Cell Cycle-Regulated Transcription of the CLB2 Gene Is Dependent on Mcm1 and a Ternary Complex Factor

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Clb2 is the major B-type mitotic cyclin required for entry into mitosis in the budding yeast Saccharomyces cerevisiae. We showed that accumulation of CLB2 transcripts in G2 cells is controlled at the transcriptional level and identified a 55-bp upstream activating sequence (UAS) containing a Mcm1 binding site as being necessary and sufficient for cell cycle regulation. Sequences within the cell cycle-regulated UASs were shown to bind Mcm1 in vitro, and mutations which abolished Mcm1-dependent DNA binding activity eliminated cell cycle-regulated transcription in vivo. A second protein with no autonomous DNA binding activity was also recruited into Mcm1-UAS complexes, generating a ternary complex. A point mutation in the CLB2 UAS which blocked ternary complex formation, but still allowed Mcm1 to bind, resulted in loss of cell cycle regulation in vivo, suggesting that the ternary complex factor is also important in control of CLB2 transcription. We discuss the possibility that the CLB2 gene is coregulated with other genes known to be regulated with the same periodicity and suggest that Mcm1 and the ternary complex factor may coordinate regulate several other G2-regulated transcripts.

In the budding yeast Saccharomyces cerevisiae, the CDC28 gene encodes a 34-kDa protein (p34CDC28) which serves as the catalytic subunit for a cell cycle-regulated protein kinase. This kinase regulates the G2-M transition, and passage through a control point in G1 known as Start, where cells prepare for DNA replication and become irreversibly committed to a further round of cell division. Changes in p34CDC28-dependent kinase activity during the cell cycle are controlled by posttranslational modifications and by its assembly into a protein complex with regulatory subunits known as cyclins. These proteins are so named after their cyclic accumulation and degradation during the cell cycle.

In the G1 phase, Cdc28 complexes with one of three functionally redundant G1 cyclins (Cln1, Cln2, or Cln3), which together are required for execution of Start and for the G1-to-S transition. Regulation of the G2-M transition by p34CDC28 requires its assembly into a complex with a separate group of cyclins known as mitotic B-type cyclins. Four mitotic B-type cyclins have been identified in S. cerevisiae: CLB1, CLB2, CLB3 and CLB4 (10, 11, 23, 28). Clb1 and Clb2 are closely related to one another and to Cdc13, a cyclin B protein essential for mitosis in Schizosaccharomyces pombe, whereas Clb3 and Clb4 more closely resemble the S. pombe B-type cyclin homolog cci1 (23). None of the mitotic cyclins in S. cerevisiae by themselves are essential for viability as they are, at least to some extent, functionally redundant (10, 11, 23, 28). Clb2, however, appears to be the most important B-type cyclin for initiation and completion of mitosis (10, 11, 28) and appears to be important in processes such as spindle elongation (17, 28) and negative regulation of bud emergence (5, 17).

In contrast to CLB3 and CLB4 transcripts, which increase early in the S phase, CLB1 and CLB2 transcripts begin to accumulate late in S phase and remain elevated until late in mitosis (10, 11, 23, 28). Increased levels of CLB2 transcripts at this time correlate with Clb2-associated kinase activity, which peaks just before and disappears immediately following anaphase (27). These observations suggest that CLB2 mRNA levels play an important role in control of Clb2 activity. It is not known, however, if levels of mitotic cyclins are controlled at the transcriptional or posttranscriptional level. Several other genes, such as CLB1, CDC5, ACE2, and SWI5, are expressed at the same time in the cell cycle as CLB2 (8, 16, 28), raising the possibility that these genes are coregulated. Our understanding of G2-specific transcription, however, is based exclusively on studies of the SWI5 gene. Regulation of SWI5 transcription is dependent on a cell cycle-regulated upstream activation sequence (UAS) which binds the Mcm1 transcription factor and a second protein, the SWI5 factor (SFF), which binds only as part of a ternary complex with Mcm1 (18). This is similar to the recruitment of ternary complex factors by the mammalian counterpart of Mcm1, the serum response factor SRF (7, 29).

Through its interactions with cell type-specific coactivators and corepressors, Mcm1 also regulates genes not under cell cycle control, such as cell type-specific pheromone and receptor genes. It is therefore likely that Mcm1 has no intrinsic cell cycle-regulated activity and that the regulatory component of the SWI5 transcription complex is provided by SFF.

