Deconvolution of Chromatin Immunoprecipitation-Microarray (ChIP-chip) Analysis of MBF Occupancies Reveals the Temporal Recruitment of Rep2 at the MBF Target Genes

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MBF (or DSC1) is known to regulate transcription of a set of G1/S-phase genes encoding proteins involved in regulation of DNA replication. Previous studies have shown that MBF binds not only the promoter of G1/S-phase genes, but also the constitutive genes; however, it was unclear if the MBF bindings at the G1/S-phase and constitutive genes were mechanistically distinguishable. Here, we report a chromatin immunoprecipitation-microarray (ChIP-chip) analysis of MBF binding in the Schizosaccharomyces pombe genome using high-resolution genome tiling microarrays. ChIP-chip analysis indicates that the majority of the MBF occupancies are located at the intragenic regions. Deconvolution analysis using Rpb1 ChIP-chip results distinguishes the Cdc10 bindings at the Rpb1-poor loci (promoters) from those at the Rpb1-rich loci (intragenic sequences). Importantly, Res1 binding at the Rpb1-poor loci, but not at the Rpb1-rich loci, is dependent on the Cdc10 function, suggesting a distinct binding mechanism. Most Cdc10 promoter bindings at the Rpb1-poor loci are associated with the G1/S-phase genes. While Res1 or Res2 is found at both the Cdc10 promoter and intragenic binding sites, Rep2 appears to be absent at the Cdc10 promoter binding sites but present at the intragenic sites. Time course ChIP-chip analysis demonstrates that Rep2 is temporally accumulated at the coding region of the MBF target genes, resembling the RNAPII occupancies. Taken together, our results show that deconvolution analysis of Cdc10 occupancies refines the functional subset of genomic binding sites. We propose that the MBF activator Rep2 plays a role in mediating the cell cycle-specific transcription through the recruitment of RNAPII to the MBF-bound G1/S-phase genes.
nately bind to the MCB-like motif (55). Cdc10 has been shown to bind the promoter of cdc18 constitutively (58). Given that Cdc10 forms complexes with Res1 and Res2 throughout the cell cycle (55), it is conceivable that the core MBF complex Res1-Cdc10-Res2 is bound at the promoter of the target genes throughout the cell cycle. However, activation of MBF at the G1 phase is complex. It has been shown that MBF is activated by Cdc2 in the G1 phase (17, 18, 46). On the other hand, the MBF activity is also regulated by the CDK Pef1-Pas1 (52). It is therefore likely that MBF activity is regulated by multiple signaling pathways in S. pombe.

After G1/S-phase transcription, the MFB activity is likely to be repressed by a number of negative regulators, Cig2, Nrm1, and Yox1 (1, 2, 20). The activation of MBF requires the activator Rep2 (42). The constitutively active allele cdc10-C4 is also known to be dependent on the function of Rep2 (55), suggesting that Rep2 is absolutely essential for MFB activity. Although the role of Rep2 in the activation of MFB is unclear, we have previously shown that Rep2 protein levels oscillated during cell cycle, with a peak level at G1/S phase, consistent with its role in the activation of MFB transcriptional activity (14).

Over 200 genes targeted by SBF/MFB in the S. cerevisiae genome have been identified (28). Many SBF/MFB target genes are found to contain no apparent SCB/MCB promoter motif, suggesting that SBF/MFB can bind the SCB/MCB-less sequences, possibly through the interaction with other transcription factors. Notably, transcription of many SBF/MFB target genes is not cell cycle regulated, suggesting that SBF/MBF sequences, possibly through the interaction with other transcription factors. Notably, transcription of many SBF/MBF target genes is not cell cycle regulated, suggesting that SBF/MBF also play a role in regulation of both periodic and constitutive transcription. In S. pombe, ~80 binding targets for Cdc10 and Yox1, half of which are not periodically transcribed, have been identified (1). This raises a question of whether the MBF bindings at the promoters of the cell cycle genes are distinguishable from those at the constitutive genes.

To address this question, we performed the genome-wide binding profiling of the MFB components Cdc10, Res1, Res2, and Rep2 and the RNAP-II largest subunit Rpb1, using the maskless high-resolution (~33-bp) genome tiling DNA microarray. High-resolution chromatin immunoprecipitation-microarray (ChIP-chip) analysis revealed that the majority of the MFB occupancies are associated with the Rpb1 occupancies or the Rpb1-rich loci. Deconvolution analysis identified a subset of Cdc10 occupancies at the Rpb1-poor loci. MFB binding at the Rpb1-poor loci but not the Rpb1-rich loci requires the MFB function, indicating that the Rpb1-poor loci are targets for the MFB complex. Most of the MFB-dependent G1/S-phase genes are found to be located downstream of the Cdc10 occupancies associated with the Rpb1-poor loci. On the other hand, Cdc10 occupancies associated with the Rpb1-rich loci are found to be overlapped with many actively transcribed ribosomal protein genes. They are not the binding targets for the MFB complex but are the individual components. Significantly, the MFB activator Rep2 is nearly absent at the promoter of many MFB target genes in asynchrous log-phase growth cells. Time course ChIP-chip analysis of Rep2 indicated that levels of Rep2 accumulation at the MFB target genes oscillate along with cell cycle, with a peak level at the G1/S phase, correlating with the transcription profile of the MFB target genes. Taken together, our results demonstrate that cell cycle transcription of the MFB target genes is regulated through coordination of the static subunits Cdc10, Res1, and Res2 and the dynamic subunit Rep2.

