A Competitive Transcription Factor Binding Mechanism Determines the Timing of Late Cell Cycle-Dependent Gene Expression

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SUMMARY

Transcriptional control is exerted by the antagonistic activities of activator and repressor proteins. In Saccharomyces cerevisiae, transcription factor complexes containing the MADS box protein Mcm1p are key regulators of cell cycle-dependent transcription at both the G2/M and M/G1 transitions. The homeodomain repressor protein Yox1p acts in a complex with Mcm1p to control the timing of gene expression. Here, we show that Yox1p interacts with Mcm1p through a motif located N terminally to its homeodomain. Yox1p functions as a transcriptional repressor by competing with the forkhead transcription activator protein Fkh2p for binding to Mcm1p through protein-protein interactions at promoters of a subset of Mcm1p-regulated genes. Importantly, this competition is not through binding the same DNA site that is commonly observed. Thus, this study describes a different mechanism for determining the timing of cell cycle-dependent gene expression that involves competition between short peptide motifs in repressor and activator proteins for interaction with a common binding partner.

INTRODUCTION

Temporal transcriptional control of gene expression is often achieved through the dynamic interactions of activator and repressor proteins with promoters. One of the most common ways of switching between these two modes is through competition for promoter occupancy by mutually exclusive binding to the same DNA site (reviewed in Thiel et al., 2004). However, this mechanism is only generally applicable to proteins that share overlapping DNA binding specificities and are usually from the same transcription factor family. More elaborate mechanisms must be employed if no overlap in DNA-binding site is possible, although little is known about such mechanisms.

Cell cycle-dependent gene expression provides an excellent model to study temporal transcriptional control mechanisms. Indeed, among multiple regulatory mechanisms of cell-cycle progression, one of the most important control points is at the transcriptional level and leads to the expression of key regulators in a cyclical manner (Simon et al., 2001; reviewed in Wittenberg and Reed, 2005). Peaks of gene expression can be identified at various points in the cell cycle, but two important control points in Saccharomyces cerevisiae are at the G2/M phase and M/G1 phase transitions (reviewed in Wittenberg and Reed, 2005). The MADS box transcription factor Mcm1p plays an important role in the transcriptional regulation of genes expressed at both of these control points. At G2/M phase, Mcm1p controls the expression of a group of genes known as the CLB2 cluster, which contains targets such as CLB2, CDC20, and SPO12 (Althoefer et al., 1995). Mcm1p forms a complex on CLB2 gene cluster promoters with the forkhead transcription factor Fkh2p (Koranda et al., 2000; Kumar et al., 2000; Pic et al., 2000; Zhu et al., 2000; reviewed in Breeden, 2000) and the coactivator Ndd1p (Koranda et al., 2000; Loy et al., 1999). This complex is regulated by cell cycle-dependent kinases that cooperate to give maximal activation through sequential phosphorylation of Fkh2p and Ndd1p (Darieva et al., 2003, 2006; Reynolds et al., 2003; Pic-Taylor et al., 2004). Mcm1p also forms higher-order complexes on early cell cycle box (ECB) elements at promoters of genes expressed at the M/G1 phase transition such as CLN3 and MCM3 (Mai et al., 2002). However, in contrast to the CLB2 gene cluster, no positively acting partner protein(s) has yet been found that cooperates with Mcm1p to regulate these M/G1 phase genes. More recently, the closely related homeodomain repressor proteins Yox1p and Yhp1p were shown to play a repressive role at ECB-containing promoters (Pramila et al., 2002). Yox1p and Yhp1p interact directly with Mcm1p as well as forming protein-DNA contacts with a binding site adjacent to the Mcm1p recognition element in the ECB (Pramila et al., 2002). Moreover, similar Yox1p-binding sites can be found in the promoters of a subset of genes from the CLB2 gene cluster such as CDC20 and SPO12. Importantly, loss of Yox1p and Yhp1p alters the precise timing of the expression of these genes in the cell cycle, but not of other genes in the CLB2 gene cluster such as CLB2 itself (Pramila et al., 2002). This suggests that...
Yox1p also plays a key role in controlling the timing of expression of a subset of CLB2 cluster genes, restricting their expression to a later time point in the cell cycle than other genes of the CLB2 gene cluster. It is unclear whether similar control mechanisms operate in mammalian cells, but homeodomain proteins are known to interact with SRF, the mammalian homolog of Mcm1p (Grüneberg et al., 1992; Chen and Schwartz, 1996). Moreover, forkhead proteins play an important role in cell-cycle control in mammals (reviewed in Laoukili et al., 2007; Lam et al., 2006), and at least two of these, Foxo3 and FOXK1, can directly affect SRF function (Liu et al., 2005; Freddie et al., 2007). Thus, it is important to understand the relationships and functions of the interactions between forkhead, homeodomain, and MADS box proteins.

