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The essential transcription factor Reb1p interacts with the *CLB2* UAS outside of the G_2/M control region

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ABSTRACT

Regulation of *CLB2* is important both for completion of the normal vegetative cell cycle in *Saccharomyces cerevisiae* and for departure from the vegetative cell cycle upon nitrogen deprivation. Cell cycle-regulated transcription of *CLB2* in the G_2/M phase is known to be brought about by a set of proteins including Mcm1p, Fkh2/1p and Ndd1p that associate with a 35 bp G_2/M-specific sequence common to a set of co-regulated genes. *CLB2* transcription is regulated by additional signals, including by nitrogen levels, by positive feedback from the Clb2–Cdc28 kinase, and by osmotic stress, but the corresponding regulatory sequences and proteins have not been identified. We have found that the essential Reb1 transcription factor binds with high affinity to a sequence upstream of *CLB2*, within a region implicated previously by others in regulated expression, but upstream of the known G_2/M-specific site. *CLB2* sequence from the region around the Reb1p site blocks activation by the Gal4 protein when positioned downstream of the Gal4-binding site. Since a mutation in the Reb1p site abrogates this effect, we suggest that Reb1p is likely to occupy this site *in vivo*.

INTRODUCTION

Progress through the cell cycle is mediated by the ordered appearance and disappearance of particular cyclins, a process controlled in turn by regulated transcription of their respective genes and regulated proteolysis of the cyclin proteins. In *Saccharomyces cerevisiae*, the mitotic cyclin Clb2p, which is coordinately expressed with Clb1p during the G_2/M phase of the cell cycle (1–3), associates with the cyclin-dependent kinase Cdc28p and acts to bring about mitosis (4) and isotropic bud growth (5). The central distinct role of *CLB2* among the cyclins is suggested by the observation that expression of *CLB2* alone, in the absence of the other three mitotic cyclins *CLB1*, *CLB3* and *CLB4*, is sufficient for cell cycle progression, while deletion of *CLB2* (even in the presence of the other three mitotic cyclins) causes morphological changes associated with delayed mitosis (1–3). Clb2p function is controlled in several ways: by regulated expression of the *CLB2* gene, by regulated localization and degradation of Clb2 protein, and by regulation of the activity of the Clb2–Cdc28 kinase (6–10).

Cell cycle regulation of *CLB2* transcription plays an important part in regulation of Clb2 activity. Transcription of *CLB2* during G_2/M is brought about by at least three proteins: the essential Mcm1 protein (11,12), Fkh1 and Fkh2 proteins (6,13–17), and Ndd1 protein (18). Mcm1, Fkh2/1 and Ndd1 proteins associate with an essential regulatory site in the *CLB2* promoter, referred to as the G_2/M-specific site (see Fig. 1), both *in vitro* and *in vivo* (11,12,19). Current evidence suggests that Fkh2p plays a more important role in *CLB2* regulation than Fkh1p, but the specific role(s) of the Fkh proteins remains to be defined (14,17,19). Moreover, sequences similar to the Mcm1p/Fkh2/1p/Ndd1p-binding site in *CLB2* are found in the upstream regions of a set of genes transcribed during the G_2/M phase (20,21).

Among these regulators, Ndd1 protein is the best candidate to be directly responsible for G_2/M-regulated expression of *CLB2*. The *NDD1* gene, which was first identified as a high copy suppressor of a *cdc28-1N* mutant strain (18), is perfectly poised for direct regulation of *CLB2*: *NDD1* is transcribed during S phase; Ndd1 protein is phosphorylated in G_2/M (14,16); and Ndd1p is recruited to *CLB2* in a cell cycle-specific manner (14,19). Neither Mcm1p nor Fkh2p is as likely to be directly responsible for cell cycle regulation. Both Mcm1 protein, a MADS box protein with homology to the serum response factor, and Fkh2p, one of four proteins in yeast containing a forkhead domain, are involved in regulation of many genes with different patterns of expression (19,22). Furthermore, both Mcm1p and Fkh2p are found upstream of the *CLB2* gene throughout the cell cycle (19). However, Fkh2p may play a role in cell cycle regulation, since cell cycle-regulated phosphorylation of Fkh2p has been observed (16).

In addition to its role in the normal cell cycle, Clb2p is implicated in a second role in an environmentally triggered departure from vegetative growth at G_2. In low nitrogen, diploid dimorphic yeast strains undergo a transition at G_2 from vegetative growth to pseudohyphal development, in which cells become elongated and buds do not septate (23,24). At least two signal transduction pathways mediate this process, one of the end results of which is expression of the *MUC1/*

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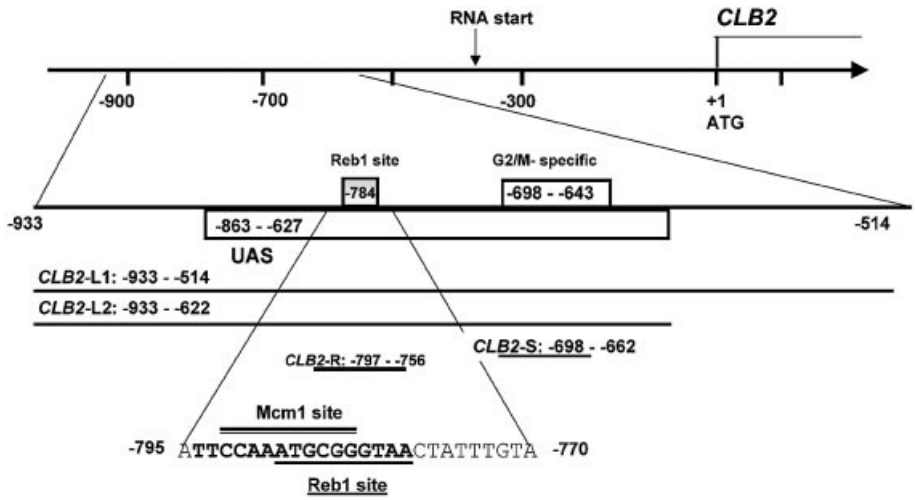

Figure 1. Diagram of the regulatory sequences of the *CLB2* gene. Sequences from –863 to –627, called UAS, are sufficient to confer full expression on a reporter gene (18). The G_2/M-specific site from –698 to –643 is bound by Mcm1p, Fkh2p and Ndd1p and is found in a set of G_2/M-regulated genes. The Reb1p site at –784 was identified in the course of these experiments; the sequence protected from DNase I digestion (–795 to –770) as well as the Reb1p-binding site (underlined) and putative Mcm1p-binding site (overlined) are shown. DNA fragments used in the binding experiments in this study are illustrated here: *CLB2-L1* (–933 to –514), *CLB2-L2* (–933 to –622), *CLB2-S* (–698 to –662) and *CLB2-R* (–797 to –756).

