A novel Mcm1-dependent element in the *SWI4*, *CLN3*, *CDC6*, and *CDC47* promoters activates M/G₁-specific transcription

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We have identified a novel promoter element that confers M/G₁-specific transcription in *Saccharomyces cerevisiae*. This element, which we call an ECB (early cell cycle box), was first identified in the *SWI4* promoter, but it is also present in the promoter of a G₁ cyclin *CLN3*, as well as in the promoters of three DNA replication genes: *CDC6*, *CDC47*, and *CDC46*. Transcripts from all five of these genes oscillate during the cell cycle and peak at the M/G₁ boundary, as do isolated ECB elements in reporter constructs. The ECB element contains an Mcm1 binding site to which Mcm1 binds in vitro, and an Mcm1-VP16 fusion, which places a constitutive activator on Mcm1-binding sites in vivo, can deregulate ECB-containing promoters. Mcm1 is a transcription factor that is also required for minichromosome maintenance. We provide evidence that the replication defect of *mcm1* mutants can be suppressed by ectopic *CDC6* transcription. Periodic expression of *SWI4* and *CLN3* may be important for cell cycle progression, as we find that these genes are both haploinsufficient and rate limiting for G₁ progression. We suggest that ECB-regulated gene products play critical roles in promoting the initiation of S-phase, both by regulating *CLN1* and *CLN2* transcription and as components of the initiation complex on origins of replication.

[Key Words: ECB; *SWI4*; *CLN3*; *CDC6*; *CDC47*; cell cycle; transcription]

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Nearly 100 genes have been identified in *Saccharomyces cerevisiae* that are transcribed at specific stages of the cell cycle. Only a minority of these gene products have been shown to play critical roles in cell cycle progression; they have been identified as unstable proteins that are rate limiting for specific processes in the cell cycle. These gene products are transcribed when they are required, and high-level constitutive expression of them is often deleterious to the cell. Understanding how these proteins function and how their expression is regulated is key to understanding the control of the cell cycle.

Swi4 is a transcription factor critical for the G₁-to-S transition, which associates with Swi6 to form the complex that activates transcription of *HO* and the G₁/S-specific cyclins *CLN1* and *CLN2* (for review, see Breeden 1996). *SWI4* transcription is periodic, and it peaks just before the peak of transcription of its target genes (Breeden and Mikesell 1991, 1994). Stabilization or constitutive transcription of *SWI4* increases the level of

CLN1 and *CLN2* transcription throughout the cell cycle (Breeden and Mikesell 1994) and leads to the aberrant budding morphology that is typical of cells overproducing *CLN1* and *CLN2* (Breeden and Mikesell 1991; Lew and Reed 1993).

CDC6, *CDC46*, and *CDC47* are all transcribed at about the same time as *SWI4* (Bueno and Russell 1992; Zwierschke et al. 1994; Dalton and Whitbread 1995). These proteins are required for the initiation of DNA synthesis (for review, see Stillman 1996), and defects in these genes cause a high rate of minichromosome loss that can be suppressed by increasing the number of replication origins on the DNA (Brewer and Fangman 1987; Hogan and Koshland 1992). The periodic transcription of *CDC6* is clearly important because high-level constitutive expression of Cdc6 (Bueno and Russell 1992; Piatti et al. 1995), or its *Schizosaccharomyces pombe* homolog Cdc18 (Kelly et al. 1993), delays M phase and loss of function of these proteins prevents DNA synthesis but allows a lethal M phase to proceed on schedule.

Certainly the best known of the stage-specific regulators are the cyclins. In the budding yeast, nine cyclins have been identified that bind and activate the Cdc28 kinase (Cross 1995). This single cyclin-dependent kinase

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(CDK) exerts primary control over the major transitions in the cell cycle, so the successive expression of different classes of cyclins must provide the specificity for these transitions. Cln3 stands out among the cyclins because even small changes in its expression have the effect of either speeding or delaying the transition from G₁ to S phase (Nash et al. 1988; Cross 1988, 1989). The only known activity of the Cln3 kinase is in activating the burst of G₁/S-specific cyclin transcription that is mediated by the Swi4/Swi6 and Mbp1/Swi6 transcription complexes (Dirick et al. 1995; Stuart and Wittenberg 1996), but neither the kinase substrate nor its mechanism of activation in late G₁ has been identified.

In this paper we described a new cell cycle-regulated promoter element that is present in the promoters of a group of genes: *SWI4*, *CLN3*, *CDC6*, *CDC47*, and *CDC46*, which are coordinately regulated at the M/G₁ boundary. This early cell cycle box (ECB) element contains an Mcm1-binding site, and we show that Mcm1 controls ECB gene expression. We provide evidence suggesting the importance of this transcriptional regulation in timing the entry into S phase and in DNA replication.

Results

Early timing of *SWI4* transcription is conferred by a novel cell cycle regulatory element

The *SWI4* promoter contains three MCB (*Mlu*I cell cycle box) elements that are known to confer G₁/S-specific transcription to many genes. However, deletion of these three known cell cycle regulatory elements does not eliminate the periodicity or the early timing of transcription (Foster et al. 1993). To identify the novel promoter element that is responsible for this early peak of *SWI4* transcription, we first showed that the 90-bp region 3' to the MCB elements (*SWI4* 90 bp) was sufficient to confer this regulation (Fig. 1, top). We then used mutagenic PCR to identify the sequences responsible for the transcriptional activity of the 90-bp fragment. The mutations that reduced transcription were clustered within an 8-bp inverted repeat (Fig. 1, bottom), and disruption of these palindromic sequences, or the spacing between them, causes up to a 50-fold drop in transcript levels. The center of the palindrome is a consensus binding sequence for the transcription factor Mcm1, which acts as an activator and a repressor in different promoter contexts (Jarvis et al. 1989; Passmore et al. 1989; Ammerer 1990; Elble and Tye 1991). To determine whether the specific residues crucial for Mcm1 binding are required for the upstream activation sequence (or UAS) activity of this 90-bp fragment, we made a GG → CC mutation within the Mcm1 consensus binding sequence. This change causes a 14-fold drop in transcription compared to wild-type DNA. The minimal Mcm1-binding site has been defined by site selection in vitro to require only the 8 bp indicated by the hatched bar in Figure 1 (Wynne and Treisman 1992). This sequence is clearly important, but we also see 5- and 10-fold reductions in UAS activity when single-base substitutions are made in the bases im-