Despite the importance of Yox1p in the regulation of cell cycle-dependent gene expression, little is known about how it represses Mcm1p transcription factor complexes. With regard to the Mcm1p-Fkh2p complex, a simple competition model between Yox1p and the activator Fkh2p for a common DNA-binding site seemed unlikely, as the Yox1p and Fkh2p DNA-binding sites are positioned on the opposite sides of the Mcm1p-binding motif (e.g., see Figure 1A). Thus, to begin to understand the repressive process, we investigated the molecular mechanisms through which Yox1p interacts with Mcm1p. Unexpectedly, Yox1p binding to Mcm1p is mutually exclusive with Fkh2p binding despite the spatial separation of their DNA recognition elements. Our data therefore reveal an atypical mode of repression that involves competition between an activator protein and a repressor protein for interaction with a common “combinatorial partner” rather than for a common DNA recognition element.

RESULTS

Mapping the Determinants for Formation of the Yox1p-Mcm1p Ternary DNA-Bound Complex

Yox1p negatively regulates a number of genes whose expression peaks transiently during the M phase of the cell cycle, including MCM3, which is usually expressed at the M/G1 border (Pramila et al., 2002). However, Yox1p also appears to be important for repressing genes that are expressed earlier in M phase such as SPO12. The SPO12 promoter contains a putative Yox1p-binding site juxtaposed to a Mcm1p-binding site (Figure 1A). Hence, to establish whether a Yox1p-Mcm1p complex forms on alternative promoters in addition to MCM3 and, if so, whether Mcm1p and Yox1p are sufficient for complex formation, we carried out an immobilized template-binding assay using a recombinant version of Mcm1p (Mcm1p(1–98)), in vitro-translated full-length Yox1p, and a fragment spanning the putative Yox1p-binding element in the SPO12 promoter. Mcm1p(1–98) spans the DNA-binding domain and is sufficient for cell viability (Christ and Tye, 1991). Although binding of Yox1p to the SPO12 promoter was readily detectable in the presence of Mcm1p, little binding of Yox1p was seen when Mcm1p was not added, or the DNA was omitted from the reaction (Figure 1B).

To map the region(s) of Yox1p required for complex formation with DNA-bound Mcm1p, we first carried out gel retardation analysis with a short region of Yox1p centered on the DNA-binding homeodomain region (Figure 1C). Yox1p(151–274) bound weakly to the SPO12 promoter in the absence of Mcm1p, but cooperative strong binding was detected upon addition of Mcm1p (Figure 1D, compare lanes 3 and 5). The identity of the Yox1p-containing complexes was confirmed by the partial supershifts/loss of binding of Flag-tagged Yox1p, which is seen upon inclusion of an anti-Flag antibody (Figure 1D, lanes 5 and 6). In contrast, addition of Fkh2p to the binding reaction did not promote the formation of Yox1p DNA-bound complexes (Figure 1E).

Further deletion analysis of Yox1p demonstrated that the region preceding the homeodomain (amino acids 151–175) was critical for the formation of Mcm1p-dependent ternary DNA-bound complexes (Figure 1F, compare lanes 2 and 4 with lanes 6 and 8). Importantly, deletion of this region of Yox1p did not affect its intrinsic DNA binding properties, as both Yox1p (151–274) and a protein lacking amino acids 151–175 (Yox1p(176–274)) bound to the SPO12 promoter with equivalent affinities when titrated at higher protein concentrations (Figure S1B available online). Thus, amino acids in the region immediately flanking the N terminus of the homeodomain of Yox1p play a critical role in promoting ternary complex formation with Mcm1p and the SPO12 promoter.

Several other yeast transcription factors use sequences in addition to their DNA-binding domains to enhance the formation of complexes with Mcm1p through direct protein–protein interactions (Boros et al., 2003; Mead et al., 1996; Tan and Richmond, 1998). Thus, one likely role for Yox1p sequences that are located outside of the homeodomain and are involved in ternary complex formation is binding to Mcm1p. This was tested by in vitro pull-down assays using recombinant MBP-Mcm1p fusion proteins and either in vitro-translated or recombinant forms of Yox1p. Both full-length Yox1p and the truncated Yox1p(151–274) could form complexes with Mcm1p (Figure S1C). However, further truncation of Yox1p to remove amino acids 151–175, Yox1p(176–274), resulted in a substantial loss of interaction with Mcm1p (Figure 1G). Importantly, the inclusion of ethidium bromide in the binding reaction did not stop this interaction, demonstrating that the binding was not mediated through interactions with contaminating nonspecific DNA (Figure S1D). Hence, amino acids located in the immediate N-terminal flanking region of the homeodomain of Yox1p are important for protein–protein interactions with Mcm1p.

A putative Yox1p-binding site flanks the Mcm1p-binding site in the SPO12 promoter (Figure 1A; Pramila et al., 2002). To probe the importance of this site for Yox1p binding, a mutant form of the SPO12 promoter was analyzed that contained a disrupted Yox1p-binding site (Figure 2A). Whereas Yox1p-Mcm1p complexes could readily be detected on the wild-type SPO12 promoter, no Yox1p-Mcm1p complexes were detectable when the putative Yox1p-binding site had been mutated (Figure 2B, compare lanes 3 and 8). In contrast, Mcm1p binding was unaffected and Fkh2p-Mcm1p complexes could form with equal efficiency on either the wild-type or the mutant SPO12 promoter (Figure 2B, compare lanes 1 and 5 with lanes 6 and 10).

