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High Functional Overlap Between MluI Cell-Cycle Box Binding Factor and Swi4/6 Cell-Cycle Box Binding Factor in the G1/S Transcriptional Program in *Saccharomyces cerevisiae*

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ABSTRACT

In budding yeast, many genes are induced early in the cell cycle. Induction of these genes has been predominantly attributed to two transcription factors, Swi4-Swi6 (SBF) and Mbp1-Swi6 (MBF). Swi4 and Mbp1 are related DNA-binding proteins with dissimilar target sequences. For most G1/S-regulated genes that we tested in a *cdc20* block-release protocol for cell-cycle synchronization, removal of both Swi4 and Mbp1 was necessary and sufficient to essentially eliminate cell-cycle-regulated expression. Detectable SBF or MBF binding sites (SCBs or MCBs) in the promoters or available genome-wide promoter occupancy data do not consistently explain this functional overlap. The overlapping ability of these transcription factors to regulate many promoters with very similar cell-cycle kinetics may provide robustness to the G1/S transcriptional response, but poses a puzzle with respect to promoter-transcription factor specificity. In addition, for some genes, deletion of Mbp1 or Swi4 enhances transcription, suggesting that these factors can also function as transcriptional repressors. Finally, we observe residual G1/S transcriptional regulation in the absence of Swi4 and Mbp1.

COMMITMENT to the cell cycle in eukaryotic cells occurs late in G1 at a point termed Start (Pringle and Hartwell 1981). Start coincides with a peak in transcription of >200 genes including the G1 cyclins *CLN1* and *CLN2*, the B-type cyclins *CLB5* and *CLB6*, and the mating-type switch endonuclease *HO*, along with many other genes involved in DNA synthesis, budding, and spindle pole body duplication (Cho *et al.* 1998; Spellman *et al.* 1998). Timely G1-specific transcription requires the G1 cyclin Cln3 (Tyers *et al.* 1993; Dirick *et al.* 1995; Stuart and Wittenberg 1995) and the cyclin-dependent kinase, Cdc28 (Koch *et al.* 1996). Cln3-Cdc28 promotes transcriptional activation by formation of the RNA polymerase II holoenzyme at the TATA boxes of the G1 cyclins *CLN1* and *CLN2*, among others (Cosma *et al.* 2001). Cln3 activates cell-cycle initiation in a dosage-dependent manner (Cross 1988; Tyers *et al.* 1993). Regulation of cell size, pheromone sensitivity, and budding by Cln3 are dependent on the transcription factors MluI cell-cycle box binding factor (MBF) and Swi4/6 cell-cycle box binding factor (SBF) (Wijnen *et al.* 2002). Cln1 and Cln2 activation may represent the key event of Start. Cln1,2-Cdc28 complexes are involved in activating a number of pathways, including activation of B-type cyclins, bud emergence, and microtubule organizing center duplication (reviewed in Cross 1995).

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Much of the Start transcriptional program depends on two related transcription factors, MBF and SBF (reviewed by Koch and Nasmyth 1994). MBF is composed of two proteins: Swi6, the *trans*-activating component, and Mbp1, the DNA-binding component. Mbp1 recognizes the MluI cell-cycle box (MCB), ACGCG (Koch *et al.* 1993). One study has found that Stb1, a Swi6-binding protein involved in Start transcription (Ho *et al.* 1999), is a specific regulator of MBF-dependent transcription (Costanzo *et al.* 2003). In addition to its role as a G1/S transcriptional activator, a role for MBF as a repressor during other stages of the cell cycle has been proposed (Koch *et al.* 1993).

SBF is composed of two proteins: Swi6 and Swi4, a homolog of Mbp1 that acts as the DNA-binding component of SBF. SBF recognizes the Swi4/6 cell-cycle box (SCB) CRCGAAA (Breeden and Nasmyth 1987; Andrews and Herskowitz 1989b; Taba *et al.* 1991). Consistent with the roles of SBF and MBF as DNA-binding factors that coordinately regulate the broad G1/S transcriptional regulatory program, three genome-wide location analyses show binding of MBF and SBF to a broad range of targets, in which SCB and MCB sites are enriched (Iyer *et al.* 2001; Simon *et al.* 2001; Harbison *et al.* 2004).

Whi5, a negative regulator of cell-cycle Start, was identified by small cell size in *whi5* deletion strains (Jorgensen *et al.* 2002; Zhang *et al.* 2002). Whi5 binds to SBF and acts as a repressor of G1-specific transcription. Dissociation of Whi5 from SBF is dependent on Cln3,

and in the absence of Whi5 the requirement for Cln3 in transcriptional activation is much reduced (Costanzo *et al.* 2004; De Bruin *et al.* 2004). A similar role for Whi5 as a repressor of MBF-dependent transcription has also been proposed (Costanzo *et al.* 2004).

The single deletion mutants *swi4* and *mbp1* are viable in *Saccharomyces cerevisiae*. However, the double deletion mutant *mbp1 swi4* is inviable with arrest occurring in G1. The *mbp1 swi4* lethality can be rescued by constitutive expression of *CLN2*, suggesting that the lethality stems primarily from failure to express G1 cyclins (Koch *et al.* 1993). A role for Cln3p as an activator of MBF and SBF has been proposed (Wijnen *et al.* 2002). However, *cln3* deletion mutants are viable with delayed expression of MBF- and SBF-regulated genes whereas *mbp1 swi4* strains are inviable, suggesting additional activators of MBF and SBF. *BCK2* and *CTR9* encode candidate SBF and MBF activators that are essential in the absence of Cln3 (Epstein and Cross 1994; Di Como *et al.* 1995; Koch *et al.* 1999). Furthermore, in *cln3* deletion strains *CLN1* and *CLN2* are sufficient for transcriptional activation via a model of positive feedback (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991; Nasmyth and Dirick 1991; Tyers *et al.* 1993; Dirick *et al.* 1995; Stuart and Wittenberg 1995; Costanzo *et al.* 2004; De Bruin *et al.* 2004), in which Cln1,2 can inactivate Whi5 and/or directly activate SBF/MBF, thus driving their own transcription.

Despite the substantial amount of information that has been accumulated about SBF and MBF and the control of G1/S-regulated transcription, some mysteries remain. In some cases, it has been noted that removal of either Swi4 or Mbp1 has at best minor effects on the transcriptional activation of putative SBF or MBF target genes, respectively (Koch *et al.* 1993; Cross *et al.* 1994). In some circumstances Swi4 and Mbp1 may be functionally redundant, either because of the presence of both SCBs and MCBs in the promoters of some genes (*e.g.*, *CLN2*; Stuart and Wittenberg 1994) or because of cross-binding of SBF and MBF to MCBs and SCBs, respectively (Koch *et al.* 1993; Partridge *et al.* 1997; Taylor *et al.* 2000). Functional redundancy of Swi4 and Mbp1 has never been tested directly, primarily because the lethality of *swi4 mbp1* double mutants precludes simple analysis.

Here we directly analyze overlap of Swi4 and Mbp1 in the G1/S transcriptional regulation of multiple genes. We wished to distinguish between (i) Swi4- or Mbp1-specific regulation, so that the appropriate single deletion would abolish regulation; (ii) overlapping function of Swi4 and Mbp1, so that only the double deletion would abolish regulation; or (iii) Swi4- and Mbp1-independent regulation.

MATERIALS AND METHODS

Strains and plasmids: All yeast strains used are described in Table 1. JB04-15D is a segregant of a cross between 2147-7C and K3294. JB03-19C is a segregant of a cross between 2147-7C and K2299. Plasmid pI411 was created by inserting ~900 bp of the *CLN3* promoter fused to 1.6 kb *CLN2* coding sequence with three HA tags at the 3′ end between the *Sal*I and *Sac*II sites in the multiple cloning site (MCS) of pRS304. A *Spe*I digest of pI411 was used to transform 2147-7C by integration at the *CLN2* locus to create JB13. Plasmid pKL035 was created by inserting ~700 bp of the *GAL*-inducible promoter fused to 1.6 kb *CLN2* coding sequence with three HA tags at the 3′ end between the *Sal*I and *Sac*II sites in the MCS of pRS404. A *Spe*I digest of pKL035 was used to transform 2147-7C by integration at the *CLN2* locus to create JB14. JB21-2C was created in a similar way to JB13 by first transforming strain K2299 with *Spe*I-digested pI411 and then crossing the resulting transformed strain to JB04-15D and selecting segregants. All integrations were confirmed by an *Ssp*I Southern blot.