Figure 1. Localizing the novel cell cycle regulatory element in the *SWI4* promoter. The central diagram depicts the *SWI4* promoter (Foster et al. 1993) in relation to the ATG (+1). Below is shown the relative position of the 90-bp promoter fragment (*SWI4* 90 bp), which harbors one inactivated MCB as well as novel sequences sufficient to confer early cell cycle regulation to the *lacZ* transcript (referred to as ECB). The plot shows the oscillation of the *SWI4* 90 bp-driven *lacZ* transcript compared to that of *CLN1*. For this plot, the *lacZ* and *CLN1* transcripts were measured through three synchronous cell cycles by S1 protection. They were quantitated by PhosphorImaging, normalized to the constitutive *MATa1* mRNA, and plotted as a function of time after release from the G₁ arrest induced by α -factor. The bottom diagram shows six PCR-generated mutants and one site-directed mutant (denoted by the asterisk); the level of transcript from each is listed to the right. The transcriptional activity of each mutant is an average value obtained from three independent transformants. These values were normalized to *MATa1* transcript levels and are presented as the percentage obtained from their wild-type counterparts (pBD1478 for the PCR-generated mutants and pBD1577 for the site-directed mutant). The hatched bar above the sequence identifies the minimal Mcm1-binding site identified by site selection in vitro (Wynne and Treisman 1992), and the black bars flanking it show the additional sequences required for full UAS activity based on this mutagenesis. pBD1900 has an additional A → G change 33 bp 3' to the ECB element in a region that is unlikely to contribute to its phenotype.

mediately flanking the minimal Mcm1-binding site. This emphasizes the importance of these adjacent sequences for transcription activation.

To assess the potential regulatory role of this UAS element within the *SWI4* promoter, we introduced point

mutations within this sequence and recombined them into the genomic *SWI4* promoter. Although *SWI4* is an essential gene in the W303 strain background, loss of the element is not a lethal mutation, because transcription persists in this strain. However, loss of only this novel UAS element causes a measurable delay in *SWI4* transcription during the cell cycle (Fig. 2) and a concomitant delay in *CLN1* and *CLN2* transcription (data not shown). The transcript generated from this promoter mutant is still periodic, but it peaks at least 5 min later than wild-type *SWI4*. The cell cycle regulation that persists is almost certainly conferred by the MCB elements because these elements are known to activate cell cycle-regulated transcription, which peaks in late G₁ (Gordon and Campbell 1991; McIntosh et al. 1991). The delay of transcription observed with the mutant UAS indicates that this sequence is required for the early wave of *SWI4* transcription. Thus, we will refer to it as an ECB element. It is interesting to note that loss of the ECB element also causes the start site for *SWI4* transcription to change (Fig. 2). Thus, both the timing of transcription and the start site selection seem to be governed by the ECB element in exponentially growing cells.

Cell size control and the length of the G₁ interval are also affected by point mutations in the *SWI4* ECB. This phenotype is easily detected in poor carbon sources such as glycerol (Fig. 3) and raffinose (data not shown), where the cell cycle is slow and wild-type cells maintain a very small cell size. Under these conditions, *swi4ecb* cells have about a 30% larger volume than their wild-type

Figure 2. Loss of the ECB element of *SWI4* causes a delay of *SWI4* transcription and a change in the transcription start site. Wild-type (A) and *swi4ecb* mutant (B) strains (BY2195) were released from α -factor arrest as in Fig. 1, and *SWI4* and *SIR3* mRNA levels were measured by S1 protection analysis. *SWI4* transcripts, normalized to the invariant *SIR3*, are plotted in C.

Figure 3. *swi4ecb* mutants spend more time in G₁ and are larger than wild-type cells. Profiles of DNA content and Coulter channelizer measurements of cell volume were carried out for the isogenic *SWI4* and *swi4ecb* mutant strains, growing exponentially in glycerol. The volume measurements are shown in femtoliters (10^{-15} liters) on the x-axis, and the y-axis reflects the frequency with which cells of a given volume were present in the population.

counterparts, and they spend a larger proportion of their time in G₁ than wild-type cells do. The simplest interpretation of this result is that *SWI4* is rate limiting for G₁ progression and that ECB-dependent transcription is an important component of its regulation under these conditions. However, we do not see this dramatic shift in size in exponentially growing cells using glucose as a carbon source. This is probably attributable to the additional layers of regulation exerted on *CLN1* and *CLN2* that are glucose mediated (Tokiwa et al. 1994; Baroni et al. 1994; Barral et al. 1995).

