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G_1 Transcription Factors Are Differentially Regulated in *Saccharomyces cerevisiae* by the Swi6-Binding Protein Stb1

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Stage-specific transcriptional programs are an integral feature of cell cycle regulation. In the budding yeast *Saccharomyces cerevisiae*, over 120 genes are coordinately induced in late G_1 phase by two heterodimeric transcription factors called SBF and MBF. Activation of SBF and MBF is an upstream initiator of key cell cycle events, including budding and DNA replication. SBF and MBF regulation is complex and genetically redundant, and the precise mechanism of G_1 transcriptional activation is unclear. Assays using SBF- and MBF-specific reporter genes revealed that the *STB1* gene specifically affected MBF-dependent transcription. *STB1* encodes a known Swi6-binding protein, but an MBF-specific function had not been previously suspected. Consistent with a specific role in regulating MBF, a *STB1* deletion strain requires SBF for viability and microarray studies show a decrease in MBF-regulated transcripts in a *swi4*Δ mutant following depletion of Stb1. Chromatin immunoprecipitation experiments confirm that Stb1 localizes to promoters of MBF-regulated genes. Our data indicate that, contrary to previous models, MBF and SBF have unique components and might be distinctly regulated.

In the budding yeast *Saccharomyces cerevisiae*, commitment to enter the mitotic cell cycle occurs during late G_1 phase at a point called Start which is analogous to the restriction point in mammalian cells (42). Start is marked by the coordinate induction of a large subset of genes that promote entry into the mitotic cell cycle (49, 51). Approximately 12% of yeast genes are cell cycle regulated, and expression of almost half of these genes peaks at the G_1/S phase transition (27, 29). Transcriptional activation of genes in late G_1 phase is largely dependent on two heterodimeric transcription factors called SBF and MBF (reviewed in reference 54). These two complexes share a common regulatory subunit, Swi6, which is tethered to DNA via its binding partners, Swi4 and Mbp1. Activation of SBF and MBF transcription initiates key cell cycle events, including budding, DNA synthesis, and spindle pole body duplication. The SBF (SCB-binding factor) complex activates transcription mainly through a *cis*-acting sequence element called SCB (for Swi4/6 cell cycle box). Genes activated by SBF include those encoding G_1 cyclins (*CLN1*, *CLN2*, *PCL1*, and *PCL2*), the *HO* endonuclease gene, *SWE1*, which encodes a protein kinase, and a number of genes required for cell wall biosynthesis (29, 54). The MBF (MCB-binding factor) complex recognizes the MCB (for *Mlu1* cell cycle box) element and activates G_1-specific transcription of the S-phase cyclin genes, *CLB5* and *CLB6*, and many genes required for DNA synthesis, including *CDC9*, *POL1*, *RNR1*, and *CDC21* (29, 54). SBF and MBF have been shown (via genome-wide chromatin immunoprecipitation [ChIP] experiments) to bind to 235 gene promoters (29). Interestingly, this group of genes includes only 21% of the genes known to be induced at the G_1/S transition (27, 29). However, among those SBF and/or MBF targets are 16 transcription factors which in turn may potentially regulate or influence the expression of thousands of genes, including those expressed in late G_1 phase (27, 29).

Passage through Start and activation of SBF and MBF both require the cyclin-dependent kinase (Cdk), Cdc28, and one of three G_1 cyclins, Cln1, Cln2, or Cln3. Although any one of the three G_1 cyclins is sufficient to drive Start, genetic studies indicate a key role for Cln3 in activating SBF and MBF (53, 59). ChIP analyses have shown that promoter binding of SBF is crucial for the subsequent recruitment of the Srb mediator, TFIIB, as well as Kin28 (9). The Srb/mediator complex is recruited to promoters in the absence of Cdc28 kinase activity, whereas PolII, TFIIB, and Kin28 are only recruited in its presence (9). There is no evidence, however, that Cln3-Cdc28 acts to directly phosphorylate or interact with components of SBF or MBF (59). Moreover, a *cln3*Δ mutant strain is viable and still undergoes SBF- and MBF-dependent transcription, albeit at a larger cell size, indicating that alternative mechanisms must function to activate SBF/MBF (53). Our laboratory and others have uncovered a number of alternative activators of G_1 transcription (11, 16, 25, 37, 58), but the precise mechanisms of activation and regulation of SBF and MBF remain unclear.

SBF and MBF transcriptional regulation is complex, and the distinction between SBF-controlled genes and MBF-controlled genes is not absolute. For example, *CLN2* expression is not entirely abolished in mutants lacking *SWI4* or SCB elements within the *CLN2* promoter (10, 52) and *CLN1* expression is regulated via SBF binding to MCB promoter elements (44). Consistent with redundancy in their roles, *SWI4* and *MBP1* are

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TABLE 1. *S. cerevisiae* strains used in this study[a]

Strain	Genotype	Reference or source
BY263	*MAT***a** *trp1 leu2 his3 ura3 lys2 ade2*	38
BY184	*MAT*α *swi4*Δ*HIS3 SCB*::*lacZ*	3
BY185	*MAT*α *swi6*Δ*HIS3 SCB*::*lacZ*	3
BY551	*MAT***a** *mbp1*Δ*TRP1*	This study
BY805	*MAT*α *stb1*Δ*URA3*	25
BY806	*MAT*α *stb1*Δ*TRP1*	25
BY822	*MAT***a** *stb1*Δ*TRP1 cln3*Δ*URA3*	25
BY1106	*MAT*α *stb1*Δ*URA3 bck2*Δ*KAN*	This study
BY1110	*MAT***a** *bck2*Δ*KAN*	This study
BY1298	*MAT***a** *stb1*Δ*TRP1*	This study
BY1693	*MAT*α *stb1*Δ*TRP1 swi4*Δ*HIS3* + pBA314	This study
BY1694	*MAT*α *stb1*Δ*TRP1 swi4*Δ*HIS3* + pBA417	This study
BY1695	*MAT*α *stb1*Δ*TRP1 swi4*Δ*HIS3* + pBA1044	This study
BY1830	*MAT*α *stb1*Δ*TRP1 mec1* + pBA1220	This study

[a] Strains listed are isogenic to the parent BY263 unless otherwise indicated. BY263 is of S288C origin.

not essential genes; however, a strain lacking both genes arrests in G_1 phase (32). Several observations suggest that the genetic redundancy of *SWI4* and *MBP1* reflects a functional redundancy at the level of DNA binding. First, Swi4 and Mbp1 share 50% identity in their DNA binding domains (32, 49). Second, isolated DNA binding domains from Swi4 and Mbp1 bind to both SCB and MCB sequences in vitro (32, 44) and Swi4 and Mbp1 bind overlapping sets of gene promoters in wild-type cells (29, 49). Finally, genome-wide ChIP studies have shown that Mbp1 and Swi4 share 34% of their target genes, suggesting that these proteins are at least partially redundant in wild-type populations (49). Despite their similarities, several differences exist between *SWI4* and *MBP1*. For example, cells possessing null mutations in *swi4* are viable but are slow growing and enlarged and exhibit defects in cell integrity and bud emergence (20). *swi4*Δ deletion mutants are also temperature sensitive and arrest primarily as unbudded cells with one nucleus and 2 N DNA content (20). Unlike *swi4*Δ mutants, *mbp1*Δ mutants appear to be similar to wild-type cells (32). Furthermore, *swi4*Δ *swi6*Δ double mutants are inviable and arrest prior to DNA synthesis while *mbp1*Δ *swi6*Δ mutants proliferate and phenotypically resemble *swi6*Δ mutants (32). Given these differences, it is possible that the regulatory mechanisms required for activation of SBF and MBF also differ. However, the genetically and functionally redundant nature of G_1 control mechanisms has made it difficult to unravel the mechanisms of SBF and MBF regulation.

In this paper, we implicate Stb1 as a specific regulator of MBF-dependent transcription. *STB1* encodes a Swi6-interacting protein which was previously identified as a regulator of Start transcription (25). However, the precise function of *STB1* in G_1 transcription is not known and an MBF-specific function was not previously appreciated. Consistent with a specific role in regulating MBF, a *STB1* deletion strain requires the SBF subunit, Swi4, for viability and microarray studies showed a decrease in MBF-regulated transcripts in a *swi4*Δ mutant following depletion of Stb1. ChIP experiments confirmed that Stb1 localizes to promoters of MBF-regulated genes. Our data suggest that contrary to previous models, MBF and SBF might have unique components and might be distinctly regulated.

MATERIALS AND METHODS

Strains and media. *S. cerevisiae* strains used in this study are listed in Table 1. Standard methods and media were used for yeast growth and transformation and strain construction (23). Minimal medium (synthetic dextrose [SD]) with appropriate amino acid supplements was used for maintaining plasmids in yeast transformants and for genetic selection (23). All gene disruptions were achieved by homologous recombination at their chromosomal loci using standard PCR-based methods (35).

Plasmids. The plasmids used in this study are listed in Table 2. Plasmid pBA314 was constructed by digesting pBA313 (36) with *Bgl*II. The resulting *SWI4* fragment was then cloned into a *Bam*HI-digested YEp13 vector. The *STB1* gene was amplified by PCR from plasmid PBA1010 (25) using the primers 5′STB1*Bgl*II (5′-CCGGAGATCTATCACGCGAAAATGCAAG-3′) and 3′STB1*Bgl*II (5′-CCGGAGATCTGCCGTCAACGATCAATCA-3′) to generate a plasmid expressing full-length *STB1* from the *MET25*-repressible promoter. The PCR product was digested with *Bgl*II and cloned into a *Bam*HI-digested p415 *MET25* vector (40) to create plasmid pBA1044. Plasmid pBA1220 was constructed through digestion of pBA1010 (25) with *Eco*RI and *Not*I. The resulting *STB1* fragment was then cloned into an *Eco*RI/*Not*I-digested pRS426 vector (48). A plasmid expressing an N-terminal fusion of glutathione *S*-transferase (GST) to full-length Stb1 was constructed by amplification of the *STB1* gene by PCR as described above. The PCR product was digested with *Bgl*II and cloned into a *Bam*HI-digested pGEX-3X vector (Pharmacia), resulting in plasmid pBA1598.

To generate a plasmid expressing an N-terminal His tag fused to full-length Stb1, the *STB1* gene was amplified by PCR as described above and the PCR product was digested with *Bgl*II and cloned into a *Bam*HI-digested pRSET-B vector (Invitrogen), resulting in plasmid pBA1622. Using the primers 5′STB1*Bgl*II (see above) and 3′STB1ΔC*Bgl*II (5′-GGGGGAAGATCTTGTTCCATGATGT GATGAC-3′), a plasmid expressing an N-terminal His-tagged Stb1ΔC fusion protein was created by amplification of a fragment of the *STB1* gene (lacking the final 630 nucleotides) from plasmid pBA1010 (25). The PCR product was digested with *Bgl*II and cloned into a *Bam*HI-digested pRSET-B vector (Invitrogen), resulting in plasmid pBA1623. A plasmid expressing *STB1*ΔC from the *MET25*-repressible promoter was constructed through amplification of *STB1*ΔC, digestion with *Bgl*II, and cloning into a *Bam*HI-digested p415 *MET25* vector, thereby creating plasmid pBA1627.