FLO11 gene (24–28). While there is much still to decipher between the signal and the end result, Rua *et al*. (24) suggest that *CLB2* may be the crucial target of the signal transduction pathways. This is based on two observations. First, deletion of *CLB2* triggers pseudohyphal development in the absence of environmental signals (29). Secondly, overproduction of *CLB2* is refractory to pseudohyphal development in the presence of a constitutively active signaling pathway (29). Moreover, *CLB2* transcription in a diploid yeast cell is regulated by nitrogen availability (30): limitation of nitrogen results in a 5-fold decrease in *CLB2* transcript in diploids. Since either low nitrogen availability or deletion of *CLB2* can produce the transition from diploid vegetative growth to pseudohyphal development (24), nitrogen regulation of *CLB2* transcription is likely to be important in this decision. It is unknown how nitrogen-dependent regulation is mediated.

There is evidence that regulation of *CLB2* transcription may involve sequences outside the known G_2/M-specific regulatory sequences (see Fig. 1) and/or proteins in addition to Mcm1p, Fkh2/1p and Ndd1p. First, Loy *et al.* (18) found that a 240 bp *CLB2* upstream regulatory sequence (UAS) from –863 to –627 (relative to the ATG) was sufficient to confer full G_2/M-regulated expression on a reporter gene, whereas the 55 bp G_2/M-specific sequence from –698 to –643, which is bound by Mcm1p, Fkh2p, and Ndd1p, was not (see Fig. 1). We refer to the sequences from –863 to –627 as the *CLB2* UAS. One interpretation of these results is that factors in addition to Mcm1p/Fkh2p/Ndd1p aid in expression/regulation of *CLB2* and bind outside the G_2/M-specific site. Secondly, Loy *et al*. (18) identified an activity associated with *CLB2* UAS sequences that exhibited properties expected of a repressor, since the activity was reduced in extracts from cells arrested in M phase, when *CLB2* is transcribed (18). The protein responsible for this binding activity was not identified, although some experiments indicated that it might be Mcm1p. Thirdly, there is evidence that *CLB2* transcription may also be regulated by other signals outside of the cell cycle, such as by nitrogen, as discussed above (30), by hypertonic stress (31), and by active Cdc28p–Clb2p in a positive feedback loop (32).

Since *CLB2* expression is central to growth and cell cycle progression and is subject to multiple other regulatory circuits, identification of the proteins involved in its regulation is crucial. We present evidence here that the essential Reb1 protein is responsible for a major *CLB2* UAS DNA-binding activity, found in extracts. We also show that *CLB2* sequences containing the Reb1p site block activation by Gal4 protein, an effect relieved by a point mutation in the Reb1p site. Thus, we suggest that Reb1p binding or its binding site is functional *in vivo*.

MATERIALS AND METHODS

Strains, plasmids and media

Yeast proteins were purified from EJG573, derived from JHRY20-2Ca [see Martzen *et al*. (33)], which is *MAT**a**, his3Δ200, leu2-3,112, ura3-52, pep4Δ::URA3, cyhR, MCM1-(HA)$_3$*. Both MHK7.2.4 and YT1091, which are derived from EG123 [see Kuo *et al*. (34)], are *MAT**a**, ura3-52, leu2-3, 112, trp1-1*, with *mcm1Δ::TRP1*; MHK7.2.4 carries YCplac33 bearing *URA3* and *MCM1(1–98) (HA)$_3$*, while YT1091 carries YCplac33, *URA3* and *MCM1 (1–286) (HA)$_3$*. Yeast strains bearing a deletion of the chromosomal *reb1* gene were obtained from John Warner (35,36). Both J342 and J343 are derived from W303 (J47A) of genotype *Mat**a** ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100*. J342 is *reb1Δ::LEU2* and carries plasmid pBM272-41 with P_{GAL} *REB1, HIS3*; J343 is derived from J342 by addition of plasmid pAB43 with *URA3* and *Kluyveromyces lactis REB1*.

The P_{GAL} *CYC-lacZ* reporter constructs were derived from $pASG_{int}$ (37), a gift from A. Johnson. The **a**-specific genes operator, called asg, which is a binding site for the Mcm1 and α2 proteins, was removed by digestion with SalI and SphI, and

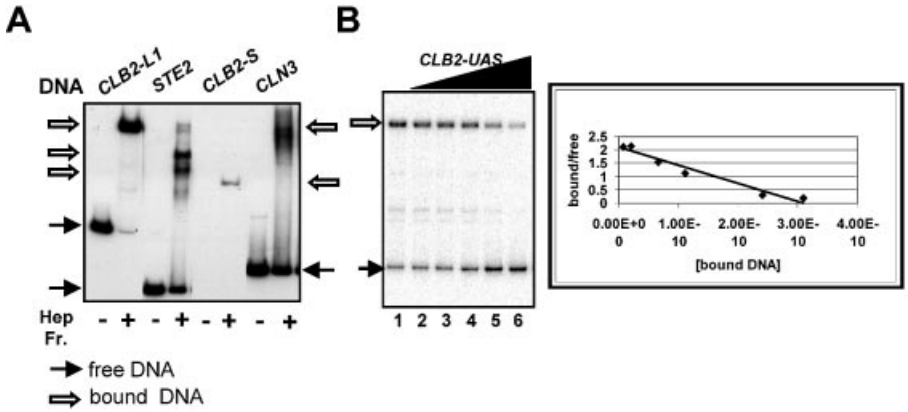

Figure 2. DNA binding of the heparin–agarose-purified yeast proteins assessed by EMSA. (**A**) The binding activity of heparin–agarose-purified yeast proteins for *CLB2-L1* DNA (–933 to –514), which contains the UAS sequences, and *CLB2-S* DNA (–698 to – 662) containing the Mcm1p/Fkh2p/Ndd1p-binding sites, as well as *STE2* and *CLN3* UAS sequences, containing known Mcm1p-binding sites. The *CLB2* DNAs are illustrated in Figure 1. Bound and free DNAs are indicated by arrows; the free *CLB2-S* DNA is not shown. (**B**) Binding of yeast heparin–agarose-purified proteins to 5×10^{-11} M labeled *CLB2-L2* DNA (–933 to –622) was competed with unlabeled *CLB2* DNA sequences from –825 to –622. Binding affinity was assessed by Scatchard analysis.

