes, we investigated the importance of *CLNs* in determining the timing and extent of *CLN2* transcription. This study led to two surprising observations. First, we show that transcriptional activation of *CLN2* occurs

with identical kinetics in wild-type cells and cells in which *CLN3* was the only functional *CLN* gene. In addition, cells lacking functional *CLN* genes or having *CLN2* as the only functional *CLN* gene induce *CLN2* transcription with indistinguishable kinetics. Together, these observations demonstrate that *CLN2* does not contribute to the timing of its own transcriptional activation and has little or no influence on the extent of activation in cycling cells. We conclude, therefore, that the positive feedback loop for *CLN* gene activation does not function as originally envisioned. We propose that *CLN3*, which is expressed early in G_1 phase, activates transcription of *CLN1* and *CLN2* as well as other SCB- and MCB-regulated genes. *CLN1* and *CLN2* then presumably act to directly perform Start, which ultimately leads to inactivation of their transcription. However, we also find that *CLN3* is not unique in its ability to stimulate *CLN1* and *CLN2* transcription, because *CLN2*, when constitutively expressed under the regulation of the *CLN3* promoter, is an efficient activator of both transcription and Start.

Results

Expression of CLN2 driven by either UAS1 or UAS2 promotes proliferation in the absence of CLN1 and CLN3

Our previous studies identified two regions of the *CLN2* promoter that are capable of conferring cell cycle-dependent transcriptional activation: UAS1, which contains both SCB and MCB elements, and UAS2, which contains an as yet unidentified regulatory motif (Stuart and Wittenberg 1994). Each of these sequences was competent to direct cell cycle-regulated transcription in synchronous cultures when evaluated in the context of either a plasmid-borne reporter fusion or when driving *CLN2* in its endogenous chromosomal location. Whereas UAS1 is able to drive peak levels of *CLN2* expression in a *CDC28*-dependent fashion, transcriptional activation by UAS2 is *CDC28*-independent. To perform a functional test of the activity of these promoter elements, the endogenous *CLN2* gene in a *cln1 Δ cln3 Δ GAL1-*CLN3* strain was replaced with a functional *CLN2* open reading frame (ORF) regulated either by a wild-type promoter or by one of the mutant promoters: Δ UAS2, in which UAS2 had been deleted, *mcb/scb*, in which inactivating point mutations had been generated in the MCB and SCB sequences of UAS1, or *mcb/scb* Δ UAS2, in which both UAS1 and UAS2 had been mutated. All of these strains were viable and had comparable sizes and growth rates while growing on galactose-based medium owing to expression of *CLN3* from the *GAL1* promoter. The *GAL1* promoter was repressed by addition of glucose to the cultures, making them dependent upon their endogenous *CLN2* gene for cell cycle progression. Under these conditions *CLN2* regulated by a wild-type promoter or by promoters that had either an intact UAS1 or UAS2 was sufficient to confer viability and allow cells to proliferate. In contrast, *CLN2* under the regulation of the*

mcb/scb Δ UAS2 promoter was not expressed sufficiently to allow cell cycle progression, and these cells terminally arrested as unbudded G_1 cells (data not shown). Similar results were obtained when the *CLN2* genes with mutant promoters were integrated ectopically at *LEU2* rather than at the normal chromosomal locus (data not shown).

These results suggested that the use of *GAL1-CLN3* would be useful in facilitating experiments designed to evaluate the transcriptional activation provided by individual *CLN2* promoter elements as a function of cell size. Variations in the degree of *CLN* expression have a profound influence on the cell size and cell cycle parameters associated with Start (Cross 1988; Nash et al. 1988; Hadwiger et al. 1989; Lew et al. 1992). Maintaining these strains on galactose-containing medium induces overexpression of *CLN3*, allowing them to transit Start and bud at a small and comparable size. Repression of the *GAL1* promoter results in a very rapid loss of *CLN3* mRNA and protein (Cross 1990; Tyers et al. 1992). This is demonstrated by the fact that cells lacking functional *CLN* genes and expressing *CLN3* from the *GAL1* promoter undergo a first cycle arrest in G_1 prior to Start following repression of the *GAL1* promoter (Richardson et al. 1989; Cross 1990). Thus, following repression of the *GAL1* promoter the cultures transiently become enriched in small unbudded G_1 cells, allowing the isolation of comparably sized populations of cells that have different combinations of functional *CLN* genes.

Mutation of UAS1 or UAS2 delays activation of CLN2 transcription in the absence of CLN1 and CLN3

Our previous studies of *CLN2* transcription led us to hypothesize that UAS2 might be specifically required early in G_1 phase for the timely activation of *Cdc28*-dependent transcription of *CLN* genes. To assess the involvement of UAS1 and UAS2 in establishing the timing and extent of transcriptional activation of *CLN2* we analyzed *CLN2* expression in small G_1 cells that lacked functional *CLN1* and *CLN3* genes. Following repression of the *GAL1-CLN3* gene by addition of glucose, small G_1 cells were isolated by centrifugal elutriation and inoculated into fresh YEPD. *CLN2* mRNA, cell size, and budding index were then analyzed at 15-min intervals. For any growth condition and *CLN* genotype there is a reproducible relationship among cell size, the activation of *CLN2* transcription, and budding (Dirick and Nasmyth 1991; Lew et al. 1992). Therefore, the timing of transcriptional activation of the *CLN2* gene subsequent to elutriation is influenced strongly by the size of the cells in the elutriated population. Because it is not always possible to isolate populations of identical size, we have presented the results of these experiments in terms of cell size (volume). As expected, all of the strains had a low level of *CLN2* mRNA in the small G_1 cells (Fig. 1A, see 0-min samples; Fig. 1B, top). In cells carrying *CLN2* under control of the wild-type promoter, mRNA began to accumulate reproducibly at a cell size of 32 fl (Fig. 1A,

top; Fig. 1B, top). In contrast, when driven by either the mcb/scb or ΔUAS2 promoter, activation of the *CLN2* gene was delayed significantly and *CLN2* mRNA failed to accumulate until a cell volume of ~55 fl was achieved (Fig. 1A, middle and bottom; Fig. 1B, top). The delay in transcriptional activation caused by either of these promoter mutations suggests that both promoter elements contribute to *CLN2* expression. This is despite the fact that only SCB and MCB elements are necessary to attain peak levels of *CLN*-dependent expression of *CLN2* (Fig. 1A, middle, asynchronous samples). As predicted from

