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# Transcriptional Coregulation by the Cell Integrity Mitogen-Activated Protein Kinase Slt2 and the Cell Cycle Regulator Swi4

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**In *Saccharomyces cerevisiae,* the heterodimeric transcription factor SBF (for SCB binding factor) is composed of Swi4 and Swi6 and activates gene expression at the $G_1$/S-phase transition of the mitotic cell cycle. Cell cycle commitment is associated not only with major alterations in gene expression but also with highly polarized cell growth; the mitogen-activated protein kinase (MAPK) Slt2 is required to maintain cell wall integrity during periods of polarized growth and cell wall stress. We describe experiments aimed at defining the regulatory pathway involving the cell cycle transcription factor SBF and Slt2-MAPK. Gene expression assays and chromatin immunoprecipitation experiments revealed Slt2-dependent recruitment of SBF to the promoters of the $G_1$ cyclins *PCL1* and *PCL2* after activation of the Slt2-MAPK pathway. We performed DNA microarray analysis and identified other genes whose expression was reduced in both *SLT2* and *SWI4* deletion strains. Genes that are sensitive to both Slt2 and Swi4 appear to be uniquely regulated and reveal a role for Swi4, the DNA-binding component of SBF, which is independent of the regulatory subunit Swi6. Some of the Swi4- and Slt2-dependent genes do not require Swi6 for either their expression or for Swi4 localization to their promoters. Consistent with these results, we found a direct interaction between Swi4 and Slt2. Our results establish a new Slt2-dependent mode of Swi4 regulation and suggest roles for Swi4 beyond its prominent role in controlling cell cycle transcription.**

In the budding yeast, *Saccharomyces cerevisiae*, the transcription factor SBF (SCB binding factor) induces cell-cycle-dependent expression of a large group of genes at the $G_1$/S-phase transition or Start (reviewed in reference 8). SBF is a heterodimeric complex composed of two proteins, Swi4 and Swi6, which bind the repeated upstream regulatory sequence CACGAAA (SCB, for Swi4,6-dependent cell cycle box [8, 32]). Biochemical studies show that Swi4 is the component of SBF that specifically binds the SCB sequence through an N-terminal helix-turn-helix DNA-binding domain (55). In contrast, Swi6 has no DNA binding activity but is present in SBF because of its interaction with Swi4 via the carboxy-terminal regions of the two proteins (3, 48). Swi6 is also a component of the transcription complex MBF (MCB-binding factor) that is composed of Swi6 and the DNA binding protein Mbp1 and binds the promoter element ACGCGT (MCB, for *Mlu*I cell cycle box [8]).

DNA microarray experiments provide an overview of cell-cycle-regulated genes in budding yeast; more than 200 genes show peak expression in late $G_1$ phase ($G_1$ genes [12, 52]). Most of the $G_1$ genes have at least one SCB or MCB element in their upstream sequences, implicating SBF and MBF in the induction of many $G_1$ genes. The $G_1$ group of genes includes the Cdc28-associated $G_1$ cyclins *CLN1* and *CLN2* and the Pho85-associated $G_1$ cyclins *PCL1* and *PCL2.* These four cyclins activate their cognate cyclin-dependent kinase (Cdk) in late $G_1$ and are required for $G_1$-to-S phase progression (reviewed in reference 2). SBF is essential for the expression of the $G_1$-specific *HO* gene and is required for maximal $G_1$-specific induction of *CLN1, CLN2, PCL1, PCL2*, and various cell wall genes (8, 28, 46). Recently, about 200 additional putative targets of SBF and MBF were identified using yeast intergenic arrays coupled with chromatin immunoprecipitation (ChIP) (29).

SBF-mediated gene expression is controlled at multiple levels, including binding of SBF to SCBs, changes in the subcellular localization of Swi6, and the activation of SBF at Start. In vivo footprinting and ChIP experiments show that SBF is bound to the SCBs upstream of three different SBF-dependent genes throughout late M and $G_1$ phases (13, 23, 33). SBF binding to the SCBs of the *HO* promoter depends on chromatin remodeling events (13, 34), suggesting that chromatin remodeling may be a feature of SBF binding to the upstream regulatory regions of a variety of genes. The binding of SCBs by SBF is not coincident with SBF-mediated transcription; rather, a second event must occur for SBF activation, and the Cln3-Cdc28 Cdk plays an important role in this process (19, 54). In fact, DNA microarray experiments show that expression of most $G_1$ genes is induced by overexpression of *CLN3* (52). However, the mechanism of Cln3-dependent activation of SBF remains unclear, and direct interaction of Cln3 with SBF has not been reported.

Strains lacking SBF arrest in $G_1$, and many $G_1$ genes have at least one copy of the SCB element in their promoters; therefore, a key role of SBF is to promote $G_1$-specific transcription. However, there are fewer than 300 genes whose transcription

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peaks at Start but more than 1,155 genes whose promoters contain matches to the SCB consensus sequence (http://cgsigma.cshl.org/jian/). A comparison of the number of SCB sites upstream of $G_1$ genes with the frequency of SCB sites upstream of a control group of non-cell-cycle-regulated genes, reveals that SCBs are found more frequently upstream of non-cell-cycle-regulated genes than MCB sites or sites for a $G_2$-specific transcription factor (MCM/SFF sites [52]). This analysis suggests that SCB elements and SBF may regulate the transcription of many genes other than those induced at Start.

One pathway that may regulate SBF outside of Start is the protein kinase C (*PKC1*) pathway (reviewed in reference 25). *PKC1* encodes an essential serine-threonine-specific protein kinase that is the yeast homolog of members of the mammalian PKC family of genes (37). Pkc1 activates a mitogen-activated protein kinase (MAPK) cascade that consists of (i) the MEKK (MAPK kinase kinase) Bck1, (ii) the redundant MEKs (MAPK kinases) Mkk1 and Mkk2, and (iii) the MAPK Slt2/Mpk1. *pkc1* mutants have thin cell walls and an osmoremedial sensitivity to a variety of cell wall stresses such as heat shock. Strains carrying a deletion of *BCK1*, *SLT2*, or both *MKK1* and *MKK2* are sensitive to high temperature but are viable at 25°C (35). Since *PKC1* mutants are inviable, *PKC1* must have other roles besides activation of the Slt2-MAPK pathway. Consistent with these genetic results, recent studies show that *PKC1* is required for both the depolarization and the repolarization of the actin cytoskeleton upon cell wall stress (18). However, components of the *PKC1*-MAPK pathway module are required only for repolarization; both *slt2* and *bck1* mutants have a depolarized actin cytoskeleton, with delocalization of actin cortical spots, abnormal accumulation of secretory vesicles, and defects in polarized cell growth (14, 18, 43). These studies suggest that the *PKC1*-MAPK pathway is required for cell polarization and for maintenance of cell wall integrity, while an as-yet-uncharacterized *PKC1*-dependent pathway is required for depolarization of the actin cytoskeleton. Slt2 kinase is activated by heat shock (31) and hypo-osmotic shock (17) during periods of polarized growth, such as bud formation and mating projection formation (59), and in response to actin perturbation (24).

In many systems, the major targets of MAPK cascades are transcription factors, and activation of the cascade leads to altered gene expression (reviewed in reference 56). Indeed, recent DNA microarray work showed transcriptional modulation of 90 genes in a strain expressing an activated allele of *PKC1* (27, 47). Currently, only two transcription factors have been identified as targets of Slt2: the MADS-box transcription factor Rlm1 (20, 58) and SBF (39). A genome-wide survey for genes whose expression was altered after expression of a constitutively active $MKK1^{S386P}$ allele for 4 h identified 25 affected genes (30). Twenty-four of the $MKK1^{S386P}$-induced genes were partially dependent on *RLM1*. This pattern suggests that, after 4 h of Slt2 activation, Rlm1 may mediate the majority of the Slt2 effects. However, the phenotypes of *rlm1*Δ strains are much less severe than those seen in *PKC1*-MAPK pathway mutants (57), suggesting that Rlm1 is not the only important transcriptional target of Slt2. In contrast, *swi4* and *swi6* mutant strains share phenotypes similar to those of strains mutated for *PKC1* pathway genes. Some *swi4*Δ strains show a temperature-sensitive growth defect that is suppressed by sorbitol (28, 39), and both *swi4*Δ and *swi6*Δ strains are sensitive to cell wall stressors (28). Further, like *PKC1*-MAPK pathway mutants, *swi4* mutants also exhibit defects in both bud emergence and projection formation (22, 39). These phenotypic similarities suggested that SBF might be a major transcriptional target of Slt2. Indeed, genetic studies, coimmunoprecipitation experiments, and kinase assays have established SBF as a target of Slt2 kinase (39).

Although the role of Slt2 in SBF regulation is not known, several observations suggest that Slt2 may activate SBF toward a subset of target genes. First, genetic evidence suggests a role for the *PKC1*-MAPK pathway at Start (41, 43), and this role may be discharged through SBF. Second, the Pkc1 pathway and Slt2 are required for maximal heat shock-dependent induction of only a subset of SBF-dependent $G_1$ genes, including *PCL1* and *PCL2* (39), and cell wall genes (28). Finally, overexpression of the $G_1$ cyclins *PCL1* and *PCL2*, but not *CLN1* or *CLN2*, suppresses the cell lysis defects of a *slt2*Δ strain (39).

In this study, we test the hypothesis that Slt2 may act to modulate SBF to induce transcription of only a subset of SBF-dependent genes. We began by exploring the regulation of the SBF- and Slt2-dependent gene, *PCL1*. Our genetic approaches, coupled with DNA microarray analysis and ChIP experiments, establish a new Slt2-dependent mode of Swi4 regulation and suggest roles for Swi4 beyond cell cycle control.

## MATERIALS AND METHODS

**Plasmid constructs.** The *PCL1* promoter-*lacZ* reporter plasmid, prPCL1$_{-751\text{-}146}$ (pBA537), was previously described as pΔSS-HCS26 (46). To construct a *PCL2* promoter-*lacZ* reporter construct, the *PCL2* promoter from −982 to −82 bp from the start site was amplified by using primers with the following sequences: 5′-AACGCGTCGACCGTAATTCTATCGATGGACC-3′ and 5′-AACGCGTCGACGGAGAATTATAAAGTG-3′. The PCR product was digested with *Sal*I and cloned into *Sal*I-digested pΔSS*Bgl*II to create plasmid prPCL2$_{-982\text{-}82}$ (pBA1306).

To generate a vector expressing an N-terminal fusion of glutathione *S*-transferase (GST) to full-length Slt2, the *SLT2* gene was PCR amplified from the previously described plasmid pFL44-SLT2-HA (BA1019) (59) by using the primers 5′SLT2*Bam*HI(5′-CGTGGATCCTGTAGTGAAAAATTCGAATTT-3′) and 3′SLT2*Eco*R1 (5′-CTCCTAATTCCGTCCTAAAAATATTTTCTATC-3′). The PCR product was digested with *Bam*HI and *Eco*RI and cloned into the *Bam*HI/*Eco*RI sites of the vector pGEX-4T-2 (Pharmacia) to create pBA1382. The integrity of all PCR products was confirmed by sequence analysis.