**MATERIALS AND METHODS**

**Strains and culture manipulations.** The strains used in this study are listed in Table 1. The res1Δ-HA cdc10-V50 strain was obtained from a crossing between the res1Δ-HA and cdc10-V50 strains. The rpb1Δ-HA strain was constructed using the PCR-mediated approach described in reference 3. Log-phase growth cells bearing a chromosomal copy of hemagglutinin (HA)-tagged cdc10Δ, rep2Δ, res1Δ, res2Δ and rpb1Δ in yeast extract with supplements (YES) medium at ~30°C were subjected to treatment with formaldehyde (Sigma, St. Louis, MO) at a final concentration of 10% (vol/vol) for 10 min. Fused cells were subsequently used for ChIP-chip analysis. For determination of the Res1 occupancies in the cdc10 mutation background, res1Δ-HA cdc10-V50 cells that were grown at 36°C for 4 h were subjected to ChIP-chip analysis. For determination of the Rep2 or Rpb1 occupancies upon hydroxurea (HU) stress, rep2Δ-HA or rpb1Δ-HA cells that were treated with HU at a final concentration of 8 mM for 4 h were subjected to ChIP-chip analysis. For determination of the temporal Rep2 occupancies during cell cycle progression, rep2Δ-HA cdc25-22 cells were synchronized by a block-release protocol (15) and sampled at various time points for ChIP-chip analysis.

**Com munoprecipitation and Western blot assays.** For the testing of the physical interaction of proteins in vivo, communoprecipitated and Western blot analysis was carried out. Typically, ~400-ml cultures at an ~0.4 optical density (OD) were harvested and resuspended in 400 μl lysis buffer (50 mM HEPES [pH 7.5], 140 mM NaCl, 1 mM EDTA [pH 7.5], 1% Triton X-100, 0.1% sodium deoxycholate, 1× anti-protease cocktail). The galactose-coupled antibodies were added to lysate to enrich the target protein complexes. Antibodies and the target protein complexes were subsequently recovered by a brief spin at 5,000 rpm for 1 min at 4°C and washed with 1 ml lysis buffer and a series of washing buffers. The bead-associated proteins were subject to Western blot analysis. In Western blot analysis, primary antibodies SC-7392 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) against HA, 8WG16 (Covance, Inc., CA) against the phosphorylated Ser2 in the CTD were used at 5,000- to 2,000-fold dilutions according to the manufacturer’s instructions. Secondary antibodies against rabbit or mouse IgG coupled with horseradish peroxidase (HRP) (GE Healthcare, Piscataway, NJ) were used at an ~5,000-fold dilution for enhanced chemiluminescence (ECL) detection systems (GE Healthcare).

**ChIP.** To enrich protein-bound DNA fragments in vivo, ChIP was applied using cells bearing the HA-tagged proteins after treatment with formaldehyde. Cell lysates were prepared as for the communoprecipitation. Lysates were sonicated three times at 30% of maximal strength for 30 s (Branson Power Company, Danbury, CT) prior to ChIP. In ChIP analysis, ~20 μl preblocked agarose-coupled anti-HA antibodies was added to 200 μl lysate, and the mixture was rotated overnight at 4°C. The beads were eluted as in communoprecipitation. DNA-protein complexes in the ChIP eluents and the whole-cell extract (WCE) lysates were heated at 65°C overnight to reverse the cross-links in ChIP eluents. The resulting DNA (10 ng) was linearly amplified using the GenomePlex am-

**TABLE 1. List of strains used in this study**

| ID* | Relevant genotype | Comment |
|-----|------------------|---------|
| LJJ188 | h− leu1-32 ura4-D18 cdc10-3HA-His6::ura4+ | Lab stock |
| LJJ2031 | h− leu1-32 ura4-D18 cdc10-3HA-His6::ura4+ | Lab stock |
| LJJ2168 | h− leu1-32 ura4-D18 res1-3HA-His6::ura4+ | Lab stock |
| LJJ2112 | h− leu1-32 ura4-D18 rep2-3HA-His6::ura4+ | Lab stock |
| LJJ1902 | h− leu1-32 ura4-D18 rep2-3HA-His6::ura4+ | Lab stock |
| LJJ2753 | h− leu1-32 ura4-D18 cdc25-22 res2-3HA-His6::ura4+ | Lab stock |
| LJJ2598 | h− leu1-32 ura4-D18 rpb1-3HA-His6::ura4+ | This study |
| LJJ2167 | res1-3HA-His6::ura4+ cdc10-V50 | This study |

* ID, strain identification.
Deconvolution and assignment of major Cdc10 occupancies. Assignment of Cdc10 occupancies to the probable promoters was based on the genome annotation (34, 57). A probable promoter is defined as the upstream region up to 1 kb from the start codon or 200 bp from the transcription start site (TSS) when the 5' untranslated region (5'-UTR) is greater than 1 kb. Of 145 Cdc10 occupancies, ~50 are found to be located at the probable promoters. The remaining occupancies are located at the nonpromoter regions, such as intragenic or convergent intergenic sequences.

Deconvolution of Cdc10 binding sites (i.e., the region of five consecutive probes or ~165 bp centered at the apex of Cdc10 occupancies) was based on the r test, which assesses whether Cdc10 levels at each binding site (i.e., 10 measurements for each binding site [five consecutive probes in two repeats]) are significantly higher than the Rpb1 levels at the same site. Deconvolution analysis identified 37 out of 145 Cdc10 binding sites whose enrichment levels are significantly higher than the Rpb1 levels (P value of <0.001) (see Table S1 in the supplemental material). Of the 37 Rpb1-poor loci, 25 are located at the probable promoter, based on annotation.

