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Multiple Levels of Regulation of the G1 Transcription Factor SBF

Doctor of Philosophy 2000

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ABSTRACT

In all eukaryotic cells the commitment to enter the cell cycle in late G1 is a highly regulated event characterized by a burst of gene transcription. In budding yeast, the heterodimeric transcription factor SBF is composed of Swi4 and Swi6 and is required for the induction of a large group of genes early in the cell cycle. In this thesis, I describe a novel set of mechanisms that regulate and modulate the activity of SBF.

During the cell cycle, Swi4 binding to DNA is a highly regulated event restricted to only late M and G1 phases. Through a series of genetic and biochemical experiments, I determined that the inhibition of Swi4 binding to SCBs in the absence of Swi6 is intrinsic to Swi4 and is mediated through the C-terminal region of Swi4. Further, the C-terminal region of Swi4 could interact in vitro with the N-terminal DNA-binding domain of Swi4. My data suggest that intramolecular interactions with the C-terminal region of Swi4 physically prevent the DNA-binding domain from binding SCBs. Upon the addition of Swi6, the interaction of the C-terminal region of Swi4 with Swi6 alleviates this inhibition, allowing Swi4 to bind DNA.

SBF-dependent transcription involves the cyclin-dependent kinase Cln3-Cdc28. However, in the absence of Cln3, SBF-dependent transcription still occurs. I present direct
evidence linking the *PKC1*-MAPK pathway to SBF activation. Further, I found that activation of Slt2 by heat shock leads to the transcriptional induction of the SBF-dependent genes *PCL1* and *PCL2* but not *CLNI* and *CLN2*.

Through a series of genetic, biochemical and DNA microarray analyses I show that Slt2 likely modulates the activity of Swi4 to regulate the transcription of 28 genes, many of which are not cell cycle-regulated or SCB-regulated. This work suggests that the *PKC1*-MAPK pathway may utilize Swi4 outside of the cell cycle to elicit part of the transcriptional response necessary for cell integrity. My experiments also suggest that the heat shock expression of Slt2/Swi4-dependent genes may not require Swi6.
Acknowledgements

First and foremost, I would like to thank my supervisor Dr Brenda Andrews for her support during my five years in her lab. I especially would like to thank her for not letting me give up on the purification of Swi4 and making me march back to the cold room. I would also like to thank all the past and present members of the Andrews’ lab who have made my stay at graduate school memorable (and I do mean memorable). I wish you all the best of luck in your future endeavors.

I would also like to thank my parents who have supported me through my many years of university. I promise this is it!! Most importantly I wish to thank Doug, whom without his love and support I doubt that this thesis would be done. You have picked me off both the floor and the ceiling many times this year…so Doug this thesis is as much yours as mine.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Δ</td>
<td>deletion (symbol for a gene knockout)</td>
</tr>
<tr>
<td>α-factor</td>
<td>alpha-factor mating pheromone</td>
</tr>
<tr>
<td>APC</td>
<td>anaphase promoting complex</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>CDC (cdc)</td>
<td>cell division cycle</td>
</tr>
<tr>
<td>Cdk</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>Clb</td>
<td>B-type cyclins (Clb1-6)</td>
</tr>
<tr>
<td>Cln</td>
<td>G₁ cyclins (Cln1-3)</td>
</tr>
<tr>
<td>CFW</td>
<td>calcoflour white</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CTR</td>
<td>carboxy-terminal region</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>Fig.</td>
<td>figure</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>HS</td>
<td>heat shock</td>
</tr>
<tr>
<td>HTH</td>
<td>helix-turn-helix</td>
</tr>
<tr>
<td>kD</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBF</td>
<td>MCB binding factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MCB</td>
<td>MluI cell cycle box</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>NX</td>
<td>no cross-linking</td>
</tr>
<tr>
<td>pA</td>
<td>protein A-sepharose</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>p.f.u.</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PRE</td>
<td>pheromone response element</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>SBF</td>
<td>SCB binding factor</td>
</tr>
<tr>
<td>SCB</td>
<td>Swi4/Swi6-dependent cell cycle box</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium-dodecylsulfate</td>
</tr>
<tr>
<td>SWI4</td>
<td><em>italics</em>, capital letters, refers to the gene</td>
</tr>
<tr>
<td>Swi4</td>
<td>non-italics, first letter capital, refers to the gene-product or the protein</td>
</tr>
<tr>
<td>TAR</td>
<td>transcriptional activation region</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>ts</td>
<td>temperature-sensitive</td>
</tr>
<tr>
<td>WCE</td>
<td>whole cell extract</td>
</tr>
<tr>
<td>Wt</td>
<td>wild-type</td>
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</tbody>
</table>