**Strains and medium.** Yeast strains used in this study are listed in Table 1. Standard methods and media were used for yeast growth and transformation. Most of the yeast strains used in this study are isogenic derivatives of BY263 (an S288C derivative [45]). Disruption and epitope tagging of *SLT2* was achieved by homologous recombination at its chromosomal locus using a PCR-based method (38). The *slt2*Δ strain was verified by PCR and phenotypic assays. The GFP(S65T)-*SLT2* allele was verified by PCR and the functionality of the Slt2 fusion protein was confirmed by Western blotting, kinase assays, and complementation of *slt2*Δ phenotypes.

**β-Galactosidase assays.** To assay the activity of *CYC*::*lacZ* reporter genes in log-phase cultures, yeast transformants were grown in 5 ml of SD-URA medium to an optical density at 600 nm ($OD_{600}$) of 0.6 at 30°C. For heat shock experiments, cultures were grown to an $OD_{600}$ of 0.6 at 30°C and then transferred to test tubes that had been preincubated at 39°C, and cultures were incubated at 39°C for 30 min. To arrest cells with α-factor, cells were grown in minimal medium supplemented with 1 M sorbitol. Once the cultures reached an $OD_{600}$ of 0.3, α-factor (Procyon Biopharma) was added to the cultures to a final concentration of 5 μM for 2 h before harvesting. The cells were harvested by centrifugation at 3,000 × *g* for 5 min, the cell pellets were quickly frozen in liquid nitrogen, and the pellets were stored at −80°C. Liquid β-galactosidase assays were performed on the frozen pellets as described previously (44). Data are presented as the mean values from triplicate experiments.

**Cell cycle synchronization, heat shock, and ChIP.** For heat shock experiments, 50-ml cultures of cells were grown to an $OD_{600}$ of 0.6. One culture was held at

TABLE 1. Yeast strains used in the study[a]

| Strain | Genotype | Source or reference |
|---|---|---|
| BY263 | *MAT***a** *trp1Δ63 ura3-52 lys2-801 ade2-107 his3Δ200 leu2-1* | 44 |
| BY107 | *MAT*α *swi6ΔHIS3* | 46 |
| BY108 | *MAT***α** *swi4ΔHIS3* | 46 |
| BY1321 | *MAT***a** *swi4ΔHIS3 TRP*$^+$ | This study |
| BY891 | *MAT***a** *swi6ΔHIS3* | This study |
| BY1342 | *MAT***a** *slt2ΔkanMX6* | This study |
| BY1343 | *MAT***a** *SLT2-GFP (S65T)::kanMX6* | This study |
| BY551 | *MAT***a** *mbp1ΔTRP1* | 26 |
| BY332 | *MAT***a** *ste12ΔLEU2 his4 leu2 trp1 ura3 can1* | I. Sadowski |

[a] All strains listed are isogenic to the parent strain BY263 with the exception of BY332.

30°C, while two other cultures were shifted to 39°C for either 30 or 60 min. Samples of cells were cross-linked with 1% formaldehyde for 15 min with shaking at either 30°C or 39°C. For cell cycle or α-factor treatment experiments, 500-ml cultures were grown at 30°C to an $OD_{600}$ of 0.3, and α-factor (Procyon BioPharma) was added directly to the medium to a final concentration of 5 μM. Cultures were incubated with α-factor for 2 h until at least 95% of the cells were arrested in $G_1$ phase as determined by microscopy. Cells were pelleted and then resuspended in fresh yeast extract-peptone-dextrose (YPD) medium. Samples (50 ml) were taken before the arrest, before the release, and every 10 min after release and then cross-linked with 1% formaldehyde for 15 min at 30°C. In both cases, cross-linking was quenched by the addition of glycine to 125 mM. Cells were pelleted at 3,000 × *g* for 5 min and washed twice with ice-cold TBS (150 mM NaCl, 20 mM Tris-HCl; pH 7.6), and whole-cell extracts were prepared for use in ChIPs basically as described previously (53). Cell lysis was performed in 400 μl of lysis buffer (50 mM HEPES-KOH [pH 7.5], 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, and one EDTA-Free Protease Inhibitor Pellet [Boehringer Mannheim] for every 50 ml) and 400 μl of glass beads. The suspension was sonicated four times for 10 s each time (resulting in an average fragment size of 0.5 kb) and clarified by centrifugation for 15 min at 14,000 × *g*. Protein concentration for each sample was determined by Bradford assay (Bio-Rad). Immunoprecipitations were performed with 1 mg of extract at 4°C overnight with rotation on a nutator and either 15 μl of protein A-Sepharose (pA) alone or 15 μl of pA plus 10 μl of affinity-purified Swi4 polyclonal antibody or 15 μl of pA plus 5 μl of affinity-purified Swi6 polyclonal antibody. Precipitates 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 TBS. Finally, the samples were processed for DNA purification as described previously (53).

**PCR analysis of immunoprecipitated DNA.** PCRs were carried out in 25-μl volumes. Serial dilutions of the immunoprecipitated material and the input material for the whole-cell extracts (WCEs) were performed to ensure the PCRs were performed in the linear range. Typically, PCRs were performed with 1/2,000 of the immunoprecipitated material and 1/20,000 of the input material for WCEs. PCR was performed using Platinum *Taq* polymerase (Gibco-BRL) with 25 pmol of each primer and 0.5 μCi of [α-$^{32}$P]dATP. For multiplex PCR with the *PCL2*, *PCL1*, *PHO5*, and *CLN1* promoter primer pairs, 4.0 mM $MgCl_2$ was used; for PCR with only one primer pair, 1.5 mM $MgCl_2$ was used. The promoters were amplified using a cycling program of an initial 2 min of denaturation at 95°C; followed by 25 cycles of 30 s at 95°C, 30 s at 53°C, and 60 s at 70°C; and a final extension step of 5 min at 70°C. The gene-specific primers were designed as 20-mers with a roughly 50% GC content. The PCR primer sequences used for the amplification of promoter regions in *PHO5*, *PCL1*, *PCL2*, *CLN1*, *RLM1*, *YLR110c*, *CWP1*, *SRL1*/*YOR248c*, and *GIC1* are available upon request. The PCR products were separated on a 5% polyacrylamide gel, dried, and exposed to Kodak Biomax-MR film.

**Fluorescence microscopy.** Localization of Slt2-GFP(S65T) and DNA DAPI (4′,6′-diamidino-2-phenylindole) staining of live yeast cells was performed using cultures of BY263 and BY1343 grown to early log phase in YPD. Cultures were then treated with 5 μM α-factor for 2 h and washed with a buffer containing 0.1 μg of DAPI/ml. Cells were observed at a magnification of ×100 using Nomarski optics or fluorescence through a fluorescein isothiocyanate (FITC) filter to observe GFP(S65T). Photographs were taken with 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.).

**DNA microarray analysis.** Yeast strains were grown in rich medium at 25°C to an $A_{600}$ of 0.3 to 0.6. For heat shock experiments, cells were grown to an $A_{600}$ of 0.3 at 25°C and then shifted to 39°C for 45 min. Cells were harvested by centrifugation and then quickly frozen in liquid nitrogen. Poly(A)$^+$ mRNA was isolated as described previously (42). Alternatively, total RNA was isolated by hot phenol extraction as described previously (4) with minor modifications. Total RNA was precipitated from the final aqueous layer and run over a Qiagen RNeasy column to remove smaller RNAs. The final eluate was used as the source of mRNA for fluorescently labeled cDNA. In order to generate labeled cDNA, ca. 2 to 3 μg of mRNA or 50 μg of total RNA was incubated with Superscript II reverse transcriptase (Gibco-BRL) and Cy3- or Cy5-dCTP (Mandel) at 42°C for 2 h as described elsewhere (http://www.oci.utoronto.ca/microarrays).

DNA microarrays consisting of ~97% of the known or predicted genes of *Saccharomyces cerevisiae* were prehybridized with a 1:1:20 solution of tRNA (10 mg/ml)–single-stranded DNA (10 mg/ml)–DIG Easy Hyb solution (Roche). The prehybridization mix was heated to 65°C for 5 min, cooled to 37°C, and applied to the DNA microarray. The microarray was covered with a glass coverslip and incubated at 37°C for ca. 1 h. Prior to hybridization, DNA microarrays were washed in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and dried by centrifugation. Labeled cDNAs were applied to the microarrays and allowed to hybridize for 12 h at 37°C. Microarrays were subjected to three 15-min washes with 0.1× SSC–0.1% sodium dodecyl sulfate (SDS) at 45°C, rinsed quickly in 0.1× SSC, and scanned with a GSI Lumonics scanner (Watertown, Mass.). Clustering analysis was carried out using the Cluster program found at (http://rana.stanford.edu/clustering [21]). Microarray data represent the averages of three independent experiments with the wild-type strain (wt/wt at 25°C) and two independent experiments with the *slt2*Δ strain (*slt2*Δ/wt at 25°C), the *swi4*Δ strain (*swi4*Δ/wt at 25°C), and the *bck1*Δ strain (*bck1*Δ/wt at 25°C). The following experiments were also performed in duplicate: wt/wt, 39°C; *slt2*Δ/wt, 39°C; *swi4*Δ/wt, 39°C; and *slt2*/*slt2*, 25°C versus 39°C (data not shown; see Results). Microarray data are available in full on the Andrews lab website (http://lambda.med.utoronto.ca).

**Batch affinity chromatography.** GST and GST-Slt2 were purified from *Escherichia coli* harboring appropriate expression plasmids as previously described (45). GST and GST-Slt2 were bound to glutathione-Sepharose 4B beads (Pharmacia) at concentrations of 1 and 3 μg/μl of beads, respectively. For affinity chromatography with in vitro-transcribed and translated Swi4 and Swi6, 20 μl of GST or GST-Slt2 beads was incubated with 18 μl of lysis buffer (100 mM Tris-HCl [pH 8.0], 1 mM EDTA, 10 mM $MgCl_2$, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 0.5% Triton X-100; two EDTA-Free Protease Inhibitor cocktail tablets [Boehringer Mannheim] per 50 ml) and 2 μl of either in vitro-translated Swi4 or Swi6 (5) for 2.5 h at 4°C. The beads were harvested by centrifugation at 2,000 rpm in an Eppendorf centrifuge for 2 min, and the unbound supernatant was collected. The beads were washed three times each in 100 μl of lysis buffer. After the final wash, the beads were resuspended in 20 μl of 1× SDS gel sample buffer and boiled. The bound and unbound supernatant fractions were separated by SDS–6% polyacrylamide gel electrophoresis (PAGE). The gels were fixed, treated with Amplify (Amersham), dried, and exposed to X-ray film.