To further assess the DNA-binding site requirements for Yox1p recruitment to DNA, we moved the Yox1p site away...
from the Mcm1p site by either two or five nucleotides while keeping the sequence of the Yox1p-binding motif intact. The formation of Yox1p-Mcm1p complexes decreased upon insertion of two nucleotides between the Yox1p- and Mcm1p-binding sites and was further diminished upon insertion of five nucleotides (Figure 2C).

These data indicate that Yox1p-DNA interactions are essential for the formation of DNA-bound Yox1p-Mcm1p complexes and that the correct spacing of the two binding sites is critically important for efficient complex formation.

**Identification of Amino Acids Critical for Yox1p Interaction with Mcm1p**

Other proteins that interact with Mcm1p to form ternary DNA-bound complexes such as MATα2 (Mead et al., 1996; Tan and Richmond, 1998) and Fkh2p (Boros et al., 2003) use short peptide motifs for direct binding to Mcm1p in which aromatic residues are the major interaction determinants. There are three aromatic residues in the region between amino acids 151 and 174 in Yox1p (Figure 3A), and hence we tested their importance for complex formation with Mcm1p. MBP pull-down assays with Yox1p
proteins mutated at either F153, F155, or F163 showed much-reduced interaction with Mcm1p, indicating an important role for these individual Phe residues (Figure 3B). Furthermore, the ability of these mutant versions of Yox1p to form complexes with Mcm1p on the SPO12 promoter was severely reduced (Figure 3C, lanes 7–10), although their autonomous DNA binding ability was unaffected (Figure 3C, lanes 2–5). Moreover, titration experiments with recombinant Yox1p proteins confirmed the reduced ternary complex forming ability of the Yox1p mutant proteins on an alternative binding site derived from the MCM3 promoter (Figure S2B).

Collectively, these results demonstrate that aromatic residues in the region preceding the homeodomain of Yox1p play critical roles in complex formation with Mcm1p.

**The Determinants for Phox1-SRF and Yox1p-Mcm1p Complex Formation Differ**

Yox1p was originally identified based on the premise that it was the most related yeast protein to human Phox1, which interacts with the MADS box protein SRF (Pramila et al., 2002). Complex formation between Phox1 and SRF is thought to be driven by the homeodomain (Grueneberg et al., 1992), and single amino acid mutations, such as E114H, were shown to disrupt functional interactions between Phox1 and SRF on reporter genes (Simon et al., 1997). However, Phox1 shows little homology with Yox1p in the region immediately N terminal to the homeodomain, which was mapped as important for Yox1p binding to Mcm1p. To probe whether the homeodomain might also be involved in the Yox1p-Mcm1p interaction, we introduced an amino acid substitution (E196H) into the equivalent position in the homeodomain of Yox1p. However, this mutant protein was still able to efficiently form complexes with Mcm1p both in solution (Figure S2E) and on DNA (Figure S2F). In contrast, the mutant protein Yox1p(N226Q), which was predicted to be unable to bind DNA based on similarities to Phox1 (Grueneberg et al., 1992), was unable to form ternary complexes with Mcm1p on DNA (Figure S3D), although it could still bind to Mcm1p in solution (Figure S2D).
Thus, the mechanisms used by Phox1 and Yox1p to bind to MADS box proteins differ substantially, with Phox1 primarily using the homeodomain whereas Yox1p uses a unique region preceding the homeodomain.

F155 Plays a Critical Role in Yox1p Function In Vivo
To establish whether the interactions that we detect in vitro are important for the in vivo function of Yox1p, we examined the effect of introducing wild-type (WT) Yox1p and Yox1p(F155A), expressed under the control of its own promoter, from the YCplac22 vector into a yox1Δ yhp1Δ double-mutant strain. The expression levels of both forms of Yox1p were identical (Figure 4A), and the overall cell-cycle profiles were similar for both strains when released from a factor arrest (Figure S3A). However, differences were detected in the levels of expression of several potential Yox1p-Mcm1p-regulated genes. The expression of the G2-M phase-expressed genes SPO12 and CDC20 and the M-G1 phase-expressed gene MCM3 are severely perturbed, with both earlier onset (28 versus 42 min for SPO12) and higher levels of expression being detected in the strain expressing Yox1p(F155A) (Figure 4B). These genes all contain potential Yox1p-binding sites in their promoters. In contrast, the expression of the late G1 phase-expressed gene CLN2 and the G2-M phase-expressed gene CLB2, which do not contain Yox1p-binding sites in their promoters, were not derepressed and displayed more similar timing of cyclical expression in cells expressing Yox1p(F155A) compared with those expressing wild-type Yox1p (Figure 4B). The premature and higher than normal expression of a subset of G2-M and M-G1 phase-expressed genes in the presence of Yox1p(F155A) is similar to the effects seen when both YOX1 and YHP1 are deleted (Figure S3B; Pramila et al., 2002). This effect is consistent with a loss-of-function mutation of Yox1p, although additional residual activities of Yox1p(F155A) appear likely due to the extended activation in comparison to complete loss of Yox1p.