Growth and synchronization procedures: YEP medium was used for all experiments, supplemented with the appropriate carbon source as indicated below. Yeast cultures were shaken at 250 rpm, in a volume no more than 20% of the container maximum at 30°. Cell-cycle synchronization was achieved by the *cdc20 GALL-CDC20* block release in 2147-7C, JB13, JB03-19C, JB04-15D, and JB21-2C by growing cells to early log phase in YEP + galactose (3%) and then filtering them and growing them in YEP + glucose (2%) for 3 hr to arrest cells in metaphase. *GALL* is a truncated version of the *GAL1* promoter that shows inducible but significantly lower expression than the full-length *GAL1* promoter (Mumberg *et al.* 1994). Cells were released from the block by filtering back into YEP + galactose (3%).

Northern blot analysis: Procedures for Northern blot analysis of mRNA were as described previously (McKinney *et al.* 1993). RNA was harvested from synchronized cultures every 15 min for 2–3 hr. Probes were generated by polymerase chain reaction using Research Genetics (Birmingham, AL) primer pairs for each gene tested. DNA fragments were radiolabeled by random-prime labeling using a Prime-It II kit (Stratagene, La Jolla, CA), and transcript levels were visualized and quantitated using a Molecular Dynamics (Sunnyvale, CA) STORM PhosphorImager system. Each blot contained a mutant time course and the relevant wild-type control time course run below it (2147-7C for JB03-19C and JB04-15D; JB13 for JB21-2C). All blots were probed with *TCM1* as a loading control. *TCM1* expression has been previously shown to be non-cell-cycle regulated and useful as a loading control for Northern blots (McKinney *et al.* 1993; Oehlen *et al.* 1996); lack of cell-cycle regulation of *TCM1* was also observed in the genome-wide study of Spellman *et al.* (1998). To quantitate expression levels, each lane was background subtracted and normalized to the *TCM1* signal. Duplicate, and in some cases triplicate, Northern blots for each gene gave highly reproducible expression profiles. For calculating expression levels, a single representative Northern blot was used for each gene. To calculate relative expression (Figure 1), normalized expression levels for each mutant were expressed relative to the peak expression for its particular control. For simplicity, in Figure 1 only one control time course [wild type (WT)] is shown for each gene. To provide a single quantitative measure of the degree of regulated expression in these experiments, we analyzed peak:trough ratios of expression, calculated as follows. For the two control strains (2147-7C and JB13) (Figure 2A), ratios of expression were defined as peak divided by trough value, where the peak is normalized to 1. The trough value is defined as the raw trough value plus an error (σ). σ is defined as the square root of the $n-1$ average of squared differences in trough values in two replicate Northern blots of strain 2147-7C for 25 genes. The ratio of two variables is obviously very large when one is small, but intuitively the

denominator should not be allowed to get smaller than the measurement error. A precise formula can be derived for the average of the logarithm of two variables, each distributed with a mean and standard deviation. In the context of actual measurements this implies that intensities (after background subtraction) are positive, and that the standard deviation should be added to small quantities before taking the ratio and the logarithm. For the ratios of expression of 2147-7C the average of two replicate Northern blots for each gene was used. The ratios of expression found by microarray hybridization (Spellman *et al.*, 1998; Figure 2A) were calculated by averaging expression ratios from four different methods of cell synchronization. To calculate ratios of expression in mutant strains relative to that in control strains (Figure 2B), the following was done: Mutant expression was defined as the normalized peak value of the mutant minus the average normalized trough value of the mutant. Control expression was defined as the normalized peak value of the control minus the average normalized trough value of the control. The ratio of expression was defined as mutant expression divided by control expression. We recognize that this metric, while convenient, does ignore some kinetic details of precise peak timing and peak width (see RESULTS). In all G1/S-regulated genes tested for which any regulated expression was detected in the various mutant strains, the peaks of expression remained at approximately the same point in the cell cycle as those in the wild-type strain. In none of the genes tested was there significant unregulated expression or peak expression at any point in the cell cycle other than G1/S. The raw quantitation of all the Northern blot data used is available upon request.

Analysis of published genome-wide binding data sets: To compare the binding of MBF and SBF found in previous published genome-wide location analyses (Figure 2D; Figure 3) we used the supplementary data available from three data sets (Iyer *et al.* 2001, http://genome-www.stanford.edu/chromatinip/; Simon *et al.* 2001, http://web.wi.mit.edu/young/cellcycle; Harbison *et al.* 2004, http://web.wi.mit.edu/young/regulatory_code). For the first data set (Iyer *et al.* 2001), the figure3_data.xls file was used to identify MBF-bound, SBF-bound, and MBF + SBF-bound ORFs, including divergently transcribed ORFs, which satisfied the authors' criteria. For the second data set (Simon *et al.* 2001), the all_genes.xls file was used to sort out all ORFs with *P*-values <0.001 for binding of Mbp1 (or Mbp1 + Swi6) and Swi4. For the third data set (Harbison *et al.* 2004), the pvalbygene_forpaper_abbr.xls file was used to sort out all ORFs with *P*-values <0.001 for binding of Mbp1 (or Mbp1 + Swi6) and Swi4. For all lists any duplicate ORFs were removed and care was taken to correct any alternate ORF names. For all comparisons the systematic yeast gene names were used. A UNIX shell script was used to compare the MBF-only-, SBF-only-, and MBF + SBF-bound gene lists from the three data sets. To determine the conservation of MBF and SBF binding sites across *sensu strictu* Saccharomyces species, we used the yeast regulatory map available from the third data set (Harbison *et al.* 2004) at http://jura.wi.mit.edu/fraenkel/regcode/.

MCB and SCB consensus site counts: To count the number of MCBs and/or SCBs in the promoters of the genes tested, a regular expression search of the upstream intergenic sequence of each gene was performed. For this analysis, we defined MCB as ACGCG and SCB as C*R*CGAAA (where *R* represents A or G).

Chromatin immunoprecipitations: ChIP was performed as described (Wilmes *et al.* 2004), with some modifications. Cells from an untagged strain (2147-7C), a Swi4-Myc-tagged strain (JB05-1B), and a Mbp1-Myc-tagged strain (JB06-1A) were synchronized by the *cdc20 GALL-CDC20* block release at midlog phase. Cells were formaldehyde fixed 40 min after release, which is at approximately the peak of G1/S-regulated gene expression in this protocol. One-tenth of the whole-cell extract was set aside for input DNA. Mbp1-Myc and Swi4-Myc were immunoprecipitated overnight with a 1:250 dilution of 9E11 monoclonal antibody (Genetex). For amplification by PCR, one-fiftieth of the immunoprecipitated DNA and one-fiftieth of the input DNA were used as template. Multiplex PCR was performed using oligos for the non-G1/S-regulated *URA3* as a control along with G1/S-regulated promoter-specific oligos. PCR was performed using PuReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Arlington Heights, IL) in a RoboCycler 96 temperature cycler (Stratagene), separated on 1.5% agarose gels, and stained with 1× Sybr Green (Molecular Probes, Eugene, OR). Primer sequences are available upon request.

Coulter counting: Mode cell volume measurements were made using a Beckman Coulter Z Series Z2 Coulter particle count and size analyzer. All cultures were grown to midlog phase in YPD and sonicated before analysis. For each strain, triplicate counts were obtained from the same culture and averaged. Mode cell volume for each strain was then obtained from those averaged size profiles.

RESULTS

Construction of a set of strains allowing cell-cycle synchronization in the absence of Swi4, Mbp1, or both: To examine the roles of MBF and SBF as transcriptional regulators we have made use of single deletion strains of *mbp1* and *swi4*. In addition, we have created double deletion strains of *mbp1 swi4*. To construct the normally inviable double mutant strain, we made use of the observation of Koch *et al.* (1993) that *mbp1 swi4* mutants were viable when *CLN2* was placed under control of a constitutive promoter. We observed reasonably efficient rescue of viable *mbp1 swi4* mutant strains by inclusion of an integrated construct containing *CLN2* under the control of the *CLN3* promoter, P_{CLN3}-*CLN2* (Valdivieso *et al.* 1993; Levine *et al.* 1996).