For affinity chromatography with insect cell-derived SBF, 20 μl of either GST or GST-Slt2 beads were incubated with 10 μl of lysis buffer and 5 μg of partially purified SBF (0.5 μg/μl) (5) for 2.5 h at 4°C. The beads were harvested by centrifugation at 2,000 × *g* for 2 min, and the unbound supernatant was collected. The beads were washed three times each in 100 μl of lysis buffer. After the final wash, the beads were resuspended in 20 μl of 1× SDS sample buffer and boiled. The bound and unbound supernatant fractions were separated by SDS–6% PAGE. The proteins were transferred to nitrocellulose, and Swi4 and Swi6 proteins were detected by Western blotting with either anti-Swi4 or anti-

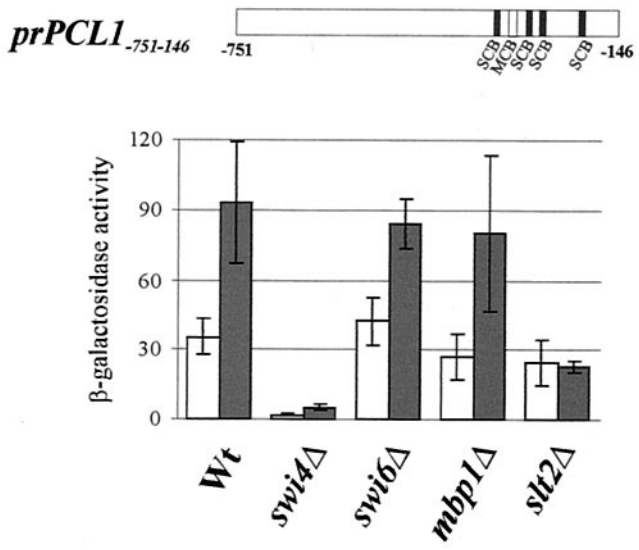


FIG. 1. Slt2/Swi4-dependent and Swi6-independent activation of a *PCL1-lacZ* reporter gene. Schematic diagram of the *PCL1* upstream regulatory region used to create a *CYC*::*lacZ* reporter construct. Locations of consensus SCB and MCB motifs are indicated. Wild-type (Wt, BY263), *swi4*Δ (BY108), *swi6*Δ (BY107), *mbp1*Δ (BY551), and *slt2*Δ (BY1342) strains were transformed with the prPCL1$_{-751\text{-}146}$ reporter plasmid and grown at 30°C to log phase (open bars) or grown at 30°C to log phase and then heat shocked for 30 min at 39°C (filled bars). Cell lysates were made and β-galactosidase activity (Miller units) was determined. Activity depicted is the mean of three experiments; the error bars show the standard deviation for the three experiments.

Swi6 antibodies and detected using enhanced chemiluminescence as described previously (5).

**Northern blot analysis.** Yeast strains were grown at 30°C in YPD medium to an $OD_{600}$ of 0.4. For α-factor treatment, cells were treated with 5 μM α-factor (Procyon BioPharma) for 2 h. Cells were pelleted at 3,000 × *g* in a 4°C centrifuge for 3 min, and RNA was isolated and Northern blotting was done as described previously (45). The probes used for the Northern blot analysis were a 600-bp *Eco*RI-*Hin*dIII fragment of the *ACT1* gene (44), PCR products containing the coding sequence of *PCL1* (45), *YLR110c, SRL1*, and *GIC1*. Sequences of PCR primers are available upon request. Probes were labeled using random-primed synthesis with Klenow DNA polymerase in the presence of [α-$^{32}$P]dATP. For RNA quantitation, Northern blots were exposed on a Molecular Dynamics screen and scanned with a Molecular Dynamics PhosphorImager and analyzed with ImageQuant software (version 3.3).

## RESULTS

**Heat shock induction of PCL1 requires Slt2 and Swi4 but not Swi6.** Upon heat shock, Slt2 is required for a two- to threefold increase in transcript levels for numerous cell-cycle-regulated genes, including the $G_1$ cyclins *PCL1* and *PCL2* (28, 39). To characterize the molecular mechanism of the Slt2-dependent transcriptional response, we turned to an in vivo assay to analyze the roles of Swi6 and Swi4 in the Slt2-dependent transcription of *PCL1*. A *PCL1* reporter gene was constructed, and sequences spanning from −751 to −146 relative to the *PCL1* ATG were placed upstream of a *CYC*::*lacZ* reporter gene on a yeast vector to create the reporter plasmid prPCL1$_{-751\text{-}146}$ (Fig. 1). To assay the contribution of SBF and Slt2 to the upstream activating sequence (UAS) activity of the prPCL1$_{-751\text{-}146}$ reporter, we transformed the wild type and *swi4*, *swi6*, *mbp1*, and *slt2* mutant strains with the prPCL1$_{-751\text{-}146}$ plasmid and the control plasmid *CYC1*::*lacZ* and then measured β-galactosidase activity. None of the transformants containing the control reporter gene produced β-galactosidase activity (data not shown), while a wild-type strain containing the prPCL1$_{-751\text{-}146}$ reporter gene had significant β-galactosidase activity at 30°C (Fig. 1). Upon heat shock, the β-galactosidase activity was induced twofold (Fig. 1). Thus, the behavior of the *PCL1* reporter gene in response to heat shock mirrors that of the endogenous *PCL1* gene (39). As previously shown, expression of the prPCL1$_{-751\text{-}146}$ reporter gene was completely dependent on Swi4 (Fig. 1 [46]), under normal growth conditions; heat shock-dependent induction of the *PCL1* reporter was also largely dependent on Swi4 (Fig. 1). Surprisingly, we discovered that the upstream activation sequence (UAS) activity from the promoter of prPCL1$_{-751\text{-}146}$ was independent of Swi6 (Fig. 1). Even though there is one consensus MCB element in the *PCL1* promoter, deletion of *MPB1* did not affect prPCL1$_{-751\text{-}146}$ reporter activity. Furthermore, heat shock-dependent induction of the *PCL1* reporter gene was completely eliminated in the *slt2*Δ strain (Fig. 1), a finding consistent with previous Northern blot experiments (39). The Swi4-Slt2-dependent but Swi6-independent regulation of the reporter gene was also seen using an upstream segment of the *PCL1* promoter lacking all consensus SCB and MCB elements but containing degenerate matches to the SCB sequence (−751 to −363; data not shown). In addition to the reporter gene assays, Northern blot analysis was also performed to assay *PCL1* transcript levels in log phase cultures of wild-type, *swi4*Δ, and *swi6*Δ strains (see Fig. 6). These blots confirmed that expression of *PCL1* was dependent on Swi4 but independent of Swi6.

**Localization of Swi4 and Swi6 to the promoters of PCL1 and PCL2 during $G_1$ phase, pheromone-treatment, and heat shock.** *PCL1* gene expression peaks in late $G_1$ (52) and the promoter of *PCL1* contains multiple SCB elements (46). Although Swi6 is only required for the proper cell cycle-dependent expression of a number of SBF target genes, we were surprised to find that the heat shock-dependent induction of *PCL1* was independent of Swi6 (see above). Studies to date show that both Swi4 and Swi6 are required for SCB-driven gene expression; in the absence of Swi6, Swi4 is unable to bind SCBs both in vitro (5) and in vivo (23, 33).

To directly examine the association of Swi4 and Swi6 with the *PCL1* and *PCL2* promoters, we performed ChIP assays by using affinity-purified Swi4 and Swi6 polyclonal antibodies. Cells were synchronized in early $G_1$ with α-factor and then released into fresh medium to allow the cells to progress through a synchronous cell cycle. Samples were taken at 10-min intervals following release from α-factor and were analyzed for position in the cell cycle by propidium iodide staining and fluorescence-activated cell-sorting. Cells begin to traverse the $G_1$-S boundary 20 min after release (data not shown). For each time point, the cells were fixed with formaldehyde and chromatin was isolated using either the Swi4 or Swi6 antibodies. The abundance of specific DNA sequences within the immunoprecipitates was measured by using PCR and appropriate primer pairs. Each reaction mixture contained four sets of primers, which enabled us to simultaneously measure the relative abundance of Swi4 and Swi6 at the promoters of *PCL1, PCL2, CLN1*, and *PHO5*. For *PCL1*, *PCL2*, and *CLN1*,