To establish whether the effects of the F155A mutation on SPO12 and MCM3 gene expression were due to lack of promoter binding by Yox1p(F155A), ChIP analysis was performed. As expected, cyclical binding of wild-type Yox1p could be detected on the SPO12 and MCM3 promoters, with the timing of reduction of Yox1p binding in G2-M phases corresponding to the timing of increased expression of SPO12 and MCM3 (compare Figures 4B and 4C). However, promoter binding was largely abolished in strains expressing Yox1p(F155A) (Figure 4C).

Collectively, these data therefore establish that mutation of the Mcm1p-interacting region of Yox1p reduces its recruitment to promoters and thereby compromises temporal Yox1p-mediated repression of promoter activity.

Yox1p and Fkh2p Binding to Mcm1p Involves a Common Binding Pocket
Mcm1p contains a hydrophobic pocket found on the surface of the MADS DNA-binding domain (Tan and Richmond, 1998), and mutation of this pocket on Mcm1p by the introduction of a V69E mutation disrupts interactions with Fkh2p (Boros et al., 2003). To establish whether the same pocket on Mcm1p is involved in binding Yox1p, we first tested protein-protein interactions between Yox1p and the V69E mutant form of Mcm1p. In comparison to wild-type Mcm1p(WT), Mcm1p(V69E) exhibited reduced interactions with both Yox1p and Fkh2p (Figure S4A). Furthermore, the formation of DNA-bound ternary complexes...
containing Mcm1p and either Yox1p or Fkh2p was severely attenuated in the presence of Mcm1p(V69E) (Figure S4B).

However, interactions between Yox1p and the surface of the MADS DNA-binding domain are likely to be subtly different than those mediated by Fkh2p, as only Fkh2p can form detectable interactions with the mammalian protein SRF, albeit weakly (Figure S4C).

Together, these results demonstrate that both Yox1p and Fkh2p bind to a region centered on a hydrophobic pocket on the surface of the MADS box DNA-binding domain of Mcm1p.

**Mutually Exclusive Binding of Yox1p and Fkh2p to Mcm1p**

As both Yox1p and Fkh2p bind to a region centered on the same hydrophobic pocket on Mcm1p, this raised the possibility that they bind in a mutually exclusive manner to Mcm1p. We therefore tested this by titrating increasing amounts of Yox1p into binding reactions containing Mcm1p and Fkh2p proteins.

Indeed, increasing concentrations of Yox1p caused a loss of Fkh2p-Mcm1p ternary complex formation, involving both full-length Fkh2p(1–862) and the truncated Fkh2p(1–458) version, with concomitant formation of an Mcm1p–Yox1p complex (Figures 5B, S4E, and S4F). Similarly, increasing amounts of full-length Fkh2p could outcompete Yox1p binding to Mcm1p on the SPO12 promoter in a dose-dependent manner (Figure 5C), and Fkh2p(1–458) could block Yox1p binding (Figure S4F, lanes 8 and 9). This finding is surprising as Mcm1p is dimeric, and in theory, both Fkh2p and Yox1p would be expected to bind simultaneously; however, there was no sign of a higher-order complex containing Fkh2p, Yox1p, and Mcm1p, even when recombinant proteins were used to eliminate the nonspecific bands on the gel (Figure S4F). To rule out potential effects transmitted through the DNA, we performed gel retardation experiments in the presence of the isolated forkhead DNA-binding domain of Fkh2p (Fkh2p(325–458)) (Figure S4F). This truncated protein is unable to cooperatively form complexes with Mcm1p (Boros et al.,...
Yox1p and Fkh2p Compete for Mcm1p Binding

Figure 5. Yox1p and Fkh2p Binding to Mcm1p Are Mutually Exclusive

(A) Schematic illustration of full-length and truncated Yox1p proteins.

(B–D) Gel retardation analyses using the SPO12 promoter fragment and (B) in vitro-translated Fkh2p(1–862) in the presence of recombinant Mcm1p(1–96) and increasing amounts of recombinant Yox1p proteins (1, 5, and 10 molar equivalents; indicated by a triangle above the lanes), (C) in vitro-translated Yox1p(151–274) in the presence of recombinant Mcm1p(1–96) and increasing amounts of in vitro-translated Fkh2p(1–862) proteins (1, 2, and 4 molar equivalents; indicated by a triangle above the lanes), or (D) the indicated combinations of recombinant versions of Mcm1p, Fkh2p(325–458), and increasing amounts of Yox1p(151–274) or Yox1p(176–274) proteins (1 and 2.5 molar equivalents; indicated by a triangle above each set of lanes; lanes 6 and 9 contain the same quantity as lanes 5 and 8). The bands corresponding to Mcm1p and Fkh2p alone (black arrows), binary Yox1p-Mcm1p or Fkh2p-Mcm1p complexes (gray arrows), or ternary Mcm1p-Fkh2p-Yox1p complexes (white arrow) are indicated. Complexes containing Yox1p alone are indicated by #. ^, a higher-order complex. *, a nonspecific band arising from the rabbit reticulocyte lysate. See also Figure S4.