A portion of the *STB1* gene (lacking the first 210 amino acids) was amplified by PCR from plasmid pBA1010 (25) using the primers 5′STB1ΔN*Bgl*II (5′-GG GGGAAGATCTATGCTTGGTTTAAGTAATGTCC-3′) and 3′STB1ΔN*Bgl*II (5′-GGGGGAAGATCTTCAATCAGTGAGTTTGTCAT-3′) to generate a plasmid expressing a truncated form of *STB1* (*STB1*ΔN) from the *MET25*-repressible promoter. The PCR product was digested with *Bgl*II and cloned into a *Bam*HI-digested p415 *MET25* vector, resulting in plasmid pBA1626. Plasmids pBA1624 and pBA1625, containing the *MEC1* and *RNR1* genes, respectively, were isolated from a *LEU2*-YCp50-derived yeast genomic DNA library (8) (American Type Culture Collection, Manassas, Va.) (ATCC no. 77162).

TABLE 2. Plasmids used in this study

Plasmid	Description	Reference or source
pLGΔSS	*CYC1*::*lacZ* (lacking UAS)	21
pBA251	4× *SCB*::*lacZ*	2
pBA487	4× *MCB*::*lacZ*	56
pBA314	2μm *SWI4-URA3*	This study
pRS425	2μm *LEU2* vector	48
pBA417	2μm *SWI4-LEU2*	36
pBA1010	2μm *STB1-LEU2*	25
pBA1044	p415 *MET25* + *STB1*	This study
pBA1220	2μ *STB1-URA3*	This study
pBA1282	pRSET-B + *STB1*ΔN	25
pBA1598	pGEX-3X + *STB1*	This study
pBA1622	pRSET-B + *STB1*	This study
pBA1623	pRSET-B + *STB1*ΔC	This study
pBA1624	CEN *MEC1-LEU2*	This study
pBA1625	CEN *RNR1-LEU2*	This study
pBA1626	p415 *MET25* + *STB1*ΔN	This study
pBA1627	p415 *MET25* + *STB1*ΔC	This study

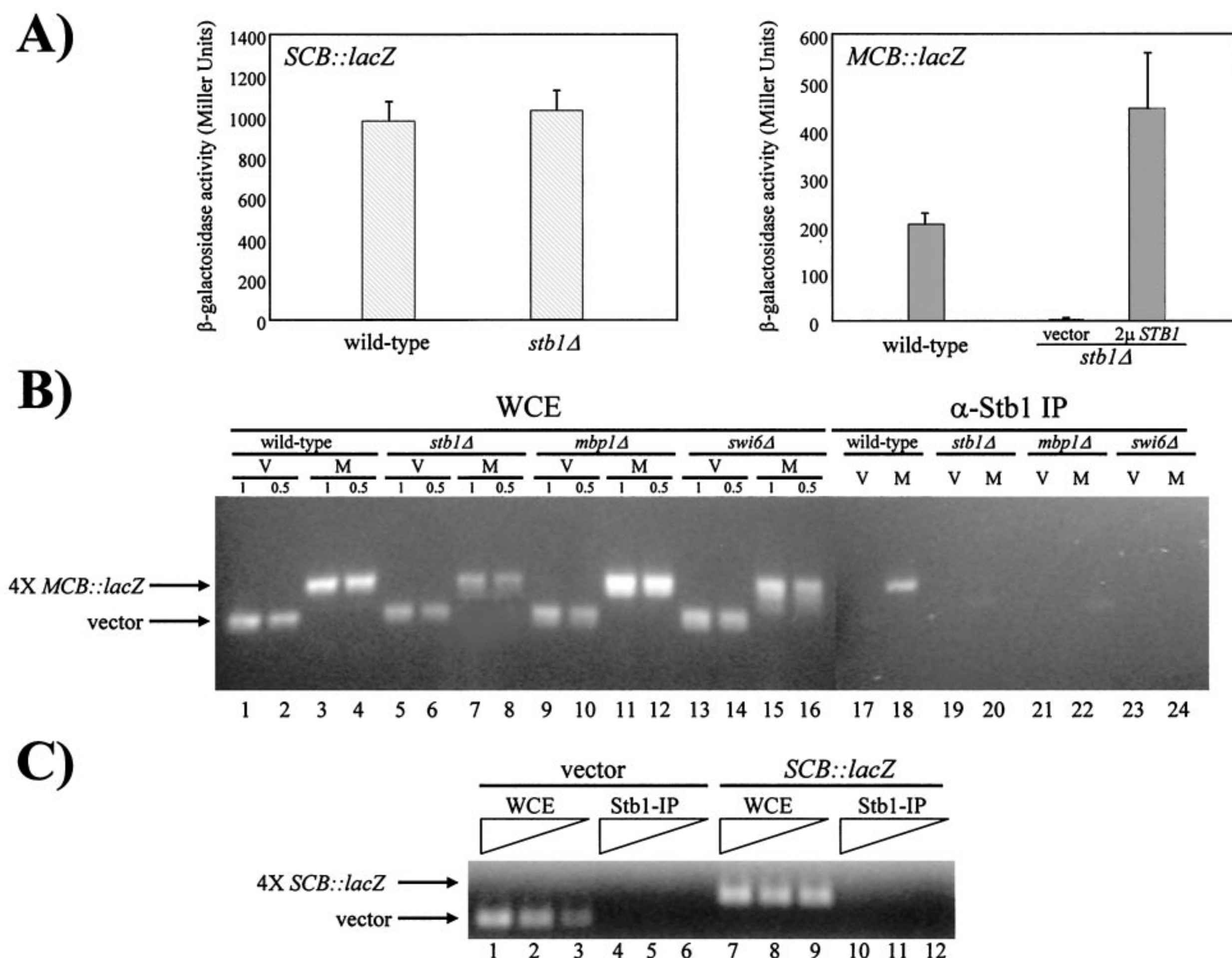


FIG. 1. A *stb1*Δ mutant is defective in *MCB*::*lacZ* but not *SCB*::*lacZ* expression. (A) Wild-type (BY263) and *stb1*Δ (BY806) cells were transformed with the *SCB*::*lacZ* plasmid (pBA251, striped bars) or the *MCB*::*lacZ* plasmid (pBA487, solid bars). In *MCB*::*lacZ* assays, *stb1*Δ cells were also transformed with a 2μm *STB1* (2μ *STB1*) plasmid (pBA1010) or a vector plasmid (pRS425). Strains were grown at 30°C to log phase, cell lysates were prepared, and β-galactosidase activity was measured. Depicted activity values represent the means of three experiments, and error bars indicate standard deviations for three experiments. (B) Stb1 localizes to MCB promoter elements. Wild-type (BY263), *stb1*Δ (BY806), *mbp1*Δ (BY551), and *swi6*Δ (BY185) strains containing a vector plasmid (pLGΔSS) (V) or an *MCB*::*lacZ* construct (pBA487) (M) were grown to mid-log phase and cross-linked with formaldehyde. Whole-cell extracts (WCE) were prepared (lanes 1 to 16) and subjected to immunoprecipitation (IP) using α-Stb1 affinity-purified antibodies (α-Stb1 IP) (lanes 17 to 24). PCR was performed on immunoprecipitated samples and on twofold serial dilutions (1× and 0.5×) of the WCE samples to amplify associated DNA. Primers used in this assay hybridized to the 3′ end of the *URA3* gene and to sequences upstream of *lacZ* in both vector (pLGΔSS) and *MCB*::*lacZ* (pBA487) plasmids. (C) Stb1 does not localize to SCB promoter elements. Wild-type (BY263) strains containing a vector plasmid (pLGΔSS) or an *SCB*::*lacZ* construct (pBA251) were grown to mid-log phase and cross-linked with formaldehyde. Whole-cell extracts (WCE) were prepared (lanes 1 to 3 and 7 to 9) and subjected to immunoprecipitation (IP) using α-Stb1 affinity purified antibodies (α-Stb1-IP) (lanes 4 to 6 and 10 to 12). PCR was performed on immunoprecipitated samples and on twofold serial dilutions of the WCE samples to amplify associated DNA. The primers used to amplify associated DNA were the same as those described for panel B.

β-Galactosidase assays. Liquid β-galactosidase assays were performed on frozen pellets as described previously (38). Data are presented as the mean values from triplicate experiments.

ChIP assays. Cultures (10 ml) of cells were grown to an optical density at 600 nm (OD_{600}) of 0.6. Strains containing *MCB*::*lacZ* or *SCB*::*lacZ* reporter plasmids (Fig. 1B and C) were grown in SD-URA medium, while strains which did not harbor a plasmid (see Fig. 6) were grown in yeast extract-peptone-dextrose (YEPD) medium. Formaldehyde cross-linking and preparation of whole-cell extracts were performed as previously described (4). Using approximately 2 mg of extract and 5 μl of affinity-purified Stb1 polyclonal antibodies, immunoprecipitations were performed as described elsewhere (4, 25). Precipitates derived from strains containing the *MCB*::*lacZ* reporter, *SCB*::*lacZ* reporter, and vector plasmids (Fig. 1B and C) were washed four times for 10 min each time in 1 ml of lysis buffer and four times for 10 min each time in 1 ml of Tris-buffered saline (4). Precipitates derived from strains that did not harbor a plasmid (see Fig. 6) were washed twice for 10 min each time in 1 ml of lysis buffer and twice for 10 min each time in 1 ml of Tris-buffered saline. Finally, the samples were processed for DNA purification and PCR amplification of immunoprecipitated DNA was carried out as previously described (4). The PCR primers used for the amplification of promoter regions in *PHO5*, *RNR1*, and plasmid pLGΔSS were as follows: PHO5-F (5′-CCTGGCGACTATGGTATTTC-3′) (4), PHO5-R (5′-TTCACTGACAGTCTGCAAGG-3′) (4), RNR1-F (5′-TCAATGCTGAACTTTCTATGG-3′), RNR1-R (5′-TATTCTAAAACGTGAGCTGCA-3′), ΔSS-F (5′-GATGCGGCCAGCAAAACTAA-3′), and ΔSS-R (5′-ATATGATCATGTGTCGTCGC-3′). PCR products were separated on 2% agarose gels.

Stb1 Western blotting analysis. A *stb1*Δ (BY806) strain harboring *STB1* (pBA1044), *STB1*ΔN (pBA1626), *STB1*ΔC (pBA1627), or a vector (p415 *MET25*) and a *stb1*Δ*swi4*Δ mutant (BY1695) harboring a *STB1* plasmid (pBA1044) were grown in SD-[LEU, MET] medium at 30°C to an OD_{600} of 0.5. Log-phase samples (10 ml) were taken for analysis. BY1695 cells were then diluted to an OD_{600} of 0.1 into SD-LEU medium with 5 mM methionine and grown at 30°C. Samples (10 ml) were taken at 2, 4, and 8 h time points. Samples (10 ml) of wild-type

(BY263) and *stb1Δ* (BY806) strains (grown in the presence of 5 mM methionine for 8 h) were also harvested to determine endogenous Stb1 protein levels. Protein extracts and Stb1 Western blotting was performed as previously described (25).