This report concerns the mechanism which controls CLB2 expression during the cell cycle. We show that CLB2 transcription plays a major part in the control of CLB2 mRNA levels in the cell cycle. Furthermore, we identified a UAS from the CLB2 promoter that is necessary and sufficient for cell cycle control and requires the Mcm1 transcription factor together with an associated ternary complex factor for activity. We discuss the possibility that this transcription factor complex functions in the coregulation of other genes expressed late in the cell cycle.
MATERIALS AND METHODS

Yeast strains and media. Cells were routinely grown at 30°C in YEPD medium (1% yeast extract, 2% Bacto Peptone, 0.1 mg of adenine per ml, 2% glucose). All other yeast manipulations were done as described previously (7). The yeast strains used in this study are listed in Table 1.

Deletion analysis of the CLB2 promoter. A CLB2 promoter fragment beginning at the ATG initiator methionine and extending to position −2,992 was generated by PCR and subcloned into pPLG-178 (13) as a SalI-BamHI fragment (sites introduced by PCR); this generated a fusion between the CLB2 5′-flanking region and the lacZ gene, which reads 5′-ATG-GAGAGGGCTATATTTGACCGAGGGAGGATCT-3′, with a half-life of 10 min instead of 30 min. The lacZ gene was initially constructed in pDL1460 as previously described (18). Rearrangement was performed by growing 1 liter of the appropriate strain in YEPD at 30°C by growing W303-1a [his3-11,15 trp1-1 ade2-1 leu2-3,112 ura3-100] to 10^7 cells per ml and using a filtrate as a source of α-factor (from Ed Heimer, Hoffmann-La Roche Inc., Nutley, N.J.) as described previously (7). Internal deletions of the CLB2 promoter were generated by linearization of pRS316 with NsiI digestion with BstUI exonuclease (Stratagene). All deletions were sequenced on both strands to determine endpoints. Transcription start sites in the CLB2 promoter were determined by primer extension analysis as described previously (6), with poly(A)+ RNA from S129.

Yeast reporter genes and indicator strains. For analysis of UAS sequences defined from deletion analysis of the CLB2 promoter, fragments were cloned upstream of a ubi7HacZ reporter which expresses β-galactosidase (β-gal) activity with a half-life of 10 min instead of >20 h (3). All CLB2::ubi7-lacZ reporter genes were initially constructed in pDL1460 as described previously (18). Repporter genes were excised with XhoI-NcoI, blunted with T4 DNA polymerase, inserted into the SacI site of pUCUra3, and integrated at the URA3 locus by homologous recombination as described previously (7). All integration events were confirmed by Southern blot analysis. Liquid culture determination of yeast β-gal has been described previously (7).

Cell synchrony experiments and Northern RNA analysis. α-Factor synchronization was performed by growing 1 liter of the appropriate bar− strain in YEPD at 30°C to 10^7 cells per ml. α-Factor (from Ed Heimer, Hoffmann-La Roche Inc., Nutley, N.J.) was added to 0.1 μg/ml (3 to 5 μg/ml for bar+ cells), and 2 h later the cells were washed twice with 200 ml of fresh YEPD and resuspended in 1 part fresh YEPD to 1 part conditioned YEPD medium (made by growing W303 1a [bar+ strain] to 2 × 10^7 cells per ml and using a filtrate as conditioned medium). Cell synchrony and release from α-factor arrest were routinely monitored by β-tubulin staining of mitotic spindles. Total yeast RNA was prepared by a modification of the bead-beat method (10). RNA samples (20 μg) were electrophoresed in 1% agarose-formaldehyde gels, transferred by capillary action onto Amersham Hybond hybridization filter membranes, and fixed onto filters with UV light by using a Stratallinker (Stratagene). Filters were prehydrated for 1 h at 23°C in 50% formamide–5× Denhardt's solution–6× SSPE (1× SSPE is 0.1 M NaCl, 10 mM NaH2PO4, and 1 mM EDTA [pH 7.7]). 1% sodium dodecyl sulfate–100 μg of bovine, sonicated salmon sperm DNA per ml and probed in prehybridization buffer with a radioactive probe (random primed) at 10 ng/ml (specific activity, >10^6 cpm/μg of DNA). The gel-isolated DNA fragments used to generate probes were CLB2 (1.3-kb internal fragment of the CLB2 gene generated by PCR), lacZ (first 350 bp of the lacZ gene in pPLG-178 generated by PCR), H2A/PriI (2.3-kb SclI fragment from YpTRT1 [20]), and URA3 (1.1-kb HindIII fragment from pUCUra3 [7]). Filters were washed to final stringency with 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate at 60°C for 60 min. Quantitation of signals on filters after probing was performed with a Molecular Dynamics PhosphorImager.