Identification of overrepresented de novo motifs at the binding sites using motif discovery scan. For determining motifs that were enriched at the Cdc10 promoter binding sites, we retrieved ~150-bp sequences located at the apex (~75 bp) of 25 Cdc10 occupancies at the Rpb1-poor probable promoter regions. These binding sequences were ranked based on the level of Cdc10 enrichment. The 15 top-ranked sequences were used to detect the motif, with the width ranging from 6 bp to 9 bp, and the remaining sequences were used to refine the identified motifs. All motifs identified can be aligned with the MCB2 motif. No specific motifs were found to be enriched in the Cdc10 binding sites at the Rpb1-rich loci. On the other hand, we retrieved the upstream sequences (150 bp) of the Cdc10-bound genes at Rpb1-rich loci, we obtained homo-D-like motifs. In this analysis, 88 promoter sequences were used: the top 30 were applied for motif identification, and the remaining for refining. The top 8 motif matrices are listed in Table S6 in the supplemental material. Sequence logos were obtained based on the matrix using the statistic package R.

To test the performance of the discovered motifs, we applied receiver operating characteristic (ROC) curve analysis. Using the matrix of individual motifs, the position-specific scoring matrix (PSSM) score is calculated based on the formula PSSM = \( \sum_{i} \log p_{i}/\sum_{j} p_{j} \) where \( p_{i} \) is the probability of the letter \( j \) at the \( i \)-th position in the motif and \( f_{j} \) is the background frequency of the letter \( j \). The Cdc10 promoter binding sites or the promoter of the Cdc10 intragenic binding sites was set as the true discovery, and the background was the randomly selected promoter sequences (i.e., 3,600 randomly selected 150-bp promoter sequences). The non-specific (or randomly bound) motif would display a curve at the upper-left of the diagonal, showing a true discovery rate and low false-positive rate.

Determination of average profiles of Rep2, Rpb1, and Res1 accumulation at the genes whose promoters are bound by Cdc10 in HU-treated or synchronized cells. The putative transcription unit (i.e., from the Cdc10 binding site to the stop codon of the open reading frame [ORF]) of the 34 Cdc10-bound genes was defined. Rep2, Rpb1 and Res1 accumulation at the putative transcription unit (i.e., from the Cdc10 binding site to the stop codon of the ORF) at various time points (see Table S7 in the supplemental material) are available in the GEO database under the accession number GSE9912 (http://www.ncbi.nlm.nih.gov/projects/geo/) and at the authors' website (http://pombe.gis.a-star.edu.sg).

RESULTS

Cdc10 occupancies are found at both the intergenic and intragenic regions in the genome. The core MFB complex consists of Cdc10, Res1, and Res2, of which Cdc10 is an indis-
ppensable component (7). To identify the genomic binding sites for the MBF complex, we first determined the binding sites for Cdc10 in cells bearing a sole chromosomal copy of the cdc10-HA allele by the use of the maskless high-resolution (~33-bp) genome tiling 50-mer oligonucleotide microarray. Standard protocol for ChIP-chip analysis was employed (see Materials and Methods). The level of fold enrichment (or binding affinity) was calculated based on the ratio between signal intensities derived from the chromatin-enriched (ChIP) DNA and whole-cell extract (WCE) DNA. ChIP-chip experiments were independently repeated for reproducibility, and all ChIP-chip datasets used in this study were LOWESS (59) and quantile normalized (5) for comparison between ChIP-chip experiments (see Materials and Methods). The enrichment level was calculated as the fold increase in comparison with the array median level, which was set to 1 (or 0 in log scale). Using the occupancy identification algorithms (see Materials and Methods), we identified ~217 Cdc10 occupancies in each of the two ChIP-chip experiments, the majority (145 occupancies) of which were found to be overlapped in two repeated experiments (Fig. 1A; see also Table S1 in the supplemental material). Notably, the overlapping occupancies exhibited a higher enrichment level than that of the nonoverlapping ones (1.62 versus 1.41; P value of <4.74e−13), suggesting that the overlapping occupancies represent the major binding sites for Cdc10 in the genome. Hence, we focused on the analysis of the major or overlapping Cdc10 occupancies, referred to herein as Cdc10 occupancies.

The MBF complex is known to bind the promoter MCB (MluI-like cell cycle box)-like motifs and regulates transcription of a set of MBF-dependent G1/S-phase genes (10, 36, 40, 42, 51, 54, 55, 60). Based on genome annotations (34, 57), we found that only approximately one-third (50) of the 145 Cdc10 binding sites were located at the probable promoters (see Materials and Methods). The remaining two-thirds of the Cdc10 occupancies were located at the nonpromoter regions, such as convergent intergenic regions or intragenic regions (see Fig. S1 in the supplemental material). We hypothesized that intragenic Cdc10 occupancies originated from the interaction with the RNAP-II complex, because most of the intragenic Cdc10 occupancies covered the entire coding regions, resembling those of RNAP (see below). To test if Cdc10 intragenic binding was a result of physical interaction with the RNAP complex, we performed coimmunoprecipitation assays between Cdc10 and Rpb1. By using the anti-Rpb1 antibody 8WG16, Cdc10 was detected in the Rpb1-coimmunoprecipitated protein complexes (Fig. 1B). This physical association was also apparent in a reciprocal coimmunoprecipitation test (Fig. 1C). We therefore concluded that the intragenic Cdc10 occupancies were a result of association with RNAP, though the function remains to be elucidated.