xiii
The events of DNA replication, mitosis and cytokinesis define a fundamental periodicity in the eukaryotic cell cycle. Precise coordination of the unidirectional transitions between these stages is critical to cell integrity and survival. Loss of appropriate cell cycle regulation is involved in the development of numerous diseases including both hereditary and spontaneous cancers. Although oncogenic defects have been identified which target all stages of the cell cycle, the step that is most frequently deregulated is at the G<sub>1</sub>/S boundary (Bartek et al., 1999; Ford and Pardee, 1999). Indeed, in all eukaryotic cells, the commitment to enter the cell cycle in late G<sub>1</sub> at the “Restriction Point” in mammalian cells or at “Start” in yeast cells, is a highly regulated event. Passage through “Start” or the “Restriction Point” is accompanied by a burst of gene transcription which commits the cell to the cell cycle (for review see Lania et al., 1999; Sanchez and Dynlacht, 1996).

At the restriction point or Start, the cell must sense signals regarding cell size, nutrient availability, cellular integrity and extra-cellular environmental stresses. All of these signals converge on late G<sub>1</sub> transcription factors to either activate transcription and drive entry into the cell cycle or repress transcription and block entry into the cell cycle. Understanding the mechanisms of integration of signaling pathways regulating G<sub>1</sub> transcription factors is key to our understanding of the molecular basis of diseases in which cell cycle regulation is perturbed.

The use of the budding yeast *Saccharomyces cerevisiae* as a model organism to understand the cell cycle has been pivotal to our understanding of transcriptional control of the cell cycle. In *S. cerevisiae*, two G<sub>1</sub>-transcription factors, SBF and MBF, are responsible for the burst of transcription in late G<sub>1</sub>. In my thesis I elucidate novel mechanisms of regulating and modulating the activity of the G<sub>1</sub> transcription factor SBF. In this chapter, I first present a brief overview of the yeast cell cycle, followed by a survey in depth of the function, structure and
regulation of SBF at Start. I also discuss roles for SBF outside the cell cycle with particular emphasis on the PKCI-MAP Kinase cell wall integrity pathway.

SBF and Regulation of the *S. cerevisiae* Cell Cycle

I. A. Overview of the *S. cerevisiae* Cell Cycle

Most eukaryotic cell cycles are divided into four major phases: G1, the gap between mitosis and the onset of DNA replication; S phase, the period of DNA synthesis; G2, the gap between S and M phases; and M phase or mitosis. *S. cerevisiae* is a particularly powerful model organism for studying the cell cycle since the phase of the cell cycle can be visually assessed by bud morphology (see Figure 1-1; reviewed by Lew, 1997). Below I give a brief outline of the key events of each stage of the cell cycle and the role of the cyclins.

i) **Start**: In budding yeast cells, the major regulatory event governing the cell cycle is “Start,” the point of cell cycle commitment. This landmark event in yeast is analogous to the “restriction point” in mammalian cells (Pardee, 1989). Start is defined as the point after which cells no longer respond to anti-mitogenic signals such as pheromones that arrest cells in G1 (Hartwell et al., 1974). Passage through Start is an irreversible decision; after Start, the cell must complete the cell cycle. Hence, regulating entry into Start is crucial for cell survival. Cells enter “Start” only after they have reached a critical size and have adequate nutrients provided that no pheromone is present (reviewed in Lew, 1997).

ii) **Post-Start Events and S phase**: Passage through Start initiates three major processes. First, DNA replication is initiated from numerous origins of replication (reviewed in Dutta and Bell, 1997). Second, spindle pole bodies assemble and duplicate shortly after Start and the short microtubules act as organizing centres for the upcoming cellular events of mitosis (reviewed in Lew, 2000). Third, bud formation is initiated by the coordinate rearrangement and polarization of
Figure 1-1 Schematic Overview of the *Saccharomyces cerevisiae* Mitotic Cell Cycle.