Figure 1. Mutation of either UAS1 or UAS2 delays transcriptional activation of *CLN2*. *cln1 CLN2 cln3 leu2::GAL1-CLN3* strains in which *CLN2* was regulated by wild-type promoter, DSY725 (*CLN2*, □), a promoter with UAS2 deleted, DSY722 (ΔUAS2, ■), or a promoter with MCB and SCBs mutated, DSY723 (mcb/scb, ●), were grown in YM-GAL and small G₁ cells were isolated by centrifugal elutriation and inoculated into YEPD. (A) *CLN2* and *ACT1* mRNA were assayed by RNase protection from samples of cells obtained from the asynchronously growing culture (Asynch) and at the indicated time points following inoculation into fresh YEPD. (B) The ratio of *CLN2*/*ACT1* mRNA (top) and percent budded cells (bottom) from the samples in part A plotted as a function of mean cell volume. The graphic plots of both RNA and budding data are for times 0–240 min for DSY725 and DSY723 and times 0–300 min for DSY722.

their ability to promote transcriptional activation of *CLN2*, cells carrying *CLN2* driven by either the mutant or wild-type promoters as their only functional *CLN2* gene can execute Start and progress into the budded phase of the cell cycle. From these experiments we conclude that both UAS1 and UAS2 are activated in a Cln-independent, and presumably Cdc28-independent, fashion but that only UAS1 can be induced to a high level by Cln-associated kinase activity. In addition, the correlation between the kinetics of bud emergence and the extent of *CLN2* expression support the notion that a threshold amount of *CLN2* protein is required to induce Start and the events that follow the Start transition.

Transcriptional activation of the CLN2 promoter in the absence of functional CLN genes depends on both UAS1 and UAS2

The experiments presented above demonstrate that both UAS1 and UAS2 can contribute to the expression of *CLN2* when a functional *CLN2* protein is produced. This was not surprising because we expected that even low levels of *CLN2* expression from these promoter elements early in G₁ would activate Cdc28 kinase, thereby providing a positive signal for transcriptional activation of *CLN2* and subsequent cell cycle progression. To evaluate the capacity of specific promoter elements to function in the absence of Cln/Cdc28 protein kinase, strains were constructed in which *CLN1* and *CLN3* were mutationally inactivated and the endogenous *CLN2* gene replaced by a nonfunctional allele of *CLN2* (*cln2x/s*; see Materials and methods). The *cln2x/s* allele was placed under control of a wild-type promoter, a ΔUAS2 promoter, or a mcb/scb promoter. Again, the strains were rendered viable by introduction of a single integrated copy of *GAL1-CLN3* and were maintained on galactose. Glucose was added to asynchronous galactose-grown cultures and small G₁ cells were isolated by centrifugal elutriation. Despite the inability of these cells to synthesize functional Cln proteins or to pass Start and bud, the *cln2x/s* mRNA increased in abundance over time (Fig. 2A,B). This was true whether *cln2x/s* was driven by a wild-type or a mutant promoter. As was observed in the previous experiment using a functional *CLN2* ORF, transcriptional activation of *cln2x/s* from either of the mutant

promoters was delayed significantly relative to the wild-type promoter (Fig. 2, A, cf. middle and bottom with top, and B). The ability of UAS2 to activate *cln2x/s* transcription during G₁ through a Cln/Cdc28-independent mechanism was expected based on previous work (Stuart and Wittenberg 1994). However, the ability of MCB and SCB sequences to support transcription under these conditions was somewhat surprising. Nevertheless, it is consistent with our previous observation that transcripts expressed from this promoter accumulate in the absence of Cln/Cdc28 activity. What is more remarkable about the results of this experiment is that transcription of the *CLN2* promoter is induced with similar kinetics and to a similar extent in cells lacking functional *CLN* genes and in cells having a functional *CLN2* gene (cf. Figs. 1 and 2). This was an unexpected result because the positive feedback hypothesis predicted that accumulation of Cln2/Cdc28 kinase would stimulate a burst of feedback-activated *CLN2* transcription. This point is considered in detail below.

CLN3 is a potent activator of CLN2 expression that acts primarily through UAS1

Experiments described above establish that both UAS1 and UAS2 are important for activation of *CLN2* transcription either in cells lacking all *CLNs* or in cells having *CLN2* as their sole functional *CLN* gene. However, wild-type cells express *CLN3* and accumulate both Cln3 protein and Cln3/Cdc28 protein kinase during early G₁ phase (Tyers et al. 1993). To evaluate the degree to which individual *CLN2* promoter regions contribute to transcriptional activation in the presence of *CLN3* activity, we made use of *cln2x/s* alleles regulated by either wild-type or mutant promoters in strains having an inactivated *CLN1* gene and a functional *CLN3* gene. A single integrated copy of *GAL1-CLN3* was again used to support growth of *cln1 cln2x/s* strains and to allow the isolation of small G₁ cells comparable to those used in the previous experiments. Small G₁ cells were isolated as described previously, and the accumulation of *cln2x/s* mRNA was monitored over time (Fig. 3A) and evaluated as a function of cell size (Fig. 3B, top).

This experiment led to several important observations. First, cells expressing *CLN3* as their only functional *CLN* gene accumulate *cln2x/s* mRNA and bud at a smaller size than cells expressing solely *CLN2* (cf. Figs. 3A and 1A). Mutational inactivation of UAS2 has a slight, but reproducible, effect on *cln2x/s* expression in the presence of *CLN3* (Fig. 3A, middle; B, top). Therefore, although UAS2 does influence the timing of *CLN2* expression in the presence of *CLN3*, that effect appears to be modest. Presumably *CLN3* can drive high-level *cln2x/s* transcription through the MCB and SCB promoter sequences of UAS1, thus masking the relatively weak activating potential of UAS2. Consistent with this assumption, mutational inactivation of SCB and MCB elements profoundly reduces expression of *cln2x/s* (Fig. 3, A, bottom; and B, top). The most probable cause of the failure of UAS2 to promote detectable expression of

Figure 2. UAS1 and UAS2 contribute to activation of the *CLN2* promoter in the absence of functional *CLN* genes. *cln1 cln2x/s cln3 leu2::GAL-CLN3* strains in which the *cln2x/s* ORF was regulated by a wild-type promoter, FC1492-2D (*CLN2*, □), a promoter with UAS2 deleted, DSY740 (Δ UAS2, ■), or a promoter with MCB and SCB mutations, DSY738 (*mcb/scb*, ●), were grown in YM-GAL. *CLN3* expression was repressed and small G₁ cells were isolated by centrifugal elutriation and inoculated into YEPD. (A) Total RNA isolated at the indicated time points was assayed by RNase protection for *cln2x/s* and *ACT1* mRNA. (B) The ratio of *cln2x/s/ACT1* mRNA from the sample in part A are presented as a function of mean cell volume. All of the RNA data for times 0–300 min are plotted.

cln2x/s in the presence of *CLN3* is that cells having functional *CLN3* execute Start and bud prior to attaining a size at which expression of the *mcb/scb* promoter becomes significantly activated (cf. Fig. 3B with Fig. 1B and 2B). When the same cells were analyzed following inoculation into YM-*raffinose* rather than YEPD (a condition that for strains of this *CLN* genotype results in a pro-

tracted G₁ phase and budding at an increased cell size, see below, Fig. 5A), expression of *cln2x/s* driven by the *mcb/scb* promoter was observed prior to the appearance of budded cells (data not shown). We conclude that the ability of *CLN3* to induce transcriptional activation of *CLN2* depends entirely, or almost entirely, on SCB and/or MCB elements. The results of this experiment cast doubt on a major role of *UAS2* in mediating *CLN2* activation during early G₁ phase in cycling cells.