If the intragenic Cdc10 occupancies were originated through the association with RNAP-II, these intragenic occupancies would be superimposable with those of RNAP-II. To test this possibility, we performed ChIP-chip analysis to determine the Rpb1 occupancies for comparison between Cdc10 and Rpb1 levels at the Cdc10 binding sites. Clearly, high-level similarity of occupancy profiles between Cdc10 and Rpb1 was observed at the intragenic binding sites (Fig. 1D, see binding sites 73 and 75; for a complete set of Cdc10 binding sites, see Fig. S1 in the supplemental material). Surprisingly, about half of the Cdc10 annotation-based promoter bindings were also overlapped/superimposed with the Rpb1 occupancies, suggesting that not all Cdc10 annotation-based promoter bindings are associated with the bona fide promoters (Fig. 1D, see binding sites 69 and 76). This was because many of the Cdc10 annotation-based pro-
motif bindings were a result of Rpb1 occupancies at the 3′-end of the upstream gene.

**Deconvolution analysis reveals a subset of Cdc10 promoter binding sites at the Rpb1-poor loci.** To improve the prediction of promoter binding sites, we employed the deconvolution analysis to separate Cdc10 binding sites at the Rpb1-poor loci from those at the Rpb1-rich loci. This was based on the assumption that the level of Rpb1 occupancies at the promoter region should be relatively low. In the deconvolution analysis, we employed the t test to identify promoter loci at which the level of Cdc10 occupancies was significantly higher than that of the Rpb1 occupancies (see Materials and Methods). To this end, we found that the Cdc10 loci at 108 (~74%) out of 145 Cdc10-bound loci were not any higher than the levels of Rpb1 occupancies (see Table S1 in the supplemental material). Cdc10 loci at these loci were found to be correlated with the Rpb1 levels (correlation coefficient = 0.57; P value = 1.3e−10), supporting the idea that these occupancies are a result of association with RNAP-II (Fig. 2A, gray dots). The levels of Rpb1 occupancies at these loci were relatively high (median Rpb1 level = 1.49 in log2 scale) (Fig. 2B). Therefore, these loci were designated Rpb1-rich loci. On the other hand, the level of the Cdc10 occupancies at the 37 (~26%) Cdc10-bound loci was found to be significantly higher than the level of Rpb1 occupancies (P < 0.001) (Fig. 2A, red dots). No apparent correlation between Cdc10 and Rpb1 levels was observed at these loci. Clearly, the Rpb1 levels at these loci were very low or nearly depleted (median level = −0.15 in log2 scale, below the array median). Thus, these loci were designated Rpb1-poor loci.

After deconvolution, half of the 50 Cdc10 annotation-based promoter binding sites were found to be associated with Rpb1-rich loci (see Table S1 in the supplemental material). None of these loci were found to be associated with the promoter of the MBF-dependent G1/S-phase genes. Significantly, most of the G1/S-phase genes were found to be associated with the remaining 25 Cdc10 binding sites at the Rpb1-poor loci. These Cdc10 binding sites at the Rpb1-poor loci were subsequently assigned to 34 downstream genes, most of which were cell cycle-transcribed genes (Fig. 2C; Table 2). To examine if genes whose coding sequences were bound by Cdc10 (at the Rpb1-rich loci) were transcriptionally regulated by Cdc10, we assigned these Cdc10 binding sites to 88 genes based on the coding sequences that were overlapped with the boundaries of Cdc10 occupancies (see Materials and Methods) (see Fig. S9 and Table S2 in the supplemental material). Many of the intragenic Cdc10 binding sites were found to be associated with the actively transcribed ribosomal protein genes (RPGs).

**MCB2-like motifs are enriched at the Cdc10 promoter binding sites but not at the intragenic binding sites.** It has been shown that the core MBF complex binds to the MCB-like motifs at the promoter of a number of the MBF-dependent G1/S-phase genes (10, 36, 40, 42, 51, 54, 55, 60). To identify the motifs that were preferentially bound by the MBF at the promoter, we performed the motif discovery scan (35) to identify the de novo motifs that were significantly enriched in the MBF binding sites. DNA sequences of ~150 bp in length at each promoter binding site were used in the motif discovery scan (see Materials and Methods). To this end, we found 8 motifs whose information content or relative entropy (a measurement of binding affinity) was greater than 1 (Fig. 3A). All of the 8 motifs whose width ranged from 6 to 9 nucleotides were found to be related to a motif known as MCB2 (19, 38, 47). We therefore designated these motifs MCB2-like motifs. It was clear that the genomic performances of all MCB2-like motifs were very similar (Fig. 3B), suggesting that MBF prefers to bind MCB2 or MCB2-like promoter motifs in the genome.

On the other hand, we found that the MBF intragenic binding sites enriched no apparent motifs (data not shown), consistent with our idea that these intragenic occupancies are not associated with the MCB-like motifs. We hypothesized that the MBF intragenic binding was a result of physical interaction between MBF components and an unknown factor(s) that regulates the transcription of the RPGs. If this were the case, we would expect that the unknown transcriptional regulator(s) has the unique binding motifs at the promoter of genes whose coding sequences were bound by Cdc10. To test this possibility, we collected DNA sequences at the promoter rather than those underlying the MBF intragenic binding sites for a motif discovery scan (35). The analysis showed that the top 8 motifs whose width ranged from 6 to 9 nucleotides overlapped one another and resembled a motif known as homo-D (Fig. 3C). The homo-D motif has been shown to be associated with the promoter of many RPGs in S. pombe (53). Our analysis indicated that the enriched homo-D motif was specific to the
promoter of genes whose coding sequences were bound by Cdc10 (Fig. 3D). Hence, we concluded that MCB2-like motifs were the preferred promoter binding sites for MBF. On the other hand, MBF may also bind to the intragenic sequences through the interaction with the homo-D-binding factor(s).