DNA microarray analysis. Yeast strains were grown in SD-[LEU, MET] medium at 30°C to an OD_{600} of 0.5. The cells were then diluted to an OD_{600} of 0.1 in SD-[LEU, MET] and SD-LEU medium supplemented with 5 mM methionine and grown for 8 h at 30°C (OD_{600}, approximately 0.6). Cells were harvested by centrifugation and quickly frozen in liquid nitrogen. Total RNA and poly(A^+) RNA were isolated as previously described (28). DNA microarrays consisting of approximately 97% genome coverage were probed with differentially labeled cDNA pools from a *stb1Δ swi4Δ MET25pr-STB1* strain (BY1695) grown in the absence or presence of 5 mM methionine as previously described (28). Arrays were obtained from the Ontario Cancer Institute Microarray facility (www.microarrays.ca). Hybridized arrays were scanned using a Gene Pix 4000B scanner (Axon Instruments).

Northern blot analysis. Yeast strains were grown as described above. RNA was isolated, and Northern blotting was performed as described previously (39). Probes used for Northern blot analysis included a 600-bp *Eco*RI-*Hin*dIII fragment of the *ACT1* gene (38) and a 1.7-kb *Bgl*II-*Eco*RI fragment of the *RNR1* gene (14). For RNA quantitation, Northern blots were exposed on a Molecular Dynamics screen, scanned using a Molecular Dynamics PhosphorImager, and analyzed using ImageQuant software, version 3.3 (Molecular Dynamics).

***stb1Δ* synthetic lethal screen.** A *stb1Δ* strain (BY806) harboring a high-copy *STB1-URA3* plasmid (pBA1220) was subjected to ethyl methanesulfonate mutagenesis as described elsewhere (60). Surviving cells were screened for sensitivity to 5-fluoroortic acid (5-FOA) at 25°C. 5-FOA sensitivity indicates dependence on *STB1* for viability and reflects the lethality generated by deletion of *STB1* in combination with extragenic mutations (i.e., synthetic lethality). *STB1* deletion strains harboring multiple extragenic mutations were identified by mating ethyl methanesulfonate-generated mutants to a wild-type strain (BY263). Diploids were grown in the presence of 5-FOA to promote loss of the *STB1-URA3* plasmid and were subsequently sporulated. Two mutants (*sls1* and *sls2*) were identified by tetrad analysis that segregated 2:2 for viability, indicating that these *stb1Δ* strains contained a single extragenic mutation and required *STB1* for viability (data not shown). Synthetic lethal mutants were characterized genetically as previously described (60). Mutants were cloned by complementation using a yeast genomic DNA library in a *LEU2*-YCp50 derivative (8). Using a tagged *MEC1* strain (data not shown), allelism of *sls1* with *MEC1* was confirmed by tetrad analysis.

Protein expression and purification and far-Western binding analysis. Full-length Stb1, Stb1ΔN, and Stb1ΔC proteins were expressed from *Escherichia coli* harboring the expression plasmids pBA1622, pBA1282, and pBA1623, respectively. Protein expression was induced for 2 h at 30°C following addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Whole-cell extracts were prepared by harvesting 5-ml cultures and resuspending the pellets in 0.5 ml of 1× sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 1% 2-mercaptoethanol). Volumes (10 μl) of whole-cell extracts were subjected to polyacrylamide gel electrophoresis (PAGE) (7.5% polyacrylamide) and transferred to nitrocellulose by means of a semidry transfer apparatus (Bio-Rad). Immunoblots were probed as described previously (25). Swi6 labeling and far-Western assays were performed as described below. GST-Stb1 was purified as previously described (39) from *E. coli* harboring the expression plasmid pBA1598. Recombinant kinase complexes were expressed and purified as described previously (41, 50). Using 1 μl of kinase/μg of substrate, approximately 1 μg of GST-Stb1 was phosphorylated in vitro. The reaction mixtures contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM NaF, 0.1 mg of bovine serum albumin/ml, 1 mM ATP, and 1 mM dithiothreitol in a volume of 20 μl. Following incubation at 30°C for 3 h, an additional 1 μl of kinase was added and the reaction mixtures were incubated at 30°C for a further 19 h. Unphosphorylated and phosphorylated GST-Stb1 was subjected to PAGE (7.5% polyacrylamide) and transferred to nitrocellulose by means of a semidry transfer apparatus (Bio-Rad). Immunoblots were probed as described previously (25). To produce full-length Swi6, the plasmid template pBA513 (3) was used as recommended in the instructions for the T7 TnT coupled reticulocyte lysate system (Promega). Far-Western assays using [^{35}S]methionine (Mandel)-labeled Swi6 were carried out as previously described (22).

Microscopy. Cells were grown in YEPD medium to log phase and observed at a magnification of ×630 with Normarski optics and a Micromax 1300y high-speed digital camera (Princeton Instruments, Trenton, N.J.) mounted on a Leica DM-LB microscope. Images from the camera were analyzed with Metaview software (Universal Imaging, Media, Pa.). Where indicated, the percentage of budded cells in each sample was determined by counting at least 300 cells per sample.

FACS. The DNA content of strains BY263, BY805, BY1106, and BY1110 was analyzed by fluorescence-activated cell sorting (FACS) as described elsewhere (55).

RESULTS

MCB-driven UAS activity is dependent on *STB1*. Owing to the complexity and genetic redundancy of SBF and MBF regulation, it is difficult to assign Start-specific transcription to SBF or MBF on the basis of consensus promoter elements in an upstream region. However, the observed redundancy in SBF and MBF function can be overcome by studying the ability of synthetic SCB and MCB elements to activate transcription of a reporter gene. *CYC1*::*lacZ* reporter genes lacking an upstream activating sequence (UAS) and driven by multiple synthetic SCB or MCB oligonucleotides are specifically dependent on SBF or MBF, respectively (2, 56). For example, deletion of *MBP1* does not affect *SCB*::*lacZ* expression and, conversely, deletion of *SWI4* causes only a modest defect in *MCB*::*lacZ* expression (56).

To determine the requirement for Stb1 in G_1-specific transcription, *MCB*::*lacZ* and *SCB*::*lacZ* reporter gene plasmids were transformed into wild-type and *stb1Δ* mutant strains and β-galactosidase activities were measured in vitro (Fig. 1A). The SCB sequence was able to function efficiently as a UAS in both wild-type and *stb1Δ* cells (Fig. 1A). Conversely, the MCB sequence was unable to function as a UAS in the *stb1Δ* mutant strain. A 200-fold decrease in β-galactosidase activity was observed in a *stb1Δ* mutant compared to that seen in wild-type cells (Fig. 1A). β-Galactosidase activity was restored in *stb1Δ* mutants upon overexpression of *STB1* (Fig. 1A). We interpret the large difference in *STB1* requirement for the activation of transcription from the MCB sequences as an indication of the specific requirement for *STB1* in this process.

Stb1 localizes to MCB promoter elements. Since *STB1* appeared to be required for MCB-dependent reporter gene expression, we directly examined the association of Stb1 with MCB synthetic promoter elements. ChIP assays were performed using affinity-purified Stb1 polyclonal antibodies (Fig. 1B). Wild-type, *stb1Δ*, *mbp1Δ*, and *swi6Δ* cells harboring the *MCB*::*lacZ* construct or a control vector were harvested during exponential growth phase. The cells were fixed with formaldehyde, and chromatin was immunoprecipitated using Stb1 antibodies (25). The abundance of specific DNA sequences within the immunoprecipitated material was measured using PCR and a vector-specific primer pair flanking the synthetic MCB elements. The *CYC1*::*lacZ* vector lacking a UAS was used as a negative control, since it does not contain any MCB elements. MCB elements and the control vector were detected in the input whole-cell extracts (Fig. 1B, lanes 1 to 16). Using cross-linked extracts derived from a wild-type strain (Fig. 1B, lane 18), specific PCR enrichment of a 380-bp fragment containing MCB promoter elements was detected in ChIPs, and this enrichment was dependent on the presence of *STB1*, *MBP1*, and *SWI6* (Fig. 1B, lanes 20, 22, and 24). *STB1* is transcribed in *mbp1Δ* and *swi6Δ* as well as *swi4Δ* mutants (reference 25 and data not shown), indicating that the inability of Stb1 to localize to MCB elements in the absence of MBF is not due to inadequate expression of *STB1*. Vector DNA was not enriched in the ChIP assay performed using affinity-purified Stb1 antibod-

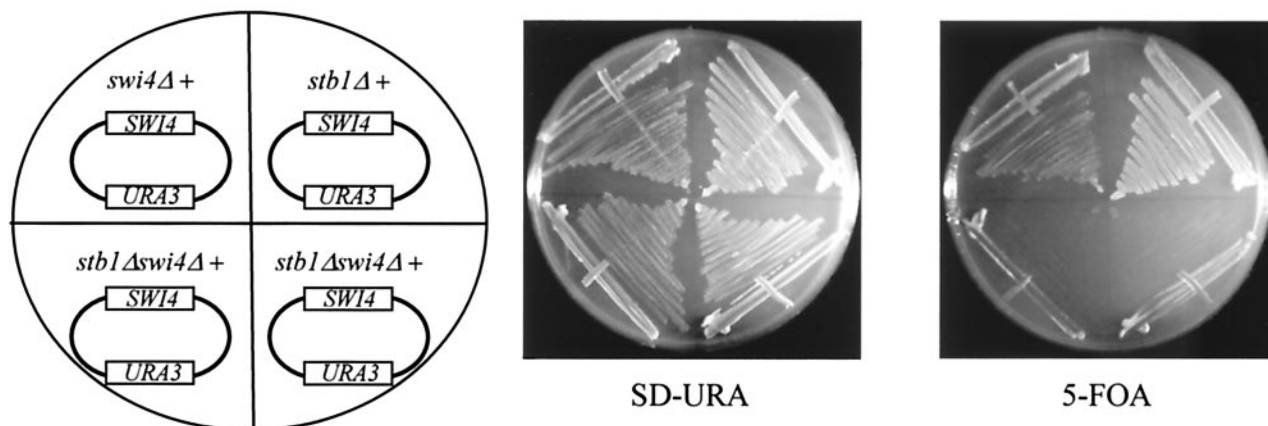
ies (Fig. 1B, lanes 17, 19, 21, and 23). Furthermore, DNA enrichment was not detected in ChIP assays performed using non-cross-linked lysates (data not shown). Similar ChIP assays were performed using a wild-type strain harboring an *SCB*::*lacZ* construct (Fig. 1C). In contrast to our findings with MCB promoter elements, no PCR enrichment of vector or SCB synthetic elements was observed in ChIP assays performed using affinity-purified Stb1 antibodies (Fig. 1C, lanes 4 to 6 and lanes 10 to 12). We therefore conclude that Stb1 specifically localizes to MCB elements in an MBF-dependent manner.