To facilitate looking at mRNA levels throughout the cell cycle, all three deletion strains mentioned above along with all their respective controls were made synchronizable by the deletion of *cdc20* and the addition of *CDC20* under the control of the galactose-inducible promoter (*GALL-CDC20*) (Table 1). In the absence of Cdc20, cells arrest in metaphase due to inability to degrade the separase inhibitor Pds1 and the B-type cyclins Clb5 and Clb2 (Zachariae and Nasmyth 1999; Shirayama *et al.* 1999; Wasch and Cross 2002). Hence, galactose withdrawal from these strains results in a metaphase block, which is rapidly released upon readdition of galactose, giving a synchronous cell cycle. This M-phase block-release protocol may be preferable for the present purposes to the G1/S block-release protocols used previously to examine Swi4- and Mbp1-dependent expression (Koch *et al.* 1993; Cross *et al.* 1994; Stuart and Wittenberg 1994), since it allows a synchronized M/G1 transition in which regulated expression of *SIC1* can be observed to confirm synchrony, and also because it separates the block release

TABLE 1

Yeast strains used in this study

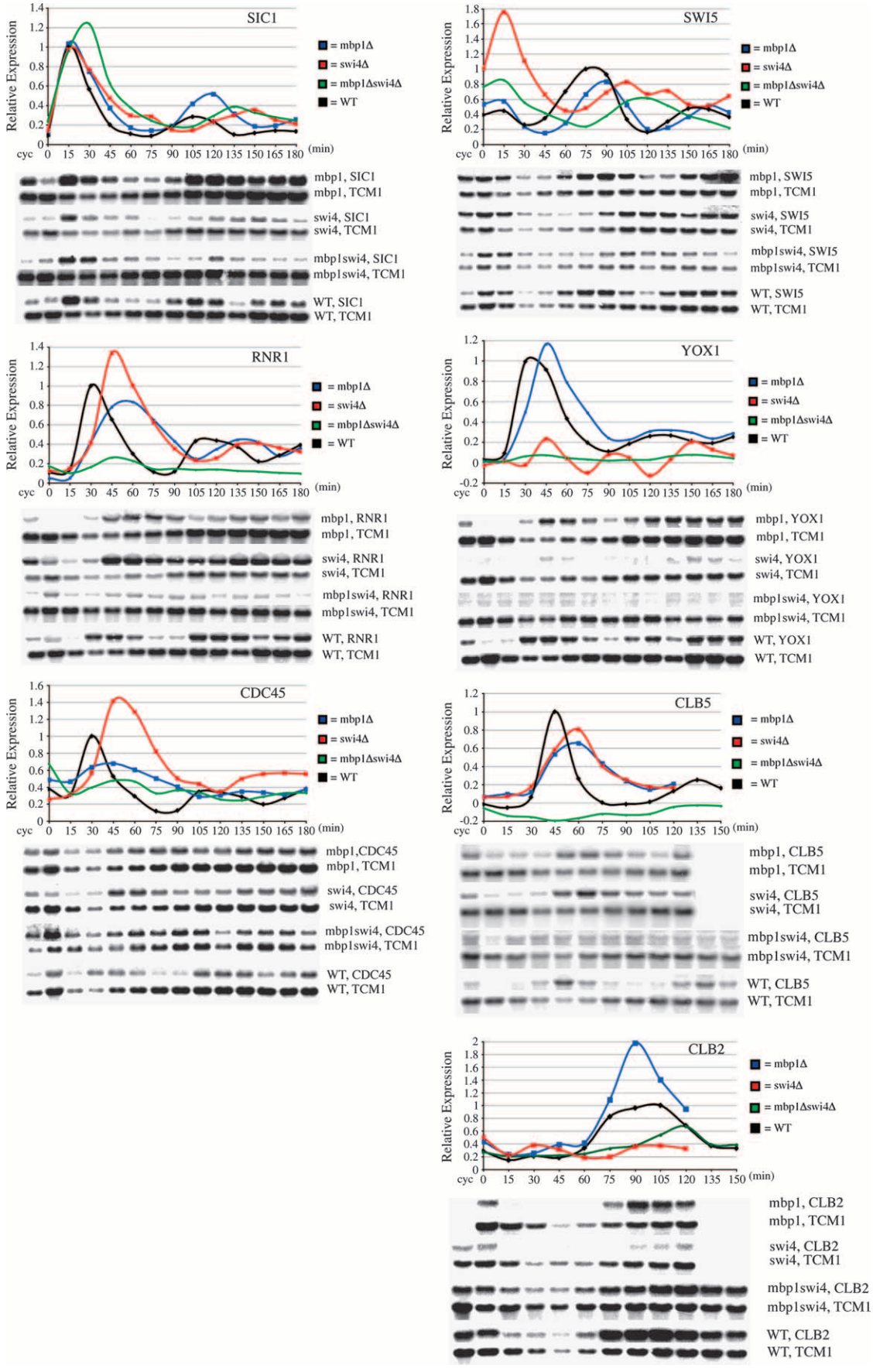
Name	Background	Genotype	Source
K2299	W303	*MAT***a** *swi4::LEU2 GAL ade2 trp1 leu2 his3 can1 ura3*	Nasmyth and Dirick (1991)
K3294	K1107	*MAT***a** *mbp1::URA3 HMLa HMRa ho-βgal HIS4 ade2 can1 met his3 leu2 trp1 ura3*	Koch *et al.* (1993)
2147-7C	W303	*MAT*α *cdc20::LEU2 ade2::ADE2::GALL-CDC20 trp1 leu2 ura3 his3 can1*	This study
JB04-15D	W303/K1107	*MAT*α *cdc20::LEU2 ade2::ADE2::GALL-CDC20 mbp1::URA3 trp1 leu2 ura3 his3 can1*	This study
JB03-19C	W303	*MAT*α *cdc20::LEU2 ade2::ADE2::GALL-CDC20 swi4::LEU2 trp1 leu2 ura3 his3 can1*	This study
JB13	W303	*MAT*α *cdc20::LEU2 ade2::ADE2::GALL-CDC20 CLN2::TRP1::*P_{CLN3}*-CLN2 trp1 leu2 ura3 his3 can1*	This study
JB21-2B	W303	*MAT***a** *cdc20::LEU2 ade2::ADE2::GALL-CDC20 mbp1::URA3 swi4::LEU2 CLN2::TRP1::*P_{CLN3}*-CLN2 trp1 leu2 ura3 his3 can1*	This study
JB05-1B	W303	*MAT*α *cdc20::LEU2 ade2::ADE2::GALL-CDC20 SWI4-18MYC::TRP1 leu2 ura3 his3 can1*	This study
JB06-1A	W303	*MAT*α *cdc20::LEU2 ade2::ADE2::GALL-CDC20 MBP1-18MYC::TRP1 leu2 ura3 his3 can1*	This study
HTLU-14A	W303	*MAT***a** *URA3 ade2 trp1 leu2 his3 can1*	This study
HTLU-2B	W303	*MAT*α *URA3 ade2 trp1 leu2 his3 can1*	This study
2819-12C	W303	*MAT***a** *URA3 ADE2 trp1 leu2 his3 can1*	This study
2819-6C	W303	*MAT*α *URA3 ADE2 trp1 leu2 his3 can1*	This study
2891-4A	W303	*MAT***a** *mbp1::URA3 ade2 trp1 leu2 his3 can1*	This study
2891-11C	W303	*MAT***a** *mbp1::URA3 ade2 trp1 leu2 his3 can1*	This study
2891-5C	W303	*MAT*α *mbp1::URA3 ADE2 trp1 leu2 his3 can1*	This study
2891-13B	W303	*MAT*α *mbp1::URA3 ADE2 trp1 leu2 his3 can1*	This study

temporally from the G1/S transition where most Swi4- and Mbp1-dependent expression is thought to occur.