RESULTS

The CLB2 5′-flanking region and mapping of 5′ ends of CLB2 transcripts. We sequenced the CLB2 promoter region (Fig. 1A) and determined the major transcription start sites by primer extension analysis (Fig. 1B). The major transcription start sites mapped by this method were the same regardless of the primer used for extensions. Although the 5′ untranslated region is unusually long (362 nucleotides for transcripts generated from the major start site), no introns were found in this region and the ATG defining the initiator methionine is the first in the CLB2 transcript. Long 5′ untranslated leader sequences (>200 nucleotides) have been previously reported for other cell cycle-regulated transcripts (20). The first 889 bases of this sequence are shown.

5′-flanking sequences are required for cyclic accumulation of CLB2 transcripts. CLB2 mRNA levels increase late in the S phase, reach maximum levels late in G2, and decline rapidly as cells complete mitosis (10, 23, 28). To determine if 5′-flanking sequences are required for periodic accumulation of CLB2 transcripts, we constructed a CLB2 promoter-lacZ fusion gene and integrated it at the URA3 locus. Cells were synchronized with α-factor, RNA levels were analyzed by Northern blot analysis, and progression through the cell cycle was monitored by assessing mitotic spindle formation. This analysis showed that lacZ transcripts from the CLB2-lacZ gene were regulated with kinetics almost indistinguishable from those of the endogenous CLB2 gene (Fig. 2), indicating that cyclic regulation requires a promoter-5′ untranslated region. Transcription start sites used in the CLB2-lacZ fusion were shown to be the same as in the natural CLB2 gene (data not shown). This analysis does not distinguish between transcriptional control and the possibility that the long 5′ untranslated region plays a role in influencing transcript stability. A 55-bp UAS is sufficient for cell cycle-regulated transcription. To identify regulatory sequences within the CLB2 promoter, we constructed a series of deletions and assayed pro-
moter function by measuring β-gal activities from CLB2 promoter-lacZ fusion genes. This analysis defined a region from positions −362 to −131 as being important for CLB2 promoter activity (Fig. 3). Deletion of these sequences resulted in reduction of β-gal activity to 11% of the wild-type level (Fig. 3) and loss of cell cycleregulation (data not shown). Sequences essential for cell cycleregulation therefore lie within the −2362 to −2131 region. Smaller deletions within this region also resulted in substantial decreases in promoter activity but to a lesser extent than the −2362 to −131 deletion (Fig. 3), suggesting that multiple elementswithin this region contribute to the overall control of CLB2 transcription.

To test if sequences defined in the previous experiment are sufficient and necessary for cell cycleregulation of CLB2 transcription, we inserted a 232-bp fragment corresponding to the −2362 to −2131 region of the CLB2 promoter upstream of a ubiYlacZ gene (half-life of β-gal, 10 min [3]) and integrated the gene fusion at the URA3 locus. If the CLB2 UAS is involved in cell cycleregulation of the CLB2 promoter, it should be capable of conferring such regulation on a reporter gene in a heterologous promoterenvironment. To address this question, cells were synchronized with α-factor and transcript levels were determined by Northern blot analysis. This analysis showed that ubiYlacZ transcripts were cell cycleregulated in a pattern indistinguishable from that of endogenous CLB2 transcripts (Fig. 4B), suggesting that multiple elements within this region contribute to the overall control of CLB2 transcription.
sequences necessary and sufficient for cell cycle regulation localized to a 164-bp region between −362 and −199. These results are summarized in Fig. 4A. Although this excluded Mcm1 sites at positions −175 and −194 as being necessary for regulation, it was still possible that the −322 Mcm1 site was involved. It should be emphasized that of these sites, at least the −194 and −322 sites are capable of binding Mcm1 in vitro (30).

To test if the −322 Mcm1 site (CCGAATCAGG) and surrounding sequences are sufficient for cell cycle regulation, a 55-bp synthetic oligonucleotide (positions −336 to −282; Fig. 5A) was inserted upstream of a ubiYlacZ gene, which was then integrated at the URA3 locus. Figure 5 shows that these sequences are sufficient to drive cell cycle-regulated transcription in a pattern indistinguishable from that of endogenous CLB2 transcripts. This element therefore functions as a cell cycle-regulated UAS in vivo.

We specifically mutated sequences within the −322 Mcm1 site (CCGAATCAGG) and surrounding sequences are sufficient for cell cycle regulation, a 55-bp synthetic oligonucleotide (positions −336 to −282; Fig. 5A) was inserted upstream of a ubiYlacZ gene, which was then integrated at the URA3 locus. Figure 5 shows that these sequences are sufficient to drive cell cycle-regulated transcription in a pattern indistinguishable from that of endogenous CLB2 transcripts. This element therefore functions as a cell cycle-regulated UAS in vivo.