An *STB1* deletion strain requires *SWI4* for viability. The results of β-galactosidase and ChIP assays suggest that *STB1* functions to activate MBF-dependent transcription specifically. However, previous efforts examining SBF- and MBF-dependent expression of chromosomally encoded genes failed to reveal any defects in a *stb1*Δ mutant (25). The lack of transcriptional defects might be explained by functional redundancy between MBF (which requires Stb1) and SBF. We therefore reexamined *STB1* genetic interactions. First, we constructed a *stb1*Δ *swi4*Δ double mutant (Fig. 2). This strain lacks functional SBF, and G_1 transcription is entirely dependent on MBF; thus, a *stb1*Δ *swi4*Δ mutant may be more sensitive to defects in MBF regulation. A *stb1*Δ*TRP1* mutant strain was mated to a *swi4*Δ*HIS3* strain harboring a high-copy-number *SWI4-URA3* plasmid. All haploid double mutants isolated by tetrad dissection contained the high-copy *SWI4* plasmid, and double mutants were subsequently tested for sensitivity to 5-FOA (Fig. 2A). Expression of the *URA3* gene causes lethality when cells are grown in the presence of 5-FOA (23); cells that are dependent on *SWI4* for viability are sensitive to 5-FOA, since they cannot lose the *SWI4-URA3* plasmid. Unlike *stb1*Δ and *swi4*Δ single mutants, *stb1*Δ *swi4*Δ double mutants were sensitive to 5-FOA, indicating that *SWI4* was required for viability of the double mutant (Fig. 2A). We then rescued the 5-FOA sensitivity by expressing *SWI4* or *STB1* from *LEU2*-based plasmids (Fig. 2B). Complementation of *stb1*Δ *swi4*Δ 5-FOA sensitivity confirmed that viability of the double-mutant strain is dependent on *SWI4* and *STB1*. Thus, consistent with an MBF-dependent function, *STB1* is required for viability in the absence of *SWI4.* Furthermore, expression of *CLN1* partially rescued the 5-FOA sensitivity of the *stb1*Δ *swi4*Δ double mutant (data not shown), suggesting that the lethality of *stb1*Δ *swi4*Δ mutants stems from inadequate G_1 cyclin levels. However, similar to *swi4*Δ *mbp1*Δ mutants (32), the rescued double-mutant cells grew more slowly than either *stb1*Δ or *swi4*Δ single mutants and had abnormal morphology, suggesting that deletion of *STB1* and *SWI4* affects additional genes (data not shown). These results provide genetic evidence that *STB1* is required for MBF-dependent transcription.

An *stb1*Δ *bck2*Δ double mutant accumulates in the G_1 phase of the cell cycle. In an effort to reveal the genetic pathway defined by *STB1*, we expanded our genetic tests to include other known regulators of Start transcription. The Cln3-Cdc28 kinase is required for efficient activation of transcription at Start (12, 53, 59). However, *CLN3* is not absolutely required for activation of SBF- and MBF-dependent gene expression. In the absence of *CLN3*, activation of Start transcription is delayed and other regulators, including *STB1* and *BCK2*, are required for proper cell cycle progression and G_1-specific transcription. Indeed, both *stb1*Δ *cln3*Δ and *bck2*Δ *cln3*Δ double mutants show pronounced G_1 accumulation and a severe growth defect (11, 16, 25, 58). We extended this analysis by assessing the phenotype of a *stb1*Δ *bck2*Δ double-mutant strain (Fig. 3). When cultured in rich or minimal medium, the growth rates of both *stb1*Δ and *bck2*Δ mutants were comparable to that of the wild-type strain, whereas the *stb1*Δ *bck2*Δ double mutant grew much more slowly (Fig. 3A and data not shown). Analysis of cell morphology and DNA content in log phase cultures revealed that *stb1*Δ *bck2*Δ double-mutant cells accumulated in G_1 phase as predominantly large unbudded cells (Fig. 3B and C). These observations support the notion that in addition to acting in a parallel pathway to *CLN3* (25), *STB1* might also function in parallel to *BCK2* to regulate Start transcription.

MBF-dependent transcription is defective in a *stb1*Δ *swi4*Δ strain. If *STB1* is required for MBF-specific transcription, defects in MCB-driven gene expression may be revealed in the absence of functional SBF. Thus, to obtain a view of gene expression patterns in a *stb1*Δ *swi4*Δ mutant, we examined the genome-wide transcriptional consequences of depleting *STB1* expression in a *swi4*Δ mutant. To accomplish this, viability of the *stb1*Δ *swi4*Δ double-mutant strain was maintained through ectopic expression of *STB1* from the repressible *MET25* promoter (Fig. 2B). Western blot analysis revealed that *STB1* levels expressed from the *MET25* promoter were drastically reduced (compared to those of endogenously expressed *STB1*) but not eliminated in the presence of 5 mM methionine (Fig. 4A, lanes 5 and 6). Limited expression of *STB1* was sufficient for proliferation of the double-mutant strain (data not shown). The ability to limit rather than eliminate *STB1* expression was useful, since it allowed us to isolate total RNA from living cells. Under these conditions, transcriptional defects are attributable to decreased Stb1 levels rather than cell death.

Cultures were grown to mid-log phase in the absence or presence of 5 mM methionine, and total RNA derived from these cultures was subjected to DNA microarray analysis (Fig. 4B). Expression levels of genes such as *ACT1*, which is neither cell cycle periodic nor regulated by methionine, remained unchanged (Fig. 4B). A 1:1 hybridization ratio was also observed for *SWI4*, since it was deleted from both samples (Fig. 4B). As expected, a large number of genes exhibited differential expression patterns. The majority of these genes function either directly or indirectly in methionine biosynthesis or metabolic pathways (Fig. 4B). However, differential expression of genes, other than those involved in methionine-regulated pathways, was also observed. In particular, expression of *RNR1* and *CDC21* was repressed three- and fivefold, respectively, in the presence of 5 mM methionine (Fig. 4B). *RNR1* and *CDC21* both function in DNA replication and metabolism; *RNR1* encodes the large subunit of the ribonucleotide reductase complex and *CDC21* encodes thymidylate synthase. Cell cycle expression of both *RNR1* and *CDC21* is dependent on MBF, since the late-G_1-specific periodicity of these genes is abolished in *swi6*Δ and *mbp1*Δ mutants but is unaffected in *swi4*Δ mutants (32, 56). We interpret modest transcriptional defects as significant, since *STB1* expression was reduced but not eliminated in the *stb1*Δ *swi4*Δ mutant. Furthermore, although periodic transcription is abolished, strains lacking *MBP1* produce constitutive levels of mRNA from MCB-driven genes such as *CDC21* (32). Given these results, we did not expect to observe



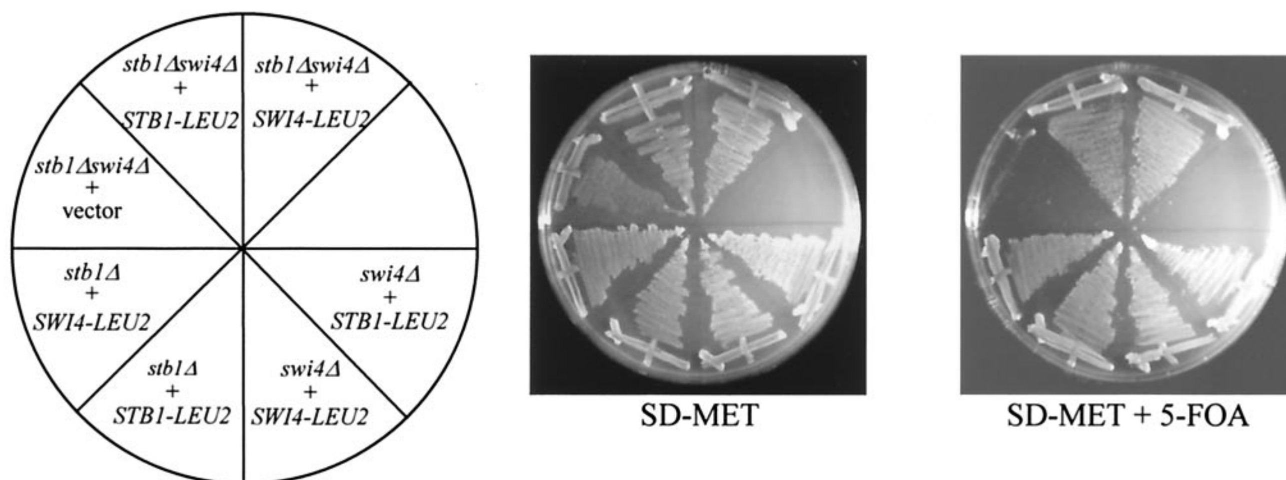


FIG. 2. *SWI4* is required for viability of a *stb1*Δ deletion strain. (A) *stb1*Δ (BY1298), *swi4*Δ (BY184), and *stb1*Δ *swi4*Δ (BY1693) mutants harboring a high-copy *SWI4-URA3* plasmid (pBA314) were grown on SD-URA medium or on SD medium containing 5-FOA. (B) *stb1*Δ (BY1298), *swi4*Δ (BY184) and *stb1*Δ *swi4*Δ (BY1694 and BY1695) mutants containing a *SWI4-URA3* plasmid and harboring either a *SWI4-LEU2* (pBA417), *MET25pr-STB1-LEU2* (pBA1044), or vector (pRS425) plasmid were grown in the absence (SD-MET) or presence (SD-MET + 5-FOA) of 5-FOA.

large differences in the abundance of MBF-dependent transcripts in *stb1*Δ *swi4*Δ mutants. Thus, depletion of *STB1* in the absence of *SWI4* was sufficient to cause transcriptional defects in at least two previously characterized MBF-regulated genes as determined by microarray analysis.

Northern blot analysis confirmed results obtained from DNA microarray experiments (Fig. 4C). Wild-type, *stb1*Δ, *swi4*Δ, and *stb1*Δ *swi4*Δ strains (harboring a *MET25pr-STB1* plasmid) were grown to exponential phase in medium lacking methionine. Cultures were subsequently grown in medium containing 5 mM methionine, and *RNR1* expression was analyzed in samples harvested at time points 4 and 8 h after addition of methionine. *RNR1* transcript levels remained constant in wild-type, *stb1*Δ, and *swi4*Δ strains (Fig. 4C). However, consistent with our microarray experiments, the *stb1*Δ *swi4*Δ double-mutant strain exhibited a gradual decrease in *RNR1*