To assess the synchrony of the *mbp1 swi4* P_{CLN3}*-CLN2 cdc20 GALL-CDC20* strain upon *cdc20* block release, we examined expression of two cell-cycle-regulated genes expressed at different parts of the cell cycle, neither of which is thought to be under control of SBF/MBF. Expression of the cyclin-dependent kinase inhibitor *SIC1* is induced in M/G1 by Swi5p (Toyn *et al.* 1996). *SIC1* expression was regulated in a cell-cycle-dependent manner in the *mbp1 swi4* double mutant similar to that in the respective control strain, indicating that the double mutant strain exits mitosis and activates Swi5 on schedule upon release of the *cdc20* block. In addition, accumulation of *SIC1* transcript in a second weaker peak after release in both wild type and the *mbp1swi4* mutant indicates successful completion of a second mitosis following release of the block (Figure 1). Likewise, the *SWI5* transcription factor itself, which is transcribed in S, G2, and M (Nasmyth *et al.* 1987) under control of Ndd1/Mcm1p/Fkh1,2 (Lydall *et al.* 1991; Althoefer *et al.* 1995; Koranda *et al.* 2000; Zhu *et al.* 2000), was regulated in a normal cell-cycle-dependent manner in the *mbp1 swi4* double mutant (Figure 1). These controls indicate that the cell cycle proceeds with relatively normal kinetics in the *mbp1 swi4* mutant following release of the mitotic *cdc20* block through the next mitosis, as indicated by probing expression of genes controlled by transcription factors other than SBF/MBF, thus validating this assay for examination of genes thought to be under direct control of SBF/MBF.

Examination of a subset of genes in the G1/S program for Swi4/Mbp1 requirements: To dissect the specificity and/or redundancy of SBF and MBF for transcriptional regulation of G1/S targets, we looked at expression profiles of *cdc20 GALL-CDC20* synchronized cultures of *mbp1*, *swi4*, and *mbp1 swi4* P_{CLN3}*-CLN2* strains (Table 1) by Northern blot analysis. We analyzed expression levels in the *cdc20* block-release protocol of 23 G1/S transcripts in all these strains (Figure 2), as well as four additional transcripts analyzed only in WT and the single *mbp1* and *swi4* mutants (*CLN1*, *CLN2*, *PCL1*,

Figure 1.—Northern blot analysis of control genes and representative G1/S-regulated genes. Northern blots of total RNA from *cdc20 GALL-CDC20*-synchronized cultures for each selected gene and the corresponding *TCM1* loading control are shown for *mbp1* (JB04-15D, blue), *swi4* (JB03-19C, red), *mbp1 swi4* (JB21-2B, green), and WT (2147-7C or JB13, black) strains over time. Total RNA from unsynchronized cultures is also shown (*cyc*). Quantitation of expression from each blot is also shown as expression level relative to the peak of expression of the WT control as a function of time from block release. For quantitation, all probes in each strain were normalized using *TCM1* probed on the same blot. For the *CLB2* and *CLB5* genes, no 165- or 180-min time point was collected for any strain and no 135- or 150-min time point was collected for *mbp1* or *swi4* single deletion strains.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Relative Expression
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= swi4Δ
= mbp1Δswi4Δ
= WT
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swi4, SIC1
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WT, TCM1
SWI5
Relative Expression
= mbp1Δ
= swi4Δ
= mbp1Δswi4Δ
= WT
cyc 0 15 30 45 60 75 90 105 120 135 150 165 180 (min)
mbp1, SWI5
mbp1, TCM1
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RNR1
Relative Expression
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= mbp1Δswi4Δ
= WT
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= WT
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WT, TCM1
CLB2
Relative Expression
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WT, TCM1

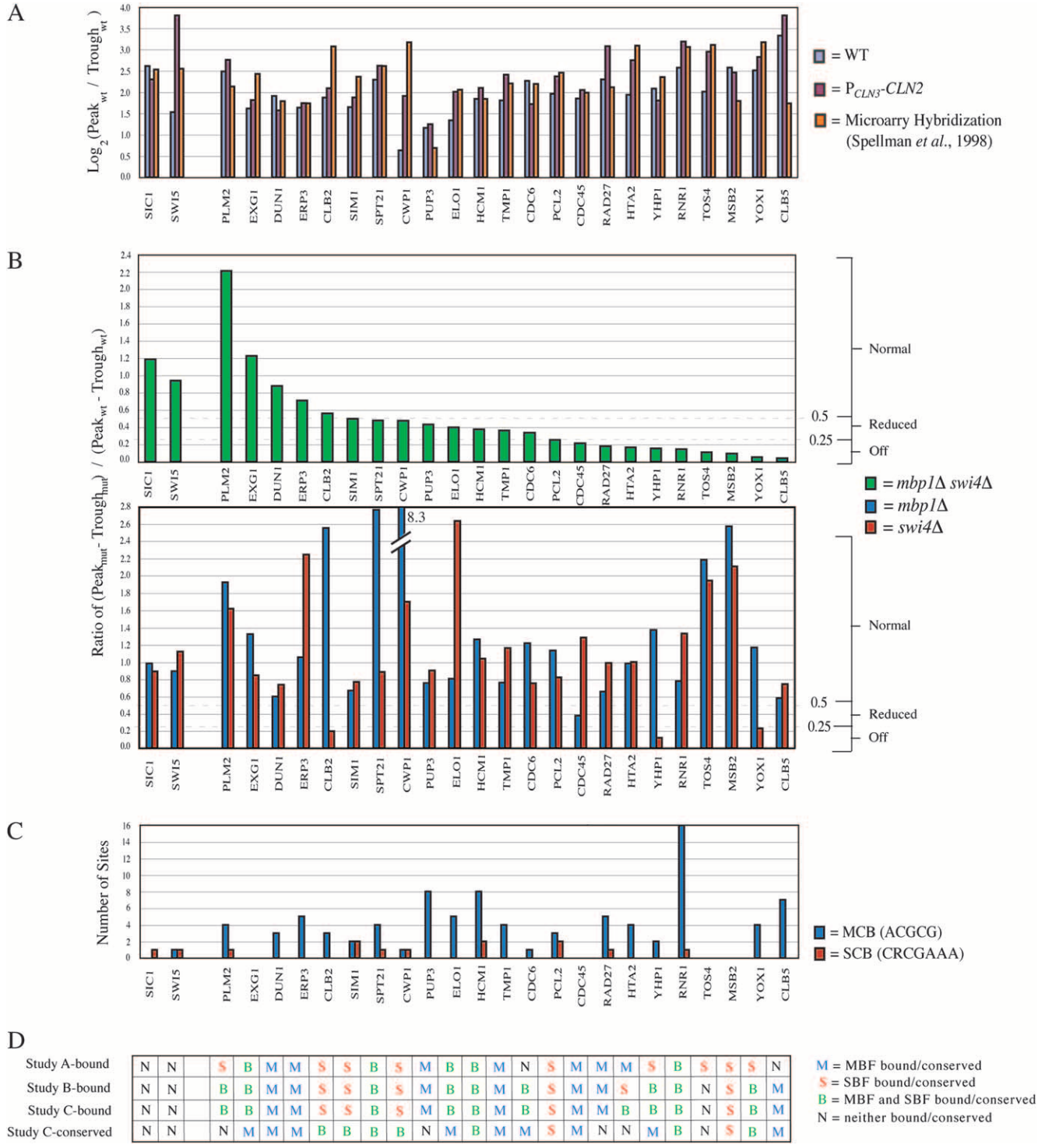

FIGURE 2.—Comparison of expression data in mutants, consensus binding sites, and genome-wide binding data. (A) Log_2 of the ratio of expression of genes in WT (2147-7C, blue), P_{CLN3}-*CLN2* (JB13, purple), and by microarray hybridization (SPELLMAN *et al.* 1998, orange). Ratios of expression for WT are the averages of two experiments. For each gene, the ratio of expression for WT and P_{CLN3}-*CLN2* is defined as $Log_2(Peak/(\sigma + Trough))$, where the trough value is normalized to the peak value, which is set to one. σ is the error in measurement of the trough value. The ratio of expression for microarray hybridization is the average of the log_2 of the expression ratios from four different methods of cell synchronization (SPELLMAN *et al.* 1998). The ratios of expression of the two control genes (*SIC1* and *SWI5*) are shown on the left. (B) Ratio of expression of genes in *mbp1 swi4* (JB21-2B, green), *mbp1* (JB03-15D, blue), and *swi4* (JB03-19C, red) all relative to WT (2147-7C or JB13). For each gene, the ratio of expression is defined as the difference of peak expression minus trough expression of the mutant divided by the difference of peak expression minus trough expression of the WT control, as determined by Northern blot. A ratio of expression >0.5 is called *normal*, from 0.25 to 0.5 is called *reduced*, and below 0.25 is called *off*. The genes are ordered (for A–D) in decreasing ratio of expression for the *mbp1 swi4* double mutant. The ratio of expression of *CWP1* in the *mbp1* mutant is 8.3 and its bar has been cut off for space. The ratios of

and *CLB6*; data not shown). These transcripts were selected on the basis of the following criteria: A number of previously identified canonical SBF and MBF targets were selected to test along with a random sampling of transcripts found to be at least moderately cell-cycle regulated (Spellman *et al.* 1998) and weighted in favor of those with a consensus with respect to Mbp1 and/or Swi4 binding in the genome-wide binding data (Iyer *et al.* 2001; Simon *et al.* 2001; Harbison *et al.* 2004). Sample data for some of these transcripts are presented in Figure 1, and a compilation of the data for 23 G1/S transcripts and the two controls discussed above (*SIC1* and *SWI5*) is shown in Figure 2B.