The cell cycle of *S. cerevisiae* is marked by two major cellular events, DNA replication in S phase and chromosome segregation in Mitosis. These two events are separated by two Gap phases G₁ and G₂. Entry into the cell cycle or passage through “Start” occurs in late G₁. As shown in the bottom half of the figure, progression through the cell cycle is easily monitored in yeast by the state of the bud. Cell cycle progression is driven by five successive waves of cell cycle-regulated transcription. The height of each wave is proportional to the numbers of genes induced in each wave. It is postulated that the various waves of transcription are regulated by distinct sets of transcription factors. The transcription factors proposed to be responsible for each wave of transcription are indicated at the top of the diagram (Spellman et al., 1998).
the actin cytoskeleton (reviewed in Lew, 2000). Cell polarization allows for delivery of secretory vesicles to the site of bud growth. This allows for specific delivery of cell wall components required for formation of the new bud and maintains the integrity of the cell wall.

iii) G2 and M phase: In contrast to higher eukaryotes, the nuclear envelope of S. cerevisiae is not broken down during mitosis. Rather, during G2 the nucleus migrates to the bud neck and during mitosis half of the nucleus squeezes through the neck. The short spindle poles formed in G1/S elongate during mitosis and perform their masterful coordination of faithful segregation of the chromosomes. Cytokinesis occurs shortly after the completion of nuclear division, producing a larger mother cell and a smaller daughter cell. The difference in size means that daughter cells must undergo a period of cell growth prior to entering the next cell cycle and dictates that the mother cell will enter the subsequent cell cycle earlier than the daughter cell.

I. B. Cyclin Dependent Kinases and Regulation of Cell Cycle

Nearly thirty years ago, classic cell fusion experiments showed that late G2 or M phase cells contain an M phase-promoting factor (MPF) capable of accelerating the onset of mitosis in G2 cells (Johnson and Rao, 1970; and reviewed in Nasmyth, 1996). Similarly, S phase cells contain S phase-promoting factor (SPF) capable of accelerating S phase in G1 nuclei (Rao and Johnson, 1970). Genetic and biochemical experiments determined that both MPF and SPF are kinases whose activity is modulated by interaction with an unstable regulatory subunit called a cyclin. We now know that every eukaryotic cell cycle is driven by successive waves of cyclin dependent kinase (Cdk) activity.

In S. cerevisiae, the major Cdk governing the cell cycle is Cdc28, which associates with three G1 cyclins (Cln1 to Cln3) and six B-type cyclins (Clb1 – Clb6) at defined times during the cell cycle (reviewed in Mendenhall and Hodge, 1998). The Cdk Pho85 associates with members
of a family of 10 cyclins named PCLs and also plays a role during the cell cycle (reviewed in Moffat et al., 2000). Three G1-specific cyclins, Pcl1, Pcl2 and Pcl9, activate Pho85 during G1 phase (Espinoza et al., 1994; Measday et al., 1994), but the precise roles of these complexes during G1 is not well defined (see section II A ii).

I. C. Cell Cycle-Regulated Transcription

One main control over the waves of cyclin activity occurs at the level of cyclin gene expression since cyclin transcription is often confined to discrete periods of the cell cycle. The associated Cdk activity is correlated with the transcriptional activation of the gene encoding the cyclin regulatory component. Cell cycle-regulated transcription is not limited to the cyclin genes. Rather, cell cycle-dependent mRNA fluctuations have been observed for genes involved in many cellular processes. Both yeast and mammalian cells have evolved to transcribe specific sets of genes at specific times throughout the cell cycle corresponding to when the activity of the gene is required (for review see Lania et al., 1999; Sanchez and Dynlacht, 1996). It has been postulated that there are many advantages to cell cycle-regulated expression of genes. First, transcribing genes only when the gene products are required conserves cellular energy since proteins are only synthesized when they are needed. Second, cell cycle-regulated expression is an important mechanism for confining the activity of a gene product to a specific stage of the cell cycle. In this case, transcriptional control must be coupled with mRNA instability and an unstable gene product.