religation in the presence of 5′ TCGACCCGGGTACCTG-TACAGCATG 3′. *CLB2* sequences from –942 to –721 relative to the ATG (PCR amplified with appropriate restriction ends) were inserted between the SalI and SphI sites. This Reb1p site, AATGCGG**g**TAACTATTTGTA, which matches the consensus site defined by Liaw *et al*. (38), was mutated to AATGCGG**T**TAACTATTTGTA, called Reb1p site-m1 below. Plasmids were integrated into the *ura3-52* allele in EMP20 *MAT**a**/MAT*α*, ura3-52/ura3-52, his3*Δ*200/his3*Δ*200, ade2-101ochre/ade2-101ochre.* Four strains derived from EMP20 are reported here: CVS1055 carries the original pASG plasmid; CVS1056 carries the pASG Δasg site; CVS1060 has pASG Δasg *CLB2* –942 to –731; and CVS1067 carries pASG Δasg *CLB2* –942 to –731 Reb1p site-m1. Integration at the *URA3* locus was checked by PCR amplification using one oligonucleotide complementary to chromosomal *URA3* sequences and the other complementary to plasmid-specific *lacZ* sequences.. The resulting PCR products were subjected to sequence analysis to confirm their identity.

Medium was made as described by Sherman (39) with either 2% dextrose or 3% galactose as the carbon source. For the experiment described in Figure 5, cells were grown in medium containing 2% galactose plus 1% dextrose and shifted to medium with only 2% dextrose.

DNAs

CLB2 sequences in the experiments shown in Figures 2, 3 and 4 came from CHR XVI, nucleotides 770 727–771 028, which is –933 to –514 relative to the *CLB2* ATG and includes the UAS sequence shown by Loy *et al*. (18) to confer G_2/M-regulated expression on a reporter gene. The *CLB2*, *CLN3* and *STE2* DNAs were previously described (22). Plasmid pFBW32 with *CLB2* sequences –933 to –514, cloned into Bluescript KS+ (Stratagene), was described previously (22); *CLB2* DNA fragments derived from this clone are labeled by the base pairs relative to the *CLB2* ATG (*CLB2-L1* –933 to –514; *CLB2-L2* –933 to –622; *CLB2-S* –698 to –662; *CLB2-R* –797 to –756). The 200 bp competitor used in Figure 2B, obtained by PCR amplification from pFBW32, contained –825 to –622. Oligonucleotides used in the binding and competition experiments reported in Figure 3B were 62 bp; the wild-type oligonucleotide contains sequences corresponding to *CLB2* –797 to –756 (agacagatcttattccaaatgcgggtaactatttgtataatatgtt-tacatggatcctgatc); other competitors were based on the same sequence with the mutations shown in Figure 3D. A competitor with a 26 bp *CLB2* sequence from –795 to –770 (attccaaatgcgggtaactatttgta) was used in Figure 3C.

DNA binding assays

DNA binding assays contained 24 mM Tris–HCl pH 7.5, 5 mM Tris–HCl pH 8, 160 mM NaCl, 3.8 mM $MgCl_2$, 1.3 mM dithiothreitol (DTT), 1.6 mM $(NH_4)_2SO_4$, 5 mM spermidine, 50 μg/ml bovine serum albumin (BSA), 20.5 mM EDTA [similar to Lydall *et al*. (40)], 0.25 mg/ml poly (dIdC:dIdC), 16% glycerol, 0.05 mg/ml calf thymus or salmon sperm DNA, 30 mM β-glycerophosphate, 1 mM Na orthovanadate, 20 nM okadaic acid, 1 mM NaF and $0.2–2 \times 10^{-10}$ M labeled *CLB2* DNA. [The labeled *CLB2* DNA was at $<0.5 \times 10^{-10}$ M for experiments involving competition by unlabeled DNA fragments (Figs 2B, and 3B and C).] Binding reactions were incubated at room temperature for 15 min and subjected to electrophoresis in 5% acrylamide, 0.2% bisacrylamide gels containing 0.5× TBE and 10% glycerol; gels were dried and exposed on a phosphoimager. Quantitation of bands was done using the Image-Quant program.

Yeast protein preparation

Yeast EJG573 strain (3.2 l) was grown in YPD medium to $OD_{600} = 3$, harvested, washed twice with cold H_2O, frozen at –70° C, washed with 10 ml of buffer ZA and resuspended in 10 ml of buffer ZA [0.2 M Tris–HCl pH 8.0, 0.8 M $(NH_4)_2SO_4$, 10 mM DTT, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 1.4 mM β-mercaptoethanol, 0.1% Triton X-100, 13% glycerol, 1 mM EDTA (41)] with protease and phosphatase inhibitors (1 mM NaF, 50 mM β-glycerophosphate, 0.4 mM Na-orthovanadate, 20 nM okadaic acid, 10 μg/ml leupeptin, 10 μg/ml pepstatin). Cells were lysed with glass beads as described (42), followed by centrifugation at 9300 *g* for 25 min to remove insoluble material from the crude extract