Because of the history of strain construction in this project, we used different controls for the single *mbp1* and *swi4* mutants than for the double *mbp1 swi4* mutant. For the single mutants, the control strain was a simple *MBP1 SWI4* wild type, while for the double *swi4 mbp1* mutant, which was rescued by P_{CLN3}-*CLN2*, the control strain was *MBP1 SWI4* P_{CLN3}-*CLN2*. To compare among all the experiments, it is important to establish that in the wild-type context, P_{CLN3}-*CLN2* did not significantly affect G1/S-regulated gene expression. Figure 2A demonstrates that this is the case: The peak/trough ratios for the 25 genes tested are generally quite comparable with or without P_{CLN3}-*CLN2* (*CWP1* displays a significant effect of P_{CLN3}-*CLN2* on its peak/trough ratio relative to that of wild type and the effect of this on the ratios of expression in mutants is discussed below; a less dramatic effect of P_{CLN3}-*CLN2* is also seen in *SWI5*). These peak/trough ratios are also generally comparable to the genome-wide data of Spellman *et al.* (1998) (shown in Figure 2A), obtained using several different methods of cell-cycle synchronization.

In Figure 2B, the data are condensed for each transcript as the relative cell-cycle-regulated induction (peak/trough) for each gene in the *mbp1 swi4* double mutant compared to that in wild type (Figure 2B, top, green bars) or in the *mbp1* or *swi4* single mutants compared to that in wild type (Figure 2B, bottom, blue and red bars, respectively). Fifteen of the G1/S targets showed evidence of being under redundant positive control of *MBF* and *SBF* since their peak/trough expression ratios were not significantly reduced by single deletion of *MBP1* or *SWI4* but were significantly reduced by concurrent removal of both *MBP1* and *SWI4*. Seven genes, including the G1 cyclins *CLN1*, *CLN2*, *PCL1*, and the B-type cyclin *CLB6* (not shown) and *YHP1* and *YOX1* (Figure 1; Figure 2B) had significantly reduced peaks of expression after removal of *SWI4* but were unaffected by removal of *MBP1*. Only one gene tested, *CDC45*, was found to have peak expression reduced significantly by a single deletion of *MBP1* but to be unaffected by deletion of *SWI4*.

Interestingly, the genes tested exhibited varying degrees of residual control in the *swi4 mbp1* double mutant. In Figure 2B, we indicate semi-arbitrary "normal," "reduced," and "off" cutoffs of >50%, <50%, and <25% of wild type as visual aids; 10 genes are in the off class, 8 are in the reduced class, and 5 are in the normal class. The G1/S-regulated genes in the normal class, *PLM2*, *EXG1*, *DUN1*, and *ERP3*, clearly demonstrate the existence of functional G1/S cell-cycle control by other factors beyond Mbp1 and Swi4, and a similar conclusion can be reached from the broad class of genes showing reduced but still clearly detectable regulation in the *swi4 mbp1* double mutant (*CLB2*, though within the normal class, is a unique case; see results hereafter and discussion).

While the quantitative measure we chose to characterize expression was peak/trough ratio, for many genes there was an apparent delay in peak expression due to removal of *SWI4* or *MBP1* (see Figure 1 for examples). Since these differences are almost all single 15-min timepoint differences in these time courses, we cannot accurately quantitate the actual peak displacement, but we note that these differences suggest a degree of independent function of Swi4 and Mbp1 at many promoters, at least at early times during the induction.

Mbp1 and Swi4 can act as transcriptional repressors at some promoters: Three genes showed increases in peak expression following removal of Mbp1: *CLB2*, *SPT21*, (each about threefold elevated) and *CWP1*, showing a remarkable eightfold increase in peak expression. As shown in Figure 2A, the peak:trough ratio of *CWP1* measured in the wild-type control is reproducibly and significantly lower than that of all other genes tested and this fact contributes to the eightfold increase in peak expression of *CWP1* in the *mbp1* strain. In all cases, an essentially normal pattern of cell-cycle regulation of these genes was observed; the main difference was the sharply increased peak value. This result suggests that Mbp1 is acting as a negative regulator of expression

expression of the two control genes (*SIC1* and *SWI5*) are shown on the left. (C) The number of MCBs (ACGCG) and SCBs (CRCGAAA) in the intergenic region upstream from each gene is shown. For divergently transcribed genes sharing the same upstream intergenic region, any sites are listed for both genes. The numbers of sites in the promoter regions of the two controls (*SIC1* and *SWI5*) are shown on the left. (D) For each gene, the binding status of MBF and SBF is shown for three genome-wide binding data sets: study A (Iyer *et al.* 2001), study B (Simon *et al.* 2001), and study C (Harbison *et al.* 2004). M (blue) indicates only MBF binding, S (red) indicates only SBF binding, B (green) indicates both MBF and SBF binding, and N (black) indicates neither MBF nor SBF binding. Also shown (bottom row) for each gene is the presence of MBF binding sites and/or SBF binding sites that are conserved across *sensu strictu* Saccharomyces species according to one data set (Harbison *et al.* 2004). For this row, M (blue) indicates a conserved MBF binding site, S (red) indicates a conserved SBF binding site, B (green) indicates conserved MBF and SBF binding sites, and N (black) indicates neither conserved MBF nor conserved SBF binding sites. The two control genes (*SIC1* and *SWI5*) are shown on the left.