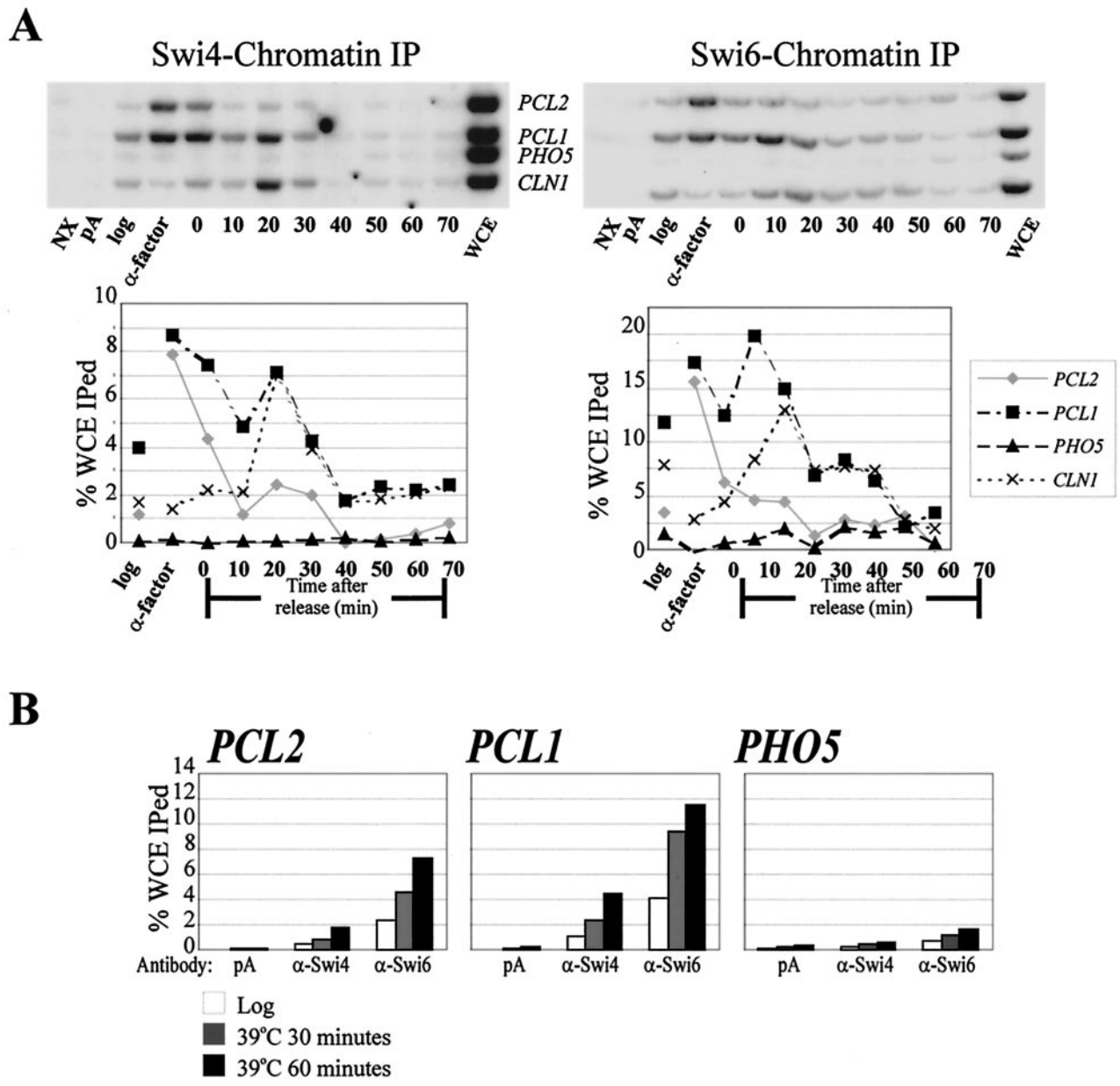


FIG. 2. Swi4 and Swi6 localization to the promoters of *PCL1* and *PCL2* during the cell cycle and after α-factor and heat shock treatment. (A) Cell cycle ChIP using affinity-purified Swi4 antibodies (left panel) and Swi6 antibodies (right panel). A wild-type strain (BY263) was grown to mid-log phase and then blocked in $G_1$ with α-factor. Cells were inoculated into fresh medium lacking α-factor, and samples were taken every 10 min. Samples taken of the unsynchronized log-phase culture (log), the $G_1$ arrested cells (α-factor), and the 10-min time points were cross-linked with formaldehyde. WCEs were made, and ChIP was done using pA alone or pA plus affinity-purified Swi4 or Swi6 antibodies as indicated. In the top panels multiplex PCR was performed to amplify the promoter regions of *PCL2, PCL1, PHO5*, and *CLN1* in each of the ChIP samples, in an immunoprecipitation with extract which was not cross-linked (NX), and in WCEs. The graphs depict the results of phosphorimager analysis of each PCR product. The results are expressed as a percentage of the product in the WCE. The experiment shown is representative of three independent experiments with Swi4 antiserum and two with Swi6 antiserum. (B) Heat shock ChIPs. Cultures of a wild-type strain (BY263) grown at either 30°C (white bars) or 30°C, followed by heat shock at 39°C for 30 min (gray bars) or 60 min (black bars) were cross-linked with formaldehyde. Lysates were made, and ChIP was performed using pA or with affinity-purified Swi4 antibodies (α-Swi4) or affinity-purified Swi6 antibodies (α-Swi6). PCR was performed to amplify the promoters of *PCL1*, *PCL2*, and *PHO5* in the ChIP samples and in WCE. The bar graphs depict the results of phosphorimager analysis of each PCR product. The results are expressed as a percentage of the product in WCE.

the primer pairs were designed to straddle any recognizable SCB or MCB elements. *PHO5* was chosen as a negative control since there are no detectable SCB or MCB elements in its promoter, its transcription is not cell cycle regulated (52), and there have been no reports of Swi4- or Swi6-dependent transcription of *PHO5*. ChIP using either affinity-purified Swi4 or Swi6 antibodies did not enrich for *PHO5* promoter DNA above that seen in negative control assays using pA alone (Fig. 2A) or

in a ChIP assay using non-crossed-linked lysates (NX, Fig. 2A). Further, ChIP with *swi4*Δ or *swi6*Δ strains and affinity-purified Swi4 or Swi6 antibodies, respectively, did not enrich for *PCL1*, *PCL2*, or *CLN1* promoter DNA (data not shown), while enrichment of these promoter regions was seen in the ChIPs from a wild-type strain. Taken together, these assays suggest that our ChIPs are specifically enriched for DNA bound by Swi4 and Swi6 in vivo.

The localization of Swi4 and Swi6 to the *CLN1* promoter peaked in late $G_1$, coincident with the normal cell-cycle-dependent activation of genes by SBF (Fig. 2A, 20-min time point). SBF also interacted with the promoter of *CLN2* at the same time (data not shown). Thus, our ChIP assays, using either Swi4 or Swi6 antibodies, provide a representative snapshot of what is occurring in vivo during the cell cycle. The cell cycle expression of *PCL1* is similar to that of *CLN1* and *CLN2*, and we found that peak localization of Swi4 to the *PCL1* promoter also occurred at 20 min. Expression of *PCL2* peaks earlier than that of *PCL1* and *CLN1* and is partially dependent on Swi5 (1). We did not detect a strong Swi4 or Swi6 association with the *PCL2* promoter during the cell cycle in our ChIP experiments. We may have failed to capture peak binding of SBF to the *PCL2* promoter in the time points used in our assay. Alternatively, SBF binding to the *PCL2* promoter may not be required for cell cycle regulation of *PCL2*.

Having established a ChIP assay for SBF localization, we next examined SBF promoter localization under conditions that activate the Slt2-MAPK pathway. Upon α-factor treatment, expression of the *CLN1*, *CLN2*, and *PCL1* cyclin genes is repressed. In contrast, expression of the *PHO85* cyclin *PCL2* is immediately induced in response to α-factor treatment and by overexpression of *STE12*, the transcription factor activated by the pheromone response pathway (44, 47). Our ChIP assay showed that Swi4 and Swi6 localization to either the *CLN1* promoter (Fig. 2A) or the *CLN2* promoter (data not shown) remained low during α-factor treatment. However, localization of Swi4 and Swi6 to the *PCL2* promoter dramatically increased (Fig. 2A). Remarkably, even though the *PCL1* gene is not expressed during α-factor treatment, Swi4 and Swi6 localization to the *PCL1* promoter also increased upon α-factor treatment. Therefore, increased association of SBF to the *PCL1* promoter during α-factor treatment is not sufficient for *PCL1* expression. Nonetheless, we conclude that pheromone treatment promotes increased association of SBF to the promoters of a subset of genes.

We next asked whether heat shock also affected the localization of SBF to the promoters of *PCL1* and *PCL2*. ChIP experiments were performed on cross-linked cells that were grown at 30°C or cells that were grown at 30°C and heat shocked at 39°C for either 30 or 60 min. We used PhosphorImager analysis to compare the PCR product of the ChIP to that of the WCE. Heat shock increased the localization of both Swi4 and Swi6 to the promoters of *PCL1* and *PCL2* (Fig. 2B) but not to the promoter of *CLN1* (data not shown). In summary, under two conditions that activate the MAPK Slt2, heat shock and α-factor treatment, SBF localization to the promoters of *PCL1* and *PCL2* increases.

**α-Factor-dependent induction of PCL2 expression is dependent on Slt2 and SBF.** Since we saw increased localization of SBF to *PCL* promoters during pheromone treatment, we next investigated the role of Slt2 in this process. First, we asked whether SBF and Slt2 are required for the pheromone-dependent induction of *PCL2* expression. We used both a reporter gene construct containing sequences 5′ to the *PCL2* transcriptional start (Fig. 3A) and Northern blot analysis (Fig. 3B) to assay *PCL2* expression. The sequences upstream of *PCL2* contain a consensus binding site or PRE (pheromone response element) for Ste12 (reviewed in reference 40). As expected, the α-factor-dependent induction of both the *PCL2* reporter gene and the endogenous *PCL2* gene were fully dependent on Ste12 (Fig. 3A and B). However, induction of *PCL2* during α-factor treatment was also dependent on Swi4 and Swi6 and partially dependent on Slt2 (Fig. 3; also see Discussion). Our results suggest that there may be an activation event specific to SBF on the promoter of *PCL2* during the pheromone response.

Immunolocalization studies have been conducted using a C-terminally tagged Slt2 expressed from a plasmid vector (31). In these experiments, Slt2-HA was nuclear during growth at 24°C and present throughout the cell when cells were grown for extended periods of time at 39°C. Our work predicts that Slt2 will also be nuclear upon α-factor treatment; therefore, we assayed Slt2 localization in live cells treated with α-factor. To do this, we fused green fluorescent protein (GFP) to the C terminus of Slt2 at its endogenous locus to generate Slt2-GFP. Genetic tests and in vitro kinase assays confirmed that Slt2-GFP was fully functional (data not shown). Under normal growth conditions at 30°C, specific localization of Slt2-GFP was not detectable, even though Western blot analysis showed that Slt2-GFP was expressed (data not shown). We presume that the Slt2-GFP signal was either too low or was too dispersed throughout the cell for detection. However, upon α-factor treatment for 1 h, cells expressing the genomically tagged Slt2-GFP had distinct nuclear staining, as well as clear staining at the shmoo tip in some cells (Fig. 3C). We conclude that, upon α-factor treatment, Slt2 is localized to the nucleus, where it may act directly on components of SBF, Swi4 and Swi6.

**Localization of SBF to the promoters of PCL1 and PCL2 upon heat shock and pheromone treatment is partially dependent on Slt2.** Since we saw increased binding of SBF to the promoters of *PCL1* and *PCL2* in two conditions that activate Slt2, we next tested the localization of SBF to *PCL* promoters in a *slt2*Δ strain. We used Swi4 and Swi6 polyclonal antibodies to immunoprecipitate chromatin from formaldehyde cross-linked wild-type and *slt2*Δ cells that were either in log phase, heat shocked at 39°C for 60 min, or treated with α-factor for 2 h. Since both heat shock and α-factor treatment cause cell lysis in *slt2* mutant cells, both the wild-type and *slt2*Δ cells were grown in medium containing sorbitol. The addition of sorbitol did not affect α-factor induction of *PCL2* promoter activity or heat shock induction of *PCL1* promoter activity (data not shown). In cells grown at 30°C, Swi4 and Swi6 associated with the *PCL1* and *PCL2* promoters at similar levels in both wild-type and *slt2*Δ cells (Fig. 4, log columns). Upon heat shock of wild-type cells, the localization of Swi4 and Swi6 to both the *PCL1* and *PCL2* promoters increased (Fig. 2B and 4). However, in *slt2*Δ cells, the enhanced localization of Swi4 and Swi6 to the promoters of *PCL1* and *PCL2* was reproducibly reduced (Fig. 4B). As described above, α-factor treatment of wild-type cells also resulted in increased localization of Swi4 and Swi6 to