The CDC6, CDC47, and CLN3 promoters also contain functional ECB elements

To identify other genes that might be coordinately regulated with *SWI4*, we searched the yeast database with the ECB sequence identified by our mutagenesis and found two closely spaced matches within the *CLN3*, *CDC6*, and *CDC47* promoters and one in the *CDC46* promoter. Interestingly, the transcripts of all four of these genes are periodic and their levels peak at about the same time as *SWI4* (Bueno and Russell 1992; Zwerschke et al. 1994; Dalton and Whitbread 1995; see Fig. 6, below), although in the case of *CLN3* the significance of this expression pattern has not been investigated (Wittenberg et al. 1990; Fernandez-Sarabia et al. 1992; Tyers et al. 1993; Oehlen and Cross 1994). Figure 4 shows the position and sequence of these putative ECB elements relative to the other known promoter elements. To determine whether the ECB sites within the *CDC6* promoter confer cell cycle-regulated transcription, a 54-bp fragment containing these sites was cloned into a UAS-deficient *lacZ* reporter plasmid. As with the *SWI4* 90-bp fragment, the *CDC6* 54-bp promoter fragment confers periodic transcription to *lacZ*. Moreover, this transcrip-

Figure 4. Strong homology to the *SWI4* ECB element was found in the promoters of four other periodically expressed genes. The position of these potential ECBs are indicated by black boxes. The other known cell cycle regulatory elements (MCBs) are also depicted relative to the ATG (+1) as indicated. The untranslated leaders, where known, are unusually long (indicated with arrows). In the case of *CLN3*, the leader is ~350 bp long and contains an ATG (J.F. Partridge and L.L. Breeden, unpubl., M. Polymenis and E.V. Schmidt, in prep.). The sequences conserved within the putative ECBs are highlighted below and compared to the minimal binding site consensus determined for Mcm1 by site selection (Wynne and Treisman 1992). Also, representative binding sites for Mcm1 from the other known classes of Mcm1-regulated genes are listed. *STE3* is one of the genes that is induced in α cells by Mcm1/Mat α 1 complexes. *STE2* is induced by α -factor and activated by Mcm1/Ste12. *STE6* is one of the α -specific genes that is repressed in α cells by Mcm1/Mat α 2, and *CLB2* is one of the G_2 -specific genes activated as a result of Mcm1/Sff1 action (Herskowitz et al. 1992; Sprague and Thorner 1992; Johnson 1995).

tion precedes that of *CLN1* by ~10 min and parallels the profile of the genomic *CDC6* transcript (Fig. 5A,B).

The two putative ECB elements in the *CDC47* promoter are immediately adjacent to one another, so we tested a 38-bp fragment containing these two contiguous elements for cell cycle-regulated UAS activity. The *CDC47* ECB fragment also confers periodic transcription, and the timing of peak transcription is equivalent to that of *SWI4* and *CDC6* (Fig. 5C). Because this *CDC47*

fragment includes only ECB sequences flanked by three additional base pairs at each end, we conclude that the ECB elements are sufficient to induce a wave of transcription that immediately precedes *CLN1* and *CLN2* transcription.

The database search for ECB elements also revealed two consensus ECBs within the *CLN3* promoter. Kuo and Grayhack (1994) had shown previously that there are three binding sites for Mcm1 in the *CLN3* promoter. Two of these fit the ECB element consensus, and they are positioned ~600-bp 5' from the start site for transcription (Fig. 4). These ECB elements are also active, as judged by the fact that the *CLN3* promoter containing the putative ECB elements confers cell cycle regulated transcription in *CLN3 ECB:lacZ*, but mutation of these putative ECBs and the third Mcm1-binding site results in constitutive transcription at or below the trough levels observed with the wild-type promoter fragment (Fig. 5D). In addition, transcription from the *CLN3* ECB reporter (*CLN3 129:lacZ*) is also cell cycle regulated (Fig. 6A).

Oscillations of the endogenous *CLN3* transcript have been observed previously using both induced and elutriated synchronies in several different laboratories (Wittenberg et al. 1990; Fernandez-Sarabia et al. 1992; Tyers et al. 1993; Oehlen and Cross 1994). To confirm and extend these earlier studies, we have quantitated the oscillation of *CLN3* transcription using two different synchrony protocols (Fig. 6) and have observed a reproducible threefold fluctuation in *CLN3* transcript levels. This wave of transcription occurs after the burst of G_2 cyclin transcription, before that of *CLN1* and *CLN2* transcription (Fig. 6A,B), and at a similar cell cycle time to *CDC6* and *SWI4* (Fig. 6C,D).

CLN3 transcription peaks at the M/ G_1 boundary

To define the phase of the cell cycle during which ECB activation is maximal, we monitored the major events in the cell cycle in parallel with transcript measurements. Transcript levels for *CLN3*, *CLN1*, and *CLB2* after α factor release are plotted in Figure 7A. Parallel samples were scored for budding and for the presence of metaphase and anaphase spindles (Fig. 7B), and FACS analysis was carried out to follow the DNA synthesis cycle (Fig. 7C). The *CLN1* mRNA level peaks at 20 and 90 min, when the majority of cells are unbudded and lack spindles. It is especially clear in the first cycle that the *CLN1* peak occurs during G_1 , before significant amounts of DNA have been synthesized. The *CLB2* mRNA level peaks after S phase is complete (50 min), when the majority of cells contain spindles, and it drops at the end of M phase. The peak levels of *CLN3* mRNA occur 70 min after release from the arrest, and *CDC6* and *SWI4* mRNAs also peak at this time (data not shown). At the 70-min time point, 80% of the cells contain either metaphase or anaphase spindles (Fig. 7B), and most of the cells have had a G_2 DNA content for at least 20 min (Fig. 7C). An increase in the population of cells with G_1 DNA content is also evident at this time. Thus, *SWI4*, *CDC6*,

Figure 5. The ECB-containing promoter fragments from *CDC6*, *CDC47*, and *CLN3* are sufficient to confer cell cycle regulation that peaks 10 min before *CLN1* transcript peaks. (A) The *CDC6* ECB reporter transcript (*CDC6 54:lacZ*) compared to genomic *CDC6* and *CLN1* transcripts in an α -factor synchrony experiment carried out as in Figure 1 and plotted in B. (C) Transcripts from the *CDC47* ECB reporter (*CDC47 38:lacZ*) were monitored through three cell cycles after release from α -factor arrest. *SIR3* serves as the internal control. (D) Transcripts from the wild-type *CLN3* promoter (−678 to −123) or the equivalent ECB-deficient promoter *cln3 ecb*, driving *lacZ* transcription, were quantitated over three cell cycles by S1 protection as above.

and *CLN3* mRNA levels peak late in M phase and/or in very early G₁.