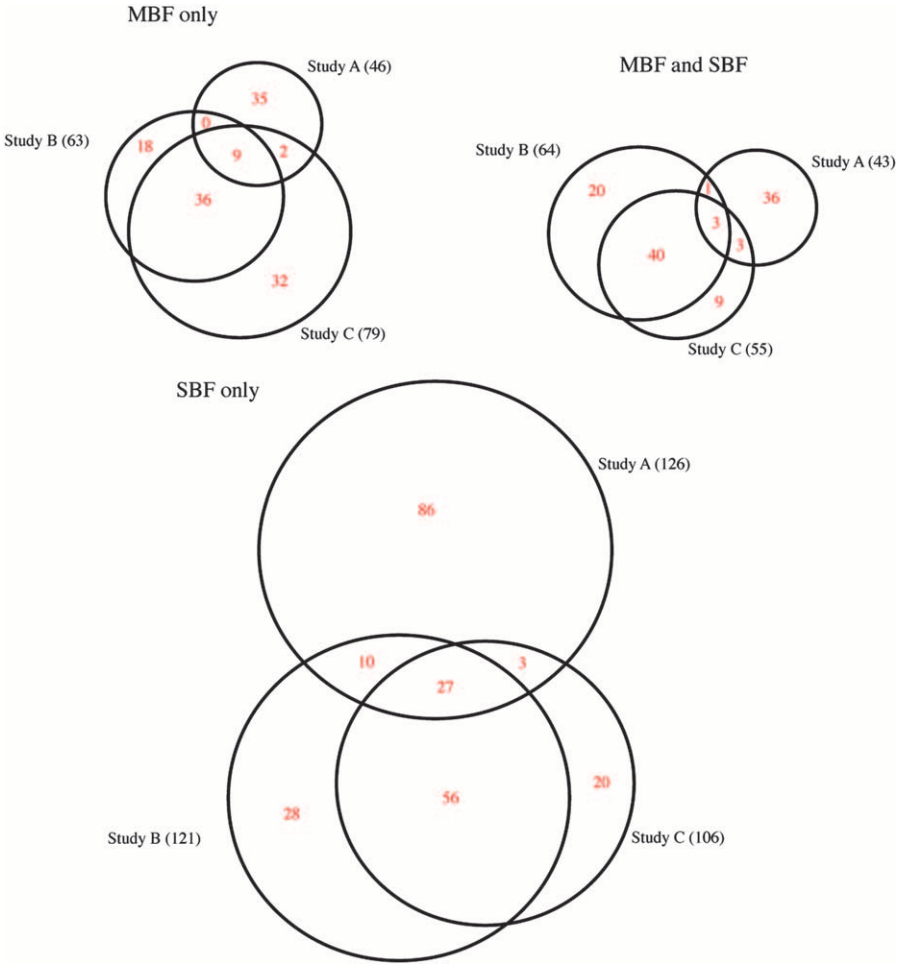

FIGURE 3.—Venn diagrams of the overlap of MBF and SBF binding in three genome-wide binding data sets. Venn diagrams depicting the overlap of MBF-only bound, SBF-only bound, or MBF- and SBF-bound genes in study A (IYER *et al.* 2001), study B (SIMON *et al.* 2001), and study C (HARBISON *et al.* 2004) are shown. All the circles representing the size of each of the nine sets (three data sets × three binding states) are drawn to scale; however, due to spatial constraints the overlaps between sets may not be to scale. The number given in parentheses next to each data set name for each binding state is the total number of genes with that binding state found in that data set. The numbers listed in red indicate the number of genes in that particular space. For example, in the MBF-only binding state, 35 (of 46) genes were found by study B (SIMON *et al.* 2001) that were not found by either of the other two data sets and 2 genes were found by study A (IYER *et al.* 2001) and study C (HARBISON *et al.* 2004) but not by study B (SIMON *et al.* 2001).

of these genes specifically at the normal peak expression time. Previously, Mbp1 was proposed to inhibit expression of its target genes during times when the genes were turned off (KOCH *et al.* 1993). Our observations here suggest in addition that Mbp1 can also be an inhibitor during the G1/S interval, when it is thought to be activated for expression of its canonical targets (KOCH and NASMYTH 1994). The three genes exhibiting elevated expression in response to *MBP1* deletion differ in their response to removal of Swi4, with *CLB2* showing very strong Swi4 dependence compared to *SPT21* and *CWP1*. Perhaps related to this, Clb2 but not the other two shows a paradoxical increase in expression in the *swi4 mbp1* double mutant compared to that in the *swi4* single mutant (see DISCUSSION).

PLM2 peak/trough ratio was increased in the absence of either Swi4 or Mbp1 and was more strongly increased in the absence of both factors, suggesting that Swi4 and Mbp1 are jointly decreasing peak *PLM2* expression. *ERP3*, *ELO1*, *TOS4*, and *MSB2* all exhibit increases in expression in the absence of Swi4 and/or Mbp1 (Figure 2B), suggesting varying patterns of repressive or activating effects of the two factors at these promoters.

Previous work showed derepression of some G1/S-regulated gene expression (*TMP1*, *POL1*, and *CLB5*) in *mbp1* G1 cells (KOCH *et al.* 1993). Under the synchronization conditions of this study, we do not see derepression of *TMP* or *CLB5* in G1 cells (Figure 1 and data not shown).

Site locations in promoters and published promoter occupancy data do not explain the pattern of Swi4/Mbp1 requirements: Three genome-wide location analyses reported the binding of MBF and SBF across all yeast promoters (IYER *et al.* 2001; SIMON *et al.* 2001; HARBISON *et al.* 2004, respectively). We attempted to use these genome-wide binding data sets to explain the results of our Northern blot analyses, with limited success. Out of the 23 G1/S-regulated genes and two control genes we assayed, 18 were assigned the same binding patterns in all three data sets (Figure 2D). Since the list of genes we assayed by Northern blot was heavily weighted in favor of genes that shared Mbp1 and Swi4 binding by the genome-wide binding data sets, the extent of shared binding in this list cannot be used to assess the overall similarity of these data sets (see Figure 3). Even among those genes with the same binding pattern in the three genome-wide binding data sets, there is little predictive power for the effect of deletion of either Mbp1 or Swi4 or for the presence or absence of a consensus binding site.

CLB5 is an example of the failure of the consensus motifs and binding data to predict function. Two genome-wide location analyses (Simon *et al.* 2001; Harbison *et al.* 2004) found that the intergenic region upstream of *CLB5* is bound by MBF but not by SBF whereas a third study (Iyer *et al.* 2001) found neither MBF nor SBF binding to the *CLB5* promoter. Northern blot analysis of yeast strains deleted for *mbp1*, *swi4*, or *mbp1 swi4* shows that expression of *CLB5* transcript is not affected by deletion of *mbp1* or *swi4* alone but is completely abrogated by a double deletion of *mbp1 swi4* (Figure 1; Figure 2B). This suggests that in the absence of MBF, SBF is able to regulate *CLB5* transcription normally despite the absence of any canonical SCB. The *CLB5* promoter contains multiple MCBs but no canonical SCBs (Figure 2C). The regulatory element that SBF is binding in the *CLB5* promoter could be the MCBs or an unknown site (for example, a highly degenerate SCB) or SBF could be acting through indirect regulation (Horak *et al.* 2002).

As an additional example, evidence from all three genome-wide location analyses (Iyer *et al.* 2001; Simon *et al.* 2001; Harbison *et al.* 2004) suggests that *RAD27*, a gene that encodes a protein involved in DNA replication or repair, is bound by MBF and not by SBF. Its promoter contains several MCBs and one SCB. Northern blot analysis of *mbp1*, *swi4*, and *mbp1 swi4* strains shows that *RAD27* expression is not perturbed by either *mbp1* or *swi4* single deletions but is strongly affected by the *mbp1 swi4* double deletion (Figure 2B). This result suggests that *RAD27*, like *CLB5*, can be regulated by either MBF or SBF.

This difference in Mbp1 and Swi4 binding among the G1/S-regulated genes we assayed underestimates the extent of the difference between the data sets across the entire yeast genome, as we used overlap among the binding data sets in initial selection of the set of G1/S-regulated genes used in our Northern blot analysis. Across the genome, the three data sets shared 11–20% of MBF-only targets, 21–25% of SBF-only targets, and 5–7% of MBF + SBF targets (Figure 3). The evident high variability among the data sets may provide the simplest explanation for why these data sets were not highly predictive of which genes would be specifically *vs.* redundantly controlled by Swi4 or Mbp1.

We note further that simple inspection of the promoter sequences for canonical SCB or MCB sites was similarly only poorly predictive of the factor(s) required for appropriate gene regulation (Figure 2; some specific examples discussed above). In this site-counting measurement, we used only a single conventional definition of the SCB or MCB consensus; while other site definitions will naturally give different numbers of sites, no alternative definitions that we have tried (in an *ad hoc*, nonexhaustive search) have given a clearly better fit to the data.

Chromatin immunoprecipitation of Mbp1 and Swi4 shows dual binding of promoters: Our data suggest that Mbp1 and Swi4 overlap functionally in the control of many genes, with little correlation to the presence or absence of recognizable SCB/MCB sites in the promoters of these genes. This could be explained by the idea of cross-binding of Swi4 and Mbp1 to MCBs or SCBs, respectively. Consistent with this idea, it was proposed that Swi4 regulates *CLN1* by binding to MCB elements in the *CLN1* promoter (Partridge *et al.* 1997). Alternatively, the effects of Swi4 or Mbp1 on regulation of some of these genes could be indirect, since other transcription factors are potential Swi4 targets (Horak *et al.* 2002). To begin to distinguish between these possibilities, we used ChIP analysis to examine the binding of Swi4 or Mbp1 to several promoters (Figure 4). We tested the specificity of binding by three criteria. First, we compared the amount of promoter-specific PCR product from anti-Myc immunoprecipitated enriched pools of DNA (IPs) from tagged *vs.* untagged strains and observed significantly higher amounts of product from the tagged strains (Figure 4, A and B). Second, we carried out the PCR reactions with inclusion of both promoter-specific oligonucleotides and oligonucleotides for amplifying a fragment of *URA3*, as a negative control (Figure 4B). No specific enrichment of *URA3* amplification in the IPs from the Swi4-tagged strain could be observed. Slight enrichment of *URA3* amplification was detected in the IPs from the Mbp1-tagged strain in a few reactions (Figure 4B). Third, we tested the IPs for enrichment of a fragment overlapping the *CLN3* promoter and coding sequence (Figure 4A). The *CLN3* promoter possesses no canonical MCBs or SCBs and is not a G1/S-regulated gene (Spellman *et al.* 1998; Mackay *et al.* 2001). No specific binding of Swi4 to the *CLN3* fragment was detected; however, there was variable enrichment of *CLN3* in IPs from the Mbp1-tagged strain. Thus, it appears that while Swi4 binding to all three G1/S promoters tested is specific, background apparently derived from nonspecific Mbp1-DNA interactions means that the data are suggestive but not conclusive of specific Mbp1 binding to the G1/S promoters. (We speculate that some "stickiness" of Mbp1 for nonspecific DNA could account for some of the variation in results in the genome-wide ChIP-chip experiments discussed above).