Yox1p Acts to Limit Promoter Occupancy by Fkh2p In Vivo

As Fkh2p is an activator protein that cyclically associates with its target promoters (Voth et al., 2007) and Yox1p is a repressor protein that displays cyclical stability and association with target promoters (Pramila et al., 2002), it was possible that competition between Yox1p and Fkh2p for interaction with Mcm1p could explain how Yox1p can repress the expression of a subset of Fkh2p target genes. We therefore tested this hypothesis by first examining the occupancy of wild-type and mutant versions of the promoter of the Yox1p/Fkh2p target gene SPO12 in vivo. To rule out indirect effects of potentially perturbing the cell cycle by affecting endogenous SPO12 expression, we integrated a plasmid containing a fusion of the SPO12 promoter linked to the E. coli LacZ gene. Strains harboring the wild-type promoter and a mutant version with a disrupted Yox1p-binding site (Figure 2A) were created, and the effect on promoter activity was determined by measuring β-galactosidase activity. As expected, the activity of the mutant promoter was increased through loss of binding of the Yox1p

2003) but at high concentrations can bind to the SPO12 promoter. In contrast to the results seen with longer forms of Fkh2p, Fkh2p(325–458) did not compete with Yox1p for Mcm1p binding but instead was able to form a higher-order complex containing Fkh2p, Yox1p, and Mcm1p (Figure 5D, lanes 3–5). Thus, interaction of Fkh2p with Mcm1p appears essential to establish competitive binding.

To further establish a role for protein–protein interactions in establishing the competitive binding mechanism, we analyzed the effects of mutant proteins and DNA-binding elements on the competition phenomenon. Hydrophobic residues in both Fkh2p (Boros et al., 2003) and Yox1p play major roles in interaction with Mcm1p. We therefore tested Yox1p(F155A) for its ability to displace Fkh2p from Fkh2p-Mcm1p complexes. In comparison to wild-type Yox1p, Yox1p(F155A) showed much-reduced ability to compete with Fkh2p for Mcm1p binding (Figure 6A, lanes 1–7), even though its intrinsic DNA binding ability is unaltered (Figure 3C). These results demonstrate the importance of protein–protein interactions in the displacement mechanism. Moreover, wild-type Yox1p was unable to compete efficiently with Fkh2p for complex formation with Mcm1p when the Yox1p-binding site on the SPO12 promoter was mutated (Figure 6A, lanes 8–11), demonstrating that DNA binding, in addition to protein–protein interactions, is required for effective competition. Furthermore, when the spacing of the Yox1p- and Mcm1p-binding sites is increased, competition with Fkh2p for binding to Mcm1p is diminished (Figure S5). This point was further underlined by the observation that a DNA-binding incompetent form of Yox1p (Yox1p(N226Q)) was unable to compete with Fkh2p for binding to Mcm1p on the wild-type SPO12 promoter fragment (Figure 6B).

Together, these results demonstrate that the formation of DNA-bound complexes between Mcm1p and either Yox1p or Fkh2p is a mutually exclusive event. Moreover, we establish that both protein–protein and protein–DNA interactions by Yox1p are essential to enable the protein to compete with Fkh2p for complex formation with Mcm1p.
repressor (Figure 7A). Next, we performed ChIP analysis to
determine promoter occupancy by Fkh2p and Ndd1p. As a con-
trol, we determined the occupancy of the endogenous
CLB2 promoter, which was unchanged irrespective of the status of
the SPO12 promoter fusion (Figures 7B and 7C). In contrast,
upon mutation of the Yox1p site, occupancy by either Fkh2p
(Figure 7B) or Ndd1p (Figure 7C) on the SPO12 promoter fusion
rose substantially.

Next, we examined the effect of overexpression of either wild-
type Yox1p or Yox1p(F155A) that is defective in Mcm1p binding
and Fkh2p displacement activity in vitro. In this case, we used
Ndd1p as a readout to indicate that correctly formed Fkh2p-
Mcm1p complexes were assembled on the promoter (Koranda
et al., 2000). Induction of wild-type Yox1p resulted in a decrease
in Ndd1p occupancy on the endogenous SPO12 promoter,
which contains a juxtaposed Yox1p-binding site (Figure 7D).
However, overexpression of Yox1p(F155A) was unable to ef-
ciently displace Ndd1p binding from the SPO12 promoter.
Importantly, both wild-type and mutant forms of Yox1p were
expressed to similar levels (Figure S6).

As a final test of our model, we performed re-ChIP analysis
to examine whether co-occupancy of the SPO12 promoter by
Fkh2p and Yox1p could be detected. However, whereas
Mcm1p binding to the SPO12 promoter could be detected in
both Fkh2p- and Yox1p-precipitated material, little binding of
Fkh2p was detectable when Yox1p was used in the first ChIP,
and conversely, little binding of Yox1p was detectable when
Fkh2p was used in the first ChIP (Figure 7E). As expected, only
Fkh2p-Mcm1p or Yox1p-Mcm1p co-occupancy was detected
at the CLB2 and MCM3 promoters, respectively (Figure 7E).
Thus, the absence of detectable complexes containing both
Fkh2p and Yox1p on the SPO12 promoter in vivo is fully consis-
tent with our model in which Yox1p and Fkh2p compete for inter-
action with promoter-bound Mcm1p.