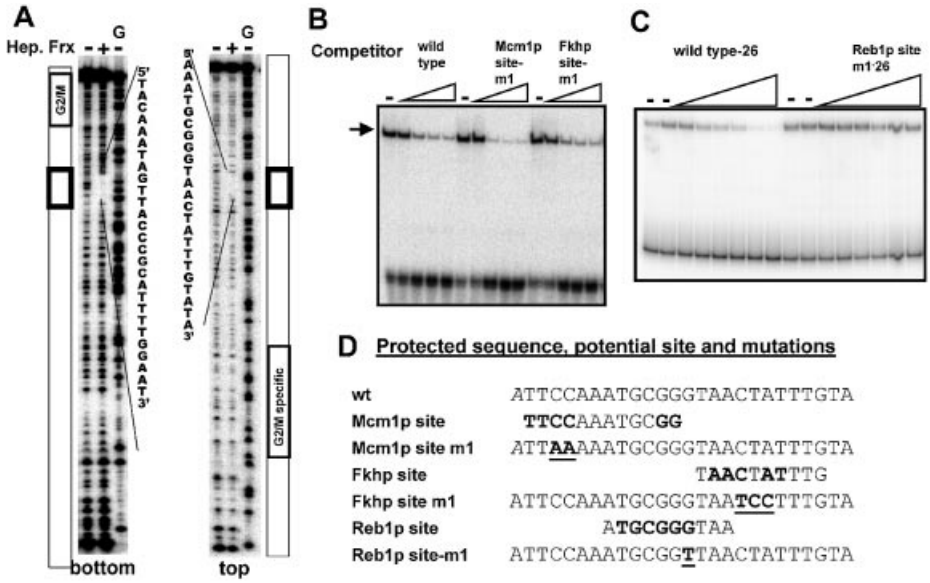

Figure 3. Identification of DNA sequences important for binding. (**A**) The binding site of the heparin–agarose-purified protein fraction was mapped by DNase I protection on each strand of the *CLB2-L2* DNA fragment. The protected region and sequence on each strand are illustrated, as well as the position of the known G_2/M-specific sequences. (**B**) Binding and competition of binding with unlabeled oligonucleotides bearing the protected DNA sequence. DNA-binding activity was examined using EMSA with labeled *CLB2-R* containing 42 bp of *CLB2* (–797 to –756). Oligonucleotides with either the wild-type sequence or mutations in the putative Mcm1p- and Fkhp-binding sites (shown in D) were also used as competitors. In each set, unlabeled competitors were at the following concentrations: 0, 2.5×10^{-10}, 5×10^{-10}, 7.5×10^{-10} and 10×10^{-10} M. (**C**) DNA binding to full-length *CLB2-L2* (–933 to –622), assessed by EMSA, is competed by an oligonucleotide bearing only 26 bp of *CLB2* (corresponding to the protected sequence –795 to –770). This oligonucleotide with a single mutation in the Reb1p (Reb1 site-m1) site fails to compete DNA binding. Competitor DNAs were present at 0, 0, 0.5×10^{-10}, 1×10^{-10}, 2.5×10^{-10}, 5×10^{-10}, 25×10^{-10} and 50×10^{-10} M. (**D**) DNA sequences of the protected region, the potential binding sites for Mcm1p, Fkh2p and Reb1p, and the mutations introduced into the oligonucleotides used in (B) and (C). For each putative binding site, the sequences that constitute a binding site are shown, with the most conserved bases in bold; the mutations introduced to impair binding are underlined.

(supernatant). $(NH_4)_2SO_4$ was added slowly to the crude extract to a final concentration of 50% saturation, followed by stirring for 20 min, and centrifugation for 45 min to obtain the pellet of precipitated proteins which was stored at –70°C. The $(NH_4)_2SO_4$ pellet was resuspended in a minimal volume (2.2 ml) of buffer C [20 mM HEPES pH 8.0, 5 mM EDTA, 7 mM β-mercaptoethanol, 20% glycerol, 5 mM DTT with 1 mM phenylmethylsulfonyl fluoride (PMSF) and additional protease and phosphatase inhibitors described above] and partially desalted by centrifugation through 2 ml of Sephadex G25 in a disposable polyprep column (Isolab). Material that flowed through the column was diluted 10-fold with buffer A-50 [50 mM Tris–HCl pH 8, 1 mM EDTA, 10% glycerol, 1 mM DTT, 50 mM $(NH_4)_2SO_4$ with protease and phosphatase inhibitors], nutated with heparin–agarose for 90 min in the cold, after which the heparin–agarose was allowed to settle, and the supernatant was decanted. The heparin–agarose was poured into a column and eluted with a gradient in buffer A from 0 M NaCl, 50 mM $(NH_4)_2SO_4$ to 2 M NaCl, 0 mM $(NH_4)_2SO_4$. *CLB2* DNA-binding activity eluted at 0.95 M NaCl, 25 mM $(NH_4)_2SO_4$.

In vitro translation

Reb1p protein was produced in the coupled T7 transcription–rabbit reticulocyte translation system from Promega. Reactions (200 µl) contained 4 µg of plasmid DNA with *REB1* under control of the T7 promoter and either [^{35}S]methionine (Amersham-AG1594, 15 mCi/ml >1000Ci/mmol) or unlabeled methionine, and additional components as per Promega Technical Bulletin 126. After incubation at 30°C for 90 min, proteins were precipitated by slow addition of an equal volume of buffer CB (20 mM HEPES pH 8.0, 0.15 M K^+ glutamate, 1 mM EDTA, 15% glycerol, 1 mM DTT) saturated with $(NH_4)_2SO_4$ (70 g added per 100 ml), incubated on ice for an additional 45 min and centrifuged in a microfuge for 30 min. The pellet containing Reb1 protein was resuspended in buffer CB (100 µl) and desalted by passage through 200–250 µl of Sephadex G25 resin equilibrated with buffer CB. The flow through was divided into 11 µl aliquots, frozen under liquid nitrogen and stored at –70°C.

β-Galactosidase assays

The LacZ overlay assay was done according to the protocol by Greg Petsko (http://www.sacs.ucsf.edu/home/HerskowitzLab/protocols/xgalagar.html) with 0.25 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal).

RESULTS

A *CLB2* UAS-binding activity that requires sequences outside the G_2/M region and that does not involve Mcm1 protein

In the course of examining crude extracts for activities that bind to DNA from the region upstream of *CLB2*, we observed strong binding to a large fragment (*CLB2-L1*) containing the entire UAS defined by Loy *et al*. (18) and partially purified this activity by heparin–agarose chromatography. As shown in Figure 2, *CLB2-L1* (–933 to –514) DNA is bound by these

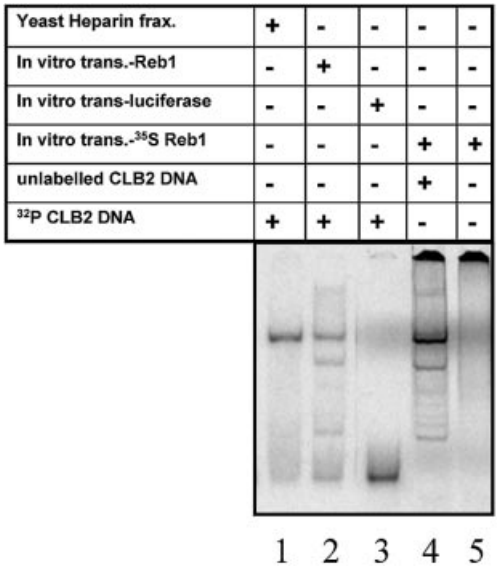

Figure 4. Reb1 protein, translated *in vitro*, binds *CLB2* upstream sequences. Reb1 protein (±[^{35}S]methionine) was made in a coupled *in vitro* transcription–translation system from a plasmid bearing the *REB1* gene under control of the T7 promoter. Lanes 1–3: labeled *CLB2-L2* DNA in EMSA using yeast heparin–agarose-purified proteins (lane 1), *in vitro* translated Reb1 protein (lane 2) and *in vitro* translated luciferase (lane 3). Lanes 4 and 5: ^{35}S-labeled Reb1 protein in EMSA using unlabeled *CLB2-L2* DNA (lane 4) or no added *CLB2* DNA (lane 5).