The *CLN2* promoter shows specific binding of Swi4 and probably of Mbp1 (Figure 4). *CLN2* transcription is at least partially Swi4 dependent (see Introduction; our data not shown). This promoter contains several MCBs and SCBs (Stuart and Wittenberg 1994), so Swi4 and Mbp1 could be binding to their cognate SCB/MCB sites or could occur through cross-binding. The finding that both Mbp1 and Swi4 are bound to the *CLN2* promoter is in keeping with a study that showed a decreased level of dimethylsulfate protection of the *CLN2* promoter in a strain lacking both Mbp1 and Swi4 compared to one lacking Swi4 alone (Koch *et al.* 1996).

TOS4 is under dual regulation by Mbp1 and Swi4 (Figure 2). Paradoxically, its promoter possesses neither

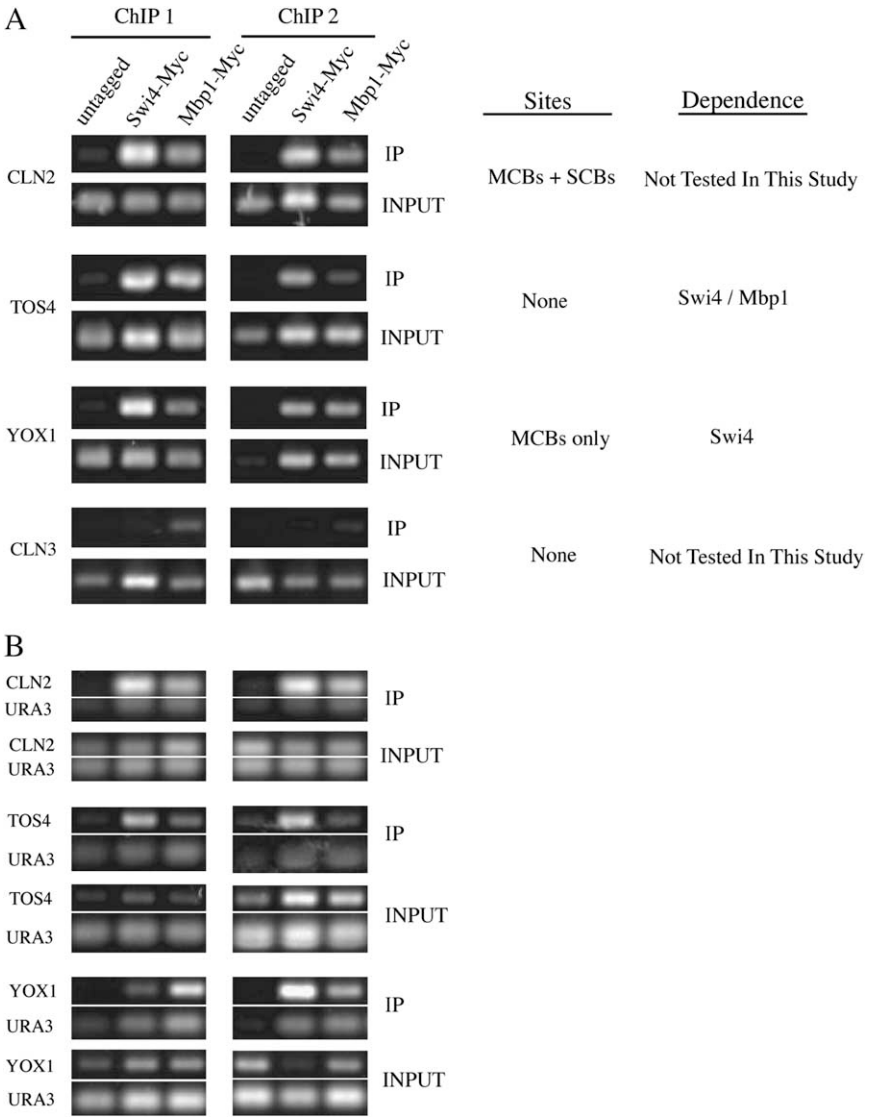

FIGURE 4.—Chromatin immunoprecipitations using tagged Swi4 and Mbp1. The results from duplicate experiments are shown. For each promoter tested, the amount of signal from an untagged control (2147-7C), a Swi4-Myc-tagged strain (JB05-1B), and a Mbp1-Myc-tagged strain (JB06-1A) is shown. All strains were *cdc20::LEU2 GALL-CDC20* and were blocked and released for 40 min before harvesting, to enrich cells at the peak time of expression of G1/S-regulated genes. "IP" indicates the signal from the anti-Myc immunoprecipitated enriched pool of DNA. "INPUT" indicates the signal from the whole-cell extracted DNA. For all ChIPs the bands shown are unsaturated and within the linear range of pixel intensities. (A) ChIPs of three G1/S-regulated genes as well as *CLN3* used as a control are shown. Also indicated is the presence or absence of MCBs or SCBs in the promoters of the genes tested. Here MCB is defined as ACGCG and SCB as CRCGAAA. Also shown is each gene's dependence on either Mbp1 or Swi4 for normal regulation as determined by Northern blot analysis (see Figure 2). "Swi4/Mbp1" means that removal of both is required to ablate regulation; "Swi4" means that removal of Swi4 is sufficient to ablate control. (B) ChIPs using multiplex PCR with oligos for three G1/S-regulated genes along with oligos for *URA3* used as a nonspecific control are shown. Regulation of *CLN2* was not fully determined in this study, since we used ectopic *CLN2* expression to rescue the *mbp1 swi4* strain, precluding analysis of the endogenous gene by Northern blot. Previous data suggest that *CLN2* is under partial control of Swi4, consistent with a 70% reduction in peak:trough ratio upon *SWI4* deletion in *CDC20*-synchronized cells in our protocol (data not shown), and also may be under partial control of Mbp1 (KOCH *et al.* 1993; KOCH and NASMYTH 1994; STUART and WITTENBERG 1994).

MCBs nor SCBs on the basis of simple counting of consensus sites; despite this, we find specific binding of Swi4, and evidence suggestive of specific binding of Mbp1, to this promoter (Figure 4). *YOX1* is Swi4 dependent (Figure 2) despite the absence of canonical SCBs from its promoter. We find specific binding of both Swi4 and probably Mbp1 to this promoter (Figure 4). It is possible that Swi4 is binding to the MCBs present in the *YOX1* promoter and regulating expression in a manner analogous to Swi4 regulating *CLN1* expression through MCBs in its promoter (PARTRIDGE *et al.* 1997). The *YOX1* homolog *YHP1* is similar to *YOX1* in that it contains only canonical MCBs in its promoter but is controlled by Swi4 (Figure 2), and this promoter may show Swi4 and Mbp1 binding similar to *YOX1* (preliminary data not shown).

In all three cases, *CLN2*, *TOS4*, and *YOX1*, the binding of Swi4 is not strikingly affected by the removal of Mbp1 and vice versa (data not shown). However, we note that the ChIP assay as we have carried it out is not a quantitative assay. Therefore, the question of quantitative regulation of the degree of Swi4 or Mbp1 binding to these promoters by the presence or absence of the other factor remains for future work.

Despite the caveats from the nonquantitative nature of these ChIP studies and the background binding problem for Mbp1, these results confirm the ability of Swi4 and probably Mbp1 to bind to promoters where simple sequence inspection makes it difficult to explain their binding. These findings could explain the functional overlap of Swi4 and Mbp1 in transcriptional regulation of these genes.