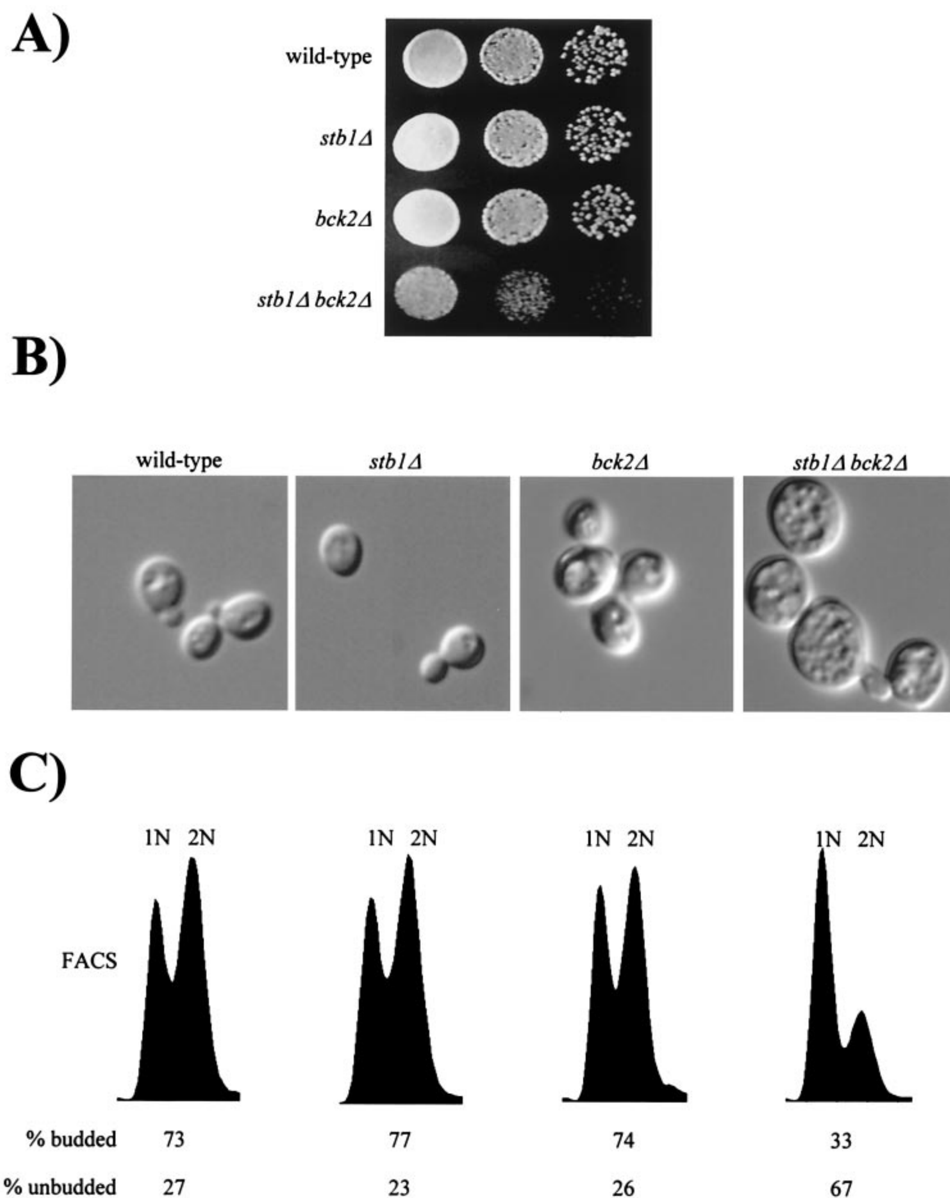


FIG. 3. Growth characteristics of the *stb1*Δ *bck2*Δ double mutant. (A) Slow-growth phenotype of a *stb1*Δ *bck2*Δ mutant strain. Tenfold serial dilutions were prepared from wild-type (BY263), *stb1*Δ (BY805), *bck2*Δ (BY1110), and *stb1*Δ *bck2*Δ (BY1106) cultures, plated onto YEPD medium, and incubated at 30°C. (B) Morphology of wild-type, *stb1*Δ, *bck2*Δ, and *stb1*Δ *bck2*Δ strains. Wild-type (BY263), *stb1*Δ (BY805), *bck2*Δ (BY1110), and *stb1*Δ *bck2*Δ (BY1106) strains were grown to mid-log phase in rich medium. The cells were viewed with Nomarski optics and photographed. (C) DNA content as measured by FACS analysis of samples shown in panel B. The positions of cells with G_1 or G_2 DNA contents are indicated by 1N or 2N, respectively. The percentages of budded and unbudded cells are indicated below the FACS profile.

expression. Following 8 h of growth in 5 mM methionine, a twofold decrease in *RNR1* transcription was detected in the *stb1*Δ *swi4*Δ strain relative to that seen with wild-type or single mutant strains (Fig. 4C).

Taken together, results from DNA microarray and Northern analyses indicate that *STB1* affects expression of at least a subset of MBF target genes, including *RNR1*. We made a genetic observation that further supports this view. In a separate series of experiments, we performed a *stb1*Δ synthetic lethal screening and isolated a *mec1* mutant that requires *STB1* for viability (Fig. 5; see Materials and Methods). *MEC1* encodes an essential kinase which is involved in the G_1, S, and G_2 cell cycle checkpoint pathways in budding yeast (reviewed in reference 13). The 5-FOA sensitivity of a *stb1*Δ *mec1* double mutant (harboring a high-copy *STB1-URA3* plasmid) was rescued by expression of *MEC1* or *STB1* from a *LEU2*-based plasmid (Fig. 5). In addition to that of *STB1* and *MEC1*, expression of *RNR1* also rescued the *stb1*Δ *mec1* phenotype (Fig. 5). Consistent with our microarray and Northern blot analyses, this result suggests that the lethality of the *stb1*Δ *mec1* mutant is due, at least in part, to inadequate expression of *RNR1*.

Microarray analyses were also employed to examine the genome-wide effects of *STB1* overexpression. Ectopic expression of both *STB1* and *MBP1* caused only modest effects on

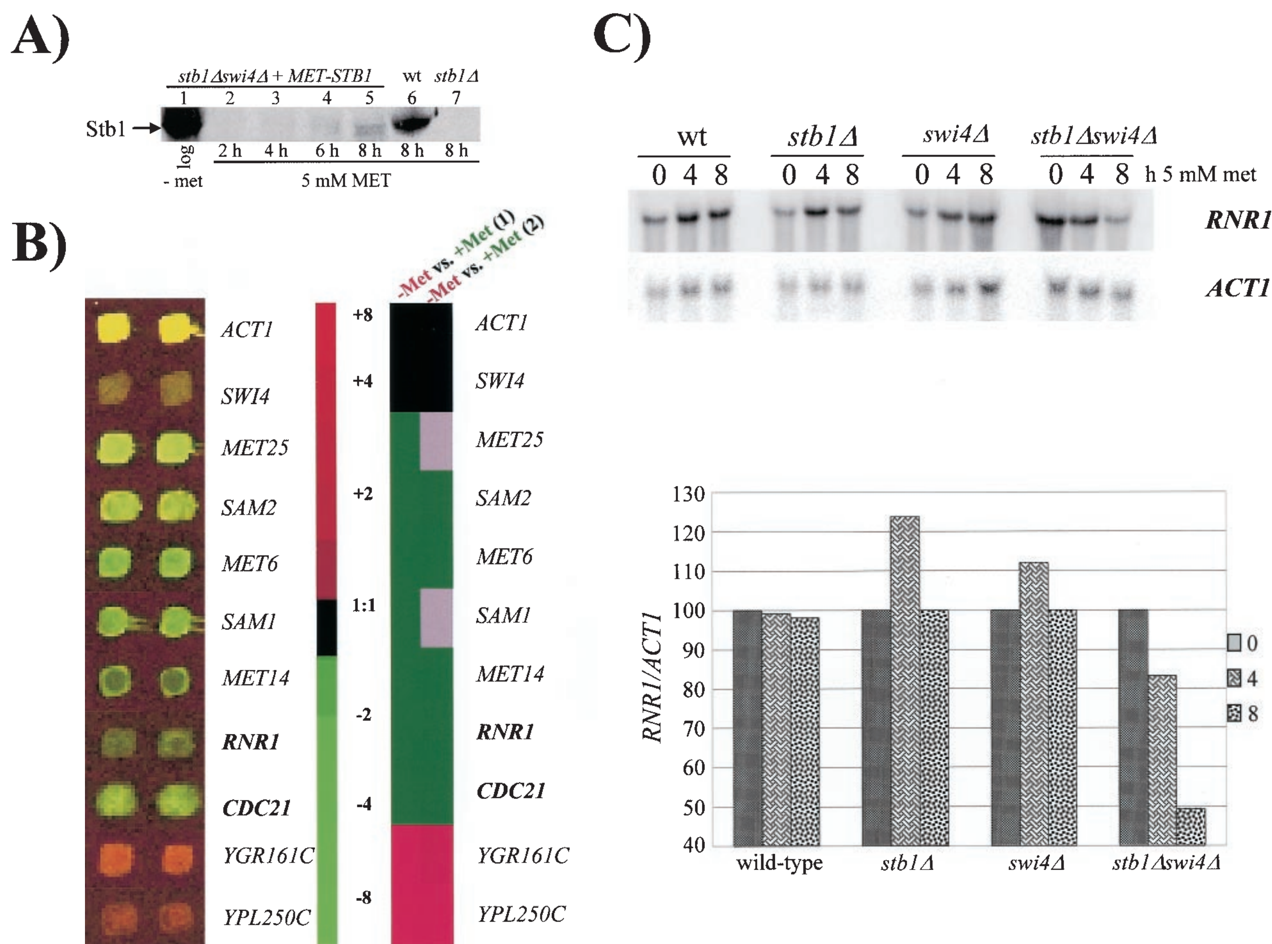


FIG. 4. MBF-regulated genes are repressed in a *stb1*Δ *swi4*Δ double mutant. (A) Anti-Stb1 Western blot analysis of extracts prepared from a *stb1*Δ *swi4*Δ strain harboring a *MET25pr-STB1* plasmid (BY1695; lanes 1 to 5), a wild-type (wt) strain (BY263; lane 6), and a *stb1*Δ strain (BY806; lane 7). Log-phase *stb1*Δ *swi4*Δ strain plus *MET25pr-STB1* cultures were grown in the absence of methionine (lane 1) and subsequently grown in medium supplemented with 5 mM methionine (lanes 2 to 5). Wild-type and *stb1*Δ cultures were also grown in medium supplemented with 5 mM methionine (lanes 6 to 7). (B) A *stb1*Δ *swi4*Δ strain containing a *MET25pr-STB1* construct (BY1695) was grown to mid-log phase in the absence of methionine. Cultures were then diluted and grown in the absence or presence of 5 mM methionine. cDNAs derived from cultures grown in the absence of methionine were labeled with Cy3 fluor, while cDNAs from cultures grown in 5 mM methionine were labeled with a Cy5 fluor. The results for a subset of genes whose expression levels changed at least threefold are shown. Green indicates methionine-dependent repression, while red indicates genes induced in the presence of 5 mM methionine. The reciprocal experiment was also performed (data not shown). (C) *RNR1* is repressed in a *stb1*Δ *swi4*Δ double mutant. Wild-type (wt) (BY263), *stb1*Δ (BY806), *swi4*Δ (BY184), and *stb1*Δ *swi4*Δ (BY1695) mutants (containing a *MET25pr-STB1* plasmid) were grown to mid-log phase in the absence of methionine ($t = 0$). Strains were subsequently grown in minimal medium containing 5 mM methionine. Aliquots were taken at 4- and 8-h time points. Total RNA was isolated from cells and probed with radiolabeled *RNR1* and *ACT1*. The histogram depicts quantitation of the Northern blot. The *RNR1* signal was quantified by phosphorimager analysis, and the values were normalized to the *ACT1* loading control before plotting.

gene expression, as might be expected due to the tight cell cycle regulation of MBF target genes (data not shown). Nonetheless, statistical analysis of these expression experiments revealed a significant correlation between *STB1* and *MBP1* overexpression profiles compared to that of relevant control experiments, consistent with a role for *STB1* in regulating MBF-dependent transcription (data not shown).