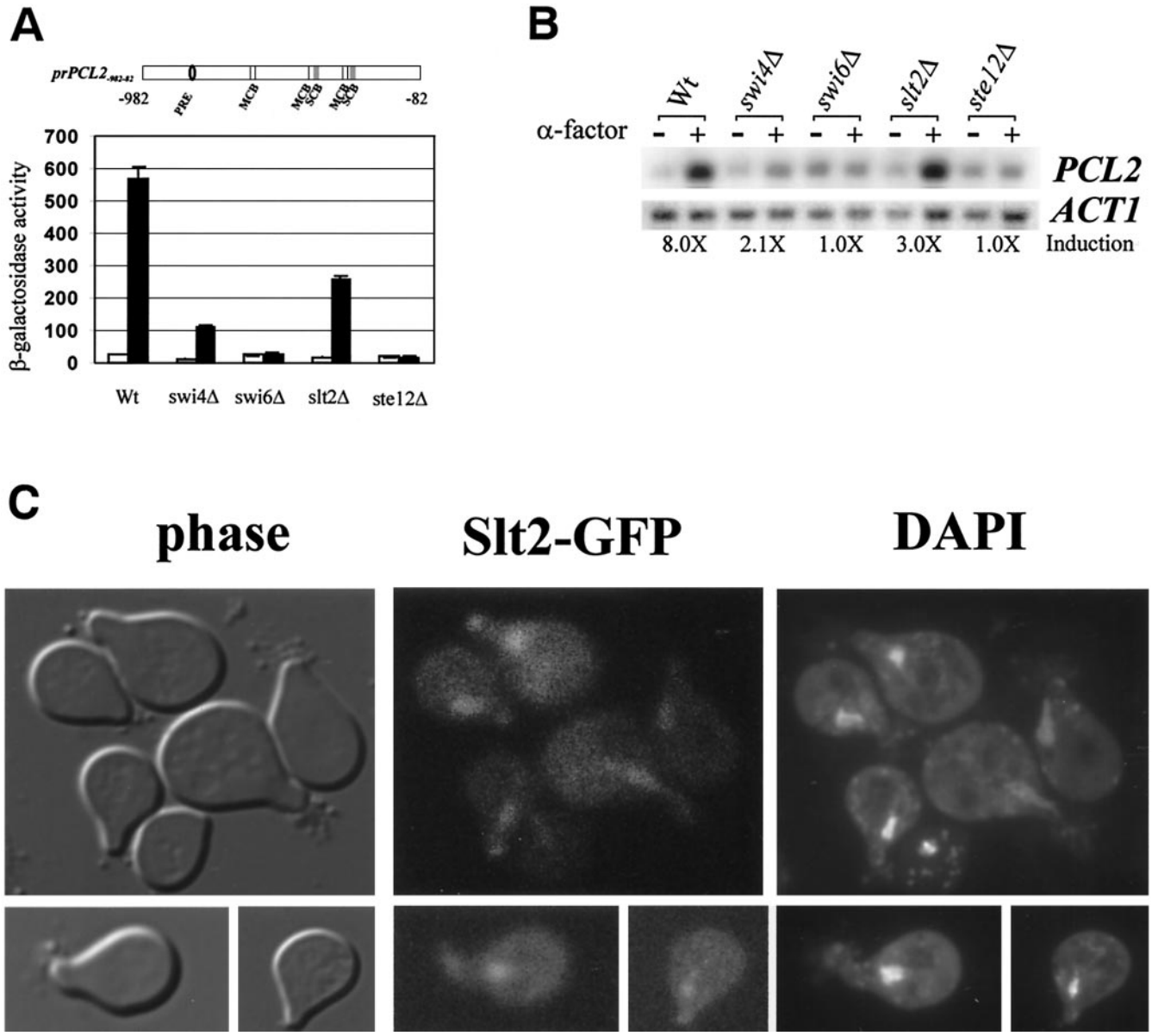


FIG. 3. Slt2 and SBF activity during α-factor treatment. (A) *PCL2* reporter gene assay. Above the graph is a schematic diagram of the *PCL2* promoter region used to create the *PCL2-lacZ* reporter construct prPCL2$_{-989-82}$. The locations of the consensus SCB, MCB, and PRE elements are indicated. The graph shows α-factor-induced expression of β-galactosidase from the prPCL2$_{-989-82}$ reporter plasmid. Wild-type (wt BY263), *swi4*Δ (BY1321), *swi6*Δ (BY891), *slt2*Δ (BY1342), and *ste12*Δ (BY332) strains were transformed with prPCL2$_{-989-82}$. The transformants were grown to mid-log phase (open bars) or grown to mid-log phase and treated with 5 μM α-factor for 2 h (filled bars). Cell lysates were made, and the β-galactosidase activity (Miller units) of each strain was determined. Activity depicted is the mean of three experiments; the error bars show the standard deviations for the three experiments. (B) *PCL2* Northern blots. The indicated strains were grown to log phase or grown to log phase and treated with 5 μM α-factor for 2 h. Total RNA was isolated and probed with *PCL2*, followed by the loading control *ACT1*. Indicated across the bottom is the fold induction of *PCL2* expression after α-factor treatment for each strain. Gel shown is representative of three independent experiments. (C) Subcellular localization of Slt2 after α-factor treatment. BY1343 cells (*SLT2*::*GFP*) were grown in rich medium to early log phase and then treated with 5 μM α-factor for 2 h. Cells were then washed once with buffer containing DAPI and once with water before examination by fluorescence microscopy at a magnification of ×1,000. Cells were exposed to fluorescent light through an FITC filter for 400 ms in order to visualize Slt2-GFP. Wild-type (BY263) cells treated identically showed no fluorescence. Photographs of the same fields viewed with Nomarski optics (DIC) and stained with DAPI to visualize cell nuclei are shown.

the *PCL1* and *PCL2* promoters (Fig. 3 and 4). As for heat shock, the localization of SBF to the *PCL1* and *PCL2* promoters after pheromone treatment was reproducibly reduced but not eliminated in *slt2*Δ cells (Fig. 4). We conclude that Slt2 contributes to the increased localization of SBF to the promoters of *PCL1* and *PCL2* upon heat shock and α-factor treatment.

**DNA microarray analysis of *swi4*Δ and *slt2*Δ mutants.** Thus far, our experiments have identified one gene, *PCL1*, whose expression depends on *SLT2* and *SWI4*. To determine whether

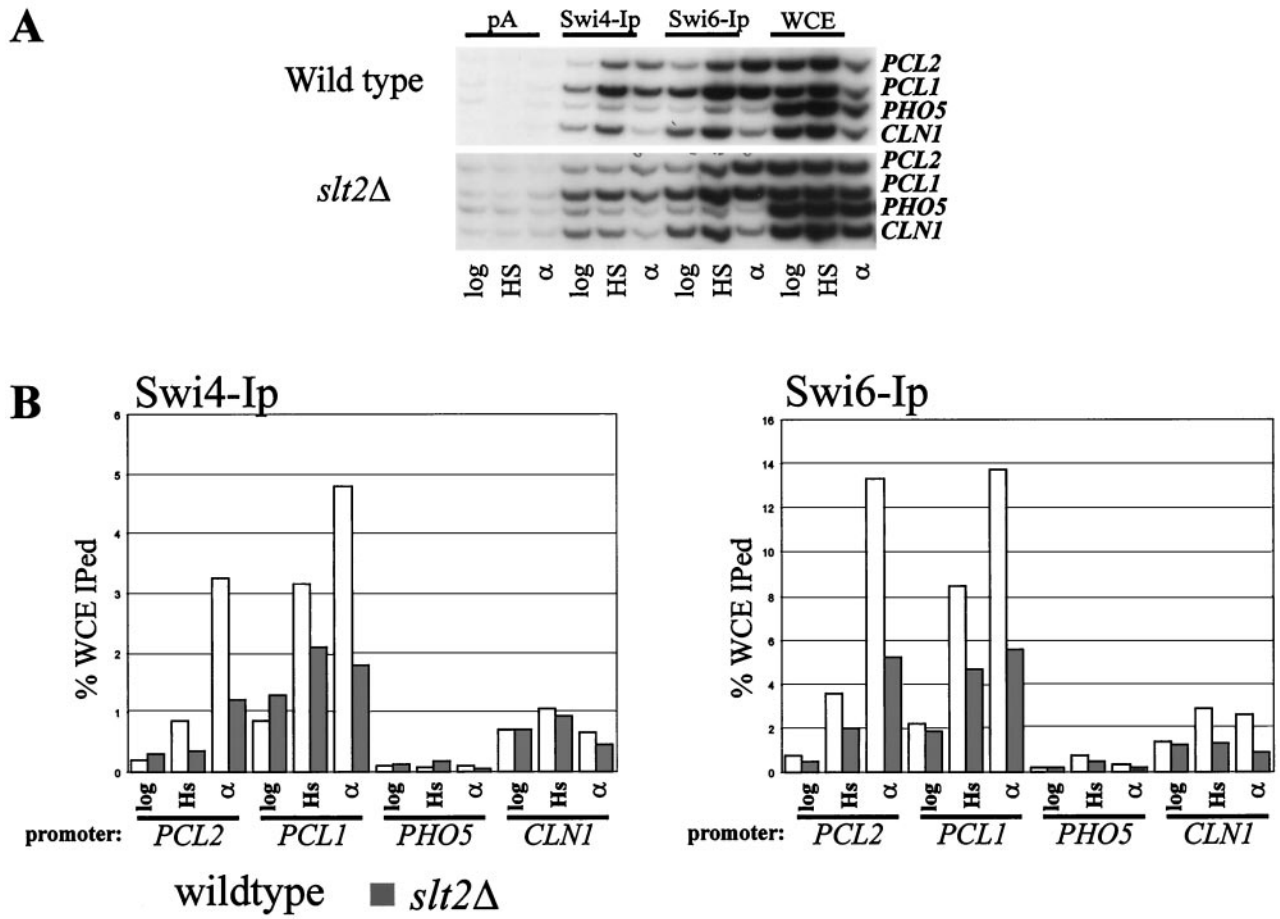


FIG. 4. Slt2 requirement for SBF localization to the promoters of *PCL1* and *PCL2*. (A) PCR amplification of ChIPs from wild-type and *slt2*Δ strains. Wild-type (BY263) and *slt2*Δ (BY1342) cultures grown at 30°C (log), heat shocked for 1 h at 39°C (HS), or treated with α-factor for 2 h (α) were cross-linked with formaldehyde. Lysates were made, and ChIPs were performed using pA alone, Swi4 antibodies (Swi4-Ip), or Swi6 antibodies (Swi6-Ip). Multiplex PCR was used to amplify the promoter regions of *PCL2*, *PCL1*, *PHO5*, and *CLN1* from each of the immunoprecipitated chromatin reactions and the WCE. (B) PhosphorImager analysis of each PCR product was performed, and the localization of Swi4 and Swi6 to the promoters of *PCL1*, *PCL2*, *PHO5*, and *CLN1* is depicted as percent WCE immunoprecipitated (%WCE Iped). Open bars, wild-type ChIPs; shaded bars, *slt2*Δ ChIPs. The data shown are representative of two separate experiments.