ECB activation is *Mcm1* dependent

To determine whether *Mcm1* can bind to the ECB element within the *SWI4* promoter, gel retardation assays were performed with the 90-bp ECB region from *SWI4*. Figure 8A shows that one prominent bandshift complex can be observed when wild-type cell extracts are incubated with the *SWI4* ECB fragment (lane 2). This complex can be competed away by addition of excess cold *CLN3*, *CDC6*, or *CDC47* ECB fragments (lanes 6–11), but it is not affected by *ecb* mutant derivatives of the *SWI4* or *CDC47* fragments (lanes 5,12). The *swi4ecb* mutant fragment was amplified from the *swi4ecb* mutant strain (see Fig. 2), and the *cdc47ecb* mutant, which substitutes AAA for TTT in both ECBs in the 38:*lacZ* reporter construct (Fig. 5C), is completely inactive in vivo (data not shown). Neither of these ECB mutants (lanes 5,12) can compete for this binding, even at 100-fold molar excess. Thus, both complex formation in vitro and the ability to activate transcription in vivo requires the ECB sequence. Furthermore, this bandshift complex can be quantitatively supershifted upon addition of antibodies specific to *Mcm1* (lane 13). This indicates that *Mcm1* is present in all of the detectable ECB-binding complexes.

To address the role of *Mcm1* in *SWI5* and *CLB2* transcription in vivo, Althoefer et al. (1995) constructed a *GAL1*-regulated *Mcm1*-VP16 fusion protein that possesses the DNA-binding specificity of *Mcm1* but should activate transcription constitutively through the VP16 domain. With this construct they were able to induce ectopic transcription of *SWI5* and *CLB2* at cell cycle stages when they are not normally transcribed. This indicates that *Mcm1* can bind and activate transcription of these promoters. However, this *GAL1:MCM1*-VP16 fusion is overexpressed and lethal to the cells. To examine the effect of an *Mcm1*-VP16 fusion on ECB-containing promoters through the cell cycle when it was expressed from its native promoter, we constructed such a fusion and integrated it into the genome of a *cdc15-1 mcm1* deletion strain. This fusion protein complements the *mcm1* deletion and supports nearly normal growth rates (data not shown). We synchronized these cells by releasing them from a *cdc15-1* arrest and found that *SWI4*, *CDC6*, and *CLN3* transcripts are present continuously throughout the cell cycle in the *Mcm1*-VP16 strain (Fig. 8B). This is not attributable to lack of synchrony because histone H2B transcription cycles normally. Rather, it appears that the cells are synchronized and that *Mcm1*-VP16 is activating these three genes throughout the cell cycle. *SWI4* and *CDC6* also have MCB elements in their promoters that are known to promote G₁/S-specific transcription (Gordon and Campbell 1991; McIntosh et al. 1991). The fact that we see continuous expression of *SWI4* and *CDC6* in *mcm1*-VP16 cells indicates that the presence of the *Mcm1*-VP16 fusion protein interferes with activation via the MCB elements. This is consistent with our results with the *swi4ecb* strain and suggests again that the ECB is the primary regulator in rapidly growing cells.

Figure 6. *CLN3* message shows periodic accumulation through three consecutive cell cycles and peaks after *CLB2* and before *CLN1*. (A) Wild-type cells were released from α -factor arrest, and the *CLN3*, *CLN1*, *CLB2*, and *SIR3* mRNA levels were measured and quantitated as before. The *CLN3* ECB reporter (*CLN3* 129:*lacZ*) was also monitored in these cells. *SIR3* serves as the internal control and the cyclin transcripts, normalized to *SIR3*, are plotted in B. (C) Temperature-sensitive *cdc15-2* cells (*MATa tyr1 leu2 ura3 his7 gal1 can1 cdc15-2* from L. Hartwell, Fred Hutchinson Cancer Research Center) were grown at 25°C in YEP glucose and arrested in late mitosis by shifting to 37°C for 180 min. Cells were released synchronously from the arrest by rapid cooling to 25°C, and *CLN3*, *CDC6*, *SWI4*, and *SIR3* mRNA levels were monitored as in Fig. 1 and are plotted in D.

Combined, these in vivo and in vitro experiments support the view that Mcm1 is a critical component of the ECB-binding complex in the *SWI4*, *CDC6*, and *CLN3* promoters. The *Mcm1*-VP16 fusion shows that a modification of Mcm1 can disrupt ECB-regulated gene expression, and the gel retardation experiments demonstrate that Mcm1 can bind ECB elements directly.