Stb1 localizes to the *RNR1* promoter. Since microarray and Northern analyses suggested that *STB1* is required for *RNR1* transcription, we directly examined the association of Stb1 with the *RNR1* promoter. ChIP assays were performed in which wild-type, *stb1*Δ, *mbp1*Δ, and *swi6*Δ cells were harvested during the exponential-growth phase. Cells were fixed with formaldehyde, and chromatin was isolated using affinity-purified Stb1 polyclonal antibodies. The abundance of specific DNA sequences within the immunoprecipitates was measured using PCR and the appropriate primer pairs. Reaction mixtures contained two sets of primers, enabling us to simultaneously measure the relative abundances of Stb1 at *RNR1* and *PHO5* promoters. For *RNR1* measurements, the primer pairs were designed to straddle previously identified MCB elements (15). Specific PCR enrichment of *RNR1* promoter DNA was detected in ChIPs from a wild-type strain (Fig. 6, lane 4). *PHO5* was used as a negative control, since there are no detectable SCB or MCB elements in its promoter and the time of maximal *PHO5* expression does not coincide with SBF and MBF activity (4, 51). DNA from *RNR1* and *PHO5* promoters was detected in formaldehyde cross-linked whole-cell extracts (Fig. 6, lanes 1

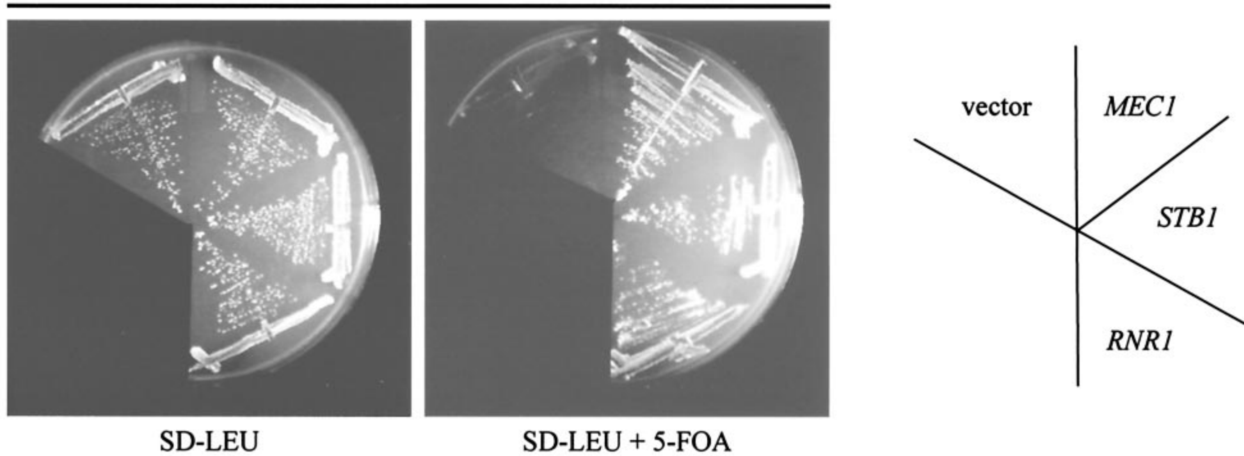


FIG. 5. *RNR1* rescues the lethality of a *stb1*Δ *mec1* mutant. A *stb1*Δ *mec1* strain (BY1830) containing a *STB1-URA3* plasmid (pBA1220) and harboring either a *STB1-LEU2* (pBA1010), *MEC1-LEU2* (pBA1624), *RNR1-LEU2* (pBA1625), or vector (pRS425) plasmid was grown in the absence (SD-LEU) or presence (SD-LEU + 5-FOA) of 5-FOA.

to 3, 6 to 8, 11 to 13, and 16 to 18). As expected, control assays using non-cross-linked lysates were not enriched for promoter-specific DNA (Fig. 6, lanes 5, 10, 15, and 20) and Stb1 antibodies did not cause enrichment of *PHO5* promoter-specific DNA (Fig. 6, lanes 4, 9, 14, and 19). Moreover, ChIP assays using *stb1*Δ, *mbp1*Δ, and *swi6*Δ strains and affinity-purified Stb1 antibodies were not enriched for *RNR1* promoter DNA (Fig. 6, lanes 9, 14, and 19). These experiments suggest that Stb1 specifically localizes to MCB elements located within the *RNR1* promoter. Consistent with our hypothesis, Stb1 requires Swi6 and Mbp1 to associate with MCB promoter elements, suggesting that Stb1 functions in the context of MBF.

Interaction with Swi6 is required for Stb1 function. Stb1 was previously characterized as a Swi6-binding protein (25), but the biological relevance of the Stb1-Swi6 interaction was not understood. We used far-Western analysis to further examine the Stb1-Swi6 physical interaction (Fig. 7A). Whole-cell extracts were prepared from bacterial cells expressing either full-length *STB1* (Stb1), truncated Stb1 protein lacking 70 amino acids at the N terminus (Stb1ΔN) (25), or a truncated form of Stb1 lacking the C-terminal 210 amino acids (Stb1ΔC). Uninduced (Fig. 7A, lanes 1, 3, and 5) and IPTG-induced (Fig. 7A, lanes 2, 4, and 6) extracts were blotted to nitrocellulose and probed with affinity-purified Stb1 antibodies or [^{35}S]methionine-labeled Swi6 (Fig. 7A). The anti-Stb1 immunoblot revealed that all three forms of Stb1 protein were expressed following IPTG induction (Fig. 7A, upper panel). Following incubation with ^{35}S-labeled Swi6, a difference in binding between full-length and truncated forms of Stb1 was observed. Unlike full-length Stb1 and Stb1ΔC, which bound Swi6 directly (Fig. 7A, lanes 2 and 6), a Stb1ΔN-Swi6 interaction was not observed (Fig. 7A, lane 4). This suggests that the first 70 N-terminal amino acids of the Stb1 protein are required for Swi6 binding. We next expressed full-length *STB1*, *STB1*ΔN, and *STB1*ΔC from the repressible *MET25* promoter in yeast transformants (Fig. 7B). Since all three forms of the Stb1 protein were expressed in both a *stb1*Δ mutant (Fig. 7B) and a wild-type strain (data not shown), we asked whether the Stb1ΔN protein was able to complement *STB1*-dependent phenotypes (Fig. 7C, D, and E). As described above, a *stb1*Δ mutant is defective in MCB-driven

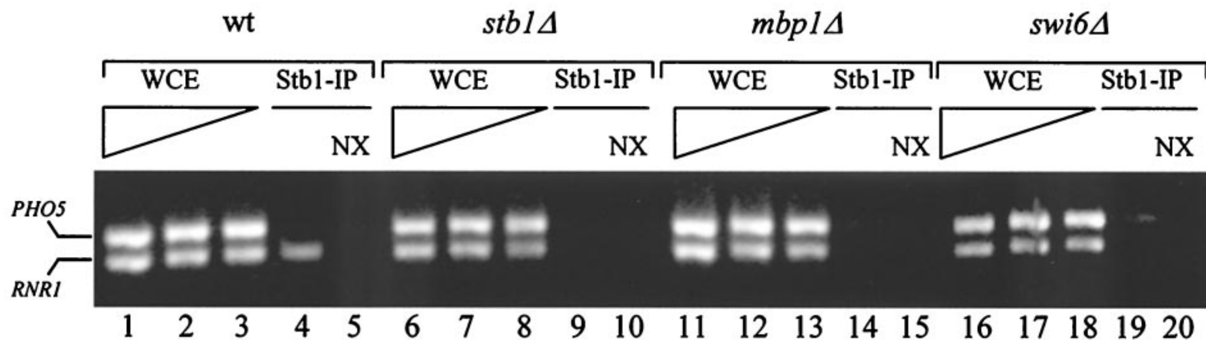


FIG. 6. Stb1 localizes to the *RNR1* promoter. Wild-type (wt) (BY263), *stb1*Δ (BY806), *mbp1*Δ (BY551), and *swi6*Δ (BY185) strains were grown to mid-log phase and cross-linked with formaldehyde. Cross-linked and non-cross-linked (NX) whole-cell extracts (WCE) were prepared and subjected to immunoprecipitation (IP) with α-Stb1 antibodies (Stb1-IP). PCR was performed on serial dilutions of the WCE samples (lanes 1 to 3, 6 to 8, 11 to 13, and 16 to 18) and on immunoprecipitated samples (lanes 4 to 5, 9 to 10, 14 to 15, and 19 to 20) to amplify associated DNA. Primers used for multiplex PCR were designed to flank the *PHO5* promoter or the MCB elements of the *RNR1* promoter.