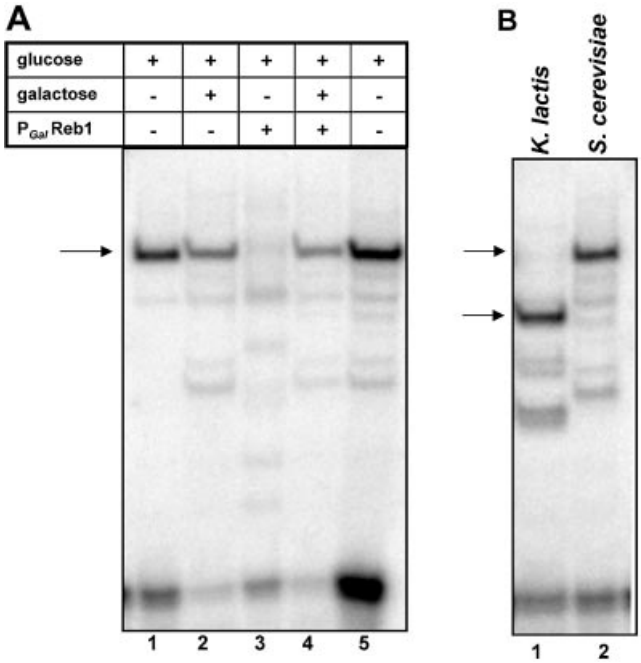

Figure 5. Alteration of either the amount or size of Reb1 protein produced in yeast alters the EMSA complex. (**A**) EMSA with labeled *CLB2-R* DNA and crude extracts made from a wild-type yeast strain (J47A) and a conditional *reb1* mutant (J342) (deleted for the chromosomal *reb1* gene and expressing *REB1* under control of the Gal promoter) (36). Yeast were grown in galactose + glucose, then shifted to glucose for 4–4.5 generations to repress *REB1*. Growth in galactose alone is toxic to strain J342, presumably because overproduction of Reb1 protein is toxic (36). Lanes 1 and 2, extracts from the wild-type parent J47A grown in glucose (lane 1) or glucose + galactose (lane 2); lanes 3 and 4, extracts from J342, carrying the conditional P_{GAL} *REB1* strain after the switch to glucose (lane 3) or grown in glucose + galactose (lane 4); lane 5, extracts from the wild-type EG573 yeast strain. (**B**) EMSA of complexes formed with labeled *CLB2-R* DNA using extracts derived from the J343 yeast strain expressing the 573 amino acid Reb1 protein homolog from *K.lactis* (lane 1) or from the J47A yeast strain expressing the 802 amino acid *S.cerevisiae* Reb1 protein (lane 2) (36).

heparin–agarose-purified yeast proteins as well as or better than the regulatory sequences of *STE2* and *CLN3*, which are known to be bound by Mcm1p (Fig. 2A) (11,12,43–45). Most of the *CLB2-L1* DNA is bound by these proteins under these conditions. Surprisingly, only a weak DNA–protein complex is observed with the smaller *CLB2-S* (–698 to –662) probe containing the known Mcm1p/Fkh2p-binding site, although similar amounts of radioactivity and DNA were present in each reaction. Since this heparin–agarose-purified protein fraction also bound to regulatory sequences of *STE2* and *CLN3*, we inferred that Mcm1p was present in this preparation. We confirmed this directly by showing that equivalent fractions from a strain expressing hemagglutinin (HA) epitope-tagged Mcm1 protein reacted with anti-HA antibody (data not shown), as observed earlier [see Kuo *et al*. (34)]. The binding to *CLB2-L1* but not to *CLB2-S* in fractions that have sufficient Mcm1p to bind other Mcm1p control regions efficiently suggested that a prominent *CLB2*-binding activity required sequences outside the cell cycle regulatory region of *CLB2*. Therefore, we continued to investigate this binding activity.

This *CLB2* DNA-binding activity was of sufficient affinity and specificity that it was likely to occur *in vivo*. Moreover, sequences sufficient for UAS activity compete efficiently for this binding, suggesting that it occurs in a biologically important region. To show this, we measured the K_D for the binding reaction with *CLB2-L2* DNA, which includes sequences from –933 to –622, and competed binding with sequences from –825 to –622 upstream of *CLB2*, which are a subset of the sequences implicated in UAS function by Loy *et al*. (18). Binding to radioactive *CLB2-L2* DNA, monitored by electrophoretic mobility shift assay (EMSA), was competed with increasing concentrations of unlabeled DNA (Fig. 2B). Based on Scatchard analysis of the data, the K_D is ~2×10^{-10} M and requires only sequences within the UAS region. Furthermore, the binding is not competed by a >100-fold mass excess of salmon sperm DNA, from which we infer that binding is specific enough to be relevant *in vivo*.

This initial observation suggests that sequences outside the G_2/M regulatory region are important for the observed DNA-binding activity, but does not provide information on whether or not proteins previously implicated in *CLB2* regulation are involved in the binding activity. For example, there are at least four sites outside of the G_2/M control region that could be bound by Mcm1 protein, including two with sequences $TTTCCN_6GG$ that match well with conserved bases in known Mcm1p-binding sites and two others that are slightly poorer matches [(12,18,22), see Mai *et al*. (46)]. These sites could mediate cooperative interactions of Mcm1p binding, or Mcm1p alone could recognize one of these sequences better than the binding site in the G_2/M-specific site. Alternatively, a completely different protein could be responsible for the observed *CLB2* UAS binding.

Although Mcm1p was known to be present in the heparin–agarose-purified preparation, and is known to bind tightly to DNA alone, we conclude that Mcm1 protein is not present in the *CLB2-L2* complex based on three lines of evidence. First, an antibody to an HA epitope tag on Mcm1 protein does not alter the *CLB2-L2* DNA–protein complex mobility in EMSA. While the HA antibody quantitatively shifted the Mcm1p–CLN3 DNA complex as expected (43), it had little, if any, effect on the protein–*CLB2-L2* complex (see Supplementary material). Secondly, a change in the size of the Mcm1 protein does not alter the mobility of the complex in EMSA. The mobility of the *CLB2* DNA–protein complexes made with extracts from two strains that differ only in the size of the Mcm1p is similar (see Supplementary material). Thirdly, the proteins that bind to the *CLN3* DNA and to the *CLB2* DNA differ from each other: the *CLB2*-binding activity is not competed by *CLN3* DNA with known Mcm1p-binding sites. Both *CLB2* and *CLN*3 compete in the 10^{-10}–10^{-9} M range for binding to themselves (data not shown).