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An Mcm1-Vp16 fusion protein activates the \textit{CLB2} promoter in vivo. To test the idea that Mcm1 binds the \textit{CLB2} UAS in vivo, we expressed an Mcm1-Vp16 fusion protein from a galactose-inducible promoter and determined if this could activate the \textit{CLB2} UAS-ubi\textsubscript{Y}lacZ reporter gene when cells were blocked at stages in the cell cycle when \textit{CLB2} transcription is usually silent.

This experiment took advantage of the unstable \textit{lacZ} derivative (ubi\textsubscript{Y}lacZ; half-life, 10 min[3]) and the strong, constitutive transcriptional activation domain of the herpes simplex virus Vp16 protein, which when fused to a heterologous DNA binding domain, activates transcription if recruited to a binding site (7).

Cells were arrested by the \textit{cdc34} temperature-sensitive mutant (late G\textsubscript{1}) or by the cell cycle inhibitor \textit{a}-factor (G\textsubscript{1}) or nocodazole (mitosis), and one-half of the culture was induced with galactose (Gal) to 2%. Extracts were prepared 120 min after the time of galactose addition. Liquid culture \textbeta-gal assays were performed as described in Materials and Methods.

\textbf{FIG. 5.} Mutational analysis of a 55-bp \textit{CLB2} UAS which is sufficient for cell cycle regulation of transcription. (A) The wild-type (WT) UAS (−336 to −282) and multiple or point mutant UAS sequences were tested for the ability to confer cell cycle regulation on a \textit{ubi\textsubscript{Y}lacZ} reporter gene (see panel B). The predicted Mcm1 binding site is in boldface type, mutations are underlined, and the specific mutant version of the UAS is noted in the right-hand column. An alignment of the \textit{SWI5} UAS (positions −327 to −273 [18]) and the \textit{CLB2} UAS (positions −336 to −282) is shown with regions of high sequence similarity outside of the Mcm1 site indicated by lines. (B) The 55-bp sequence shown in panel A was tested to determine if it could confer cell cycle-regulated transcription on a \textit{ubi\textsubscript{Y}lacZ} reporter gene together with point mutations (−318C and −317T; panel A) in a predicted Mcm1 binding site. Mutations were also introduced at other positions outside of the putative Mcm1 binding site predicted to be functionally important on the basis of similarities between the \textit{CLB2} and \textit{SWI5} UAS elements regulatory sequences in the \textit{CLB2} UAS are therefore required for cell cycle regulation, one which resembles an Mcm1 binding site and adjacent sequences which are conserved between the \textit{SWI5} and \textit{CLB2} UAS elements.

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Cells were arrested by the \textit{cdc34} temperature-sensitive mutant (late G\textsubscript{1}) or by the cell cycle inhibitor \textit{a}-factor (G\textsubscript{1}) or nocodazole (metaphase), and one-half of the culture was induced with galactose (Gal) to 2%. Extracts were prepared 120 min after the time of galactose addition. Liquid culture \textbeta-gal assays were performed as described in Materials and Methods.

\textbf{FIG. 6.} An Mcm1-Vp16 fusion protein activates a \textit{CLB2-ubi\textsubscript{Y}lacZ} reporter gene in vivo. A fusion gene consisting of the first 98 amino acids of Mcm1 was fused to the acidic activation domain of Vp16 (amino acids 412 to 490) under control of the inducible GAL1-10 promoter and integrated at the TRPI locus in S500 (\textit{CLB2\textsubscript{UAS-WT-ubi\textsubscript{Y}lacZ}:\textsubscript{ura3}), S501 (\textit{CLB2\textsubscript{UAS-318C-ubi\textsubscript{Y}lacZ}:\textsubscript{ura3}), S502 (\textit{CLB2\textsubscript{UAS-306A-ubi\textsubscript{Y}lacZ}:\textsubscript{ura3}), or S504 (\textit{CLB2\textsubscript{UAS-305G-ubi\textsubscript{Y}lacZ}:\textsubscript{ura3}). Cells were originally grown in YEPD medium plus 2% raffinose (Raff) and, when appropriate, arrested under restrictive conditions (at 37°C or in the presence of a cell cycle inhibitor [0.2 μg of \textit{a}-factor per ml or 150 μg of nocodazole per ml]) and split in two, at which time expression of the Mcm1-Vp16 fusion was induced in one of the cultures by addition of galactose (Gal) to 2%. Extracts were prepared 120 min after the time of galactose addition. Liquid culture \textbeta-gal assays were performed as described in Materials and Methods.
where activity of the uninduced (cells grown in raffinose medium) culture was very low. A point mutation in the CLB2 UAS shown to severely reduce Mcm1 binding (see Fig. 7) also showed severely reduced ubiYlacZ activity compared with the wild-type control, showing that trans-activation by Mcm1-Vp16 is dependent on its ability to bind the UAS. In contrast, activity of the −306A mutant UAS was similar to that of the wild type.