CLN3, but not CLN2, determines the timing of CLN2 transcription in cycling cells

The positive-feedback hypothesis for regulation of *CLN1* and *CLN2* transcription is derived largely from the observation that overexpression of either *CLN1* or *CLN3* from the *GAL1* promoter in *CLN*-deficient cells can induce the expression of *CLN1*, *CLN2*, and other SCB-regulated genes (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991). However, the results of our previous experiments led us to suspect that there might be a substantial difference in the efficiency with which the wild-type *CLN2* and *CLN3* genes are able to activate transcription during G₁ phase. To evaluate this possibility we directly compared the timing of transcriptional activation of *CLN2* in strains having either *CLN2* or *CLN3* as their only functional *CLN* gene. A wild-type strain and a *CLN*-deficient strain were included as controls. As in previous experiments, all of the strains carried an integrated *GAL1-CLN3* construct and were grown in the presence of galactose to eliminate cell size variations that have confounded earlier studies. Small G₁ cells were again isolated by centrifugal elutriation and the timing of *CLN2* transcript accumulation was determined. The size at which the *CLN2* promoter became transcriptionally active was virtually identical in a wild-type strain and a strain having *CLN3* as its sole *CLN* gene (*cln1 cln2x/s CLN3*) (Fig. 4, A, top, cf. *CLN1 CLN2 CLN3* with *cln1 cln2x/s CLN3*, and B, top). This was in striking contrast to the strong delay in *CLN2* activation observed in cells having *CLN2* as the only functional *CLN* gene (Fig. 4, A, *cln1 CLN2 cln3*, and B, top). The delay in *CLN2* transcript accumulation is made even more remarkable by the fact that the kinetics of accumulation in the *cln1 CLN2 cln3* strain are comparable to that of the *cln2x/s* transcript in a *CLN*-deficient strain (Fig. 4, A, bottom, cf. *cln1 cln2x/s cln3* with *cln1 CLN2 cln3*, and B, top). This is despite the drastic difference in the ability of these two strains to bud and proliferate under these conditions (Fig. 4B, bottom). The ability of *cln1 cln2x/s CLN3* strains to maximally activate the transcription of *CLN2*, and presumably *CLN1*, at the same cell size as a wild-type strain, implies that *CLN1* and *CLN2* are of little importance with regard to activating their own transcription. This is supported further by the observation that although *cln1 CLN2 cln3* strains are viable and have a functional *CLN2* gene, the expression of the *CLN2* gene is not amplified to a level greater than achieved in cells expressing no functional Cln protein. The addition of functional *CLN1* in these strains (i.e.,

Figure 3. *CLN3* activates the *CLN2* promoter through MCB and SCB elements. *cln1 cln2x/s CLN3 leu2::GAL1-CLN3* strains with the *cln2x/s* ORF under the regulation of a wild-type promoter, DSY771 (*CLN2*, □), a promoter with *UAS2* deleted, DSY761 (Δ *UAS2*, ■), or with MCB and SCB mutations, DSY760 (*mcb/scb*, ●), were grown in YM-GAL. Expression of *CLN3* from the *GAL1* promoter was repressed and small G₁ cells isolated by centrifugal elutriation were inoculated into YEPD. (A) Total RNA was assayed by RNase protection for *cln2x/s* and *ACT1* mRNA at the indicated time points. (B) The ratio of *cln2x/s/ACT1* mRNA (top) and the percent budded cells in each sample (bottom) from samples in part A were plotted against mean cell volume. The graphic plots of both RNA and budding data are for times 0–180 min for DSY760 and DSY761 and times 0–165 min for DSY771.

CLN1 CLN2 cln3) fails to alter this result (data not shown). Thus, neither the Cln1 nor the Cln2 protein, to the extent that they accumulate under these conditions, participates in transcriptional activation of the *CLN2* gene.

Although the *CLN2* promoter becomes transcriptionally active at the same cell size in both a *cln1 cln2x/s* and a wild-type strain, the transcript actually accumulates to a higher level in cells deficient in *CLN1* and *CLN2* (Fig. 4, A, top, cf. *cln1 cln2x/s CLN3* with *CLN1 CLN2 CLN3*, and B, top). This hyperaccumulation of transcript is correlated with the prolonged G_1 phase of the *cln1 cln2x/s CLN3* strain relative to wild-type cells. Thus, despite their capacity to activate *CLN2* transcription at the same size as the wild-type cells, a strain expressing only *CLN3* is compromised in the ability to bud and proceed through a new cell cycle (Fig. 4B, bottom). This is better illustrated by the experiment described below.

Addition of glucose to cycling cells that are growing in poorer carbon sources results in a G_1 phase delay that leads to an adjustment to a larger minimum budding size (Lorincz and Carter 1979). Because the extent of that delay is affected by the status of the *CLN* genes (Baroni et al. 1994; Tokiwa et al. 1994) it has been suggested that it might be imposed through modulating the timing of their transcriptional activation. Therefore, it was important for us to eliminate the possibility that shifting cells from galactose into glucose was affecting the results of these experiments. We repeated the previous experiment but repressed the *GAL1* promoter by shifting cells into raffinose rather than glucose (Fig. 5A). As observed with small G_1 cells inoculated into glucose, the accumulation of *CLN2* transcripts occurred at the same cell size and with similar kinetics in wild-type and *cln1 Δ cln2 Δ CLN3* cells. This result confirms that the failure of *CLN1* and *CLN2* to contribute to the timing of activation of *CLN2* transcription in wild-type cells is not a

Figure 4. *CLN3* activates the *CLN2* promoter but *CLN2* fails to activated positive feedback. Wild-type *CLN1 CLN2 CLN3*, DSY580 (□), *cln1 cln2x/s CLN3*, DSY771 (●), *cln1 CLN2 cln3*, DSY725 (○), and *cln1 cln2x/s cln3*, FC1492-2D (■), all containing an integrated *GAL1-CLN3* were grown in YM-RAF supplemented with 0.5% galactose. The *GAL1* promoter was repressed by incubating the cells in YM-RAF and small G_1 cells isolated by centrifugal elutriation were inoculated into YEPD. (A) Total RNA isolated from samples obtained at the indicated time points was assayed for *CLN2* and *ACT1* mRNA. The relevant genotype of each strain is indicated for each panel. (B) The ratio of *CLN2/ACT1* mRNA obtained from the samples in part A plotted as a function of cell volume (top) and the percent budded cells in each culture plotted as a function of cell volume (bottom). The graphic plots of both RNA and budding data represent times 0–165 min for DSY580 and DSY771 and times 0–225 min for DSY725 and FC1492-2D.

consequence of the carbon source upshift from galactose into glucose.