The essential Reb1 protein binds the *CLB2* UAS upstream of the G_2/M-specific regulatory region

To further characterize the DNA-binding activity, the binding site was mapped using DNase I protection analysis. As shown in Figure 3A, the observed protected region is ~26 bp long and covers similar regions of each strand. It is located between –795 and –770, ~95 bp upstream of the known G_2/M-specific sequences (see Fig. 1). Thus, this binding activity differs in both location and protein content from the previously described binding of Mcm1p/Fkh2p/Ndd1p to the G_2/M cell cycle regulatory region (11,12).

We suggest that the activity responsible for DNase I protection is also responsible for the complex detected by EMSA, based on two observations. First, a strong binding activity is found using a labeled 57 bp fragment which includes 42 bp of the protected sequences (Fig. 3B) (*CLB2-R*, from –797 to –756) and 15 flanking base pairs, and binding to this probe is self-competed by DNA at $<5 \times 10^{-10}$ M, a concentration similar to that required for competition of binding by the 200 bp UAS fragment (Fig. 2B). Secondly, the protein–DNA complex formed with the 300 bp *CLB2-L2* (–933 to –622) fragment is competed by an oligonucleotide in which the only *CLB2* sequences are the 26 bp protected from DNase I digestion (see Fig. 3C). Thus the EMSA-detected binding activity and binding detected by DNase I protection are probably due to the same binding activity, and sequences outside the protected region are not required for high affinity binding.

Within and surrounding the protected region, there are potential binding sites for Mcm1p, Reb1p and possibly Fkh-like proteins (see Figs 1 and 4D). Thus it is feasible that any of these proteins or proteins with related DNA-binding domains could interact with this sequence. For example, in yeast, in addition to Mcm1p, there are three proteins, Arg80p, Rlm1p and Smp1p (47–49), that contain MADS box DNA-binding domains. Since MADS box proteins recognize related DNA sequences (50) and in some cases act cooperatively (51), any of these proteins could recognize this site. Likewise, in addition to Fkh2p, there are three additional forkhead-containing proteins, Hcm1p, Fkh1p and Fhl1p (13,17,52,53). Reb1p, the product of an essential gene, is implicated in transcription regulation at many sites (54–56). One yeast protein, the product of YDR026, has significant homology to the myb-like Reb1p DNA-binding domains, although this protein has not been previously characterized.

To learn which (if any) of the consensus binding sites was important for the binding activity, oligonucleotides with and without mutations in the putative Mcm1p-, Fkhp- and Reb1p-binding sites were used as competitors of the DNA-binding activity (Fig. 3B and C). In the experiment shown in Figure 3B, complexes were formed with the 57 bp labeled DNA *CLB2-R*, with *CLB2* sequence from –797 to –756, and formation of the labeled complex was competed with unlabeled DNA containing the same *CLB2* sequences bearing the mutations indicated in Figure 3D. Competitor DNAs with mutations in either the Mcm1p-binding site or the putative Fkhp-binding site were as effective competitors as DNA with the wild-type sequence (Fig. 3B and D). Since the mutations in the Mcm1p-binding site alter highly conserved bases implicated in MADs box binding (47,57–60), it is unlikely that any MADS box protein is required for binding. Since introduction of three mutations into the putative Fkhp-binding site did not impair binding, it is unlikely that Fkh-like proteins are involved.

In the experiment shown in Figure 3C, complexes were formed with labeled full-length *CLB2-L2* DNA (–933 to –622), and binding was competed with DNAs containing only 26 bp homologous to *CLB2* (–795 to –770), corresponding to the protected region shown in its entirety in Figure 3D. DNA with the wild-type sequence is an effective competitor (Fig. 3C). In contrast, an oligonucleotide with a mutation in a conserved base in the putative Reb1p-binding site is severely impaired as a competitor (Fig. 3C and D). Since the sequence GCGGGT was obtained by selection of Reb1p-binding sites from random sequences (38) and is highly conserved at known Reb1p-binding sites, the specific mutation site was chosen to alter the Reb1p consensus sequence GCGG<u>G</u>T without affecting the Mcm1p-binding site (Fig. 3D). We infer that this part of the sequence is important for specific binding and that Reb1p (or its homologs) may bind *CLB2-L* fragments. Moreover, the apparent K_D calculated from oligonucleotide competition of *CLB2-L2* binding (from data in Fig. 3C, see Supplementary material) is 3×10^{-10} M, very similar to that for *CLB2* UAS competition of *CLB2-L2* binding (2×10^{-10} M, Fig. 2B). This suggests that the oligonucleotide contains the sequences important for *CLB2-L2* binding.

To provide support for this proposal, we show directly that Reb1 protein, made *in vitro* in a rabbit reticulocyte extract, is able to bind the *CLB2-L2* fragment containing the *CLB2* UAS (Fig. 4). As shown in Figure 4, the Reb1p–*CLB2* DNA complex observed with Reb1 protein translated *in vitro* (lanes 2 and 4) migrates to the same position as the *CLB2* complex with yeast heparin–agarose-purified proteins (lane1). Reb1p binding to *CLB2-L2* can be observed either with labeled *CLB2-L2* DNA and unlabeled *in vitro* translated Reb1 protein (lane 2) or with radioactively labeled Reb1 protein and unlabeled *CLB2-L2* DNA (lane 4). Both Reb1 protein and *CLB2* DNA are required for complex formation since no complex is observed either when labeled *CLB2-L2* DNA is incubated with a lysate programmed to produce luciferase (lane 3) or when radioactive Reb1 protein is examined in the absence of *CLB2* DNA (lane 5). In addition, two faster migrating complexes are seen with *in vitro* transcribed/