**Res1 and Res2 occupancies are colocalized with Cdc10 occupancies at both the Rpb1-poor and Rpb1-rich loci.** Cdc10 is known to form the core MBF complex with Res1 and Res2 at the promoter of the MBF-dependent $G_1/S$-phase genes (10, 36, 40, 42, 51, 54, 55, 60). We therefore investigated if Res1 or Res2 was colocalized with both the promoter (Rpb1-poor) and intragenic (Rpb1-rich) Cdc10 binding sites. For this reason, we performed ChIP-chip analysis to determine Res1 and Res2 occupancies by the use of cells containing a chromosomal copy of the res1-HA or res2-HA allele in two independently repeated experiments. The ChIP-chip results indicated that the majority (>73%) of the Cdc10 occupancies were overapped with the Res1 or Res2 occupancies (occupancies identified in at least one of the 2 ChIP-chip experiments) (Fig. 4A and B). Over 55% of the Cdc10 occupancies at the Rpb1-poor loci and ~80% of the occupancies at the Rpb1-poor loci were found to be colocalized with the Res1 or Res2 occupancies. These results indicate that the core MBF components bind to both the Rpb1-poor promoters and the Rpb1-rich intragenic regions.

The levels of Res1 or Res2 at the Rpb1-rich loci were found to be correlated with the Rpb1 level, suggesting that the Res1 or Res2 bindings at the intragenic regions resulted from the association with RNAP (Fig. 4C and D). We noted that the level of Res1 or Res2 occupancies at the Rpb1-rich loci was similar to that of Cdc10 occupancies (median level of 1.49 or 1.48 in log$_2$ scale). Assuming that the binding concentrations of Cdc10, Res1, and Res2 at the Rpb1-poor loci was lower than that of Cdc10. This result supports the

### TABLE 2. List of the MBF target genes

| Gene no. | ID$^b$ | Cdc10 level$^c$ | Gene | Reference$^b$ | Phase$^c$ | Cdc10-Yox1$^d$ | Product$^e$ |
|---------|-------|----------------|------|--------------|----------|---------------|-------------|
| 1       | ns68  | 3.18           | cdc22| ABC          | G$_1$    | +             | Ribonucleoside reductase |
| 2       | ns92  | 3.08           | wpl1 | B            | M        | +             | Wings apart-like homolog |
| 3       | ns92  | 3.08           | cdt1 | ABC          | G$_1$    | +             | Replication licensing factor |
| 4       | ns185 | 2.96           | Cc63,14 | A   | G$_2$    | +             | Unknown |
| 5       | ns24  | 2.84           | cdc2 | ABC          | G$_2$    | +             | WD repeat protein |
| 6       | ns24  | 2.84           | ps3  | B            | M        | +             | Mitotic cohesin complex |
| 7       | ns213 | 2.79           | am2s | ABC          | G$_1$    | +             | Transcription factor |
| 8       | ns138 | 2.77           | cdc10| B            | G$_1$    | +             | MFB transcription factor complex |
| 9       | ns134 | 2.72           | cdc18| ABC          | G$_1$    | +             | MCM loader |
| 10      | ns214 | 2.67           | CPB1C11.02 | C   | ND      | +             | Amino acid permease |
| 11      | ns98  | 2.49           | mit3 |             |          | +             | Meiosis-inducing protein |
| 12      | ns22  | 2.49           | AP14E8.02 | ABC | G$_1$    | +             | Unknown |
| 13      | ns217 | 2.41           | mpeg9|             |          | +             | Unknown |
| 14      | ns217 | 2.41           | CC70.10 | A   | G$_2$    | +             | Guanyl-nucleotide exchange factor |
| 15      | ns211 | 2.40           | ste5 | A            | G$_1$    | +             | Triacylglycerol lipase |
| 16      | ns211 | 2.40           | Cc1450.16c | A | G$_2$    | +             | DNA replication factor |
| 17      | ns88  | 2.37           | sub1 | ABC          | G$_1$    | +             | Mitotic inhibitor kinase |
| 18      | ns88  | 2.37           | mik1 | ABC          | G$_1$    | +             | Putative protein kinase |
| 19      | ns158 | 2.32           | pppk25| B            | G$_1$    | +             | NADH pyrophosphatase |
| 20      | ns143 | 2.27           | BC1778.03c |     |          | +             | Spop4-Spop6 kinase complex |
| 21      | ns143 | 2.27           | spo6 |             |          | +             | Cyclin |
| 22      | ns35  | 2.19           | cig2 | ABC          | G$_1$    | +             | Checkerpoint mediator |
| 23      | ns67  | 1.86           | mrc1 | ABC          | G$_1$    | +             | Deoxyuridine 5'-3P nucleotidohydrolase |
| 24      | ns31  | 1.82           | AC644.05c | ABC | G$_1$    | +             | MCM complex |
| 25      | ns153 | 1.71           | mcm7 |             |          | +             | Ribonucleotide reductase |
| 26      | ns153 | 1.71           | suc22| A            | G$_1$    | +             | Hexose transporter |
| 27      | ns172 | 1.66           | gnh8 | B            | G$_2$    | +             | Kleisin |
| 28      | ns197 | 1.65           | rad21 | AB  | G$_1$    | +             | Peptidyl-prolyl cis-trans isomerase |
| 29      | ns160 | 1.61           | rev1 |             |          | +             | Deoxyxycytidyl transferase |
| 30      | ns160 | 1.61           | flp39| 1.61          | G$_1$    | +             | Mitochondrial translation elongation factor |
| 31      | ns174 | 1.49           | CC553.10 | ABC | G$_1$    | +             | Mitotic inhibitor kinase |
| 32      | ns104 | 1.46           | BC1306.01c | ABC | G$_1$    | +             | Mitotic inhibitor kinase |
| 33      | ns104 | 1.46           | BC1306.02 | ABC | M        | +             | WD repeat protein |
| 34      | ns196 | 1.44           | tel1 |             |          | +             | ATM checkpoint kinase |