The most notable result of the alteration in the experimental protocol was that budding of the *cln1Δ cln2Δ CLN3* strain was delayed dramatically relative to wild-type cells (Fig. 5A, bottom) and relative to the same cells grown on glucose (Fig. 4). In contrast, wild-type cells were only affected modestly in terms of their size at budding. As observed in the glucose-grown cells, the accumulation of the *CLN2* transcript persisted in *cln1 cln2x/s CLN3* cells grown in raffinose. However, correlated with the extensive delay in budding, transcriptional activation persisted even in cells that had achieved a cell volume greater than three times the size at which transcription was initiated. This observation supports the notion that transcriptional inactivation is associated with progression into the budded phase of the cell cycle and is consistent with the observation that transcriptional repression of SCB-dependent gene expression is dependent upon *CLB* gene expression (Amon et al. 1993).

In addition to being concerned about artifacts associated with shifting cells from galactose into glucose, we considered the possibility that the results of these experiments were influenced by making cells artificially small via overexpression of *CLN3*. To evaluate the contribution of *CLN1* and *CLN2* to transcriptional activation of *CLN2* without prior overexpression of *CLN3*, *CLN2* expression was analyzed in cells synchronized by mating pheromone arrest and release. This protocol had the further advantage that cells were grown continuously in rich glucose-containing medium, thereby completely eliminating the carbon source shift utilized in the elutriation protocol. Wild-type cells and cells containing either *CLN2* or *CLN3* as their only functional *CLN* gene were arrested by mating pheromone and released into fresh medium. Samples were obtained at 15-min intervals over a time course of 90 min and analyzed for the level of *CLN2* transcripts and budding index. Because cells prepared in this manner are not normalized in terms of cell volume as a result of differences in their *CLN* gene complement, cell volume was not a useful parameter for comparison. Nevertheless, the results observed in terms of the time of transcriptional activation of *CLN2* roughly parallel those obtained relative to cell volume in the previous experiments. That is, *CLN2* is activated with comparable timing in *cln1Δ cln2Δ CLN3* cells and in wild-type cells, whereas it is activated significantly later in cells having *CLN2* as their only functional *G₁* cyclin. In addition, the *CLN2* transcript accumulates to higher levels and persists for an additional 15 min in cells lacking functional *CLN1* and *CLN2*. Finally, in each case, as observed in the experiments performed by centrifugal elutriation, transcriptional inactivation appears to be most closely correlated with entry of cells into the budded phase of the cell cycle. Thus, whereas the interpretation of these experiments is limited relative to centrifugal elutriation by the fact that cell sizes are not equivalent at the beginning of the time course, the results are completely consistent with those obtained using the elutriation protocol.

Figure 5. (A) The failure of *CLN1* and *CLN2* to contribute to transcriptional activation in the presence of *CLN3* is not affected by carbon source. Wild-type *CLN1 CLN2 CLN3*, DSY580 (□) and *cln1 cln2x/s CLN3*, DSY771 (●), both containing an integrated *GAL1-CLN3* were grown in YM-RAF supplemented with 0.5% galactose. The *GAL1* promoter was repressed by incubating the cultures in YM-RAF, and small *G₁* cells isolated by centrifugal elutriation were inoculated in YM-RAF. (Top) Total RNA isolated at 15-min intervals was assayed for *CLN2* and *ACT1* mRNA, and the ratio *CLN2/ACT1* is plotted as a function of cell volume. (Bottom) The percent budded cells in each culture plotted as a function of cell volume. The graphic representation of both RNA and budding data represent times 0–135 min for DSY580. RNA data for DSY771 are plotted for times 0–210 min. Budding data were collected and plotted for times 0–465 min for DSY771 to allow the characterization of cell size at bud emergence. (B) Wild-type *CLN1 CLN2 CLN3*, 15DaubΔ (□), *cln1 CLN2 cln3*, DSY832 (○), *cln1 cln2x/s CLN3*, DSY714 (●), growing in YEPD were arrested with α-factor pheromone and released into YEPD. Samples of each culture were collected at 15-min intervals. (Top) The ratio of *CLN2/ACT1* mRNA at the indicated times following release from α-factor arrest. (Bottom) The percent budded cells in each culture at the indicated times following release from α-factor arrest.

CLN2 regulated by the CLN3 promoter is as potent as CLN3 in activating CLN2 transcription

One interpretation of the previous experiment is that in cycling cells *CLN3* is unique in its ability to activate the other *CLN* genes. This interpretation is contradicted by the fact that *cln1* and *cln2* reporter genes can be activated effectively by overexpression of any *CLN* gene or of *CLB5* (Cross and Tinkelenberg 1991; Schwob and Nasmyth 1993). However, it has also been reported that *CLN1*, when expressed from the *GAL1* promoter, is a less effective activator of *CLN* transcription than is *CLN3* (Tyers et al. 1993). In an effort to resolve this apparent paradox we directly tested the relative potency of *CLN2* and *CLN3* with regard to their ability to activate