TABLE 2

Mode cell volume and percentage unbudded in *mbp1* and control yeast strains

Genotype	Mode cell volume (fl)	% unbudded
MBP1	46.7 ± 1.85	27.4 ± 1.65
mbp1	55.7 ± 0.71	22.2 ± 0.87

Genotype *MBP1* is the average of the mode cell volumes of strains HTLU-14A, HTLU-2B, 2819-12C, and 2819-6C. Genotype *mbp1* is the average of the mode cell volumes of strains 2891-5C, 2891-13B, 2891-4A, and 2891-11C. Each individual strain mode cell volume was determined from an average of triplicate Coulter counts. Standard deviations for the average mode cell volumes and percentage unbudded are shown.

Evidence for function of Mbp1 in a *SWI4* background: The absence of *SWI4* causes a notable phenotype, including slow growth, problems with morphogenesis, and large cell size (*e.g.*, Ogas *et al.* 1991; Jorgensen *et al.* 2002; Igual *et al.* 1996). In contrast, little or no phenotypic consequence to deletion of *MBP1* has ever been described to our knowledge. Perhaps consistent with this, of all the genes we tested, only *CDC45* was found to be specifically Mbp1 dependent for its normal transcription. This could suggest the hypothesis that Mbp1 essentially functions solely in a backup pathway for Swi4. Since cell size is a sensitive indicator of cell-cycle progression overall (Jorgensen *et al.* 2002) we tested whether removal of Mbp1 altered cell size. We found a significant cell size phenotype for *mbp1* strains (otherwise wild type) during exponential growth (Table 2). Loss of Mbp1 leads to a 20% increase in modal cell volume compared to that in controls. This volume increase is associated with a 5% increase in the proportion of budded cells, suggesting a possible delay in the replicative (budded) part of the cell cycle.

As noted above, many of the dually Swi4/Mbp1-regulated genes showed evidence for slight delays in peak expression in both the *mbp1* and *swi4* single mutants. The transcriptional delay in the *mbp1* single mutants could be consistent with the moderate overall cell-cycle delay suggested by increased cell size and proportion of unbudded cells in the mutant (Table 2).

DISCUSSION

Do Mbp1 and Swi4 overlap in function? This study was motivated by the clear importance of SBF and MBF for G1/S transcriptional regulation, combined with a number of observations showing puzzlingly small effects of deletion of the DNA-binding components of these factors on regulation of their putative targets (see Introduction). The standard view of SBF and MBF as direct and exclusive regulators of SCB-containing promoters and MCB-containing promoters, respectively, was clearly inadequate. The simple prediction that these factors might overlap in function at some promoters has been made (first by Koch *et al.* 1993), but was never tested directly by simultaneous deletion of the factors, due to inviability of the *swi4 mbp1* double mutant. We exploited the observation that the *swi4 mbp1* double mutant was viable upon ectopic expression of the G1 cyclin *CLN2* (Koch *et al.* 1993) to directly test overlap of transcriptional regulation by SBF and MBF through the cell cycle.

Our results simplify understanding of G1/S-regulated expression in yeast: Swi4 and Mbp1 are substantially redundant with each other for control of the majority (19/23) of the G1/S-regulated genes that we tested (Figure 2). Only four genes [*YOX1*, *YHP1*, *CDC45* (Figure 2B), and *PCL1* (data not shown)] show specific Swi4 or Mbp1 dependence and lack of dependence on the other factor.

Swi4- and Mbp1-independent G1/S regulation: Our data show clearly that G1/S activators of transcription that are independent of Swi4 and Mbp1 must exist. *PLM2*, *DUN1*, *EXG1*, and *ERP3* are cell-cycle regulated at least as well in the absence as in the presence of Mbp1 and Swi4 (Figure 2B). We note in addition that many of the genes that we tested show reproducible (although significantly reduced) regulation of expression in the absence of Mbp1 and Swi4 (Figure 2B), suggesting Swi4- and Mbp1-independent regulation of G1/S accumulation of many transcripts, by an unknown mechanism.

Mbp1 and Swi4 as repressors: A number of genes (*SPT21*, *CWP1*, *CLB2*, *PLM2*, *ERP3*, *ELO1*, *TOS4*, and *MSB2*) show increased peak expression in the absence of Swi4 and/or Mbp1 in various patterns, suggesting that Swi4 and/or Mbp1 may act as repressors for many genes. As noted above, this phenomenon is distinct from the previously characterized repressive effect of Mbp1 on some of its targets during G1, when the targets are normally not expressed (Koch *et al.* 1993).

CLB2 is a unique case in that its peak expression level is higher in a double *mbp1 swi4* mutant than in the single *swi4* mutant, in which expression is greatly reduced (Figure 2B). The Swi4 dependence of *CLB2* expression, combined with the ability of Clb2 to antagonize Swi4-dependent gene expression (Amon *et al.* 1993), suggests the possibility of a negative feedback loop, whereby Swi4 would activate *CLB2*, and Clb2 would subsequently inactivate Swi4. This would prevent *CLB2* expression from activating prematurely and then preventing Swi4-dependent expression from occurring at all. Swi4 dependence of *CLB2* expression may be due to the repressive effects of Mbp1 on *CLB2* transcription. Swi4 does bind to the *CLB2* promoter by ChIP (in both the published genome-wide studies and in our unpublished data); we lack clear data as to whether Mbp1 binds the *CLB2* promoter (data not shown). The near-normal cell-cycle regulation of *CLB2* in the *mbp1 swi4* double mutant (Figure 1) is presumably due to the previously

characterized Ndd1/Mcm1/Fkh1,2 regulators (Lydall *et al.* 1991; Althoefer *et al.* 1995; Koranda *et al.* 2000; Zhu *et al.* 2000; Reynolds *et al.* 2003).

A remaining puzzle for specific site recognition: For the large class of genes for which Swi4 and Mbp1 overlap in function, the mechanistic basis for the overlap is still unclear. The simplest explanation, which doubtless applies in some cases, is independent binding of Swi4 and Mbp1 to canonical binding sequences in some promoters (as may be the case for *CLN2*; Stuart and Wittenberg 1994). Another possibility is cross-binding of Swi4 and Mpb1 to canonical sequences for the other factor. There is biochemically detectable cross-binding of Swi4 to MCBs and of Mbp1 to SCBs (Dirick *et al.* 1992; Primig *et al.* 1992; Partridge *et al.* 1997; Taylor *et al.* 2000). Expression from simple SCB-element reporter plasmids is specifically dependent on Swi4 (Andrews and Herskowitz 1989a); thus, cross-binding of Mbp1 to SCBs may not be an adequate explanation in general. (The reciprocal measurement of the degree of exclusive dependence on Mbp1 for expression of MCB element reporter expression has not been reported to our knowledge.)

It is also possible that many promoters contain noncanonical binding sequences for Swi4 and/or Mbp1. The canonical sequences for binding Mbp1 or Swi4, ACGCG and CRCGAAA, respectively, are not very similar by inspection beyond the CRCG core (although the proteins use a different method of inspection than the human eye does!) In addition, both of these are rather short sequences, which occur many times in the genome presumably without effectively regulating transcription; it seems likely that any proposed hybrid sequence that could be recognized by both factors would have even less information content. This leaves us with little understanding of how these sequence-specific binding factors bind effectively to the "right" sites and not the "wrong" sites. Binding of other factors to SBF/MBF-regulated promoters may provide an environment that might encourage Swi4 or Mbp1 binding even to apparently nonconsensus sites.

The technique of measuring genome-wide promoter occupancy mapping by "Chip-ChIP" methods is a powerful and very useful method, and initially we expected that it would help to resolve many of these mysteries. Unfortunately, in the present specific experimental context we have not found the available public data sets to be useful in helping us to predict what genes will be regulated by Swi4 or Mbp1. It is also notable that multiple carefully performed studies using very similar reagents and procedures give binding occupancy data that are substantially nonoverlapping (Figure 3). We do not have any concrete suggestions to make here, but wish only to provide a caveat for the use of these very valuable data sets.

Conclusions: Gene duplication followed by partial divergence of function is a well-known theme in evolution and is frequently observed in transcription factors. This process provides fascinating challenges with respect to coevolution of binding specificities and binding sites and either divergence or conservation of functions of the related factors. Swi4 and Mbp1 present an excellent case study for working out some of these issues. Here we have shown that these factors have substantially conserved the ability to regulate the majority of their target genes. We document an apparent reversal of specificity in the case of several targets, including the main mitotic cyclin *CLB2*, for which Swi4 appears to be an activator and Mbp1 a repressor. Last, we pose a paradox with respect to specificity of DNA binding and promoter occupancy.

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