To show that this is specific for the CLB2 UAS, the same experiment was performed with a strain carrying a ubiYlacZ gene driven by the non-cell cycle-regulated RP39 UAS. In this strain, Mcm1-Vp16 failed to increase reporter gene activity, confirming that trans activation requires an intact Mcm1 binding site.

Mcm1 recruits a ternary complex factor to the CLB2 UAS in vitro. To determine if we could detect binding of Mcm1 to the cell cycle-regulated CLB2 UAS from crude whole-cell extracts, we performed band shift experiments by using the wild-type CLB2 UAS as a probe. In this assay, we detected several specific DNA-protein complexes (Fig. 7A), designated here T, M, and B (lanes b to m), which did not appear to vary with the cell cycle (data not shown). These protein-DNA complexes are all sensitive to mutations in the predicted Mcm1 binding site which would be expected to abolish or significantly reduce Mcm1 binding. For example, the −318C and −317T mutations which change essential DNA contact points for Mcm1 and abolish cell cycle-regulated UAS activity in vivo severely reduced or abrogated formation of T, M, and B complexes in vitro (Fig. 7A, lanes c, f, i, and l, and our unpublished results).

UAS mutations outside of the predicted Mcm1 binding site (−306A and −307G/−306T/−305G) which abolished cell cycle control in vivo (Fig. 5) also blocked the formation of ternary complexes in vitro (Fig. 7A, lanes d, g, j, and m, and 7B, lanes 4 and 5). Hence, a strong correlation exists between the ability of the CLB2 UAS to bind factors from crude cell extracts in vitro and its ability to function as a cell cycle-regulated UAS in vivo.

crude extract from strain S130 (MATα). Lanes: a, no extract; b, c, and d, 5 μg of extract; e, f, and g, 10 μg of extract; h, i, and j, 15 μg of extract; k, l, and m, 5 μg of extract from MATα S1 cells. Positions of free probe (F), nonspecific complexes (NS), and T, M, and B complexes are indicated. (B) T, M, and B complexes are dependent on Mcm1. Wild-type (lanes 1, 2, 6, 7, 11, and 12), −318C (lanes 3, 8, and 13), −307G/−306T/−305G (lanes 4, 9, and 14), and −306A (lanes 5, 10, and 15) CLB2 UAS binding sites (Fig. 5A) were end labeled with 32P and incubated with or without crude extract, 10 μg of S130 extract (lanes 2 to 5), 5 μg of S226 extract (lanes 7 to 10), 5 μl of insect cell rBV extract (lanes 12 to 15), or 5 μl of mock-infected insect cell extract (lane 11). No extract was added to the reactions in lanes 1 and 6. Positions of Mcm1-lof (lanes 7 to 10), specific complexes (T, M, and B, lanes 2 to 5), Mcm1-lof-TCF complexes (lane 7), and free probe (F) are shown. Note that the mobilities of the UAS-Mcm1-alone B complexes (lanes 2, 4, and 5) and UAS-rMcm1 complexes (lanes 12 to 15) are slightly different. The right-hand panel (lanes 16 to 23) shows that antibodies raised againstanMcmbind the UAS-associated complexes from crude yeast cell extracts. A wild-type CLB2 UAS probe was incubated with 5 μg of S130 extract (lane 16), a 1:20 (lane 17) dilution of preimmune serum, or a 1:2000 (lane 18) or 1:20 (lane 19) dilution of an anti-Mcm1 peptide antibody. Nonspecific or specific Mcm1 competitor peptides were added to band shift reactions as follows: 1 (lane 20) or 100 (lane 21) ng of a nonspecific peptide and 1 (lane 22) or 100 (lane 23) ng of a specific competitor peptide. The spread of complexes resulting from these experiments is indicated by the vertical bar. (C) A second protein, along with Mcm1, is recruited into a ternary complex at the cell cycle-regulated CLB2 UAS. A wild-type CLB2 UAS probe was incubated with 10 μg of S130 total crude extract (lane a), 5 μl of rBV Mcm1 extract, and/or 2.5-μl fractions of crude extract after chromatography over a Q-Sepharose column, the flowthrough at 50 mM KCl fraction (F/T), the 0.3 M KCl fraction, or the 0.1 M KCl fraction of T, M, and B complexes from the S130 crude extract are indicated (lane a) for comparison with mobility shifts in lanes b to f. B-form (Mcm1-alone) complexes which show different mobilities are indicated by the bracket (lanes c to i). Addition of crude extract or extract fractions was sufficient to shift the mobility of rBV Mcm1 to that of the endogenous Mcm1 detected in crude extracts (lane f).
To show that Mcm1 is a component of the complexes forming on the CLB2 UAS, an extract from a strain expressing only a truncated form of Mcm1 (Mcm1\textsuperscript{307G/23135}) was incubated with a probe. Under these conditions, the T, M, and B complexes disappeared on a wild-type UAS and were replaced by a faster-migrating complex, corresponding to truncated Mcm1, and a more slowly migrating ternary complex (Fig. 7B, lane 7). Truncated Mcm1 showed decreased binding to the −318C mutant (Fig. 7B, lane 8) and was unable to form ternary complexes on the −306A and −307G/−306T/−305G mutant forms (Fig. 7B, lanes 9 and 10), which we have shown to cause loss of cell cycle control in vivo (Fig. 5); these results are therefore consistent with those obtained with full-length Mcm1 (Fig. 7A and B, lanes 2 to 5).