CLN2 transcription. Comparable patterns of *CLN2* and *CLN3* expression were achieved by placing the *CLN2* ORF under the regulation of a *CLN3* promoter (*CLN2^{3p}*) (Valdivieso et al. 1993). Cells carrying this construct accumulate levels of *CLN2* mRNA that are comparable to *CLN3* under the same promoter. A *cln1 cln2x/s cln3* strain carrying single integrated copies of both *GAL1-CLN3* and *CLN2^{3p}* was directly compared with a *cln1 cln2x/s CLN3* strain with regard to the ability to activate the *CLN2* promoter and initiate budding. The results of that analysis are depicted in Figure 6 and the quantitative data plotted in parallel with the data from the wild-type and *cln1 cln2x/s CLN3* strains that was presented in Figure 4 (Fig. 6B). As was expected based upon the small mean cell size and the presence of small budded cells in an asynchronous population of cells carrying *CLN2^{3p}* as its only functional *CLN* gene, both expression of the *cln2x/s* gene and budding were initiated at a smaller cell size in the elutriated population of *CLN2^{3p}* cells relative to the wild-type or *cln1 cln2x/s CLN3* cells (Fig. 6A,B). This transcriptional activation is mediated primarily through UAS1, as an *mcb/scb* mutant *CLN2* promoter is ineffectively activated by *CLN2^{3p}*, whereas a Δ UAS2 *CLN2* promoter and a wild-type *CLN2* promoter are activated comparably (data not shown). Thus, *CLN2^{3p}* activates the endogenous *CLN2* promoter through the same *cis*-acting elements as does *CLN3*. Based upon these experiments we conclude that when comparably expressed *CLN2* is at least as effective as *CLN3* at inducing transcription and cell cycle progression. Thus, when constitutively expressed, *CLN2* is capable of prematurely inducing both activation of *CLN* transcription and Start. In contrast, when regulated by its own promoter *CLN2* induces Start but fails to amplify its own transcription effectively. This can be explained if *CLN2*-associated kinase is relatively more effective at activating Start and cell cycle progression than it is at activating *CLN* transcription (see Discussion).

Discussion

The timing of activation of CLN2 transcription in cycling cells is determined by CLN3, not by positive feedback

The ability of any one of the three *CLN* genes to induce G₁-specific transcription of *CLN1* and *CLN2* led to the hypothesis that *CLN1* and *CLN2* gene expression is regulated by a positive feedback loop. In this study we have characterized the cell size-dependent transcriptional activation of *CLN2* and the relative contributions of the two previously characterized promoter elements UAS1 and UAS2. In the course of these studies we analyzed the capacity of *CLN2*, as well as *CLN3*, to activate *CLN2* transcription via these elements. The most striking result of these experiments is that *CLN2* expressed from its own promoter does not effectively amplify its own transcription. That is, a wild-type *CLN2* gene and the nonfunctional *cln2x/s* gene are activated with identical kinetics in cells that are otherwise *CLN* deficient. This

Figure 6. *CLN2* when expressed from the *CLN3* promoter can effectively activate the *CLN2* promoter. In conjunction with the experiment presented in Fig. 4, small G₁ cells of the strain *cln1 cln2x/s cln3 HIS2::CLN^{3p}* with an integrated *GAL1-CLN3* (CWY278) were isolated by centrifugal elutriation and inoculated into YEPD. (A) Total RNA from samples isolated at the indicated time points was probed for *CLN2* and *ACT1* mRNA. The *CLN2* probe identifies *CLN2* mRNA driven both from the endogenous *CLN2* gene (*cln2x/s*) and from the integrated *CLN2^{3p}* (*CLN2^{3p}*). Note that *CLN2^{3p}* displays little variation relative to the *ACT1* mRNA during the course of the experiment. (B, top) mRNA driven by the endogenous *CLN2* gene as a ratio *CLN2/ACT1* plotted against the mean cell volume; *cln1 cln2x/s cln3 HIS2::CLN^{3p} LEU2::GAL1-CLN3*, CWY278 (○), *CLN1 CLN2 CLN3 LEU2::GAL1-CLN3*, DSY580 (□), *cln1 cln2x/s CLN3 leu2::GAL-CLN3*, DSY771 (●), the data used to plot wild-type, and *cln1 cln2x/s CLN3* mRNA were presented in Fig. 4. (Bottom) The percent budded cells in the cultures plotted against the mean cell volume, as at top, the data for wild-type and *cln1 cln2x/s* strains were presented in Fig. 4. The RNA and budding data plotted against cell volume represent data for times 0–165 min.

is despite the fact that the expression of *CLN2* results in cell cycle progression, whereas cells expressing only *cln2x/s* arrest in G₁ and cannot progress through Start. This suggests that Cln2/Cdc28 kinase plays little, if any, role in the activation of the *CLN2* promoter. Furthermore, we show that *CLN2* transcription is activated with identical timing in cells having only *CLN3* and in wild-type cells. The efficacy of *CLN3* at activating *CLN2* transcription along with the failure of *CLN2* to increase its own transcription demonstrates that *CLN2*, and probably other SCB-regulated genes including *CLN1*, are activated primarily by *CLN3*. Whether *CLN3*-associated kinase directly activates SCB-dependent transcription and Start is unclear, because in addition to *CLN1* and *CLN2*, two B-type cyclins (*CLB5* and *CLB6*) and at least two other cyclin-like genes (*PCL1* and *PCL2*) are expressed during G₁ phase. Inactivation of either pair of cyclins in combination with deletion of *CLN1* and *CLN2* results in G₁ phase cycle arrest (Schwob and Nasmyth 1993; Measday et al. 1994). We show that *CLN2* or the combination of *CLN1* and *CLN2* fail to activate their own transcription effectively in the absence of *CLN3*. In addition, inactivation of both *CLN1* and *CLN2* does not affect the timing or kinetics of *CLN2* mRNA accumulation in the presence of *CLN3*. Thus, we conclude that *CLN1* and *CLN2* do not participate in the initial activation of their own transcription. That is, the initial transcriptional activation of *CLN2* is dependent on *CLN3* but is feedback independent. Whereas we cannot categorically exclude the possibility that under some conditions *CLN1* and *CLN2* will contribute to their own activation, it is clear that in cycling cells *CLN3* alone is sufficient to determine both the timing and extent of transcriptional activation of *CLN2*.

The role of CLN2 promoter elements

When cells are rendered dependent on *CLN2* as the sole G₁ cyclin, the presence of either UAS1 or UAS2 is sufficient to allow transcriptional activation and, consequently, to promote Start and cell proliferation. In contrast, inactivation of both promoter elements precludes cell cycle progression. Whereas inactivation of either promoter element delays and limits the extent of transcriptional activation of *CLN2*, neither completely eliminates it. Because this effect is observed most easily in cells lacking functional *CLN* genes, we conclude that either UAS1 or UAS2 alone is sufficient to activate *CLN2* transcription in a *CLN*-independent manner. This is in contrast to our previous observation that an isolated UAS2 fragment is competent to induce transcription of a heterologous reporter in the absence of *CDC28* but not in *CLN*-depleted cells (Stuart and Wittenberg 1994). The basis for this discrepancy is unclear but appears to be accounted for by the context of the plasmid-borne UAS2 because, when maintained on centromeric episomal plasmids, neither an isolated UAS2 fragment nor an entire *mcb/scb CLN2* promoter display activation during an arrest induced by *CLN* deprivation.