The minichromosome maintenance defect of mcm1 strains is suppressed by constitutive CDC6 expression

Mcm1, Cdc6, Cdc46, and Cdc47 are all required for minichromosome maintenance (Maine et al. 1984; Hogan and Koshland 1992), and we have shown that *CDC6*, *CDC46*, and *CDC47* promoters all contain Mcm1-dependent ECB elements. To determine whether the role of Mcm1 in transcription of *CDC6* could be the cause of its minichromosome maintenance defect, we expressed *CDC6* from the *GAL1* promoter on a plasmid (p*GAL*:*CDC6*) in an *mcm1-1* strain. These cells were grown in raffinose, so that the *GAL*:*CDC6* gene would be expressed at a low constitutive level that was comparable to that of the endogenous *CDC6* in logarithmically growing cultures (data not shown). We then monitored the loss rate of a *CEN* plasmid from the *mcm1-1* (p*GAL*:*CDC6*) cells and compared to that of the same strain carrying the vector alone (p*GAL1*). We observed a 34% loss rate per generation with the control cells and only a 3.5% loss rate for the *mcm1-1* (p*GAL*:*CDC6*) cells. This 10-fold drop in

the chromosome loss rate that results from ectopic expression of Cdc6 suggests that the role of Mcm1 in *CDC6* transcription is the most probable cause of its minichromosome maintenance defect.

SWI4 and CLN3 are both rate limiting for the G₁ to S transition

Cln3 and Swi4 play critical roles in activating the burst of *CLN1* *CLN2* transcription in late G₁ (Nasmyth and Dirick 1991; Ogas et al. 1991; Dirick et al. 1995; Stuart and Wittenberg 1996), but the mechanism of that activation is not understood. We have shown that *SWI4* and *CLN3* transcription is ECB regulated and peaks just before the peak of *CLN1* and *CLN2* transcription. Thus, it is possible that transcriptional control of *SWI4* and *CLN3* allows these regulators to reach a level that promotes *CLN1* and *CLN2* transcription. If this is the case, expression of both proteins should be rate-limiting for G₁ progression, and even small changes in their rate of accumulation should effect the length of G₁. To test this prediction, we constructed diploid strains that were heterozygous for either *SWI4* or *CLN3*, or both. Figure 9A shows that *SWI4* heterozygotes spend more time in G₁ and that they are larger than wild-type diploid cells. This experiment shows that Swi4 is not in excess in wild-type cells and that a twofold change in *SWI4* dosage is sufficient to prolong G₁. Many studies have shown that Cln3 is a dose-dependent activator of G₁ progression (Cross

Figure 7. ECB-activated transcription peaks at the M/G₁ boundary of the cell cycle. An α -factor arrest and release was performed and samples were taken at 10-min intervals for RNA isolation (A), immunofluorescence (B), and DNA content analysis (C). (A) *CLN3*, *CLN1*, and *CLB2* RNA levels were quantitated with respect to the *SIR3* internal control as before. (B) Budding, metaphase, and anaphase cells were scored from 150 to 200 cells. (C) FACS analysis of DNA content through the cell cycle.

1988, 1989; Nash et al. 1988) and, as expected, we find that there is a clear size difference between diploids carrying zero, one, or two copies of *CLN3* (Fig. 9B.) Moreover, size analyses of double heterozygotes show that the effects of reduced *SWI4* and *CLN3* gene dosage are additive. The cells maintain a successively larger size as the combined dose of these two genes is reduced. These data suggest that *Cln3* and *Swi4* provide independent, rate-limiting functions that promote the transition to S phase, consistent with the suggestion that their transcriptional regulation is important for cell cycle controls.

Discussion

We have identified a cell cycle-regulated promoter ele-

ment (ECB) that activates transcription at the M/G₁ boundary. The ECB was identified by dissection of the *SWI4* promoter and then was found in the promoters of several other key regulators of the cell division cycle: *CDC6*, *CDC46*, *CDC47*, and *CLN3*. The transcript levels of all of these genes peak at the M/G₁ boundary, and fragments of their promoters that contain the ECB elements confer early periodic transcription. The only sequence conserved among these promoter fragments is a 16-bp palindrome (TTT/aCCnnnnnAGGAAA), and two tandem copies of this sequence are sufficient to confer early cell cycle regulation to reporter constructs. Elimination of the ECB from the genomic *SWI4* promoter causes a loss of the early wave of *SWI4* transcription. Furthermore, mutation of the ECBs from the 600-bp *CLN3* promoter fragment disrupts the cell cycle regulation of that construct.

There is an Mcm1-binding site at the center of the ECB element that is critical for its transcriptional activity. Mcm1 binds to these sites and is required for their activation. Mcm1 is a component of many transcription complexes and can act as an activator or a repressor, depending on the transcription factor with which it associates (Elble and Tye 1991). Most of the known Mcm1-regulated promoters contain sites that are imperfect 16-bp palindromes and do not bind Mcm1 or activate transcription on their own. Instead, associations between Mcm1 and other transcription factors that bind to adjacent sequences appear to be required to stabilize the complex and to confer specific regulatory properties (see Fig. 4). The ECB elements represent a new class of Mcm1-dependent promoter elements that activate transcription specifically at the M/G₁ boundary. They differ from the other Mcm1-regulated elements in that they have a different, more extensive consensus sequence within the palindrome to which Mcm1 is known to bind (Herskowitz et al. 1992), and they do not contain or require adjacent binding sites for other transcription factors. Previous studies of the Mcm1-binding sites in *CLN3* indicate that the ECB elements are sites for Mcm1 binding in vitro and that Mcm1 alone can protect the entire ECB sequence (Kuo and Grayhack 1994). Thus, it is possible that Mcm1 is the only DNA-binding component required for ECB activation. If that is the case, its M/G₁ specificity may be conferred either by modifications of Mcm1 or by another protein that binds directly to Mcm1.

A systematic determination of the bases that are most critical for M/G₁-specific ECB activation will be required before we can identify all of the likely ECB-regulated genes. However, some of the *S. cerevisiae* genes required for mating contain Mcm1-binding sites that are quite similar to ECB elements, and two of these (*STE2* and *MFA2*) are also cell cycle regulated (Oehlen et al. 1996). These promoters also contain consensus binding sites for Ste12, which cooperates with Mcm1 to increase basal transcription and to confer pheromone inducibility (Hwang-Shum et al. 1991; Errede and Ammerer 1989). However, the *STE2* Mcm1-binding site is capable of activating transcription on its own (Hwang-Shum et al.