To characterize the binding of Mcm1 to the CLB2 UAS further, we used an rBV expression vector to produce high levels of Mcm1 (rMcm1) in insect cells. rMcm1 binding activity in crude insect cell extracts was detected by band shift analysis (Fig. 7B, lanes 11 to 15) and was similar to the DNA binding specificity of Mcm1 from crude yeast extracts (Fig. 7B, compare lanes 2 to 5 to lanes 12 to 15) but generated a protein-DNA complex with slightly higher mobility (Fig. 7B). The same UAS point mutations which severely reduced yeast Mcm1 binding had similar effects on Mcm1 produced in insect cells. Extracts prepared from mock-infected insect cells showed no Mcm1-like binding activity in this assay (Fig. 7B, lane 11). Importantly, we showed that binding of Mcm1 from crude cell extracts and from a recombinant source is not affected by the −306A and −307G/−306G/−305G mutations. Thus, loss of cell cycle control caused by these mutations in vivo cannot be explained by decreased Mcm1 binding.

We also showed that a protein antigenically related to Mcm1 is present in the B complex by antibody supershift experiments (Fig. 7B, lanes 16 to 23). Antibodies raised against a peptide epitope of Mcm1 (14) specifically shifted Mcm1-dependent B complexes in band shift reactions (Fig. 7B, lanes 16 to 23); no shift was seen when preimmune serum was included instead of immune serum. The specificity of this antibody was shown by preincubation of the immune serum with peptides. The peptide used as an antigen to raise antibodies (14) blocked the supershift of B complexes (Fig. 7B, lane 23), while a second peptide, which corresponds to an epitope from the Cdc45 protein (13a), failed to block the supershifting of B complexes (lane 21) under equivalent conditions. Together, these results show that the B complex is composed of an Mcm1-UAS complex and that M and T complexes must be generated by the recruitment of other proteins, in addition to Mcm1, by protein-protein and/or protein-DNA interactions.

To formally show that factors other than Mcm1 are components of the M and T bandshift complexes, rMcm1 was incubated with protein fractions from yeast extracts depleted of Mcm1 by chromatography on a Q-Sepharose column. Mcm1-depleted extracts were then used to supplement rMcm1 in bandshift reactions as an assay for ternary complex formation. An activity collected in the low-salt flowthrough fraction (and to a lesser extent in the 0.3 M wash fraction) was found to form a ternary complex with rMcm1-DNA complexes. This activity, which we refer to as TCF\textsubscript{CLB2}, did not bind DNA in the absence of rMcm1 (Fig. 7C, lane b) and could only be recruited to the CLB2 UAS as part of a ternary complex (Fig. 7C, compare lanes b and g). The mobility of the ternary complex generated by addition of the Mcm1-depleted flowthrough fraction to rMcm1 was identical to that of the T complex from crude extracts and was abolished when the −306A mutant probe was used (data not shown). We judge this activity to be the same as that detected in crude yeast extracts on the basis of these criteria. Although rMcm1-DNA complexes display greater mobility than yeast Mcm1-DNA complexes, addition of the flowthrough fraction from the Q-Sepharose column shifted rMcm1-DNA complexes to a mobility similar to that of yeast Mcm1-DNA complexes. This is probably due to association with an unidentified protein in yeast cell extracts or to extract-dependent posttranslational modifications of rMcm1 (1).