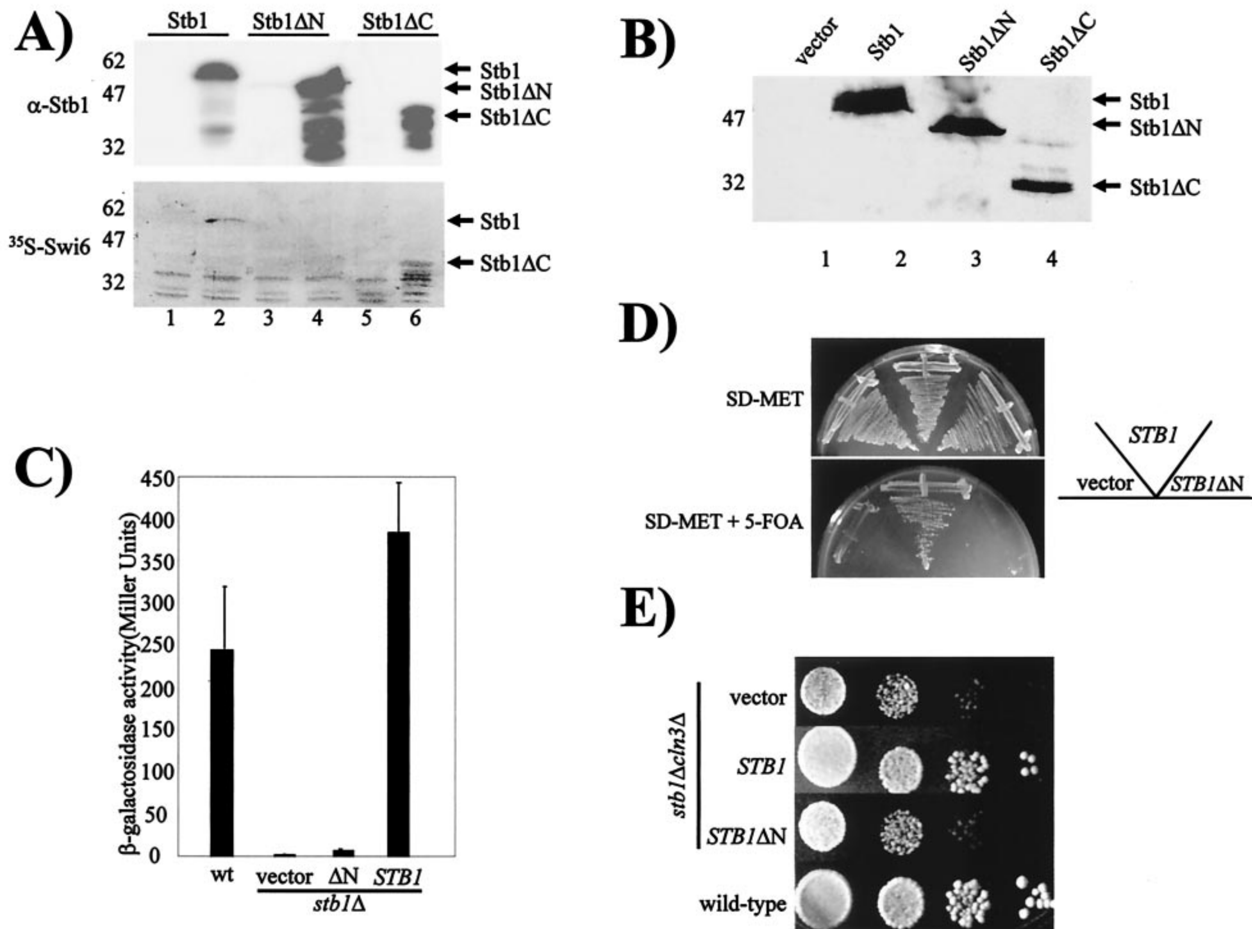


FIG. 7. Interaction with Swi6 is required for Stb1 function. (A) Uninduced (lanes 1, 3, and 5) and IPTG-induced (lanes 2, 4, and 6) bacterial whole-cell extracts were prepared from cells expressing full-length *STB1* (pBA1622; lanes 1 and 2), *STB1*ΔN (pBA1282; lanes 3 and 4) and *STB1*ΔC (pBA1623; lanes 5 and 6). Extracts were subjected to SDS-PAGE, and duplicate gels were transferred to nitrocellulose. One blot was probed with α-Stb1 antibodies (α-Stb1), while a second blot was probed with in vitro-translated [^{35}S]methionine Swi6 (^{35}S-Swi6), as indicated to the left of the photograph. (B) Anti-Stb1 Western blot analysis of extracts prepared from a *stb1*Δ mutant (BY806) harboring a p415 *MET25* vector (lane 1) or a *STB1* (pBA1044, lane 2), *STB1*ΔN (pBA1626, lane 3), or *STB1*ΔC (pBA1627, lane 4) plasmid. Cultures were grown to mid-log phase in medium lacking methionine. (C) Wild-type (wt) (BY263) and *stb1*Δ (BY806) cells were transformed with the *MCB*::*lacZ* plasmid (pBA487). The *stb1*Δ strain was also transformed with a vector (p415 *MET25*) or a *STB1* (pBA1044) or *STB1*ΔN (pBA1626) plasmid. Strains were grown at 30°C to log phase in the absence of methionine, cell lysates were prepared, and β-galactosidase activity was measured. Depicted activity values represent the means of three experiments, and error bars indicate standard deviations for three experiments. (D) A *stb1*Δ *swi4*Δ (BY1693) mutant containing a *SWI4-URA3* plasmid and harboring either a vector (p415 *MET25*) or a *STB1-LEU2* (pBA1044) or *STB1*ΔN-*LEU2* (pBA1626) plasmid was grown in the absence (SD-MET) or presence (SD-MET + 5-FOA) of 5-FOA. (E) Tenfold serial dilutions were prepared from a wild-type (BY263) and a *stb1*Δ *cln3*Δ (BY822) strain harboring a vector (p415 *MET25*) or a *STB1* (pBA1044) or *STB1*ΔN (pBA1626) plasmid, plated onto medium lacking methionine, and incubated at 30°C.

UAS activity (Fig. 1A). Unlike expression of full-length *STB1*, which restores *MCB*::*lacZ* activity to wild-type levels, *STB1*ΔN does not complement the defect in MBF-dependent reporter gene expression (Fig. 7C). Furthermore, expression of *STB1*ΔN does not complement the 5-FOA sensitivity of a *stb1*Δ *swi4*Δ double mutant, demonstrating that full-length Stb1 is required for viability in the absence of Swi4 (Fig. 7D). The inability to complement these phenotypes suggests that the MBF-specific function of *STB1* is dependent on its interaction with Swi6. Consistent with these observations, Stb1ΔN also fails to complement the slow growth phenotype of a *stb1*Δ *cln3*Δ double mutant (Fig. 7E), indicating that Swi6 binding is also required for Stb1 function in G_1 cell cycle progression.

Cln-Cdc28-dependent phosphorylation inhibits the Stb1-Swi6 interaction. Previous studies have suggested that the Cln3-Cdc28 complex is a major activator of SBF- and MBF-dependent transcription (53, 59). However, Cln3 does not appear to phosphorylate or interact with components of either SBF or MBF, suggesting that Cln3-dependent activation is indirect (59). Stb1 was previously identified as a Swi6-interacting protein by affinity chromatography and coimmunoprecipitation experiments (25), and Swi6 binding is likely required for Stb1 function in G_1 phase (Fig. 7). Stb1 is also a phosphoprotein in yeast, and Ho et al. have shown that Stb1 is an excellent substrate for Cln-Cdc28 kinase complexes in vitro and in vivo (25). Thus, Cln-Cdc28 complexes might regulate MBF through

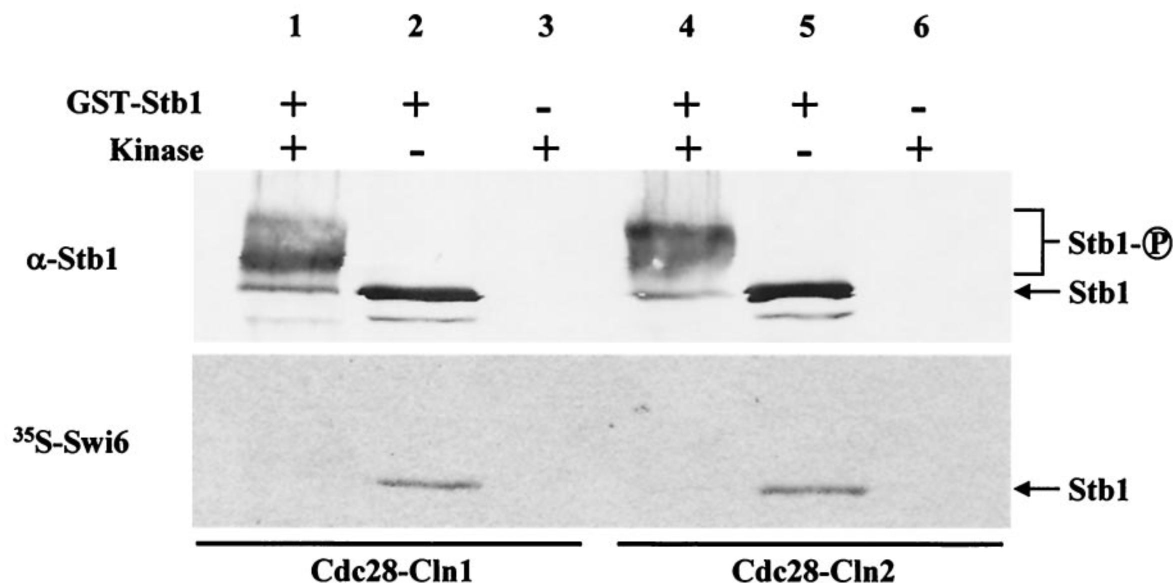


FIG. 8. Cln1- and Cln2-dependent phosphorylation of Stb1 inhibits the Swi6-Stb1 interaction in vitro. Bacterially expressed GST-Stb1 was phosphorylated in vitro with Cdc28-Cln1 (lane 1) or Cdc28-Cln2 (lane 4) purified from insect cells. Phosphorylated Stb1 (lanes 1 and 4) and unphosphorylated Stb1 (lanes 2 and 5) were subjected to SDS-PAGE. Duplicate gels were transferred to nitrocellulose. One blot was probed with α-Stb1 antibodies (α-Stb1). A second blot was probed with in vitro-translated [^{35}S]methionine Swi6 (^{35}S-Swi6).

phosphorylation of Stb1. To explore this possibility, we employed far-Western analysis to examine the effects of Cln-dependent phosphorylation on the Stb1-Swi6 interaction. Using Cln1-Cdc28 or Cln2-Cdc28 kinase purified from insect cells, purified recombinant GST-Stb1 was phosphorylated in vitro (Fig. 8). Unphosphorylated Stb1 and Stb1 phosphorylated by Cln-Cdc28 were blotted to nitrocellulose and probed with affinity-purified Stb1 antibodies or [^{35}S]methionine-labeled Swi6 (Fig. 8). Cln-dependent phosphorylation of Stb1 was confirmed by Western blot analysis, since phosphorylated Stb1 migrated more slowly than the unphosphorylated protein (Fig. 8, upper panel, lanes 1 and 4). Following incubation with ^{35}S-labeled Swi6, a clear difference in binding between phosphorylated and unphosphorylated Stb1 was observed. Unphosphorylated Stb1 was able to bind Swi6 (Fig. 8, lanes 2 and 5), whereas Stb1 which had been phosphorylated by Cdc28-Cln1 or Cdc28-Cln2 failed to interact with Swi6 (Fig. 8, lanes 1 and 4). Therefore, Cln-dependent phosphorylation inhibits the Stb1-Swi6 interaction in vitro and, hence, may play an inhibitory role in regulating MBF-dependent transcription (see Discussion).

DISCUSSION

In vivo footprinting experiments have revealed that SBF and MBF are bound to promoter elements throughout the G_1 phase (24, 33). Since transcription only occurs late in G_1 phase, DNA binding is not sufficient and specific activation of SBF and MBF must occur. So far, intense genetic scrutiny has failed to reveal activation mechanisms or regulatory differences between SBF and MBF complexes. In this paper, we describe a series of experiments whose results suggest a role for Stb1 in the regulation of MBF-specific transcription at Start.

A role for Stb1 as a component of MBF. With the exception of the *HO* gene, whose expression is completely dependent on the presence of SBF (6, 7), only synthetic SCB- and MCB-driven reporter genes exhibit a specific dependence on SBF and MBF, respectively (2, 56). We found that a strain lacking *STB1* was specifically defective in *MCB*::*lacZ* expression. *STB1* had been previously implicated as a cell cycle regulator (25); a *stb1*Δ *cln3*Δ mutant is slow growing and is severely delayed in G_1 phase, accumulating a high fraction of large unbudded cells with 1 N DNA content (25). Based on these results, it was concluded that *STB1* is required for G_1/S-phase progression and functions in a pathway parallel to that of *CLN3* to activate G_1-specific transcription. However, the precise role for *STB1* in transcriptional activation remained unclear, since conventional Northern analyses failed to reveal defects in transcript levels or cell cycle periodicity of G_1-regulated genes in a *stb1*Δ mutant (25). The *MCB*::*lacZ* defect described here represents direct evidence that Stb1 functions as a transcriptional activator.

In the absence of *SWI4*, G_1-specific transcription and cell cycle progression is dependent on MBF (32). We found that *STB1*, like *MBP1*, has an overlapping essential function with *SWI4*. Unlike the case of a *stb1*Δ *swi4*Δ mutant, no obvious additional phenotypes were found in *stb1*Δ *mbp1*Δ or *stb1*Δ *swi6*Δ strains. Although these genetic results do not preclude a role for *STB1* in regulating SBF-dependent genes, they strongly support our other experiments showing that the primary role for *STB1* in G_1 transcription involves MBF. Consistent with an MBF-specific function, we also observed a clear reduction in transcription of two MBF-regulated genes, *RNR1* and *CDC21*, when Stb1 levels were limiting. This result may indicate that *STB1* is required for transcription of only a subset of MBF targets. Alternatively, our experimental conditions may create a bias for identifying the MBF-regulated genes which are most sensitive to Stb1 levels.