Genome-wide transcriptional analysis using DNA microarrays has elegantly demonstrated the scale of cell cycle-regulated transcription during the mitotic cell cycle in budding yeast (Cho et al., 1998; Spellman et al., 1998). More than 800 genes, or more than 10% of the predicted 6220 genes of S. cerevisiae, are cell cycle-regulated. Though the boundaries are
not unequivocal, cell cycle-regulated transcription appears in five peak waves: late M/early G₁ phase, late G₁ phase, S phase, G₂ phase and M phase (Figure 1-1). It has been postulated that expression of each subgroup of cell cycle genes is largely regulated by specific transcription factors (see Figure1-1). Indeed, promoter analysis of each group of genes has identified distinct sequences within each subgroup that are related to the binding motifs of known transcription factors. DNA sequence motifs are also found in the upstream sequences of genes within subsets that do not have known DNA-binding proteins associated with them. This suggests that other, yet unidentified transcription factors may be required for faithful cell cycle-regulated transcription. Though studies of this comprehensive nature have yet to be conducted on mammalian cells, it is clear that large waves of cell-cycle transcription also occur in higher eukaryotic cells.

II. G₁-S Transcription at Start

As described earlier, passage through Start is an irreversible decision that commits the cell to entering the cell cycle (see section I A.i). Passage through Start initiates three events that require de novo synthesis of many gene products: DNA replication, spindle pole duplication and bud formation. Cell cycle DNA microarray experiments have determined that nearly 300 genes are induced in late G₁ in budding yeast (Cho et al., 1998; Spellman et al., 1998). A similar burst of transcription also occurs at the “Restriction Point” in mammalian cells (Lania et al., 1999). Below I outline the roles of some of the genes that are transcribed at the G₁-S boundary and are required for the fidelity of cell cycle progression.
II. A. Genes Transcribed at Start

i) G1 cyclins *CLN1* and *CLN2*

Cln1 and Cln2 activate the Cdk Cdc28 at Start (Tyers et al., 1993) and are required for regulating many cellular events at Start. A third G1 cyclin, Cln3, also activates the Cdk Cdc28 but the *CLN3* gene is transcribed earlier than *CLN1* and *CLN2* and has a role in regulating the activation of SBF at Start (see section IV B.(i) for more details on Cln3). *cln1Δcln2Δcln3Δ* mutants arrest before Start; however, a strain lacking any two *CLN* genes is viable (Nasmyth and Dirick, 1991). Further, hyperstable alleles of *CLN2* accelerate passage through Start (Tyers et al., 1993). Cln-Cdc28 complexes stimulate DNA replication indirectly by accelerating the degradation of the S phase inhibitor Sic1 (reviewed in Mendenhall, 1998). Sic1 inhibits the Clb-Cdc28 complexes that promote S phase and mitosis (Schwob et al., 1994). Cln-Cdc28 kinases also promote S phase by increasing the stability of the Clbs through phosphorylation of Cdh1, a protein required by the anaphase promoting complex (APC) for Clb degradation (reviewed in Jorgensen and Tyers, 1999). *CLN1* and *CLN2* have also been implicated in pre-bud site assembly (Lew and Reed, 1993), possibly through degradation of the CDK inhibitor Far1 (Shimada et al., 2000). During late M and early G1, Far1 binds to and sequesters Cdc24 in the nucleus. Cdc24 is the guanine-nucleotide exchange factor for the GTPase Cdc42, which is required for proper reorganization of the actin cytoskeleton during polarized growth. During G1, Cln-Cdc28 phosphorylates Far1 and triggers its degradation. Degradation of Far1 releases Cdc24 from the nucleus allowing Cdc24 to activate Cdc42 at the incipient bud tip and activate polarized growth (for more information about Cdc42 see section IX and Mendenhall and Hodge, 1998). Undoubtedly, there are many other roles for the Cln-Cdc28 complex in progression through Start which have yet to be determined.
ii) G₁ cyclins PCL1 and PCL2