$^a$ Cdc10 level indicates binding affinity in log$_2$ scale.

$^b$ References 44 (Peng et al.), 47 (Rustici et al.), and 43 (Oliva et al.) are indicated by A, B, and C, respectively.

$^c$ Phase indicates the pattern of cell cycle transcription. ND indicates no specific phase.

$^d$ Cdc10-Yox1 target genes (1) are indicated as “+” and others as “−”.

$^e$ The known or predicted product is based on the GeneDB database at http://www.geneDB.org. MCM, minichromosome maintenance; 5'-3P, 5'-3P, 5'-triphosphate.

$^f$ ID, gene identification.
model of the core MBF complex containing two heterodimers, Cdc10-Res1 and Cdc10-Res2, at the promoter of the MBF-dependent genes (55). The pattern of relative levels of Cdc10, Res1, and Res2 at the Rpb1-poor and Rpb1-rich loci was confirmed in ChIP-qPCR assays (see Fig. S2 in the supplemental material). Different stoichiometric ratios of the individual MBF components at the Rpb1-poor and Rpb1-rich loci suggest that the MFB binding at the promoter is mechanistically distinct from the binding at the intragenic regions.

Res1 binding at the Rpb1-poor loci, but not the Rpb1-rich loci, requires Cdc10 function. It is known that MFB binding at the promoter of some MFB-dependent genes requires the complex function, because disruption of any one of the core MFB components is sufficient to abolish its promoter bindings (10, 36, 40, 42, 51, 54, 55, 60). Given that the stoichiometric ratios of the individual MFB components at the promoters and intragenic regions were different, we wanted to test if the MFB binding would require no complex activity at the intragenic regions. To test this possibility, we performed ChIP-chip analysis to determine the Res1 occupancies in cdc10-V50 cells. cdc10-V50 encodes an H362Y mutation within the first Cdc10 ankyrin motif and fails to form the MFB complex at the restrictive temperature (31, 45). ChIP-chip analysis showed that the level of Res1 occupancies at the Rpb1-poor promoter loci was largely diminished in cdc10-V50 cells at the restrictive temperature (Fig. 5A and B; see also Fig. S3 in the supplemental material). On the other hand, the levels of Res1 bindings at the Rpb1-rich intragenic loci were not much affected (Fig. 5A and C). This result indicates that the MFB complex activity is required for the binding at the Rpb1-poor promoter loci, but not at the Rpb1-rich intragenic loci, supporting the idea that the MFB binding at the Rpb1-poor promoters is mechanistically different from the binding at the Rpb1-rich intragenic regions.

Rep2 is absent from the Cdc10 occupancies at the Rpb1-poor loci in log-phase-growth cells. It is known that Rep2 is required for transcriptional activation of the MFB-dependent genes at the G1/S phase (15, 42) and that the levels of Rep2 oscillate during the cell cycle, with a peak level at the G1/S phase (14). To investigate if Rep2 was present at both the Cdc10 promoter and intragenic binding sites, we performed ChIP-chip analysis of log-phase-growth rep2-HA cells to determine the Rep2 occupancies. We found that ~70% of Cdc10 occupancies were overlapped with the Rep2 occupancies (Fig. 6A). Interestingly, most (>90%) of the Rep2 occu-
pencies were found to be colocalized with the Cdc10 occupancies associated with the Rpb1-rich loci. On the other hand, less than 20% of the Cdc10 occupancies at the Rpb1-poor loci were found to be overlapped with Rep2 occupancies. This result suggests that Rep2 is absent from the Cdc10 promoter binding sites.

The level of Rep2 at the Rpb1-rich loci was found to be correlated with that of Rpb1 (correlation coefficient = 0.71; P value = 2.2e−16) (Fig. 6B), similar to the level of other MBF factors (see Fig. 2A and 3C and D). On the other hand, the Rep2 level at the Rpb1-poor loci showed no apparent correlation with the Rpb1 level (correlation coefficient = 0.13). Notably, the level of Rep2 occupancies at the Rpb1-poor loci was much lower than that at the Rpb1-rich loci (median level of 0.40 versus 1.51 in log2 scale; P value = 4.78e−14) (Fig. 6C). We found that the level of Rep2 occupancies at the Rpb1-poor loci was much lower than that of the core MBF component Cdc10, Res1, or Res2 (median level of 0.40 versus 2.01, 1.07, or 1.48; P value = 2.2e−16, 2.93e−06, or 1.39e−09), supporting the idea that Rep2 is absent from the Rpb1-poor loci. The pattern of the relative levels of Rep2 and other MBF components at the Rpb1-poor and Rpb1-rich loci was further confirmed in ChIP-qPCR assays (see Fig. S2 in the supplemental material).