The importance of UAS2 for transcriptional activation

of *CLN2* is affected dramatically by the presence of a functional *CLN3* gene. In cells expressing *CLN3*, elimination of UAS2 has a very modest effect on the timing and extent of *CLN2* activation. This is true whether *CLN3* is expressed at wild-type levels or overexpressed from the *GAL1* promoter, and is consistent with our previous demonstration that UAS2 makes a minor contribution to *CLN2* transcription in wild-type cells. In contrast, a dramatic increase in the accumulation of *CLN2* transcript directed by UAS2 is observed upon either elimination of *CLNs* or inactivation of *CDC28*. Because it is unlikely that Cln3 activity can directly repress UAS2, a more tenable explanation for the inactivity of UAS2 in the presence of *CLN3* is that by promoting Start, *CLN3* decreases the duration of G₁, the cell cycle phase in which UAS2 is active. This hypothesis is supported by the observation that cells possessing a functional *CLN3* have budded and entered a new cell cycle before they reach a size at which maximal UAS2-driven transcription can be detected. In addition, when the unbudded phase of G₁ is prolonged activation of UAS2 can be observed. Because the presence of *CLN3* eliminates much of the negative impact of inactivation of UAS2, we predict that regulation of transcription mediated through UAS2 occurs under as yet unestablished conditions where the timing of Start is modulated independent of *CLN3*.

CLN3 is required for effective activation of the *CLN2* promoter in G₁-phase. Although transcription of *CLN2* in cells expressing *CLN3* is essentially uninfluenced by inactivation of UAS2, it is affected profoundly by the inactivation of the SCB and MCB sequences in UAS1. Based on this observation and the low level of *CLN2* transcription driven by UAS2 in cells expressing *CLN3* from the *GAL1* promoter, we conclude that *CLN3*-dependent activation of *CLN2* is directed primarily through SCB and/or MCB sequences. Furthermore, because cells depending on *CLN2* as their only *CLN* gene require *SWI4* (Cross et al. 1994; Nasmyth and Dirick 1991) but not *MBP1* (D. Stuart and C. Wittenberg, unpubl.) for viability, we presume that *CLN3* promotes transcriptional activation of *CLN2* through SBF. The mechanism by which *CLN3* activates SBF is unclear. Alterations in the electrophoretic mobility of SBF-DNA complexes following activation of *CDC28* have been put forth as evidence that phosphorylation by Cln/Cdc28 kinase is the event that activates SBF (Tabata et al. 1991). However, regulation of SBF activation might also be exerted at the level of expression of *SWI4* (Breedon and Mikesell 1991), subcellular localization of Swi6 (Tabata et al. 1991), or through other post-translational mechanisms.

CLN2, a potent activator of its own expression, is not regulated by positive feedback

The failure of the wild-type *CLN2* gene to induce its own transcription seems to contradict previous studies in which overexpression of any *CLN* gene in cells that had been arrested by *CLN* deprivation induced transcription

of *CLN1* and *CLN2* (Cross and Tinkelenberg 1991). We show that *CLN2* expressed at a much lower level (under the regulation of the *CLN3* promoter) is also able to induce the endogenous *CLN2* promoter. This presents a paradox; *CLN2* can effectively induce *CLN1* and *CLN2* transcription but does not do so in cycling wild-type cells. This paradox can be resolved if a functional difference exists between the *CLN* gene products with regard to the activity of their associated kinases toward specific substrates. *CLN3*-associated kinase may be highly effective at activating SBF but less effective at inducing Start and cell cycle progression. *CLN2*-associated kinase may be more effective at inducing Start and its associated events. Consistent with that hypothesis, *cln1 cln2 CLN3* cells can effectively activate the *CLN1* and *CLN2* promoters but are delayed in passage through Start. Furthermore, cells that are dependent on *CLN2* accumulate *CLN2* mRNA and presumably Cln2 protein with its associated kinase activity very slowly. These cells may perform Start and bud before Cln2 kinase can accumulate sufficiently to activate SBF. This is predicted if the Cln2-associated kinase is relatively more effective at promoting Start and cell cycle progression. Because transit through Start and entry into the budded phase of the cell cycle precludes activation of SBF (Amon et al. 1993), cells dependent on *CLN2* never display the burst of *CLN1* and *CLN2* transcription observed in cells having a functional *CLN3* gene. This hypothesis also predicts that if cells dependent on *CLN2* are prevented from passing Start and as a consequence are prevented from inactivating SBF, then Cln2 protein will accumulate to a sufficient level to stimulate *CLN1* and *CLN2* transcription. This phenomenon has been reported (Cross and Tinkelenberg 1991). We show here that the *CLN2* transcript in *cln1 cln2x/s CLN3* cells accumulates for a longer period and to significantly higher levels than in wild-type cells. This is consistent with the delay observed in these cells in the execution of Start and inactivation of SBF.

Because *CLN2* is activated with identical kinetics in cells having solely *CLN3* and cells having both *CLN2* and *CLN3*, we propose that *CLN2* is a more potent activator of cell cycle progression than of its own transcription. Because of the inherent imperfection in the synchrony of populations of elutriated cells, it is possible that *CLN2* makes some contribution to its own activation but that its effect is obscured by the competing processes of transcriptional activation and repression. That is, in cells with functional *CLN2*, a portion of the population may be experiencing activation of transcription while another fraction is experiencing the transcriptional inactivation associated with cell cycle progression. Thus, a small addition to transcriptional activation provided by *CLN2* might not be apparent. In contrast, cells deficient in *CLN2* lack this added transcriptional activation but are also greatly delayed in transcriptional inactivation. As a result the rate of accumulation of *CLN2* transcript could appear to be identical in both strains. However, this scenario would predict that at least the initial rate of transcript accumulation should be

accelerated in cells having *CLN2* because transcriptional inactivation appears to be significantly delayed relative to the initial transcriptional activation. Such acceleration has not been observed. Although we cannot formally exclude the possibility that imperfect synchrony obscures a contribution of *CLN2* to its own transcription, we find it unlikely because the timing and kinetics of transcriptional activation in cells with and without functional *CLN2* appears identical in a number of different strains and under a number of different conditions (Figs. 4, 5A, and 6; data not shown).