PCL1 and PCL2 are the only members of the PCL cyclin family whose expression is induced at Start (Cho et al., 1998; Measday et al., 1997; Spellman et al., 1998). PCL1 expression peaks at the same time as that of G₁ cyclins CLN1 and CLN2. PCL2 expression peaks slightly earlier than PCL1, CLN1 and CLN2 and, though largely regulated by G₁/S transcription factors (see subsequent sections), expression of PCL2 is partially dependent on the late M/early G₁ transcription factor Swi5 (Aerne et al., 1998; Measday et al., 1997). As noted earlier, the PCL cyclins associate with and activate the Cdk PHO85. The Pho85-Pcl complexes have been implicated in a wide variety of cellular events (reviewed in Moffat et al., 2000), but the role of PCL1/2 at Start is not fully understood. Pcl1/2-associated Pho85 kinase activity is only essential for cell cycle progression in the absence of Cln1,2-associated Cdc28 kinase activity (Measday et al., 1994). Mutants defective in PHO85 or a subset of PCLs, including PCL1 and PCL2, display abnormal actin localization and bud site selection (Lee et al., 1998). This result suggests that PCL1 and PCL2 may have a role at Start in either establishing or regulating polarized cell growth that is distinct from CLN1 and CLN2. In chapter III, I explore a link between PCL1 and PCL2 expression and the PKCl-MAPK pathway (see section VII and subsequent sections for detailed discussion of the PKCl-MAPK pathway).

iii) Cell Wall Synthesis Genes

The surge of polarized growth required for bud formation necessitates the expression of numerous genes involved in formation of the cell wall. Indeed, the expression of a large group of genes involved in cell wall biosynthesis peaks in late G₁ coincident with budding (Cho et al., 1998; Igual et al., 1996; Spellman et al., 1998). This group of genes includes FKS1 (subunit for (1-3)-B-glucan synthase) and MNN1 (involved in mannosylation of proteins), both genes which
are also induced by the *PKCl*-MAPK pathway upon heat shock (Igual et al., 1996). These genes are co-expressed during the cell cycle with 92 genes whose expression peaks slightly after the peak of other G1 genes (Spellman et al., 1998). This expression pattern suggests that they may be regulated in a slightly different manner from the other G1-genomes.

iv) S Phase Cyclins *CLB5* and *CLB6* and DNA Synthesis Genes

The primary role of Clb5 and Clb6 is to initiate S phase (Schwob et al., 1994), prevent reinitiation on replication origins that have already fired (Dahmann et al., 1995) and negatively regulate Cln-Cdc28 activity (Basco et al., 1995). Consistent with these roles, *clb5Δclb6Δ* strains have a long delay in S phase initiation, but they do eventually progress through the cell cycle. This result suggests that the remaining B-type cyclins may play a redundant role with Clb5 and Clb6 (Schwob and Nasmyth, 1993). Indeed, a strain lacking all Clb activity arrests before S phase (Schwob et al., 1994). Since one of the primary events of Start is to initiate S phase, it is not surprising that many of the genes induced at Start are genes involved in DNA replication. These include the *CDC9* DNA ligase, the *POL1* DNA polymerase and the *RNR1/2* subunits of ribonucleotide reductase (Cho et al., 1998; Spellman et al., 1998).

II. B. *S. cerevisiae* G1-Transcription Factor Complexes SBF and MBF

As indicated in Figure 1.1 and illustrated in Figure 1.2, there are two transcription factor complexes required for transcription at Start, SBF and MBF (most recently reviewed in Breeden, 1996). Maximal expression of the G1 cyclins, *CLN1, CLN2, PCL1*, and *PCL2*, at Start requires the transcription factor, SBF (SCB binding factor) (Espinoza et al., 1994; Measday et al., 1994; Nasmyth and Dirick, 1991; Ogas et al., 1991). SBF is a complex composed of at least two proteins, Swi4 and Swi6, which binds the repeated upstream regulatory sequence CNCGAAA
Figure 1-2 The SBF and MBF Transcription Factor Complexes.