Figure 8. Mcm1 binds ECBs in vitro, and an Mcm1-VP16 fusion protein causes constitutive expression of ECB regulated genes in vivo. (A) Gel retardation assays using the *SWI4* ECB region as labeled probe (lane 2). Competition reactions with unlabeled wild-type (+) ECB fragments from the *SWI4* (lanes 3,4), *CLN3* (lanes 6,7), *CDC6* (lanes 8,9), and *CDC47* (lanes 10,11) promoters added in 10 and 100 m excess. Competitions were also carried out with *swi4* and *cdc47* mutant (m) ECB fragments at 100 m (lanes 5,12). Lane 13 is identical to lane 2 except that 1 μ l of Mcm1 antibody was added. (B) BY2183 (*MCM1-VP16 cdc15-1*) cells were released synchronously from a *cdc15* arrest and *CLN3*, *CDC6*, *SWI4*, histone H2B (*HTB1*), and *SIR3* mRNAs were monitored as in Figure 6. This experiment was also carried out with an isogenic *MCM1* strain, and *SWI4* transcript clearly oscillates in those cells (data not shown).

1991), so it is quite likely that the Mcm1-binding site in *STE2* and perhaps others are of the ECB class, and that they also confer cell cycle regulation to these promoters. Mating only occurs during G_1 , so it may be optimal, or perhaps even essential in some instances, to express these mating-specific genes at the M/ G_1 boundary.

Mcm1 is also required for transcription of the G_2 -specific transcripts [Lydall et al. 1991; Althoefer et al. 1995; Maher et al. 1995], which are transcribed immediately before the wave of ECB-activated transcription (see Fig. 6). The G_2 -specific promoter element includes an Mcm1-binding site and an adjacent site to which at least one other protein must bind (see Fig. 4). It will be interesting to determine how the rapid shift in the activity of Mcm1 from G_2 -specific promoters to ECBs takes place. In the case of one ECB-regulated gene, *CDC6*, the G_2 -specific Swi5 protein is also required for maximum transcription [Piatti et al. 1995]. Thus, it is possible that the *CDC6* promoter is not accessible to Mcm1 until *SWI5* is expressed in G_2 . However, there are no Swi5-binding sites in the minimal ECB element, which is sufficient to confer M/ G_1 -specific transcription, so it is unlikely that Swi5 is a direct or essential regulator of ECB gene activation.

Mcm1 was originally identified as a gene required for efficient minichromosome maintenance, but it has since been demonstrated to be a transcription factor [Maine et al. 1984; Passmore et al. 1989; Ammerer 1990]. None of its previously characterized target genes are involved in DNA replication. This has left the connection between

Mcm1 and DNA replication unexplained. However, three of the potential ECB-regulated genes: *CDC6*, *CDC46*, and *CDC47* are required for DNA replication, and mutations in these genes result in the same minichromosome maintenance defects observed with *mcm1* mutants [Maine et al. 1984; Palmer et al. 1990]. Furthermore, we have found that low-level, Mcm1-independent expression of *CDC6* suppresses the minichromosome stability defect of *mcm1-1* cells. This strongly suggests that Mcm1 is not directly involved in DNA replication but, rather, that it is required, indirectly, for the transcription of *CDC6* and perhaps other DNA replication components.

Mcm1 also controls expression of two genes, *SWI4* and *CLN3*, which are known to be critical for entry into S phase and the transcription of the G_1 cyclins *CLN1* and *CLN2* [Dirick et al. 1995; Stuart and Wittenberg 1996]. Our studies with *CLN3* and *SWI4* heterozygotes indicate that these proteins are not in excess during the cell cycle. Rather, they provide independent, rate-limiting functions that affect the length of G_1 . We speculate that the ECB-mediated transcription of these genes, peaking at the M/ G_1 boundary, helps achieve the expression levels of these critical proteins that are required for activating *CLN1* and *CLN2* transcription and subsequent entry into S phase. Consistent with this suggestion, we have shown that mutating the *SWI4* ECB elements results in delayed *SWI4* and *CLN1* transcription and an extended G_1 . Efforts are under way to establish the significance of ECB regulation of *CLN3*. In the case of *CLN3*, the fluctuation

Figure 9. *SWI4* and *CLN3* are dose-dependent activators of G₁ progression, and their effects are additive. These measurements were all carried out with exponential cultures grown in YEP glucose and isogenic strains. (A) FACS profiles of DNA content (left) and cell volume measurements (right) of *SWI4*/*SWI4* (+/+) and *swi4*/*SWI4* (-/-) diploids. *swi4/swi4* cells could not be used in this analysis because *SWI4* is essential in this strain background. (B) Cell volume measurements of wild-type (+/+), *CLN3* heterozygotes (+/-) and *cln3* deletion homozygotes (-/-). (C) Cell volume measurements of cells having one (+/-) or two (+/+) copies of *SWI4* per diploid genome and zero (-/-), one (+/-), or two (+/+) copies of *CLN3*.

in mRNA levels is much less than other ECB-regulated genes, or the other cyclins. However, numerous experiments have shown that cells are acutely sensitive to the gene dosage of this cyclin (Fig. 9; Cross 1988, 1989; Nash et al. 1988). Furthermore, it peaks at a time distinct from that of the other cyclins and consistent with its role to act with Swi4 to induce *CLN1* and *CLN2* transcription (Fig. 10).