**HU-induced Rep2 accumulation occurs at the coding region but not at the promoter of the MBF-dependent G1/S-phase genes.** We have previously shown that the Rep2 level is accumulated upon HU treatment, which is correlated with the transcription level of the MBF-dependent G1/S-phase genes (14). Therefore, we wanted to test if, in HU-treated cells, the level of Rep2 occupancies would be increased at the Rpb1-poor loci, most of which are at the promoter of the MBF-dependent G1/S-phase genes. For this reason, we performed ChIP-chip analysis to determine Rep2, Rpb1, and Res1 occupancies in cells after treatment with 8 mM HU for 4 h (see Materials and Methods). ChIP-chip analysis showed that Rep2 accumulation at the G1/S-phase genes was more apparent in HU-treated cells than in HU-untreated cells (Fig. 7A). Surprisingly, however, the accumulation of Rep2 was not limited to the promoter binding sites. Rep2 accumulation was found to be traversed into the coding region of many MBF-dependent G1/S-phase genes. The pattern of Rep2 accumulation at these cell cycle-transcribed genes resembled that of the elongating RNAP subunit Rpb1 (Fig. 7B).

Transcription factors entering the coding region from the promoter binding site upon stress are not uncommon (41). This is due largely to the increased level of transcription with which transcription factors are traversed into the coding region with the elongating RNAP-II. In this case, the peak level of accumulation signals would remain at the promoter. ChIP-chip analysis of Res1 occupancies in HU-treated cells indicates that the peak level of Res1 accumulation was apparent at the promoter binding sites, although the accumulation at the coding region was also increased (Fig. 7C). Unlike Res1 accumulation, Rep2 accumulation showed no apparent peak at the promoter, resembling that of the elongating RNAP-II. We found that the low-level Rep2 binding (i.e., below the threshold) at the Cdc10 promoter binding sites in cells untreated with HU...
recognized by the H5 antibodies was found in the Rep2-associated complexes (Fig. 7D). In the reciprocal experiment, Rep2 was also detected in the elongating RNAP-associated complexes (Fig. 7E). These results support that Rep2 plays a role in recruiting RNAP-II complex upon transcription activation of the MBF-dependent G1/S-phase genes.

Oscillated levels of Rep2 accumulation at the coding region of genes are correlated with the levels of their cell cycle transcription. We have shown previously that levels of Rep2 protein are oscillated during the cell cycle, with the peak level at G1/S phase (14). If Rep2 played a role in recruiting RNAP-II to the MBF-dependent G1/S-phase genes whose promoter was bound by the core MBF complex, levels of Rep2 accumulation at these genes would oscillate along with the cell cycle. To test this possibility, we performed coimmunoprecipitation assays to determine Rep2 occupancies in synchronized cdc25-22 cells. Small-sized synchronous cultures (e.g., <400 ml) used in Western blot analysis are found to reach the peak level of septation (or S phase) at ~120 min after release from the temperature block (14), similar to the culture of cdc25-22 strain without the rep2-HA allele (44). For ChIP-chip analysis, which requires large sample sizes, we employed the large-sized cultures (e.g., >800 ml). We found that the adjustment of temperature for large-sized cultures during release was not as quick as that for small-sized cultures, in which the peak level of septation was reached at ~140 min after release from the temperature block (Fig. 8A, see asterisk). The 10-min delay in septation was due to the slow temperature adjustment of the large-sized cultures but not to a defect of the strain in cell cycle progression.

Synchronous cells were taken at various time points after release from the temperature block at G2/M phase for a period of 130 min at intervals of 15 or 30 min for ChIP-chip analysis of Rep2. Clearly, the levels of Rep2 accumulation at the coding region of many MBF-dependent genes were oscillated along the cell cycle, with a peak level at the G1/S phase (Fig. 8B). To test if all of the genes whose promoter was bound by Cdc10 exhibited a pattern identical to that of Rep2 accumulation, we performed hierarchical cluster analysis (24) of the temporal Rep2 accumulation pattern of genes whose promoter was bound by Cdc10. To this end, most of the Cdc10-promoter-bound genes formed two clusters, based on the similarity of temporal Rep2 accumulation profiles. Genes in cluster 1 exhibited the peak level of Rep2 accumulation at G2/M phase (Fig. 8C, cluster 1). Consistent with this, 5 out of 9 genes exhibited the peak level of transcription at G2 or M phase. However, it was not clear if these genes were solely regulated by MBF or represented the MBF meiotic targets (e.g., mei3 and spo6).

On the other hand, genes in cluster 2 showed the peak level of Rep2 accumulation at G1/S phase (Fig. 8C, cluster 2). In agreement with this, all cluster 2 genes were known to exhibit the peak level of transcription at G1/S phase except for wpl1. This result supports the idea that Rep2 plays a role in recruiting RNAP-II to the MBF-bound genes for transcription. However, the timing of Rep2-mediated RNAP-II recruitment might be controlled by the core MBF complex, activation of which by CDKs (17, 18, 46, 52) permits Rep2 to recruit RNAP-II for transcription of the MBF target genes.

There were another 12 genes whose Rep2 accumulation patterns showed no apparent similarity. This was because the dynamic range of Rep2 accumulation level changes at these
genes was relatively low (~25%). In agreement with this, it was hard to determine the precise cell cycle transcription pattern for genes: only one (bc1306.01c) of these genes' cell cycle transcription patterns was detected in all three independent studies of mitotic transcription profiling (43, 44, 47).