*PCL1* is representative of a larger group of genes, we used DNA microarrays to assay for genes regulated by both *SLT2* and *SWI4*. We analyzed the genome-wide changes in transcription by using log-phase cultures of *slt2*Δ, *swi4*Δ, and *bck1*Δ strains grown at 25°C. We expected that the subtle effects of Slt2 on gene expression in log-phase cells meant that this analysis would provide an incomplete view of Slt2-Swi4-regulated genes. However, both Slt2 and Swi4 clearly have roles in the normal mitotic cell cycle, and we expected that strongly regulated genes may be uncovered (see Materials and Methods). We also assayed *slt2*Δ and *swi4*Δ cells at 39°C since transient heat shock causes a $G_1$ delay and activation of Slt2, thus biasing ourselves to a population of cells in which both Swi4 and Slt2 are essential.

We first attempted to correlate the *swi4*Δ and *slt2*Δ profiles at 39°C and found a poor degree of correlation ($\rho = 0.04$). In fact, *swi4*Δ profiles at 25 and 39°C showed an extremely low degree of correlation ($\rho = 0.12$), and the same was true for *slt2*Δ profiles at 25 and 39°C ($\rho = 0.22$). We presume this lack of correlation reflects both the pleiotropic effects of heat shock and the multifunctional nature and nonoverlapping functions of Slt2 and Swi4. Since there was little overlap between *swi4*Δ or *slt2*Δ profiles after heat shock, we instead focused on comparing the transcriptional profile from the wild-type strains to the patterns of gene expression in *slt2*Δ, *swi4*Δ, and *bck1*Δ cells at 25°C. Analysis of the gene expression profiles revealed that the *swi4*Δ signature correlated with both the *slt2*Δ and the *bck1*Δ signatures ($\rho = 0.39$ and 0.37 at 25°C, respectively), suggesting coregulation of genes by *SWI4* and the Slt2-MAPK pathway (see below for more details). As expected, the *slt2*Δ and *bck1*Δ profiles correlated to a high degree ($\rho = 0.63$ for all genes), a finding consistent with published data showing that *BCK1* encodes the MEKK for Slt2 (reviewed in reference 25). In our comparison, we did not observe altered expression of previously characterized SBF targets, including *PCL1*, *PCL2*, *CLN1*, *CLN2*, *HO*, *GAS1*, *VAN2*, *KRE6*, and *CSD2*, a result consistent with previous work (28, 29, 39). This may be due to lack of cell synchrony, and we presume that an analysis of cell

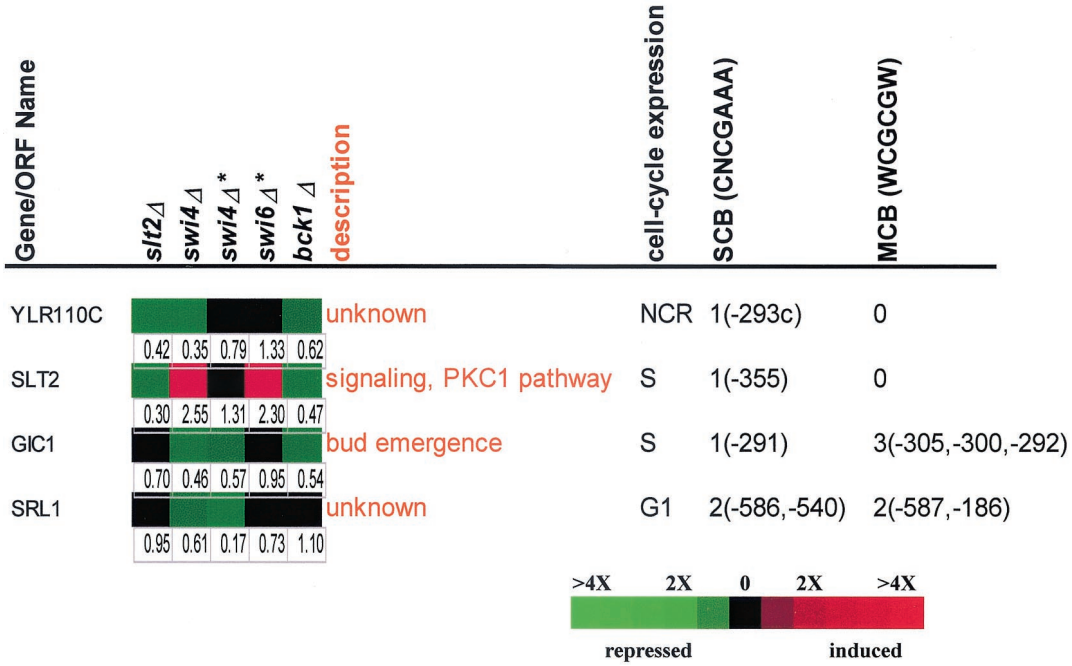


FIG. 5. DNA microarray analysis. Experiments are listed along the horizontal axis, and genes are listed along the vertical axis. Three independent DNA microarray datasets were generated (*slt2*Δ-25°C, *swi4*Δ-25°C, and *bck1*Δ-25°C) and compared to two related data sets from previously published data (*swi4*Δ and *swi6*Δ [27]). Genes whose expression levels were reduced >1.8-fold in *swi4*Δ and/or *slt2*Δ cells are shown. Three control experiments were performed with wild-type RNA (wild-type by wild-type comparison) to validate the cutoff used in the experiments with the mutant strains (see Materials and Methods). Cell cycle expression data were obtained from the Stanford Cell Cycle Expression project and Spellman et al. (52). SCB and MCB elements were found using the SCPD and SGD databases available on the internet. Numbers below the colored squares indicate the intensity ratio between the mutant strain versus the wild-type strain grown under the same conditions and represent the average values from two independent experiments. A value of 1 indicates no change; only genes that were verified by Northern blot analysis are shown.

populations synchronized in $G_1$ would enhance our ability to detect known SBF targets (12, 52). However, a focus on $G_1$-specific cells would also preclude us from identifying potential genes that are regulated outside $G_1$ phase by *SWI4* and *SLT2*, an important goal of our study.

In order to make more detailed predictions of potential Swi4-Slt2 target genes, we next compared our *swi4*Δ and *slt2*Δ profiles with previously published DNA microarray datasets (27, 47) relevant to our experiments (see below). Previous experiments include profiles of cells harboring activated alleles of *PKC1* (*PKC1-R398A*) and *RHO1* (*RHO-Q68H*), as well as *swi4*Δ and *swi6*Δ cells (27, 47). *PKC1* and *RHO1* profiles were chosen since both genes act upstream of *SLT2* and *SWI4* in a signaling cascade sensitive to cell integrity defects (25). Overall, our DNA microarrays agree well with previous studies of transcriptional effects following activation of the PKC1-Slt2 MAPK pathway (data not shown; see http://lambda.med.utoronto.ca).

We separated genes into classes based on their *SWI4*/*SWI6* dependence and their behavior in *bck1*Δ and *slt2*Δ cells or cells carrying activated *PKC1*. Of particular interest for this study were genes that showed Swi4 and/or Slt2 dependence and Swi6 independence. We selected only those genes whose expression profiles changed significantly in the mutant strains relative to the wild type (i.e., >1.8-fold; see Materials and Methods). We identified several genes whose expression appeared dependent on both *SWI4* and *SLT2*. Some of these genes encoded ribosomal protein components; this subgroup of ribosomal protein genes is unlikely to be significant, since gene-specific error models place these genes with others whose levels fluctuate highly between microarray experiments (27). However, follow-up experiments (see below) showed that our microarray analysis indeed identified additional targets of Swi4 and Slt2. Expression of *YLR110c* and *GIC1* in log-phase cultures appeared dependent on *SWI4* and *SLT2* but independent of *SWI6* (Fig. 5). *YRL110c* is a previously uncharacterized gene whose expression is not cell cycle regulated, although there is one consensus SCB element upstream of the gene. *GIC1* encodes a CRIB (Cdc42/Rac-interactive binding) motif-containing protein that associates with the Cdc42 GTPase to promote polarized cell growth (10, 11). *GIC1* transcription peaks in S phase, and *GIC1* overexpression can complement the temperature-sensitive phenotype of certain *swi4*$^{ts}$ alleles (M. Donoviel and B. Andrews, unpublished data). We also identified genes whose expression was dependent on Swi4 but independent of both Swi6 and Slt2 (data not shown and Fig. 5 [see website mentioned above]). We performed follow-up studies on one of these genes, *SRL1*, since Swi6 independence is an unusual property for Swi4-regulated genes (see below). It is of interest to note that *SLT2* was upregulated in *swi4*Δ and *swi6*Δ strains, suggesting that the *SLT2* pathway may be activated in the absence of *SWI4* or *SWI6*. This result suggests that *SWI4* and *SWI6* may play some part in downregulating the status of the Slt2-MAPK pathway (36). In summary, our DNA microarray

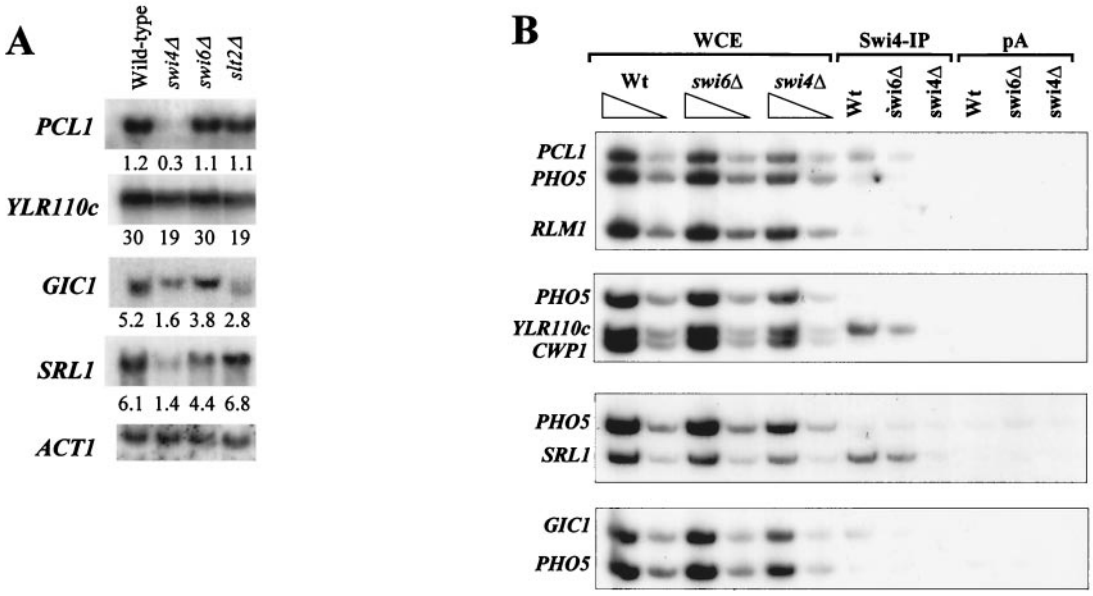


FIG. 6. Northern blot analysis and Swi4 localization to the promoters of newly identified Swi4-Slt2-dependent genes. (A) Northern blot analysis of a subset of proposed Swi4- and/or Slt2-dependent genes. Wild-type (Wt, BY263), *swi4*Δ (BY108), *swi6*Δ (BY107), and *slt2*Δ (BY1342) cells were grown to mid-log phase at 30°C, and total RNA was isolated and probed with the indicated open reading frame (ORF). A probe for *ACT1* was used as a loading control. Autoradiograms shown are representative of one of three experiments. PhosphorImager analysis was performed, and the signal of each ORF was standardized to the *ACT1* loading control (volume ORF/volume *ACT1*). The average standardized signal of three experiments is indicated beneath each lane. The standard deviation for each triplicate was <10% of the average. (B) Swi4 localization to the promoters of Swi4-Slt2-dependent genes. Wild-type (Wt, BY263), *swi6*Δ (BY107), and *swi4*Δ (BY108) cultures grown at 30°C (log) were cross-linked with formaldehyde, WCEs were made, and ChIPs were performed using pA or Swi4 antibodies (Swi4-IP). Multiplex PCR was performed as indicated for the ChIP samples and on fivefold serial dilutions of the WCE to amplify the promoter regions of the genes listed to the left of the panel. *RLM1* and *PHO5* were included as negative controls. *CWP1* was identified as a putative SBF target in a genome-wide ChIP experiment (29) but did not immunoprecipitate with Swi4 antibodies in our experiment.