As already discussed, the other group of genes that are controlled by Mcm1 and that are expressed at the M/G₁ boundary includes *CDC6* and *CDC47*, which are required for the initiation of DNA synthesis. Thus, it appears that Mcm1 is controlling the expression of two

groups of genes (see Fig. 10) that mediate progression into S phase, by activating *CLN1* and *CLN2* transcription and activating replication origins.

Materials and methods

Yeast strains and growth conditions

Wild-type cells (W303-1a) were used unless indicated otherwise (*MATa leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 ura3 ssd1-d*). Cells were grown in YEP or YC minimal medium supplemented as indicated (Sherman et al. 1994). α -Factor and *cdc15*-induced synchronies have been described (Sidorova et al. 1995). One-step gene replacements (Rothstein 1983) and lithium acetate transformation (Ito et al. 1983) were performed to introduce *cln3::URA3* (Cross 1988) and/or *swi4::HIS3* deletions into W303 homozygous diploids (BY609). The *swi4::HIS3* deletion construct (pBD965) was made by replacing the *XhoI*-*BamHI* fragment of *SWI4* with the *HIS3* gene. Replacements were confirmed by Southern blots and PCR (Mullis and Faloona 1987), and site-directed mutants (Kunkel 1985) were all sequenced before and after insertion into the genome.

The chromosomal *swi4ecb* mutant was generated in BY1963, which is isogenic with W303-1a and carries a pGAL1:*SWI4* plasmid (pBD896). First, the *SWI4* promoter region was disrupted with *URA3* using pBD1919 to produce BY2197. Then, *ScaI*-cleaved pBD1920 [*swi4ecb*] DNA was used to replace the *swi4::URA3* disruption to produce BY2197; then the pGAL1:*SWI4* plasmid was lost from these cells to produce BY2196.

The *MCM1*-VP16 fusion (pBD1938) was integrated into an *MCM1*/*mcm1::LEU2* heterozygous diploid (BY2010) at the

Figure 10. Two consecutive waves of transcription occur at the M/G₁ and G₁/S transitions to produce critical regulators of the transition to S phase. *SWI4*, *CLN3*, *CDC6*, and *CDC47* are transcribed at the M/G₁ boundary. Each of these genes contains ECB elements in their promoters, which bind the transcription factor Mcm1. Our data suggest that this wave of transcription prepares cells for the transition to S phase in two ways. It induces the expression of several proteins that bind the origin recognition complex (ORC) and are required for the initiation of DNA replication. It also induces maximal transcription of two gene products (*Cln3* and *Swi4*) that are rate limiting for the transition to S phase and are known to activate G₁/S-specific transcription of *CLN1* and *CLN2* and many other genes.

HIS3 locus. This strain was sporulated and His⁺ Leu⁺ spores were crossed with BY1782 (*cdc15-1*) to produce BY2183 (*cdc15-1 mcm1-VP16::HIS3 mcm1::LEU2*).

Plasmid constructions

pBD1478 contains promoter sequence from *SWI4* (−516 to −395) in pBD1442. pBD1442 is a UAS-deficient *lacZ* reporter construct derived from pSH144 (Foster et al. 1993) in which a *NotI* sequence has been inserted downstream of the *XhoI* cloning site for directional cloning. The *SWI4* promoter sequences in pBD1478 (*SWI4* 90 bp) were amplified from pBD1357 using oligonucleotides BD1371 (GCCAGCTCGAGTTGAAGTGACTC-GTCAC) and BD1394 (GCCAGCTAGCGGCCGCTTAG-TCCTTGTTCCCTC). pBD1357 was derived from pBD873 (Foster et al. 1993). It contains a site-directed mutation of the most 3' MCB core sequence in the *SWI4* promoter from CGCG to CTCT.

Mutation of the putative Mcm1 site within the *SWI4* ECB region was performed by site-directed mutagenesis (Kunkel 1985) of pBD873 with the mutagenic oligonucleotide BL56 to generate pBD1647. This mutated ECB region was amplified by PCR using oligonucleotides BD1394 and BL23 (GCCAGCTC-GAGCTCCCCGCTGC), and subcloned into the *XhoI*–*NotI* sites of pBD1442 to generate pBD1655.

The ECB sites from the *SWI4*, *CDC6*, and *CLN3* promoters were amplified and cloned into pBD1442. The *CDC6* 54-bp ECB sequence (−199 to −146) was amplified from genomic library DNA with added *XhoI* and *NotI* sites and subcloned into pCR II (Invitrogen) and then into pBD1442 to generate the *CDC6* ECB reporter construct pBD1637. The ECB region from *SWI4* (−469 to −395) was amplified from pBD1478 and subcloned into pBD1442, as described above, to generate pBD1577. The *CLN3* ECB sequences (−994 to −865) were amplified from pC3C-1 (from J. Roberts, Fred Hutchinson Cancer Research Center), as described above, and cloned into pBD1442 to make pBD1791. Both strands of the *CDC47* 38-bp ECB DNA sequence (−232 to −195) were synthesized with *XhoI* and *NotI* cohesive ends and cloned directly into pBD1442 to produce pBD1951.

The *Apal*–*HindIII* region encompassing the *CLN3* promoter (−1335 to +711) was subcloned from pC3C-1 into pBS KS[−] to generate pBD1865. The upstream Mcm1 sites in pBD1865 were mutated by site-directed mutagenesis to generate pBD1866. The mutagenic oligonucleotides changed nucleotides at −954, −953, and −952 from C to G, nucleotides −894 and −893 from G to C, and nucleotides −980 and −981 from G to C. The *CLN3* promoter driving *lacZ* transcription was generated by PCR amplification of *CLN3* promoter sequence from pBD1865 (−994 to −439) with flanking *XhoI* and *NotI* sites. This fragment was cloned into pBD1442, as described above, to generate pBD1790. The analogous plasmid, with all four potential Mcm1-binding sites mutated (pBD1867), was generated in an analogous manner using the mutated *CLN3* promoter sequence in pBD1866, with a mutant 5' primer to avoid regenerating the Mcm1-binding site during the PCR reaction.