**DISCUSSION**

In this study, we show that the majority of the genomic occupancies by the core MBF components Cdc10, Res1, and Res2 are located at the intragenic sequences. Only one-third (50) of them were found to be associated with probable promoters, based on the genome annotations (34, 57). We also found that Rpb1 binding signals at many actively transcribed genes tend to peak at the 3’ end. This leads to the misassignment of many intragenic Cdc10 occupancies to the promoter of the adjacent downstream genes. To prevent this erroneous assignment, we applied deconvolution analysis in which we assumed that Rpb1 levels would be relatively low at the promoter. We further found that Rpb1-poor promoter occupancies are associated with the Rpb1-poor loci, most of which are located at the promoter of the MBF-dependent genes. Of the 34 genes that are assigned to the Rpb1-poor promoter loci, 31 (~90%) were found to be overlapped with the Cdc10-Yox1 target genes that were recently identified by Aligianni et al. (1) (see Fig. S4 in the supplemental material) (P value = 2.91e−06). On the other hand, the genes associated with Cdc10 occupancies at the Rpb1-rich promoter loci showed no overlap with the Cdc10-Yox1 target genes except for one (P value = 4.60e−08). Clearly, deconvolution analysis greatly improves the true promoter binding discovery rate.

The binding of individual MBF components at the promoter of many MBF-dependent genes is known to require the MBF complex function, because disrupting any one of the MBF components is sufficient to diminish the promoter binding of other MBF components (10, 36, 40, 42, 51, 54, 55, 60). In this study, we show that Res1 binding requires the Cdc10 function (10, 36, 40, 42, 51, 54, 55, 60). In this study, we show that Res1 binding requires the Cdc10 function (10, 36, 40, 42, 51, 54, 55, 60). In this study, we show that Res1 binding requires the Cdc10 function (10, 36, 40, 42, 51, 54, 55, 60).

Cell cycle transcription at G1/S phase is likely to be regulated by the multiple pathways to ensure the activation timing of target genes. A number of negative feedback loops, such as Cig2, Nrm1, and Yox1, have been proposed to repress the MBF activity (1, 2, 20). In this study, we show that Res1 binding requires the Cdc10 function at the Rpb1-poor loci, but not at the Rpb1-rich loci, supporting the idea that Cdc10 occupancies at the Rpb1-rich loci are not the MBF complex binding targets. We propose that the MBF occupancies at the Rpb1-poor loci are MCB2-like motif associated. On the other hand, those at the Rpb1-rich loci are likely to exist as a result of physical association with RNAP-II, particularly at the actively transcribed ribosome protein genes.

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cycle transcription in *S. pombe* requires the coordination between the activation of the static subunit(s) (e.g., Cdc10) and the availability of the dynamic subunit (e.g., Rep2).

In *S. cerevisiae*, it has been shown that the cell cycle transcription is driven partly by the cell cycle-specific transcription regulatory network: cell cycle phase-specific transcription factors whose encoding genes are found to be transcribed prior to the cell cycle phase of their function (49). For example, Swi4, a component of the SBF complex, is cell cycle-specifically transcribed (26) and regulates the transcription of genes in the subsequent cell cycle phase (32, 33). We found that the transcription profile of rep2 resembles that of polo1, one of the M/G1-phase genes regulated by the forhead transcription factors Fkh2-Sep1 (see Fig. S5A in the supplemental material) (44, 47). Rep2 protein accumulates with a peak level at G1/S phase, and its activity is required for transcription of the MFB target genes. Therefore, we hypothesize that Rep2 connects the M/G1-phase and the G1/S-phase transcription. It has been shown that M-phase transcription factors Fkh2-Sep1 are responsible for the transcription of Ace2, a transcription factor that regulates a subset of G1/S-phase genes that contain the ACE2 motif (8, 9). We have evidence that Fkh2 binds at not only the promoter of ace2 genes, but also the promoter of rep2 (see Fig. SSB in the supplemental material). However, the strong phenotype impedes the synchronous analysis of cell cycle transcription in fkh2Δ mutant cells. Future studies of conditional alleles of fkh2 should be able to test this hypothesis.

ChiP-chip analysis of binding sites for MFB/SBF in *S. cerevisiae* and the essential MFB component Cdc10 and its negative regulator Yox1 in *S. pombe* has shown that many of their binding targets are the constitutively transcribed genes (1, 28). In this study, we show that the majority of the MFB occupancies in the genome are intragenic and associated with the Rpb1-rich loci. Binding of the individual MFB components at the Rpb1-rich loci is independent of the MFB complex activity, indicating that they are functionally distinct from the bindings at the Rpb1-poor promoter loci. Transcription factors that bind to the coding region are thought to play a role in regulation of transcription elongation (41). Defects in transcription elongation often confer hypersensitivity to transcription elongation inhibitor 6-azauracil (6AU) (48). In this study, we were unable to observe the hypersensitivity to 6AU in MFB-defective mutants. However, we observed fragmented nucleoli in the MFB mutants (see Fig. S6 in the supplemental material). Given that most of the intragenic MFB-associated genes encode ribosomal proteins, a defect in the transcription of these RPGs could lead to the distorted nucleolus structure (30). This result suggests that MFB may have a role in transcriptional regulation of the actively transcribed genes. Alternatively, excess MFB factors may be localized or stored at the coding region of the actively transcribed genes in the genome.

In conclusion, our results demonstrate that Cdc10 promoter binding targets can be enriched through deconvolution analysis of Cdc10 genomic occupancies using the Rpb1 occupancy profiles. Furthermore, time course ChiP-chip analysis shows that Rep2 is temporally accumulated at the coding region of the MFB target genes, possibly mediating the recruitment of RNAP-II.

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