There are two major transcription factor complexes required for transcription at Start: SBF (SCB binding factor) and MBF (MCB binding factor). SBF, which is composed of Swi4 and Swi6, binds upstream regulatory element SCBs and is required for the maximal expression of the G\(_1\) cyclins, \textit{CLN1}, \textit{CLN2}, \textit{PCL1} and \textit{PCL2}, and cell wall biosynthetic genes. MBF, which is composed of Mbp1 and Swi6, binds the upstream regulatory elements MCBs and is required for the maximal expression of genes required for DNA replication, including \textit{TMPL}, \textit{POL1} and \textit{RNR1}. Both Swi4 and Mbp1 contain DNA-binding domains that bind their respective target DNA sequences (red stripes). In contrast, Swi6, the shared component of the two G\(_1\) transcription complexes, does not bind DNA, but interacts with both Swi4 and Mbp1 through a C-terminal heterodimerization domain (green shading).
SBF

Swi4

DNA binding domain

C-terminal heterodimerization domain

MBF

Swi6

Cln1

Cln2

Pcl1

Pcl2

Cell wall biosynthetic genes

Scb

Mbp1

Tmp1

Pol1

Rnr1

McB
SBF [Swi4/Swi6-dependent cell cycle box]) (Andrews and Herskowitz, 1989; Andrews and Herskowitz, 1989; Ogas et al., 1991; Taba et al., 1991). SBF is also required for the G1-specific expression of the HO gene and various cell wall biosynthetic genes (Andrews and Herskowitz, 1989; Breeden and Nasmyth, 1985; Igual et al., 1996). Biochemical studies have revealed that Swi4 is the component of SBF that specifically binds the SCB sequence (Andrews and Moore, 1992; Primig et al., 1992). Swi4 contains an N-terminal DNA-binding domain that is sufficient for specific recognition of SCB sequences in vitro (Primig et al., 1992). In contrast, Swi6 has no DNA-binding activity but is present in the SBF complex because of its interaction with Swi4 via the carboxy-terminal regions (CTRs) of the two proteins (see section III C.) (Andrews and Moore, 1992; Koch et al., 1993; Primig et al., 1992; Sidorova et al., 1995).

Swi6 also interacts with another DNA-binding protein, Mbp1, to form the transcription factor complex MBF (MCB-binding factor), which binds the repeated upstream regulatory sequence ACGCGTNA (MCB [Mlu1 cell cycle box]) (Koch et al., 1993; Lowndes et al., 1991; McIntosh et al., 1991). MBF is required for the late G1-specific transcription of numerous genes needed for DNA replication, including TMP1, POL1 and RNR1 (Dirick et al., 1992; Elledge and Davis, 1990). Similar to Swi4 in SBF, Mbp1 is the sole component which makes contacts with DNA, while Swi6 interacts with Mbp1 through their C-terminal regions (CTR) (Sedgwick et al., 1998).

The importance of SBF and MBF for cell cycle progression is clearly demonstrated by the phenotypes of the double mutants, swi4Δmbp1Δ and swi4Δswi6Δ, which die with a terminal G1 phenotype (Koch et al., 1993; Nasmyth and Dirick, 1991; Ogas et al., 1991). Single mutants deleted for any one of SWI4, SWI6 or MBP1 are all viable. However, in contrast to mbp1Δ strains, which have normal cell cycle progression and initiation of DNA replication
(Koch et al., 1993), both swi4Δ and swi6Δ strains are slow-growing with large misshapen cells (Andrews and Herskowitz, 1989; Breeden and Nasmyth, 1987; Breeden and Nasmyth, 1987). This result suggests that some SBF-regulated gene products are essential for appropriate cell cycle progression while misregulation of MBF-regulated genes can be tolerated. This idea is supported by the genetic observation that swi6Δmbp1Δ strains are viable with a phenotype similar to swi6Δ strains (Koch et al., 1993). Interestingly, in contrast to swi6Δ strains, swi4Δ homozygous diploid strains are inviable and swi4Δ/swi4-tsl diploids arrest in G1 when grown at the restrictive temperature (Ogas et al., 1991). These observations suggest that Swi4 may have roles at Start that are separate from those of Swi6.