The relative efficiencies with which different G_1 cyclins induce Start and transcriptional activation of *CLN2* supports a model in which Cln3/Cdc28 kinase is more effective at activating SBF, whereas the Cln2/Cdc28 kinase is more effective at phosphorylating substrates such as *SIC1* that are associated with cell cycle progression (Schwob et al. 1994). The inability of wild-type *CLN2* to effectively induce SBF-dependent transcription may reflect a requirement for a higher level of this kinase to activate the relevant substrates. Cells that express *CLN2* early in G_1 , such as those containing the *CLN2^{3p}* allele, initiate the cell cycle with sufficient kinase activity to activate SBF and to induce Start. In those cells both *CLN2* transcription and cell cycle progression can be activated prematurely. In contrast, cells that are dependent upon the endogenous *CLN2* gene may accumulate kinase activity too slowly to attain a threshold of activity necessary to activate SBF prior to attaining a sufficient level to induce Start.

It is not clear why *CLN2^{3p}* activates SBF-dependent transcription earlier than *CLN3*. Perhaps the absolute level of Cln2 and Cln3 protein resulting from the same levels of transcription differ. Although Cln2 and Cln3 are both unstable proteins (Tyers et al. 1992; Cross and Blake 1993; Salama et al. 1994; Deshaies et al. 1995), Cln3 accumulates to a lower level than Cln2 in asynchronous cultures (Tyers et al. 1993). Furthermore, small differences in their stability could have profound physiological consequences if their steady-state level is near the threshold required to generate an activated Cln/Cdc28 kinase complex. Alternatively, the amount of kinase activity resulting from equivalent amounts of *CLN2* and *CLN3* may differ. Using histone H1 as a substrate, Cln3/Cdc28 kinase is at least two orders of magnitude less active than Cln2/Cdc28 (Tyers et al. 1993). Finally, because *CLN2* is not normally expressed early in G_1 it may not be subject to the same forms of regulation that affect *CLN3*. Although it is difficult to assess the contribution of each of these mechanisms, it is likely that there is more than one explanation for the difference in the ability of these two cyclins to activate transcription and Start.

New thinking about the regulation of Start in yeast

Previous thinking about Start has been based largely on and influenced by the positive feedback hypothesis. It has been suggested that *CLN3* acts as an upstream activator of *CLN1* and *CLN2* in this process (Tyers et al.

1993). We now envision progression through G₁ and passage through the G₁/S phase transition as a linear pathway in which *CLN3* acts through the SBF transcription factor to induce the transcription of *CLN1*, *CLN2*, and other G₁-specific genes. Cln1 and Cln2 then rapidly induce events associated with Start such as budding, spindle pole body duplication, DNA replication, and commitment to a mitotic cell cycle. As a consequence, SBF activity is repressed. A linear model for progression through G₁ phase and Start acts to highlight the functional differences between G₁ cyclins. Although any one of the *CLN* genes can confer viability to cells, they display qualitative and quantitative differences with regard to their ability to activate SCB-dependent transcription and Start. Why should budding yeast cells utilize such a two-tiered system for passage through Start? It may be that multiple stage-specific cyclins allow for an increased range of physiological responses to intracellular and extracellular signals. Because Cln3/Cdc28 kinase has weak activity and Cln3 is an intrinsically unstable protein, it is ideally suited to a role in sensing growth rate. Modulating its activity can influence the timing of activation of other G₁ cyclins. *CLN1* and *CLN2*, once activated, may be subject to forms of regulation distinct from those affecting *CLN3*. Elucidation of the mechanisms governing the accumulation of these G₁ cyclins and the identification of their targets remains a central goal in investigations of cell cycle regulation.

Materials and methods

Strains and media

All of the yeast strains used in this study were congenic with 15DaubΔ, a *bar1Δ ura3Δ* derivative of BF264-15D (*MATa ade1 his2 leu2-3,112 trp1-1^a*) [Richardson et al. 1989]. The relevant genotypes of the strains used in this study are provided in Table 1. Cells were grown in YM media (1% yeast extract, 2% Bacto-peptone, 30 mg/l of adenine) supplemented with 2% glucose (YEPD), galactose (YM–GAL), or raffinose (YM–RAF). For the experiments described in Figures 4 and 6, cells were initially grown in YM supplemented with 2% raffinose and 0.5% galactose. Yeast transformations and standard genetic techniques were performed as described [Rose et al. 1990]. Point mutations and deletions in the *CLN2* upstream region were described previously [Stuart and Wittenberg 1994]. The nonfunctional *cln2x/s* ORF was generated as described [Cross and Tinkelenberg 1991]. The polypeptide synthesized from this mutant gene is nonfunctional by virtue of its inability to physically interact with and activate the Cdc28 kinase [M. Valdivieso and C. Wittenberg, unpubl.]. In addition, the *cln2x/s* transcript has been shown to display regulation indistinguishable from the *CLN2* transcript under a variety of conditions [Cross and Tinkelenberg 1991; Stuart and Wittenberg 1994]. A two-step transplacement procedure was used to replace the endogenous *CLN2* gene in a *cln1 cln2 cln3 leu2::GAL1–CLN3* strain with versions of *CLN2* or *cln2x/s* that had the indicated promoter mutations [Stuart and Wittenberg 1994]. Gene replacements were confirmed initially by Southern blot hybridization and upstream and downstream regions were tested by PCR analysis and subsequent restriction mapping of the PCR products. Construction of the

chimeric *CLN2^{3p}* in which the *CLN2* ORF is placed under the regulation of the *CLN3* promoter has been described previously [Valdivieso et al. 1993]. *CLN2^{3p}* was integrated in single copy at the *HIS2* locus of a *cln1 cln2x/s cln3 leu2::GAL1–CLN3* strain. In glucose-based medium this strain is dependent on the expression of the *CLN2^{3p}* construct for viability. The *MBP1* disruption plasmid was generated by PCR amplification of genomic DNA with the oligonucleotide primers 5'-CGAG-GATCCCAACGCGCTAGGCTTACCG-3' and 5'-CGCCTC-GAGGACGAAATCTACATCAACGGT-3'. The 3.4-kb amplification product was ligated into a *Bam*HI/*Xho*I-cut Bluescript vector (Stratagene), and the internal *Eco*RI fragment was replaced with a 1.1-kb *URA3* gene. This plasmid then was cleaved with *Bam*HI and *Xho*I and used to disrupt the *MBP1* gene in DSY725, *cln1 CLN2 cln3 leu2::GAL1–CLN3* by a one-step gene replacement [Rothstein 1983]. Candidate transformants were screened by PCR amplification of the *MBP1* locus, and three independent transformants that carried the disrupted *MBP1* allele were tested for the ability to grow on glucose-based medium. Centromeric *lacZ* reporter genes used to test the ability of plasmid-borne *UAS2* and *mcb/scb CLN2* promoters have been described previously [Stuart and Wittenberg 1994].