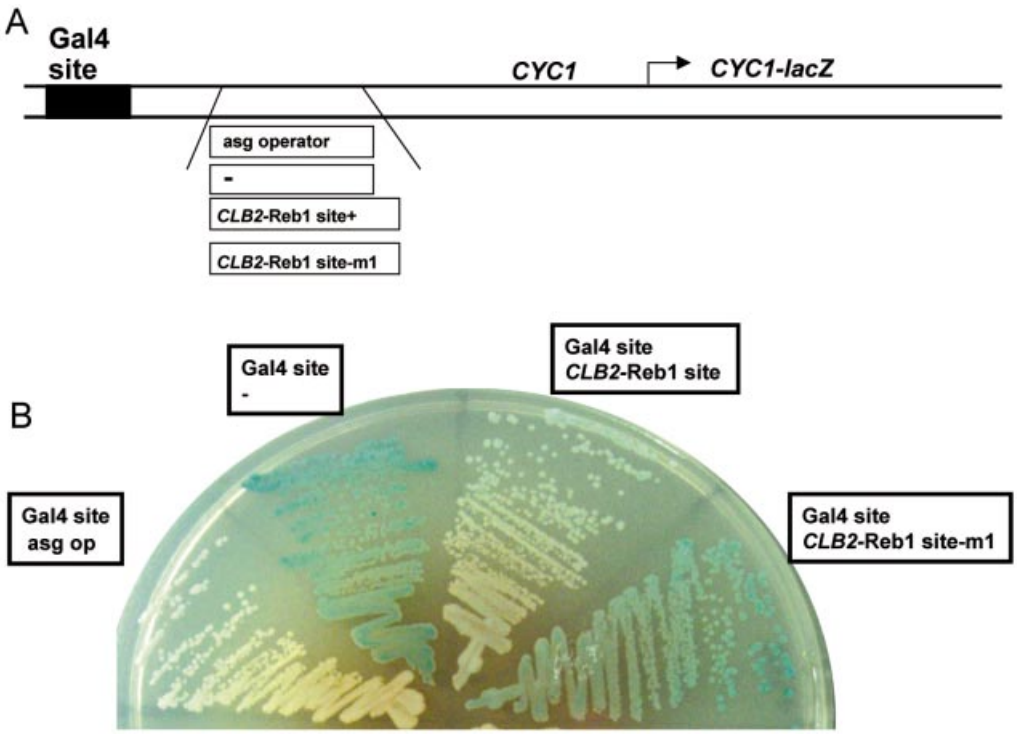

Figure 6. The *CLB2* Reb1 site modulates Gal4p activation of gene expression. (**A**) Diagram of the four reporter constructs that were integrated at the *URA3* locus and assayed in (B). The Gal4p DNA-binding site and the **a**-specific genes (asg) operator are present in the original construct (37). The asg operator was replaced by *CLB2* sequences from –942 to –721 (either wild type or bearing the Reb1p site m1 mutation) or by a linker sequence as shown. (**B**) The expression of the *CYC1-lacZ* gene from yeast strains bearing each plasmid was examined on plates containing X-gal and galactose.

translated Reb1 protein; these are most probably due to truncated Reb1 protein, since their formation requires both *CLB2* DNA and Reb1 protein. Since all reactions contain a 45-fold mass excess of salmon sperm DNA over *CLB2* DNA and the salmon sperm DNA is present in the no *CLB2* control in lane 5, the Reb1p DNA-binding activity appears to be specific for CLB2 DNA.

Reb1p is responsible for the binding activity observed in crude extracts

Two lines of evidence indicate that Reb1 protein is responsible for the DNA-binding activity that protects –795 to –770 upstream of *CLB2* and is the most prominent activity observed with yeast crude extracts (data not shown). First, if *REB1* expression is altered, the amount of *CLB2*–protein complex is altered. We demonstrated this using a strain in which expression of the *S.cerevisiae REB1* gene is under galactose control. As shown in Figure 5A, DNA binding to *CLB2-R* (–797 to –756) is severely depleted in extracts from the P_{GAL}-*REB1* conditional mutant made 3–4 generations after shifting the strains to glucose medium (in which expression of *REB1* is nearly shut off) (lane 3) in comparison with extracts made from the same strain grown in glucose plus galactose (lane 4) (in which some expression of *REB1* occurs). This is not observed in the parental strains (lanes 1 and 2). We infer either that Reb1 protein is required for the *CLB2*-binding activity or that a fairly labile gene product whose expression is under Reb1p control is required for the activity. Furthermore, we note that the major *CLB2*–protein complex from these extracts co-migrates with the major *CLB2*-binding activity found in our *pep4*Δ strain (lane 5).

Secondly, if the size of the Reb1 protein is altered, the mobility of the protein–DNA complex is also changed. We showed this by using a strain that expresses the *K.lactis* homolog of *REB1*, which encodes a 573 amino acid protein instead of the 802 amino acid protein encoded by the *S.cerevisiae REB1* gene (36). As can be seen in Figure 5B, the *CLB2-R*–protein complex observed with extracts from a strain in which *K.lactis* Reb1p is the primary source of Reb1 protein (Fig. 5B, lane 1) migrates significantly faster than the complex with the *S.cerevisae* Reb1p (Fig. 5B, lane 2). This result provides strong evidence that Reb1 protein is part of the major *CLB2*–protein complex *in vitro*.

CLB2 sequences with the Reb1p site block Gal4 transcription activation

To find out if the Reb1p site is occupied *in vivo*, we inserted *CLB2* sequences containing the Reb1p site downstream of a binding site for the Gal4 transcription activator and upstream of the TATA box adjacent to a *CYC1-lacZ* reporter gene (37). Use of this construct allows identification of sequences that either activate transcription in glucose or interfere with Gal4 activation in galactose. *CLB2* sequences containing the Reb1p site but lacking the G_2/M-specific activation region (see Figs 1 and 6A) were introduced into the reporter gene, which was then integrated into the chromosome at the *URA3* locus. Both of the control constructs, with either the Gal4 site alone or the Gal4 site and the asg operator, behave as expected. Expression

of *CYC1-lacZ* on galactose is observed in constructs bearing the Gal4-binding site alone (Fig. 6B); little/no expression of this construct is seen on glucose (data not shown). The presence of a binding site for a known repressor [the **a**-specific genes (asg) operator] reduces galactose-dependent expression of *CYC1-lacZ* (Fig. 6B) (37); the α2 protein, a strong repressor, binds the asg operator with Mcm1 protein (61,62).

We find that the *CLB2* sequences interfere with Gal4-dependent expression of *CYC1-lacZ*, and that interference activity is dependent upon an intact Reb1p-binding site. *CYC1-lacZ* expression on galactose is substantially reduced when the *CLB2* sequences –942 to –721 are inserted downstream of the Gal4-binding site. Moreover, a single mutation in a conserved base in the Reb1p-binding site (38) substantially restores galactose-dependent expression of *CYC1-lacZ*, i.e. the mutation reduces interference by these *CLB2* sequences. We have shown that this base change, a G→T mutation (seen in Fig. 3C and D), almost completely abolished Reb1p binding *in vitro* based on the inability of this DNA to compete specific binding. Thus, the Reb1p site is likely to be occupied *in vivo*. We note that the *CLB2* sequences do not cause activation in glucose, suggesting that there is no strong activation function associated with these *CLB2* sequences (data not shown).