analyses identified genes that were candidates for coregulation by *SLT2* and *SWI4* and for *SWI6*-independent regulation by *SWI4*.

**Swi6 independence of newly identified Slt2-Swi4-dependent genes.** Our *PCL1* experiments suggested that genes regulated by Slt2 and Swi4 may also be independent of Swi6. Indeed, our analysis of DNA microarray data revealed genes whose expression appears dependent on Swi4 but independent of Swi6 (Fig. 5 [27]). To further explore our microarray results, we performed both Northern blot analysis and Swi4 ChIP on the Swi4- and Slt2-dependent genes (*YLR110c* and *GIC1*) and one Swi4-dependent gene (*SRL1*) identified in our microarray experiments. Although *PCL1* was not identified in our DNA microarray analysis, we included it in our analysis.

For Northern blot analysis, we used probes for the selected genes on RNA isolated from wild-type, *swi4*Δ, *swi6*Δ, and *slt2*Δ strains cultured at 30°C (Fig. 6A). As expected from our *PCL1* promoter analysis (Fig. 1), *PCL1* expression is dependent on Swi4 and independent of Swi6. As predicted from our microarrays, *YLR110c* and *GIC1* expression was also dependent on both Slt2 and Swi4. Interestingly, like *PCL1*, *YLR110c* and *GIC1* expression was independent of Swi6. We conclude that our microarray analysis has identified additional genes that appear to be bona fide targets of Swi4 and Slt2.

To test whether the new genes that we identified in our DNA microarray experiments were direct targets of Swi4, we used our ChIP assay to determine whether Swi4 localized to their promoters. Our attempts at localizing Slt2 to the promoters of either *PCL1* or *PCL2* have been unsuccessful (data not shown). We performed Swi4 ChIPs on extracts prepared from wild-type, *swi6*Δ, and *swi4*Δ strains cultured at 30°C. Primers were designed to specifically amplify the promoter regions of selected Swi4- and/or Slt2-dependent genes and to straddle any recognizable SCB elements. As expected, Swi4 did not immunoprecipitate the negative control promoter *PHO5* or the promoter of *RLM1* (Fig. 6B), whose expression is dependent on the activation of the *PKC1* pathway and independent of Swi4 (20, 47). However, we did detect in wild-type cells Swi4 localization to the promoters of *YLR110c* and *PCL1* and the shared promoter of *SRL1* and *YOR248c*. We also detected localization of Swi4 to the promoter of *GIC1*; however, this localization was significantly weaker than the others. In *swi6*Δ strains, Swi4 still localized to the promoters of *YLR110c, SRL1* and *PCL1,* at only a slightly reduced level (Fig. 6B). Although Swi4 can bind to the promoters tested in the absence of Swi6, our experiments with the *PCL1* promoter suggest that Swi6 may be present with Swi4 in the wild-type situation. Interestingly, as summarized in Fig. 5, *YLR110c* is not classified as a cell-cycle-regulated gene by DNA microarray analysis (12, 52), suggesting that regulation of Swi4 by Slt2 may allow Swi4 to regulate genes whose expression is not restricted to late $G_1$.

**Swi4 interacts directly with Slt2 in vitro.** Activation of the *PKC1* pathway by a number of stimuli results in Slt2-dependent phosphorylation of Swi6 (39). These results have led to

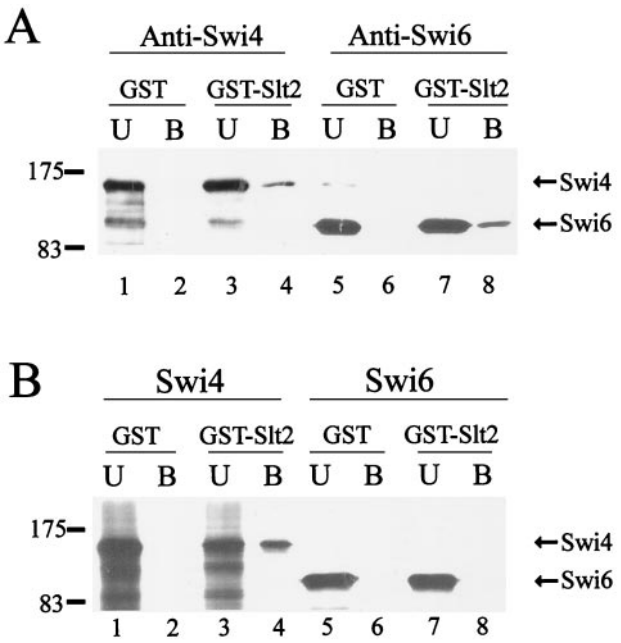


FIG. 7. Binding of Slt2 to Swi4 and Swi6 in vitro. (A) Five micrograms of partially purified SBF derived from insect cell extracts was incubated with either GST or GST-Slt2 immobilized on glutathione beads. The unbound (U) and bound (B) fractions were separated by SDS–6% PAGE. The gels were blotted and incubated with Swi4 antiserum (lanes 1 to 4) or Swi6 antiserum (lanes 5 to 8) to identify Swi4 or Swi6 proteins. The migration positions of molecular mass markers are indicated to the left (in kilodaltons). (B) Two microliters of in vitro-translated Swi4 and Swi6 were incubated with either GST or GST-Slt2 immobilized on glutathione beads. The unbound (U) and bound (B) fractions were separately by SDS–6% PAGE. The migration positions of molecular mass markers are indicated to the left (in kilodaltons).

the suggestion that Slt2 may regulate gene expression through phosphorylation of Swi6 (39). Several results suggest that phosphorylation of Swi6 by Slt2 may not be of regulatory significance. First, our promoter analysis of *PCL1*, along with DNA microarray and Northern blot analysis, suggests that expression of Swi4-Slt2-dependent genes may be partially or fully independent of Swi6. Second, our ChIP experiments showed Swi4 localization to the promoters of *PCL1*, *YLR110c*, and *SRL1* genes in the absence of Swi6 (Fig. 6B). Finally, a phosphorylation site mutant of Swi6, Swi6-SA4 (50), which cannot be phosphorylated by Slt2 in vivo, does not display any obvious cell integrity defects (data not shown). As discussed previously, Swi6 independence is an unusual feature of Swi4-dependent genes, and no other SCB-containing reporter construct has displayed Swi6 independence. Together, these results suggest that Slt2 may not modulate the activity of Swi6. Instead, Slt2 may regulate Swi4, possibly through direct interaction of Slt2 with Swi4.

To test whether Slt2 interacts directly with Swi4, we fused *SLT2-HA* to GST (*GST-SLT2*) and performed batch affinity chromatography assays. Slt2-HA was previously used to show phosphorylation of Swi4 and Swi6 and also for coimmunoprecipitation with SBF from crude yeast extracts (39). GST or GST-Slt2 was incubated with full-length Swi4 and Swi6 that was transcribed and translated in vitro (Fig. 7B). The GST-Slt2 fusion bound full-length Swi4 but did not bind full-length Swi6 (Fig. 7B). This result suggests that Slt2 can interact directly with Swi4 but not with Swi6. Previous coimmunoprecipitation experiments with crude yeast extracts showed Swi6 coimmunoprecipitation with Slt2. To test whether Swi6 coimmunoprecipitation occurs only in the context of SBF, we incubated GST or GST-Slt2 with partially purified SBF produced in insect cells (5). Both Swi4 and Swi6 were detected in the bound fraction when SBF was incubated with GST-Slt2 (Fig. 7A). Together, our batch affinity chromatography experiments demonstrate that Slt2 interacts directly with Swi4 but not with Swi6.

## DISCUSSION

We have used genetic and biochemical approaches, along with DNA microarray analysis, to describe genes whose expression is dependent on both the cell cycle transcription factor Swi4 and the *PKC1*-activated MAPK Slt2. Genes that are sensitive to both Slt2 and Swi4 appear to be uniquely regulated, which may reflect the ability of Slt2 to modulate the activity of Swi4; in particular, regulation of Swi4 by Slt2 may allow Swi4-dependent transcription in the absence of Swi6 (summarized in Fig. 8 and discussed further below). Our study also implies roles for Swi4 beyond its prominent role in controlling cell-cycle-dependent transcription.

**Swi4 and Slt2 coregulate a subset of genes.** In order to understand the transcriptional role of Swi4 and Slt2 in maintaining cell wall integrity, we performed DNA microarray analysis to identify genes that require both Slt2 and Swi4 for their expression. Through careful analysis of our microarray data and other published datasets, we identified genes whose expression was reduced in both *SWI4* and *SLT2* deletion strains (Fig. 5). Although Northern blot analysis and ChIP experiments generally support the validity of the microarray data, we are unlikely to have identified all Slt2-Swi4-dependent genes. First, we performed our microarrays using RNA probes from log-phase cells in an attempt to minimize cell cycle effects. Second, many Slt2-dependent transcriptional effects range from 1.2- to 2-fold changes (28, 30, 39), which is below the cutoff for our microarray analysis (1.8-fold). Indeed, our limits exclude the possibility of identifying known Slt2-Swi4-dependent genes, including *PCL1* and several cell wall biosynthetic genes (28).