RNA analysis

Total RNA was obtained from frozen cell pellets and subjected to RNase protection analysis as described previously [Stuart and Wittenberg 1994] except that 10 μg of total RNA per sample was analyzed. Riboprobes for *CLN2* and *ACT1* were described previously [Stuart and Wittenberg 1994]. The *CLN2* probe protects not only the full-length *CLN2* mRNA but also the 5' region of the *CLN2^{3p}* mRNA allowing both to be visualized simultaneously. Quantitation of the RNase protection data was performed with a Molecular Dynamics PhosphorImager.

Cell synchronization

Populations of small G₁-synchronized cells were obtained by centrifugal elutriation. For the experiments illustrated in Figures 1–3, cells were inoculated in 2 liters of YM–GAL and grown overnight at 30°C to a density of 5 × 10⁶ cells/ml at which time glucose was added to the culture to a final concentration of 2% and incubation was continued for 1.25 hr. Cells then were harvested, cooled to 4°C and sonicated for 2 min with the large-sized tip of a Braun Sonic 2000 sonicator to disperse clumps. The culture was maintained on ice while the cells were loaded into a Beckman J6M/E centrifuge (JE 5.0 rotor) at 4°C 4000 rpm at a pump speed of 0.8, a speed at which the cells were retained in the rotor chamber. When a suitable number of cells had been concentrated in the chamber, the pump speed gradually was increased until small G₁ cells were pumped out of the chamber. The elutriated population of G₁ cells was maintained on ice until a sufficient number had been collected, then the cells were concentrated by centrifugation and resuspended in prewarmed YEPD. The experiments illustrated in Figures 4, 5A, and 6 utilized a similar protocol with the exception that the cells initially were grown overnight in YM–RAF supplemented with 0.5% galactose. When the cultures had achieved a density of 5 × 10⁶ cells/ml, the cells were collected by filtration, resuspended in prewarmed YM–RAF and incubated for 1.5 hr. The cells were cooled to 4°C, harvested by filtration, and small unbudded G₁ cells were isolated by elutriation as described above. The elutriated cells were inoculated into prewarmed YEPD (Figs. 4, 6) or prewarmed YM–RAF (Fig. 5A). Synchronization of

Table 1. *S. cerevisiae* strains used in this study

Strain	Relevant genotype ^a	Source
15DaubΔ	<i>MATa ade1 his2 leu2-3,112 trp1-1 ura3Δns bar1</i>	Richardson et al. (1989)
FC1492-2D	<i>MATa cln1 cln2x/s cln3 leu2::GAL1-CLN3</i>	F. Cross
DSY720	<i>MATa cln1 cln2::URA3 cln3 leu2::GAL1-CLN3</i>	this study
DSY725	<i>MATa cln1 CLN2 cln3 leu2::GAL1-CLN3</i>	this study
DSY722	<i>MATa cln1 ΔUAS2 CLN2 cln3 leu2::GAL1-CLN3</i>	this study
DSY723	<i>MATa cln1 mcb/scb CLN2 cln3 leu2::GAL1-CLN3</i>	this study
DSY724	<i>MATa cln1 ΔUAS2 mcb/scb CLN2 cln3 leu2::GAL1-CLN3</i>	this study
DSY755	<i>MATa cln1 cln2::URA3 CLN3</i>	this study
DSY771	<i>MATa cln1 cln2x/s CLN3 leu2::GAL1-CLN3</i>	this study
DSY761	<i>MATa cln1 ΔUAS2 cln2x/s CLN3 leu2::GAL1-CLN3</i>	this study
DSY760	<i>MATa cln1 mcb/scb cln2x/s CLN3 leu2::GAL1-CLN3</i>	this study
DSY740	<i>MATa cln1 ΔUAS2 cln2x/s cln3 leu2::GAL1-CLN3</i>	this study
DSY738	<i>MATa cln1 mcb/scb cln2x/s cln3 leu2::GAL1-CLN3</i>	this study
DSY580	<i>MATa LEU2::GAL1-CLN3</i>	this study
CWY278	<i>MATa cln1 cln2x/s cln3 LEU2::GAL1-CLN3 HIS2::CLN2^{3p}</i>	M. Valdivieso and C. Wittenberg (The Scripps Research Institute)
DSY795	<i>MATa cln1 CLN2 cln3 mbp1::URA3 leu2::GAL1-CLN3</i>	this study
DSY714	<i>MATa cln1 cln2x/s CLN3</i>	this study
DSY832	<i>MATa cln1 CLN2 cln3</i>	this study
DSY789	<i>MATa CLN1 CLN2 cln3 leu2::GALCLN3</i>	this study
DSY833	<i>MATa cln1 mcb/scb cln2x/s cln3 leu2::GALCLN3 HIS2::CLN2^{3p}</i>	this study
DSY834	<i>MATa cln1 ΔUAS2 cln2x/s cln3 leu2::GALCLN3 HIS2::CLN2^{3p}</i>	this study

^aAll strains are derivatives of 15DaubΔ.

cells by mating pheromone was achieved by the addition of α -factor to a concentration of 100 ng/ml to cultures grown in YEPD. Following pheromone addition the cultures were incubated for 2.5 hr and the arrest was monitored by examining cell morphology. To release cultures from the arrest cells, were collected by filtration, washed once with pre-warmed YEPD, and then inoculated into pre-warmed YEPD. To test the ability of plasmid-borne *CLN2* promoter fragments to be activated during *G*₁ arrest caused by *CLN* deprivation cultures of FC1492-2D harboring centromeric, *URA3*-marked, *lacZ* reporter gene plasmids were grown in minimal medium lacking uracil and supplemented with 2% galactose to a density of 1.2×10^6 cells/ml. These cultures then were allowed to proceed through two doublings in YM-GAL prior to being arrested by the addition of glucose to a final concentration of 2%. Samples of each culture were taken at 1-hr intervals for 6 hr and total RNA was analyzed by RNase protection using probes for *lacZ* and *ACT1* as described previously (Stuart and Wittenberg 1994).

Other methods

Cell size analysis was performed on a Coulter Channelyzer that had been calibrated with latex beads. Cell volume was determined on fresh cells. Approximately 1×10^5 cells were diluted into 10 ml of physiological saline, and the mean cell volume of the population (in femtoliters) was determined. Asynchronous cultures were lightly sonicated prior to measurement. During the time course following elutriation, cell samples were collected and held on ice until the completion of the time course when all of the samples were analyzed. We have observed that as long as the cells are maintained on ice there is no significant change in cell volume for at least 5 hr. For determination of cell morphology and proportion of budded cells 200 cells from formaldehyde-fixed samples were counted for each time point.

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CLN3, not positive feedback, determines the timing of CLN2 transcription in cycling cells.

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