DISCUSSION

We have provided evidence that the essential Reb1 transcription factor binds a sequence upstream of the *CLB2* gene in a region implicated in transcription regulatory function (18). A tight, specific DNA-binding activity protects a region of *CLB2* 95 bp upstream of the previously identified G_2/M-specific site, which is bound by Mcm1p/Fkh2p/Ndd1p. Although a consensus Mcm1p-binding site is present in the protected region, neither the sequence nor the protein requirements for binding are consistent with a role for Mcm1p in this complex. In support of this, we find that (i) competition for binding to *CLB2* sequences is not impaired by mutations in the conserved bases of the Mcm1p-binding site at the protected region; (ii) the *CLB2*-binding activity is not competed by DNAs containing known Mcm1p-binding sites; and (iii) the mobility of the *CLB2*–protein complex is not affected by antibodies directed at an HA epitope tag on Mcm1p, or by the size of the Mcm1 protein present in the yeast extract. Reb1 protein is responsible for DNA binding based on the following evidence: (i) mutations in the putative Reb1p-binding site found at the protected region do impair binding, since an oligonucleotide with a mutation in the Reb1p site is unable to competitively inhibit specific DNA binding; (ii) Reb1 protein made *in vitro* or in *Escherichia coli* (data not shown) is able to bind these *CLB2* sequences and generate a protein–DNA complex of mobility similar to the yeast complex; (iii) the size of the Reb1 protein in a yeast extract affects the mobility of the protein–*CLB2* complex; and (iv) depletion of Reb1 protein from yeast cells eliminates the *CLB2*–protein complex.

It is likely that this upstream sequence of *CLB2* is bound by Reb1p *in vivo*. First, Gal4-mediated transcription activation is blocked by upstream *CLB2* sequences that contain the Reb1p site; this effect is significantly diminished by the introduction of a single base change in the Reb1p-binding site. The ability of this site to affect gene expression *in vivo* suggests that it is normally bound *in vivo*. Secondly, while this work was in progress, Lee *et al*. (63) mapped the location of 106 transcription factors in the intergenic regions of the *S.cerevisiae* genome by tagging the regulators and performing chromatin immunoprecipitation (ChIP) analysis. Their data provide independent support for the presence of Reb1p at *CLB2 in vivo*; they report a high probability that Reb1 protein is associated with *CLB2* intergenic sequences ($\rho = 2.4 \times 10^{-5}$, with $\rho = 0.001$ an acceptable score) (63).

Reb1 protein is an essential, auto-regulated DNA-binding protein that binds to many sites in the *S.cerevisiae* genome (35,64); upstream regions from 146–250 yeast genes are enriched in genomic ChIP analysis with Reb1p (63). Reb1p sites, which can have either positive or negative effects on expression, are found upstream of a number of RNA polymerase II-dependent genes, including many important metabolic genes (56,65–69), as well as upstream and downstream of rRNA genes (55,65,70,71). Reb1p, as well as its homologs in *Scizosaccharomyces pombe* and mice (72–74), is implicated in termination of rRNA transcription (55,73,75).

Reb1p may exert many of its effects on gene expression by modulating chromatin structure, based on three observations. First, Reb1 protein is implicated in the separation of silent chromatin domains from expressed domains. Reb1p-binding sites are present in insulator elements that have this effect, and a synthetic multimer of Reb1p-binding sites exhibits insulator activity (76). Secondly, the Reb1 protein in *K.lactis*, which is highly homologous to and can functionally substitute for Reb1p in *S.cerevisiae* protein, is responsible for transcriptional silencing at the mating type loci (77); this activity is primarily associated with chromatin domains. Thirdly, Reb1 protein is found associated with several proteins implicated in chromatin structure or remodeling, including Rsc2 and Rsc3, components of a histone deacetylase complex, and Eaf1, part of a complex with the histone acetyltransferase Esa1 (78,79). Interestingly, yeast with mutations in *RSC2* arrest in the G_2/M phase of the cell cycle (80–82), although the basis for this phenotype is unknown.

We can envisage several possible roles for Reb1p and this regulatory site in *CLB2* control, most of which are not necessarily mutually exclusive. Since there are overlapping Mcm1p and Reb1p consensus sites at this position, Mcm1p and Reb1p may compete for binding to this site in *CLB2*. Regulation might be mediated by competition, in which the important role of Reb1p is primarily occlusion of Mcm1 protein, or vice versa. If so, deletion of the site could easily fail to reveal its potential regulatory effects since binding of both proteins would be lost simultaneously; mutation of individual sites will be required. Moreover, the situation may be complicated by the presence of additional Mcm1p-binding sites upstream of *CLB2*.

Alternatively, the Reb1p site might be required for differential regulation such as repression in low nitrogen, osmotic regulation, activation by Cdc28–Clb2 or repression during sporulation. Reb1p might act alone or in concert with other regulators. For example, Miled *et al*. (30) found that Xbp1 (a stress-inducible transcription factor) was required for repression of *CLB2* transcription in low nitrogen. It is unknown if Xbp1 binds directly to the *CLB2* regulatory region, although Mai and Breeden (83,84) noted a consensus binding site for Xbp1 in the *CLB2* upstream region.

Another alternative is that at *CLB2*, Reb1p may work primarily by recruiting specific chromatin-remodeling agents; we can envisage three different effects these might have on *CLB2* expression. First, the Reb1p–*CLB2* recruited complex could potentiate activation by the Mcm1p–Fkh2p–Ndd1p complex. If this is correct, then Reb1p binding is the explanation for the observation by Loy *et al*. (18) that the 250 bp UAS was required to activate a reporter gene rather than simply the 55 bp G_2/M regulatory sequence. Secondly, Reb1p-recruited agents could facilitate repression of *CLB2* during the S and G_1 phases of the cell cycle. Evidence that such repression exists is based on the observation that deletion of *FKH1* and *FKH2* has three consequences: loss of cell cycle control of *CLB2* transcription, an increase in basal transcription of *CLB2* throughout the cell cycle, and suppression of the requirement for *NDD1* for viability (14). Thirdly, Reb1p-recruited complexes could insulate the *CLB2* gene from external signals commencing at adjacent genes that could result in inappropriate expression since either too much transcript, too little transcript or expression at the wrong time is likely to have consequences. Fourel *et al*. (85) propose that Reb1 protein, with a set of general regulatory factors, may act to partition the genome, in particular insulating one chromosomal domain from another.

Reb1p is likely to play an important role in the complex regulation of *CLB2* which will in turn influence the timing or progression of the cell cycle or the switch to pseudohyphal development. Identification of other proteins that affect this promoter and their interactions will allow us to understand not only the regulatory circuitry of the *CLB2* gene but also the mechanisms by which the cell cycle is regulated.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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