Our results suggest that the CLB2 and SWI5 UAS elements are not only functionally equivalent but are controlled by the same pair of trans-acting regulators, Mcm1 and ternary complex factor (TCF)/SFF. To determine if the DNA sequence specificities for TCF-Mcm1 and SFF-Mcm1 complexes are the same or related, we performed competition binding experiments in which complexes formed on a radiolabeled CLB2 UAS were challenged by various excess amounts of the wild-type or mutant unlabeled SWI5 UAS binding site (Fig. 8). The addition of the wild-type SWI5 UAS competitor at a 5-fold molar excess markedly reduced complex formation on the CLB2 probe (lane g); addition of more of the SWI5 UAS competitor, up to a 200-fold molar excess (lane d), almost completely eliminated T, M, and B complexes formation on the probe. As
another competitor, we used a mutant SW15 UAS (T308) previously reported to block Mcm1- and SFF-dependent complexes through its inability to form specific base contacts with Mcm1 (18). Little or no effect was seen when an unlabeled mutant T308 SW15 competitor was used over a 5- to 50-fold molar excess (lanes i to k), and only partial elimination of complexes was seen at a 200-fold molar excess (lane h). The inability of this mutant binding site to compete successfully for TCFCLB2 against the CLB2 probe is reminiscent of that of the SFF factor, which also cannot bind the SW15 site in the absence of Mcm1. A SW15 UAS unable to bind SFF (A296; see reference 18) is predicted to be a good competitor of T, M, and B complexes which form on a CLB2 probe under these conditions as an intact Mcm1 site in such a competitor DNA would still effectively compete for Mcm1, thereby removing Mcm1-dependent ternary complexes from the CLB2 probe. These observations are consistent with the possibility that the same, or similar, proteins in cell extracts bind the CLB2 and SW15 cell cycle-regulated UAS elements.

**DISCUSSION**

A role for Mcm1 and a ternary complex factor in CLB2 transcription. We have shown that cell cycle-regulated changes in levels of CLB2 transcripts are controlled at the level of transcription by demonstrating that a CLB2-lacZ fusion gene is regulated in a manner indistinguishable from that of the endogenous CLB2 gene. This periodicity is very similar to that of SW15, CDC5, ACE2, and CLB1, which are also expressed from the late S phase through to late mitosis (8, 16, 21, 27). A 55-bp CLB2 UAS found to be sufficient for cell cycle regulation in vivo also binds two proteins in vitro, of which we believe one is Mcm1 and the other is a ternary complex factor resembling SFF (18). The ternary complex factor (TCFCLB2) identified here exhibits no autonomous DNA binding activity but is recruited into a ternary complex by DNA-bound Mcm1. Several pieces of evidence suggest that Mcm1 binds the cell cycle-regulated UAS and regulates CLB2 transcription. First, a protein that displays DNA binding characteristics similar to those of Mcm1 and is antigenically related to Mcm1 binds the CLB2 UAS in vitro. Second, a strain expressing only a truncated derivative of Mcm1 forms a faster-migrating complex on gel shifts in the absence of a more slowly migrating complex seen in MCM1 wild-type strains. Third, an Mcm1-Vp16 fusion protein was capable of ectopically activating a CLB2-uni lacZ reporter in vivo in a sequence-specific manner. Finally, point mutations which abolished or reduced binding of Mcm1 to the CLB2 UAS in vitro had similar effects on transcription in vivo. A role for TCFCLB2 is implied by the observation that a single-base substitution which blocked ternary complex formation in vitro also severely reduced UAS activity in vivo. We were unable to detect changes in the formation of these complexes throughout the cell cycle, and so if this complex is involved in regulating UAS activity in vivo, its involvement is probably not due to the binding activity of the components in the ternary complex alone. Although the −362 to −131 UAS was shown to be necessary and sufficient for cell cycle control, deletion of this Mcm1 site did not abolish cell cycle control in the intact CLB2 promoter, but mutagenesis of all three Mcm1 sites in the CLB2 UAS resulted in complete loss of activity (our unpublished results). We interpreted this to mean that clustered Mcm1 sites in the CLB2 promoter are functionally redundant. It is unclear, however, if other promoter elements, such as those required for TCFCLB2 recruitment, are also functionally redundant. It is clear, though, that not all Mcm1 sites (those which bind Mcm1 in vitro) in the CLB2 UAS have closely associated sequences which are sufficient for cell cycle control (Fig. 4A).