**DISCUSSION**

Transcriptional regulation during the eukaryotic cell cycle is
complex. Recent studies in S. cerevisiae indicate that, rather
than two major waves of gene expression at G1/S and G2/M
phases, there are many overlapping waves of expression with
subtly different timing mechanisms, each with their associated
transcriptional regulatory circuitry. Although a limited number
of regulators have been identified, it is becoming increasingly
apparent that these can be combined in different ways to pro-
vide unique control opportunities to individual or subsets of
promoters, determining precise timings of cell cycle-dependent gene expression (Horak et al., 2002; Iyer et al., 2001; Lee et al., 2002). Here, we have uncovered an atypical mechanism of antagonism between transcriptional activators and repressors during M phase of the cell cycle. Mcm1p acts as a common platform for recruitment of the homeodomain repressor protein Yox1p and the forkhead transcription activator protein Fkh2p. These interactions with Mcm1p are mutually exclusive, and hence, the timing of recruitment of these two regulators to promoters during the cell cycle determines the transcriptional status of cyclically regulated target genes.

Interactions between Yox1p and Mcm1p are driven via a combination of protein-DNA interactions involving the homeodomain of Yox1p and through direct protein-protein interactions with the MADS box DNA-binding domain of Mcm1p. Though we have only tested the DNA-binding domain of Mcm1p in vitro, it is likely that the interactions are relevant to full-length Mcm1p as we validate our results in vivo wherein full-length Mcm1p is present. Several aromatic residues that are clustered on a short peptide in the flanking region located N terminal to the homeodomain of Yox1p were found to be important for these interactions. Many other yeast and mammalian transcription factors use short peptides that are distinct from their DNA-binding domains to bind to MADS box partner proteins (Mead et al., 1996; Tan and Richmond, 1998; Hassler and Richmond, 2001; Shore and Sharrocks, 1994). A common feature is the use of aromatic residues to drive the protein-protein interactions with the MADS box partner proteins. Indeed, the structures of the MATα2-Mcm1p and SAP-1-SRF complexes both reveal that hydrophobic residues insert into a common hydrophobic pocket found on the surface of the MADS DNA-binding domain (Tan and Richmond, 1998; Hassler and Richmond, 2001). Furthermore, molecular studies have implicated the same hydrophobic pocket as a major determinant of Fkh2p-Mcm1p interactions, most likely through insertion of an aromatic residue from Fkh2p, as there are three important aromatic residues in Fkh2p required for interaction with Mcm1p (Boros et al., 2003). In mammalian systems, the coactivator MAL also uses aromatic residues to interact with the hydrophobic pocket on SRF (Zaromytidou et al., 2006). Thus, a common mode of interaction between MADS box proteins and their interaction partners has been adopted by a heterologous array of binding partners. However, despite

Figure 7. Yox1p Displaces Fkh2p from Promoters In Vivo

(A) Reporter gene assay of SPO12 promoter activity in cells (AP18) containing either integrated pAS2000 (wild-type [WT] SPO12 promoter) or pAS2001 (mutant [mut] SPO12 promoter). Data are shown relative to the activity of the wild-type SPO12 promoter (taken as 1) and are the average (± SEM) of five independent experiments.

(B–D) ChIP assays of occupancy of either the CLB2 promoter in its normal endogenous context or the SPO12 promoter in the SPO12-LACZ fusion (B and C) or the SPO12 promoter in its normal endogenous context (D). ChIP was performed using either anti-Myc (to detect Myc-tagged Fkh2p) or anti-Pk (to detect Pk-tagged Ndd1p) antibodies and extracts isolated from logarithmically growing AP16 (B) or AP180 (C) cells containing either integrated pAS2000 (wild-type [WT] SPO12 promoter) or pAS2001 (mutant [mut] SPO12 promoter), or in (D), AP180 cells containing empty vector (Δp) or plasmids expressing either GAL1 promoter-driven wild-type (WT-pAS2573) or F155A (pAS2574) Yox1p derivatives. In (D), cells were grown in galactose-containing media for 4 hr before sample collection. Data are shown relative to the background binding to the CLB2 or SPO12 promoters in control cells (W303-1a) harboring untagged wild-type Fkh2p (B and C) or Ndd1p (D, control) (white bars) (taken as 1) and are the average (± SEM) of at least two independent experiments.

(E) Re-ChIP analysis of Mcm1p, Pk-tagged Yox1p, and Myc-tagged Fkh2p binding to the indicated promoters following a first-round ChIP with either Pk (for Yox1p) or Myc (for Fkh2p) antibodies. Data are average of two independent experiments.

(F) Model of how Yox1p represses cell cycle-dependent transcription. In the case of a subset of CLB2 cluster genes, Yox1p binding causes the displacement of the activator protein Fkh2p (top). On genes expressed at the M/G1 phase border (bottom), Fkh2p is not present at the promoter, and Yox1p might act through displacement of an unknown (co-)activator (indicated as X) or through direct repressive effects. Potential protein-DNA interactions are indicated by a dotted arrow. See also Figure S6.
these similarities, there are key differences in how some proteins interact with MADS box proteins. This is exemplified by the closest human homologs of Fkh2p and Yox1p, FOXK1 and Phox1, respectively. FOXK1 interactions with SRF are driven by the forkhead DNA-binding domain rather than a flanking peptide (Freddie et al., 2007), and interactions between Phox1 and SRF appear to be directed by the homeodomain (Grueneberg et al., 1992), although direct biochemical proof of the latter is lacking. Similarly, the homeodomain of Nkx-2.5 is sufficient for binding to SRF (Chen and Schwartz, 1996). However, it is striking that, in yeast, flanking DNA-binding sites for Fkh2p and Yox1p can be found in close juxtaposition to Mcm1p DNA-binding sites, whereas in mammals, there does not appear to be such combinatorial sites. This suggests that the role of the DNA-binding domain of MADS box-interacting proteins has been subverted through evolution from primarily mediating protein-DNA contacts in yeast to providing protein-protein interactions in higher organisms.