As expected, we identified SBF-dependent genes in our microarray analysis, which are cell cycle-regulated in $G_1$ and S phase, when SBF-dependent transcription normally occurs (data not shown; see website cited above). However, our microarray analysis and other experiments suggest a new role for Swi4 that may not be discharged through its well-characterized function in SBF. First, expression of one Swi4-Slt2-dependent gene, *YRL110c*, is not $G_1$ specific; however, our ChIP experiments show that Swi4 is localized to the promoter of *YLR110c.* These experiments suggest a role for Swi4 in regulating genes outside of $G_1$ phase. *YLR110c* and a number of other genes were also earmarked as putative SBF targets using a genomic ChIP approach (29). Second, we identified Swi4-dependent genes in our microarray experiments that were Swi6 independent (Fig. 5). We think it unlikely that the Swi6 independence reflects Swi4 misregulation (49), since dominant transcriptional effects of Swi4 in *swi6*Δ backgrounds have been reported

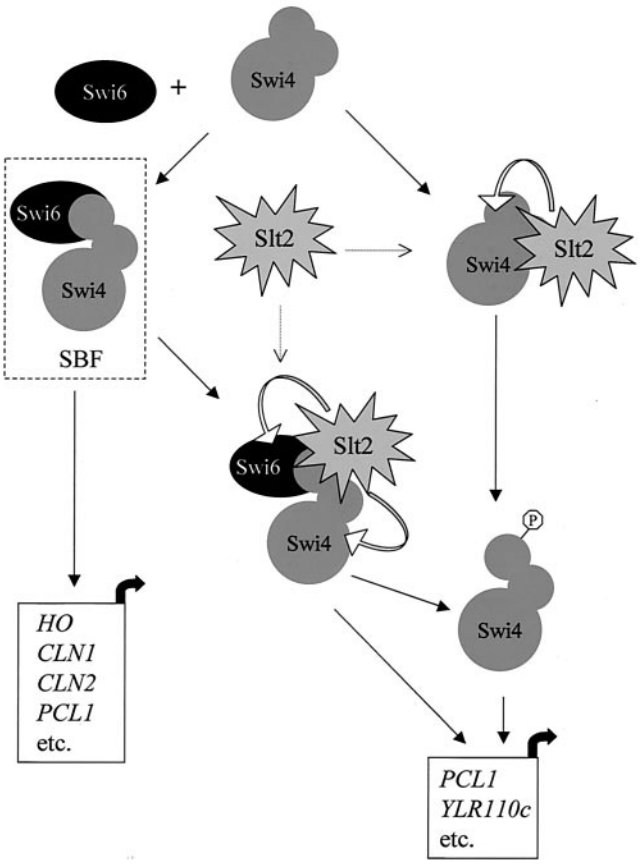


FIG. 8. Model for Slt2 and Swi4 coordinate regulation of *PCL1*-like genes. Possible modes of regulation of Swi4 by Slt2 are diagrammed. Swi4 interacts with Swi6 to form SBF (in dashed box) to control expression of $G_1$-specific transcripts (*HO*, *CLN1*, *CLN2*, *PCL1*, etc.) via binding to SCB elements. In our model, phosphorylation of Swi4 by Slt2, either on its own or complexed with Swi6, may relieve the autoinhibition of Swi4 DNA binding. Regulation of Swi4 by Slt2 may impart a unique Swi6-independent function to Swi4 that directs activation of a subset of Swi4-Slt2-dependent genes (*PCL1*, *SRL1*, etc.). Genes controlled by Swi4 and Slt2 include non-cell-cycle-regulated genes and define a novel role for Swi4 beyond its major role in controlling cell-cycle-dependent transcription.

only for strains overexpressing full-length Swi4 or expressing C-terminally truncated alleles of Swi4 (5, 49). Also, we did identify many genes with the expected dependence on both Swi4 and Swi6 (data not shown). Together with our *PCL1* experiments, our data strongly suggest that Swi4 can function independently of Swi6 and outside of $G_1$ phase, revealing a new cellular role for Swi4 (see below for further discussion).

**Role of the PKC1 pathway and SBF in pheromone response.** In addition to regulating a subset of Swi4 targets during mitotic growth, our work suggests that Slt2 may also partner with Swi4 to regulate the pheromone-dependent induction of gene expression. DNA microarrays have been used to examine genome-wide patterns of gene expression in response to pheromone treatment (47). Interestingly, pheromone treatment induces both an early transcriptional response dependent on Ste12 and a later transcriptional response dependent on *PKC1*. The late-induced genes are not enriched for Ste12 binding sites (PREs) in their promoters, nor are they induced by overexpression of *STE12*. These results suggest that the *PKC1*-MAPK pathway likely activates other transcription factors necessary for the late pheromone-induced genes.

*PCL2* was identified as an early pheromone-induced gene, whose expression was dependent on *STE12*. Our studies confirm that induction of *PCL2* expression in response to α-factor depends on Ste12 but also reveal a requirement for both Swi4 and Slt2 for the full activation of *PCL2* (Fig. 3A and B). This pattern of dependency suggests that the *PCL2* gene may be responsive to both early and late pheromone signals, requiring Ste12 for early pheromone response and requiring Slt2 and SBF for the late pheromone response. Alternatively, there may be a subset of pheromone-responsive genes whose full induction is dependent on Ste12, Slt2, and SBF.

Unlike *PCL2*, *PCL1* expression is not induced by α-factor. Nonetheless, we saw an enrichment of SBF on the promoter of *PCL1* upon α-factor treatment (Fig. 2 and 4). As described earlier, SBF localization to $G_1$-specific promoters in early $G_1$ phase is necessary but not sufficient for transcription activation. Our results show that SBF binding to promoters during the transcriptional response to pheromone treatment is also not sufficient for activation of transcription; in both cases, it is clear that SBF-dependent transcription requires an activation event. Interestingly, Slt2 has some transactivation activity (15, 51). Although we have shown that Slt2-GFP localizes to the nucleus upon α-factor treatment (Fig. 3C), our attempts at localizing Slt2 to the promoters of either *PCL1* or *PCL2* have been unsuccessful. Either Slt2 does not localize to DNA or its interaction with promoters is transient and not detectable using ChIP assays. Alternatively, SBF activation in response to Slt2 may require other proteins.

Both our *PCL2* reporter assay and Northern analysis show that the α-factor induction of *PCL2* is fully dependent on Swi6 but only partially dependent on Swi4. This is in contrast to the behavior of *PCL1*, whose log-phase and heat shock expression is independent of Swi6 (Fig. 1 and 6). One intriguing possibility is that Swi6 is required only to mediate a subset of *PKC1*-MAPK-mediated responses. Although both *swi6*Δ and *swi4*Δ strains are sensitive to variety of cell walls stressors, only *swi4*Δ strains and not *swi6*Δ strains are temperature sensitive for growth at 37°C (22, 28, 39), and overexpression of *SWI4* but not of *SWI6* can rescue the temperature sensitivity of *PKC1*-MAPK pathway mutants (28, 39). Since overexpression of *MBP1*, the other known DNA-binding partner of Swi6, does not rescue *PKC1*-MAPK pathway mutants (28, 39), cell wall defects of *swi6* mutants are likely due to inappropriate regulation of Swi4-dependent transcription. However, the possibility that Swi6 regulates other transcription factors cannot be excluded.

**Slt2-dependent regulation of Swi4.** As mentioned above, both our *PCL1*::*lacZ* reporter gene and Northern blot analyses show that *PCL1* expression is dependent on Swi4 and partially dependent on Slt2 but independent of Swi6 (Fig. 1 and 6). Swi6 independence is not unique to the *PCL1* gene. As discussed above, our microarray experiments identified other genes whose expression during log phase is dependent on Swi4 but independent of Swi6. Further, our Northern blot analysis confirmed that the log-phase levels of *PCL1* and *YLR110c* mRNA are Swi6 independent and that the log phase levels of *GIC1*

and *SRL1* mRNA are partially independent of Swi6. Surprisingly, Swi4 was also localized to the promoters of *PCL1*, *SRL1*, and *YLR110c* in the absence of Swi6. This result contrasts with a previous Swi4 ChIP study, which showed that Swi6 was required for Swi4 localization to the SCBs of the promoter of *HO* endonuclease (13), whose activity is fully dependent on Swi6 (9). In the same study, Swi6-independent localization of Swi4 to the promoter of *CLN2* was detected; however, we find that expression of *CLN2* is not affected by deletion of *SLT2* (data not shown). Clearly, Swi4 regulation is complex, and we propose that, in the absence of Swi6, Slt2 may control the binding of Swi4 to only a subset of Swi4-dependent genes.

Our work suggests that Slt2-Swi4-dependent genes are largely independent of Swi6. Consistent with this, we demonstrated that Slt2 interacts directly with Swi4 and not Swi6 (Fig. 7). A model to explain the apparent Swi6-independent regulatory properties of Swi4 is shown in Fig. 8. Both in vitro and in vivo experiments show that Swi4 has no intrinsic ability to bind SCB-containing DNA in the absence of Swi6 due to autoinhibition of DNA binding involving both the DNA-binding domain of Swi4 and the C-terminal region that interacts with Swi6 (5). Phosphorylation of Swi4 by Slt2 may relieve the intramolecular interactions that prevent Swi4 from binding DNA in the absence of Swi6. This regulation of Swi4 binding activity by Slt2 could occur in either the presence or the absence of Swi6. Consistent with this model, coimmunoprecipitation experiments and kinase assays show that Slt2 can interact with and phosphorylate both Swi6 and Swi4 in vitro (Fig. 7 and 39). Phosphorylation regulates DNA binding by the Ets-1 transcription factor by modulating its autoinhibitory mechanism (16); likewise, phosphorylation of Swi4 might affect its autoinhibition of DNA binding or the sequence specificity of DNA binding.

Another possibility is that Slt2 may regulate the interaction of Swi4 with another transcription factor, which can alleviate the autoinhibition of Swi4 DNA binding and/or alter the DNA-binding specificity of Swi4. The Swi4 homolog, Mbp1, can interact with the transcription factor Skn7 instead of Swi6, and the interaction of Mbp1 with Skn7 alters the activity and promoter specificity of Mbp1 (7). Likewise, Swi4 may also have alternative partners that regulate the binding of Swi4 to DNA.

MAPKs have been implicated in the regulation of chromatin remodeling (6). Recent studies of the SBF-dependent gene *HO* show that directed chromatin remodeling of the locus is required before Swi4 can bind the promoter (13, 34). The promoters of other Swi4-dependent genes may also require chromatin remodeling to allow Swi4 DNA binding. One of the roles of Slt2 may be to activate or coordinate chromatin remodeling on the promoters of Slt2-dependent genes to allow Swi4 to specifically bind only these promoters and not all SBF-dependent genes. In agreement with this idea, our ChIP experiments show that Slt2 is partially responsible for localizing Swi4 and Swi6 to the promoters of *PCL1* and *PCL2* upon heat shock and α-factor treatment. Currently, only three in vivo targets of Slt2 have been identified; a more complete view of in vivo substrates of Slt2 will be required to better define the dual role of Slt2 and Swi4 in activating gene transcription.

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