**Evidence that Mcm1-TCFCLB2 complexes regulate multiple genes in G2.** Mcm1 is a member of an evolutionarily conserved class of transcription factors (the MADS family [24]) which have related DNA binding and dimerization domains. Other members of this family include a regulator of arginine biosynthesis in *S. cerevisiae*, Arg80 (9); the human serum response factor SRF (22); and the products of the plant homeotic genes *deficiens* (26) and *agamous* (31). Mcm1 controls the expression of a group of divergently regulated genes. In *MATa* cells, Mcm1 forms a ternary complex with the coactivator α1 to activate transcription of a-specific genes involved in determining cell identity, such as *STE3* (4), and with the corepressor α2 to silence a-specific genes, such as *STE6* (15). In *MATa* cells, Mcm1 binds to the promoters of a-specific genes involved with *α*-factor responses with the Ste12 transcription factor. In each case, sequences flanking the Mcm1 binding site have an important role in recruitment of accessory factors to DNA, thus facilitating the formation of promoter-specific transcription complexes. We believe that the ternary complex factors (M and T) assembling on the CLB2 UAS are distinct from α1, α2, and STE12, as the TCF activity was detected in *MATa* cells (in which α1 and α2 are absent) and *MATa* cells (Fig. 7A) and is unlikely to be Ste12, as no sequence resembling a PRTE (Ste12 binding site) was identified in the CLB2 UAS. It is likely that the M and T complexes are composed of Mcm1 and distinctly different ternary complex factors, both of which are dependent on Mcm1 for recruitment to the CLB2 UAS. The possibility that the M TCF is a breakdown product of the T TCF is unlikely, as both complexes do not appear to have the same sequence requirements (−306 A mutation abolishes the T complex but not the M complex). Although mutations which abolish the T complex result in loss of cell cycle control, we know little about the role of M complexes in CLB2 control. The identification of mutants defective in M complex formation but competent to form T complexes may help address this question.

We favor the possibility that the ternary complex factor binding the CLB2 UAS in conjunction with Mcm1 is the same factor previously implicated in SW15 regulation, SFF (18). Several lines of evidence suggest that CLB2 transcription and SW15 transcription are controlled by the same (or similar) regulators and that the respective UAS elements are functionally equivalent. First, both UAS elements confer cell cycle regulation with the same periodicity; second, Mcm1 is required for regulation in both cases; third, the cis regulatory sequences flanking the Mcm1 binding site in both UAS elements are very similar; and fourth, both UAS elements bind at least one ternary complex factor, in addition to Mcm1, which has no detectable autonomous DNA binding activity. It is unclear if other genes expressed at the same time as CLB2 and SW15 (such as CDC5, CLB1, and ACE2) are also under Mcm1-TCFCLB2–SFF control. We have identified putative Mcm1 binding sites however, in the CLB2 promoter, but their functional role has not been established (our unpublished observation).

We have presented evidence which suggests that the SW15 and CLB2 genes are coregulated by a transcription factor complex consisting of Mcm1 and a ternary complex factor, SFF-TCFCLB2. Although molecular and biochemical characterization of SFF-TCFCLB2 has not been performed, we believe it most likely that SFF-TCFCLB2 is the target of regulation for this cell cycle-regulated transcription complex, as Mcm1 has not been previously shown to exhibit any intrinsic cell cycle regulation. We cannot, however, rule out the possibility that...
some other, unidentified factor is involved in periodic activation of these genes. Given the well-characterized precedent that Mcm1-regulated genes are controlled in conjunction with corepressors and coactivators, it is likely that TCF<sub>CBL2</sub> is the regulatory subunit of the complex. This would also be similar to regulation of the SRF-elk1–TCF<sub>CLB2</sub> transcription complex which binds the serum response element in the human c-fos promoter (29).

It has been previously suggested that the B-type mitotic cyclins are required for their own synthesis involving a positive autoregulatory loop (2). In this report, we have demonstrated that cell cycle regulation of CLB2 mRNAs occurs at the level of transcription. Although CLB2 mRNA is unstable, its stability does not appear to be regulated with the cell cycle (6a), and so transcriptional control is most likely the only major determinant of CLB2 transcript levels. If a positive feedback loop exists, it is likely to act on CLB2 via TCF<sub>CLB2</sub> which could involve either a direct or indirect effect of Clb-Cdc28 kinase activity on Mcm1-TCF<sub>CLB2</sub>. Moreover, a direct role for B-type and G2 cyclins has been ruled out (18). Furthermore, the ternary complex is required only for activated CLB2, and so transcriptional control is most likely the only major determinant of CLB2 transcript levels. If a positive feedback loop exists, it is likely to act on CLB2 via TCF<sub>CLB2</sub> which could involve either a direct or indirect effect of Clb-Cdc28 kinase activity on Mcm1-TCF<sub>CLB2</sub>; the decrease in CLB2 transcription seen at the end of mitosis could then be explained as a consequence of Clb destruction. An alternate hypothesis is that periodic transcription is governed by a repressor protein and that the ternary complex is required only for activated transcription. There is no evidence to support this model, and in SWI5 transcription, a role for a cell cycle-regulated repressor has been ruled out (18). Moreover, a direct role for B-type cyclins in control of G<sub>1</sub> cyclin transcription (SCB-dependent pathway) has previously been shown (2), so it is possible that mitotic cyclins have two roles in the control of transcription in the cell cycle: repression of SCB-regulated transcripts and activation of Mcm1-TCF<sub>CLB2</sub>-regulated transcripts in the late S and G<sub>2</sub> phases. We are currently investigating this possibility.

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REFERENCES