The direct competition for Mcm1p binding by Yox1p and Fkh2p was unexpected due to the clear separation of their DNA-binding sites on opposite sides of the Mcm1p DNA-binding site (Figure 7F). However, each protein contacts a region centered on the same binding pocket of Mcm1p, and as interaction with Mcm1p is essential for the recruitment of both Fkh2p (Boros et al., 2003; Hollenhorst et al., 2001) and Yox1p, only one protein can be recruited at a time. Mcm1p is a dimer and, as such, can theoretically present two identical surfaces that could accommodate two interacting proteins. However, as this clearly does not occur, it suggests that these proteins lack the flexibility to interact with both surfaces of Mcm1p, and that the structure of the DNA-bound complex adds constraints, which means that only one of these proteins is allowed to bind to one (the same) surface of Mcm1p at any given time. Alternatively, it is possible that protein binding to Mcm1p straddles both sides of Mcm1p, preventing proper access to the second binding surface. Indeed, it is possible that Fkh2p binds as a dimer with only one subunit contacting DNA, as Fkh2p dimerization has been reported previously (Hollenhorst et al., 2001). Of interest, a similar scenario has been observed in mammalian systems in which the activator proteins MAL and Elk-1 compete for binding to the same surface of the dimeric MADS box protein SRF, despite both apparently functioning as monomers (Zaromytidou et al., 2006). Further structural studies are needed to establish the exact molecular architecture of these complexes.

It was previously suggested that Yox1p might function to displace the coactivator protein Ndd1p (Pramila et al., 2002). Here, we show that this does occur but through an indirect mechanism by displacing Fkh2p and, hence, concomitant loss of Ndd1p binding (Figures 7B–7F). At the transcriptional level, this Yox1p-dependent inhibition of formation of Mcm1p–Fkh2p–Ndd1p complexes would result in a delay in the timing of activation of the CLB2 cluster genes containing the Yox1p-binding site compared to those that do not, which is exactly what is seen (Figure 4; Pramila et al., 2002). Previous work has suggested that cell cycle-dependent gene expression of YOX1 determines the timing of repression of Yox1p target promoters (Pramila et al., 2002). In this model, fluctuating Yox1p protein levels would change its availability for promoter binding and repression. Furthermore, relatively low constitutive expression of YOX1 represses the cyclical expression of the Yox1p target gene MCM3 (Pramila et al., 2002). Thus, the timing of Fkh2p binding would be determined by the levels of Yox1p present in the cell. In agreement with this model, we find that constitutive expression of YOX1 inhibited Ndd1p binding to the Yox1p-regulated Spo12 promoter (Figure 7D). However, it is also possible that additional mechanisms contribute to the regulation of Yox1p/Fkh2p competition for Mcm1p binding. For example, Yox1p is phosphorylated by cyclin-Cdk complexes (Übersax et al., 2003), and it is possible that this triggers changes in promoter occupancy and/or the proteolytic degradation and turnover of Yox1p.

Although we have established the mechanism underlying the timing of expression of a subset of CLB2 cluster genes in the cell cycle by Yox1p, it remains unclear how Yox1p regulates the timing of expression of ECB-controlled genes. No other common juxtaposed binding motifs are obvious in the promoter regions of ECB genes, and no other binding partners for Mcm1p that regulate ECB genes have been identified. However, it is tempting to speculate that the mechanism of action is similar to the regulation of Mcm1p–Fkh2p and involves the displacement of an as yet unidentified coactivator that competes with Yox1p for binding to the surface of the Mcm1p MADS box DNA-binding domain (Figure 7F). Notwithstanding this possibility, in both scenarios, Yox1p might also actively repress transcription through the recruitment of corepressor proteins that act, for example, on the local chromatin environment. In addition to its activator role via Ndd1p, Fkh2p also has a repressive role and recruits the Rpd3 corepressor to the CLB2 promoter at early points in the cell cycle (Veis et al., 2007; Voth et al., 2007). However, Yox1p is unlikely to influence this activity, as the point of Yox1p function is later in the cycle during M phase.

It is unclear why such an elaborate system for repression is utilized by Yox1p, rather than a more typically found simple steric hindrance model whereby the activator and repressor proteins compete for the same site on DNA. However, DNA binding sequence constraints probably dictate that this is not possible, as the recognition motifs for Fkh2p and Yox1p are quite different. Thus, by creating a system whereby promoter recruitment is dependent on cooperative interactions with a common binding partner, transcription factors from heterologous families are able to compete for promoter occupancy. Furthermore, by invoking a third protein (in this case Mcm1p), the repressive activity of Yox1p on Fkh2p function can be made more specific and restricted to a subset of target genes on which Mcm1p is also present and leave other genes on which Fkh2p acts independently from Mcm1p (Hollenhorst et al., 2001) unaffected.