Our observation that a *mec1* mutant requires *STB1* for viability is also consistent with an MBF-specific role for *STB1*. Since *MEC1* is required for viability in our strain background

(S288C), we infer that the *mec1* mutation we identified is a hypomorphic allele. Although we have not yet pursued a detailed molecular characterization of the *sls* allele of *MEC1*, our *RNR1* suppression data allow us to formulate a model for the *stb1*Δ *mec1* genetic interaction. The *RNR1* gene product is limiting for ribonucleotide reductase activity in yeast extracts and is therefore tightly regulated (57, 61). One form of *RNR1* regulation occurs at the transcriptional level. Ho et al. previously showed that SBF is involved in up-regulating *RNR* gene expression in response to DNA damage (26). However, periodic expression of *RNR1* (in the absence of DNA damage) is dependent on MBF rather than SBF (32, 56). In addition to transcriptional regulation, Rnr1 is also regulated at the posttranslational level (61). Rnr1 binds to a protein known as Sml1 which inhibits its activity when DNA synthesis is not required (61). During S phase, a signal is transmitted to relieve Sml1 inhibition of Rnr1; Mec1 appears to remove the inhibitory effect of Sml1 on Rnr1 during S phase, thus facilitating DNA replication (61). We suggest that the Sml1-mediated negative control of Rnr1 is not relieved in our *stb1*Δ *mec1* mutant. Hence, inhibition of Rnr1 activity coupled with reduced *RNR1* gene expression (in the absence of *STB1* [Fig. 4 and 5]) may result in cellular dNTP levels which are inadequate to support DNA synthesis.

Stb1 localizes to the *RNR1* promoter, and association with MCB element-containing promoters is indirect and dependent on the presence of Mbp1 and Swi6. Consistent with this finding, efforts to purify MBF from whole-cell extracts revealed that an unidentified protein (with a molecular weight similar to that of Stb1) copurified with Mbp1 and Swi6 (32) and Stb1 copurifies with Swi6 and Mbp1 when Swi6 is isolated from yeast extracts by means of an affinity-tagging protocol (N. Krogan and J. Greenblatt, personal communication). Although our ChIP experiments further implicate Stb1 as a component of MBF, biochemical studies have shown that Mbp1-Swi6 and Swi4-Swi6 complexes produced by in vitro translation are sufficient for DNA binding (32, 45). Thus, we propose that Stb1 localization to MCB-driven promoters functions to regulate MBF-dependent transcription rather than mediate MBF-DNA binding.

Consistent with our findings, analyses of genome-wide ChIP and microarray studies have provided additional evidence suggesting that Stb1 acts as a direct regulator of G_1-specific transcription (34). However, this strictly computational approach failed to identify the *RNR1* promoter as a significant Stb1 target, while our results clearly show Mbp1-dependent localization of Stb1 to the *RNR1* promoter. Statistical analysis of genomic binding data also suggested that Stb1 does not share common promoter targets with Swi6 (34). In contrast to this interpretation, Stb1 is a Swi6-binding protein (25) and observations from this study indicate that Stb1 association with chromatin is dependent on the presence of Swi6 and that an interaction with Swi6 is important for the cell cycle-dependent function of Stb1. It is difficult to address Stb1 specificity by means of analysis of genome-wide ChIP experiments, since both MBF and SBF were capable of binding most of the identified Stb1 target promoters in these experiments (34). Statistical analyses of genome-wide expression and binding data can provide many testable hypotheses with regard to the transcriptional circuitry of budding yeast (34). However, given the nature of genomic experiments, more-focused investigations are required to confirm specific details proposed by these models.

Swi6 is a common subunit of both SBF and MBF. Ho et al. previously showed that the Swi6 ankyrin repeat domain is required for the Stb1-Swi6 interaction (25). Mutational analyses of Swi6 ankyrin repeats identified several point mutants that retained the ability to bind DNA in the context of SBF and MBF (17). However, these mutants failed to induce SBF- and MBF-dependent gene expression, indicating that the Swi6 ankyrin repeat domain is important for transcriptional activation (17, 19, 46). In similarity to *stb1*Δ mutants, most Swi6 ankyrin repeat domain mutants showed severe defects in MCB reporter gene expression while the SCB reporter gene was less affected (17). These results imply that the Swi6-Mbp1 interaction or MBF activity might have a stronger dependence on the ankyrin domain, and this dependence may account for the observed MBF-specific function of *STB1*. Alternatively, Stb1, in addition to interacting with the Swi6 ankyrin repeat domain, may associate directly with Mbp1. Homologous ankyrin repeat domains are also found in the central region of Swi4, and Mbp1 and far-Western analysis revealed that Stb1 can bind Mbp1 directly in vitro (M. Costanzo, unpublished data). Whether nonconserved residues in the ankyrin domain of Mbp1 mediate specific interactions with Stb1 in vivo requires further investigation.

A role for *STB1* in regulating G_1-specific transcription. Based on our results, we present a model for G_1-specific transcription whereby SBF and MBF transcription factors are differentially regulated (Fig. 9). *CLN3* is the major activator of Start transcription and likely activates both SBF and MBF through Swi6 (53, 59). However, a *cln3*Δ mutant is viable and still undergoes SBF- and MBF-dependent transcription, indicating the existence of alternate mechanisms to activate G_1-specific transcription and promote entry into S phase (43, 53). Consistent with this model, Ho et al. previously showed that *STB1* functions in a pathway parallel to that of *CLN3* to regulate G_1-specific transcription (25).

In addition to *STB1*, the *BCK2* (bypass of C-kinase mutation) gene also appears to be involved in an alternative pathway(s). In similarity to a *stb1*Δ *cln3*Δ strain, *bck2*Δ *cln3*Δ double mutants grow slowly, accumulate in G_1 phase, and show reduced levels of Start transcription (11, 16, 58). Unlike that of *stb1*Δ mutants, which do not have obvious transcriptional defects (25), expression of SBF- and MBF-regulated genes is modestly delayed, but not abolished, in *bck2*Δ mutants (11). Furthermore, overexpression of *BCK2* results in transcriptional induction of several SBF and MBF target genes (11, 16, 58). These observations suggest that *BCK2* likely activates both SBF- and MBF-dependent transcription.

We found that in similarity to *bck2*Δ *cln3*Δ and *stb1*Δ *cln3*Δ strains, *stb1*Δ *bck2*Δ double mutants are also slow growing and accumulate in the G_1 phase of the cell cycle. The G_1 delay phenotype observed in these mutants suggests that there are at least three independent pathways for activation of Start transcription. Our present work suggests that *STB1*, unlike *CLN3* and *BCK2*, functions specifically to regulate MBF-dependent transcription. Therefore, in the absence of *STB1* and *CLN3* or *BCK2*, at least two SBF/MBF activation pathways are compromised, resulting in reduced levels and/or timing of Start tran-

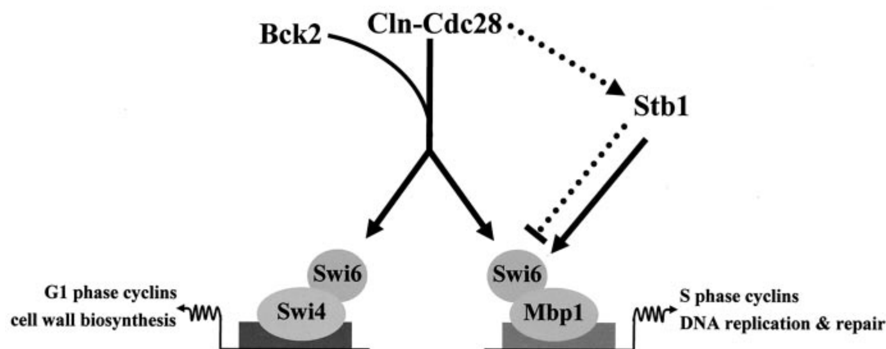


FIG. 9. A model for Stb1-dependent regulation of MBF transcription. Our model predicts that Stb1 functions in an alternative pathway to activate G_1-specific transcription. Cln3-Cdc28 is the major activator of SBF- and MBF-dependent transcription, whereas Stb1 and Bck2 function as alternate activators of Start transcription. Unlike Cln3 and Bck2, Stb1 specifically regulates MBF. Prior to Start, Stb1 is predominantly unphosphorylated and interaction with Swi6 serves to activate MBF-dependent transcription (solid lines). Immediately following the time of maximal Start-specific transcription, Cln-Cdc28-dependent phosphorylation of Stb1 mediates dissociation of the Stb1-MBF complex, resulting in termination of MBF-specific transcription (dotted lines).

scription and a delay in G_1 phase. Consistent with this prediction, activation of *RNR1*, *CLN1*, and *CLN2* transcription was significantly delayed in a *stb1*Δ *cln3*Δ double mutant (25).

Our work also suggests that phosphorylation of Stb1 might regulate its ability to associate with MBF. Stb1 is both a Cln-Cdc28 substrate and a Swi6-interacting protein (25), and our far-Western analysis revealed that Cln-dependent phosphorylation of Stb1 inhibits the Swi6-Stb1 interaction in vitro. Since Swi6 binding is likely required for Stb1 function, we propose that phosphorylation of Stb1 might play a role in down-regulating MBF-dependent transcription (Fig. 9). Previous studies demonstrated that Stb1 phosphorylation is cell cycle periodic, with maximal phosphorylation occurring after Start transcription but prior to DNA replication (25). Hence, it is possible that Cln-dependent phosphorylation mediates the dissociation of Stb1 from MBF, resulting in down-regulation of MCB-driven gene expression. SBF repression is likely mediated by Clb2-Cdc28 kinase activity (1, 47), and DNA microarray analysis has also shown that Clb2 overexpression results in repression of a large number of G_1 phase-regulated genes (51). Previous studies have also shown that Clb2 interacts with the ankyrin repeat domain of Swi4 (47). Thus, in similarity to that by Clb2, Stb1-dependent regulation of MBF transcription may also be mediated through its interaction with ankyrin repeats.

Analogy between budding yeast MBF and E2F in higher eukaryotes. Despite *MBP1* having conserved functional and sequence homologues in distantly related yeasts (5), no homologues have yet been identified in higher eukaryotes. However, members of the E2F/DP1 family of transcription factors may be considered functionally analogous to MBF (30). E2F is under the control of the retinoblastoma protein, Rb, which binds and inhibits E2F (18). The Rb-E2F interaction is regulated by phosphorylation; in noncycling cells or in early G_1 phase, Rb is hypophosphorylated and inhibits E2F activity. Conversely, in late G_1 phase, Rb is progressively phosphorylated by cyclin-Cdk complexes and, consequently, its affinity for E2F diminishes. The release of Rb triggers the activation of E2F target genes, which allows cells to progress through the G_1/S transition (18). In this report, we propose that Stb1 plays a role as an MBF-specific regulator and that analogous to Cdk-dependent phosphorylation of Rb, Cdc28-dependent phosphorylation might inhibit the interaction between Stb1 and MBF. Therefore, cyclin-Cdk complexes appear to affect E2F- and MBF-dependent transcription, at least in part, through regulation of protein-protein interactions between transcription factors and regulatory proteins such as Rb and Stb1. Rb inhibits E2F transcriptional activation, to some extent, by recruiting chromatin remodeling factors such as histone deacetylases and members of the SWI/SNF complex (18). Similarly, Stb1 was found to interact with the Sin3-Rpd3 histone deacetylase complex (31). However, our data suggest that, unlike Rb, Stb1 functions as an activator of MBF-dependent transcription. A direct role for Stb1 in transcriptional repression remains unclear.

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