The endogenous *SWI4* promoter region was disrupted with pBD1919, which has *SWI4* DNA from −508 to +334 replaced with the *URA3* gene. Then, *ScaI*-cleaved pBD1920 (*swi4ecb*) DNA was used to replace the *swi4::URA3* disruption. pBD1920 is pBSKS⁺ (Stratagene) containing *SWI4* DNA from −1980 to +1912 in which the ECB element has been mutated to CCC-GTTTACC by site-directed mutagenesis (Kunkel 1985).

CDC6 was amplified from −6 to +1681 from a *MET::CDC6* plasmid (C2526) (Piatti et al. 1995) and cloned into pCR II (Invitrogen). This fragment was digested at the primer-derived

*Bam*HI and *Kpn*I sites and recloned into pYES2 (Invitrogen) to form p*GAL1::CDC6* (pBD1857).

The Mcm1–VP16 construct fuses amino acids 1–154 of MCM1 to the 76 carboxy-terminal amino acids of VP16 (Triezeberg et al. 1988). To do this, a *Kpn*I fragment of MCM1 from pSL1497 (Bruhn and Sprague 1994) was ligated to a *Kpn*I PCR fragment containing VP16. A *Bam*HI fragment containing this construct was ligated into the *HIS3*-integrating vector pRS303 (Sikorski and Hieter 1989) to make pBD1938.

Transcript measurements

S1 protection was used to measure transcript levels as described previously (Breen and Mikesell 1991). To produce the *CLN3* probe, a *XhoI*–*HindIII* fragment of *CLN3* (−412 to +385) was subcloned into M13mp18, synthesis was primed within *lacZ* (+97 to +80) and released by cleavage with *Pfl*MI. The *SIR3* probe (+1523 to +1932) in M13mp19 was synthesized, as described above, and released by cleaving with *Hind*III. The *CLB2* probe is an *Nhe*I–*Hinc*II fragment inserted into M13mp18, primed for DNA synthesis with the M13 universal primer, and released by *Eco*RI cleavage. The *CDC6* probe (−299 to +444) is cloned into M13mp19 (BD1727), synthesized using the M13 universal primer, and liberated by cleavage with *Hind*III.

PCR mutagenesis

Mutagenesis of the *SWI4* promoter was performed by mutagenic PCR as described (Cadwell and Joyce 1992), differing only in that 7 mM MgCl₂ and an annealing temperature of 55°C were used. Products were cloned into pCR II, and recloned into pBD1442, as described above. Two independent pools of PCR-mutated plasmids were generated (BD1498 and BD1656), transformed into wild-type cells, and assayed for β-galactosidase activity as colonies on filters (Breen and Nasmyth 1985). Mutant plasmids were isolated, sequenced, and retransformed to confirm their phenotype. The autonomously replicating sequences were then eliminated from these plasmids by *Hind*III cleavage and religation; then they were integrated at the *URA3* locus.

Gel retardation assays

Crude cell extracts from wild-type cells were generated and binding reactions carried out essentially as described (Lowndes et al. 1992; Sidorova and Breen 1993). The *SWI4* ECB probe was made using PCR and spanned the region from −468 to −395. Competitor ECB DNAs were also made by PCR. These include the ECB regions from *CLN3* (−996 to −864), *CDC6* (−200 to −133), and *CDC47* (−232 to −195). *swi4ecb* mutant competitor DNA spanned the same region and was amplified from BY2195. The *cdc47ecb* mutant DNA was synthesized with substitution of TTT for AAA at positions starting at −195 and −211. The antibody (from G. Sprague) used for supershifting was directed against Mcm1 amino acids 139–155 and has been characterized previously (Jarvis et al. 1989).

Cell analysis and plasmid loss measurements

Spindle microtubules and DNA were visualized as described previously (Kilmartin and Adams 1984; Sidorova et al. 1995). The DNA content of cells was measured by FACS analysis using a Becton Dickinson FACScan, with CellQuest and LYSYS II software. The distribution of cell sizes in exponentially growing populations of yeast was made using a Coulter Counter (Model ZM) with Sampling Stand (Model IIA) and Channelyzer 256.

Samples were sonicated briefly and diluted ~100-fold in Isoton II (Coulter) before measurement. Cell volume data were accumulated until 500 cells with the modal cell volume had been measured. The data were exported from the Channelyzer to an Excel version 5.0 spreadsheet, and the point values for each distribution were smoothed by nine iterations of the following algorithm $(x_{n-1} + 2x_n + x_{n+1})/4$ and plotted.

Plasmid loss rate was measured as in Chen and Tye (1995), with BY2076, which is a *ura3* derivative of SY1672 (*a leu2-3,-112 his4Δ34 mcm1-1*) made by fluoro-orotic acid selection (Boeke et al. 1984). This strain, carrying the pRS315 (*CEN*) plasmid (Sikorski and Hieter 1989), as well as either *pGAL:CDK6* or the parent vector (*pYES2*, Invitrogen), was grown in raffinose minimal media for ~10 generations under conditions that selected only for the *pGAL*-based vectors. Then the cells were plated with or without selection for pRS315 to determine the final stability (*F*) or the fraction of cells that had lost the plasmid. Two independent experiments were carried out with essentially the same result. These data were averaged and expressed as percent plasmid loss per generation [*R*], where $R = F^{1/N}$ after *N* generations of nonselective growth.

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