As MADS box, forkhead, and homeodomain proteins are also found in higher eukaryotes, some aspects of the combinatorial regulation mechanisms documented here are likely to be conserved. For example, the MADS box protein SRF interacts with the forkhead proteins FOXK1 (Freddie et al., 2007) and FOXO4 (Liu et al., 2005) and the homeodomain proteins Phox1 (Grueneberg et al., 1992) and Nkx-2.5 (Chen and Schwartz, 1996). In these examples, interactions with forkhead proteins are repressive, whereas the homeodomain proteins work in a positive manner. However, the direct interplay involving interactions
between these proteins has not been investigated. In a more
general context, the phenomenon of sequence-specific repres-
sor and activator proteins antagonizing each other through
cmpeting for the same protein surface on a common DNA-
ound transcription factor is likely to be more widespread in
timing mechanisms in other transcriptional control networks.
Thus, these studies may have important implications for under-
standing the mechanisms by which gene expression determines
the precise timing of events in cellular processes, in addition to
cell proliferation, such as development.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis
See the Supplemental Information for details of plasmids used.

Protein Production, Pull-Down Assays, and Western Blotting
Wild-type and truncated derivatives of Yox1p and Fkh2p were produced by
 coupled in vitro transcription and translation (using rabbit reticulocyte lysates,
Promega) and subsequently analyzed and quantified by phosphorimaging.
His-tagged Mcm1p(1–98), Yox1p, and Fkh2p derivatives were expressed
 in Escherichia coli BL21 (DE3) and purified using nickel-NTA-agarose resin
(QIAGEN) according to standard procedures. MBP-Mcm1p(1–96) was pre-
pared by maltose affinity chromatography according to standard procedures.
For the experiments in Figure S4B, the Mcm1p moiety was released from the
fusion protein while still attached to the beads by cleavage with factor Xa.
Protein concentrations were estimated compared to BSA standards.
For titration experiments, a 9-, 10-, or 25-concentration fold was
used, with typical amounts of protein ranging from ~50 to 500 ng. When two
different proteins were directly compared, protein levels were normalized by
comparison on the same gel.

MBP pull-down assays with in vitro-translated and recombinant proteins
were carried out as described previously for GST pull-down assays
(Shore and Sharrocks, 1994). Where indicated, ethidium bromide (100
µg/ml) was added to the MBP-fusion protein immobilized on the beads prior to the addi-
tion of Yox1p proteins and was included in all wash buffers.

To detect epitope-tagged derivatives by western analysis, anti-Flag (Sigma)
and anti-myc (9E10; Santa Cruz) antibodies and Supersignal west dura
substrate (Pierce) were used.

Gel Retardation and Immobilized Template Assays
Gel retardation and immobilized template binding assays were performed as
described previously (Boros et al., 2003) using the binding sites as detailed
in the Supplemental Information. Complexes containing Flag-tagged Yox1p
derivatives were detected by including, 2.5 µg of anti-Flag antibody (Sigma)
in the binding reaction. His-tagged Mcm1p(1–98) was used in all gel retarda-
tion experiments apart from those in Figure S4B in which Mcm1p(1–96)
cleaved from MBP-Mcm1p(1–96) derivatives was used.

Immobile template binding assays were performed in the same buffers as
used in gel retardation assays. 14 ng of biotin-labeled DNA-binding sites were
incubated with 7 µl magnetic streptavidin beads (Dynal) for 15 min with gentle
shaking. The beads were subsequently washed in 100 µl binding buffer, and
recombinant Mcm1p(1–98) was then added and allowed to bind for 15 min
with gentle shaking. The beads were washed, and then 3[H]-labeled in vitro-
translated Yox1p was added and allowed to bind for 1 hr. After washing the beads
 twice in binding buffer, the proteins were eluted on ice for 15 min in elution
buffer (1 x PBS, 1 M NaCl) and then analyzed by 12% SDS PAGE
followed by fixing, drying, and visualization by phosphorimaging.

Yeast Growth, RNA Analysis, and Chromatin Immunoprecipitations
Details of yeast strains are provided in the Supplemental Information.

Yeast cells were grown, GAL7-promoter-driven constructs were induced,
and transformations were performed as described previously (Darieva et al.,
2003). Yeast cultures were synchronized in G1 phase by treatment with
a-factor (5 µg/ml) for 3 hr. DNA content analyses were performed using propi-
odium iodide-stained cells as described previously (Darieva et al., 2006).

RNA extraction and real-time RT-PCR analysis were carried out as
described previously (Darieva et al., 2006) using the following primer pairs:
ADS1444/1445 (CLN2), ADS1436/1437 (CLB2), ADS1704/1705 (SPO12),
ADS1926/1927 (CDC20), ADS1708/1709 (MCM3), and ADS1439/1440 (ACT7).
Details are available on request. All data were normalized to ACT7 levels in the
same cells.

Details of chromatin immunoprecipitation (ChiP) and re-ChiP assays are
provided in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures
and six figures and can be found with this article online at doi:10.1016/
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