The fact that swi4Δ and mbp1Δ single mutant strains are viable while a swi4Δmbp1Δ strain is inviable suggests that there may be cross-talk between SBF and MBF in vivo. Indeed, numerous studies have supported this idea. Regions of both the CLN1 (Partridge et al., 1997) and CLN2 (Cross et al., 1994) promoters are Swi4-dependent but do not contain consensus SCB elements. Detailed analysis of the CLN1 promoter has indicated that Swi4-dependent activation occurs through MCB-like elements with a CGCG core and in vitro studies confirmed that SBF can bind MCB-containing DNA fragments from the CLN1 promoter (Partridge et al., 1997). Conversely it has been shown that Mbp1 can bind SCBs in vitro (Koch et al., 1993; Partridge et al., 1997). Of the 300 G1-genes identified by DNA microarray analysis, nearly all have at least one copy of an MCB or SCB element or a degenerate SCB motif ACRMSAAA (where R is A or G, M is A or C, and S is C or G) (Spellman et al., 1998). It is not known whether SBF indeed acts through the degenerate SCB motif. Both in vivo and in vitro footprinting analysis have determined that Swi4 and Mbp1 contact DNA outside the core motifs, suggesting that the context of the promoter elements may contribute to the DNA-binding
specificity of Swi4 and Mbp1 (Andrews and Herskowitz, 1989; Dirick et al., 1992; Harrington and Andrews, 1996; Koch et al., 1996). In order to fully understand transcriptional activation by SBF and MBF, an in depth analysis of the sequence specificity of Mbp1 and Swi4 will be required.

II. C. G1/S Transcription Factor Complexes in Other Organisms

As regulation of “Start” or the “Restriction Point” is key to cell cycle control, many organisms have specialized G1/S transcription factors. In the fission yeast Schizosaccharomyces pombe, the G1/S transcription factor complexes are highly homologous to SBF and MBF (see next section and review by Breeden, 1996). “Start” transcription is mediated by three gene products: Cdc10 (Lowndes et al., 1992), Res1 (Caliguri and Beach, 1993) and Res2 (Miyamoto et al., 1994). Res1 and Res2 interact with Cdc10 to form heterodimeric complexes that can bind specifically to the MCB element ACGCGT (see Figure 1-3). Though both the Res1-Cdc10 and Res2-Cdc10 complexes have been implicated in Start-specific transcription, the two complexes appear to have different roles. Genetic studies have indicated that Res1 has a more important role in the mitotic cell cycle (Caliguri and Beach, 1993) while Res2 has a predominant role in meiosis and pre-meiotic replication (Miyamoto et al., 1994; Zhu et al., 1997).

In mammalian cells, Drosophila and other higher eukaryotes, the E2F and DP protein families form heterodimeric transcription factors that play a central role in expression of cell cycle-regulated genes at the G1/S boundary (for review, see Dyson, 1998). E2F-DP activity is controlled by the retinoblastoma (pRb) family of pocket proteins. There are six E2F genes, two DP genes and three pRb family members in mammalian cells, and, as expected, the family encodes proteins with diverse activities and regulation. The simplest model of E2F/pRB activity in the cell cycle is illustrated in Figure 1-3. The pRb-E2F complexes, which are thought to
A) The G1-S transcription factor complexes of *Schizosaccharomyces pombe* are highly homologous to SBF and MBF. Res1 and Res2 interact with Cdc10 to form heterodimeric complexes that bind specifically to MCB elements. As is the case with SBF and MBF, only Res1 and Res2 interact with the DNA. Genetic studies have indicated that the Res1-Cdc10 complex has a predominant role during the mitotic cell cycle, while Res2-Cdc10 has a predominant role in meiosis.

B) In higher eukaryotes, the E2F and DP protein families form heterodimeric transcription factors that play a central role in expression of genes at the G1/S boundary. In G0 or in quiescent cells, the transcriptional activity of the E2F-DP complex is repressed through interaction of pRB with E2F. Entry into the cell cycle leads to phosphorylation of pRB by Cyclin E- and Cyclin D-dependent CDKs. Phosphorylation of pRB inhibits the interaction of pRB with E2F, which leads to de-repression of the E2F-DP complex and active transcription.
A) *S. pombe*

\[ \text{ACGCGT} \]

**Mitosis Specific Complex**

\[ \text{ACGCGT} \]

**Meiosis Specific Complex**

B) Higher Eukaryotes

\[ G_0 \]

Active Repression

\[ \text{Cyclin E/D CDK} \]

De-repressed and Activated Transcription
predominate in quiescent or early G₁ cells, act as repressors of transcription since pRb masks the E2F transactivation domain. Cyclin D- and Cyclin E-dependent phosphorylation of pRb inhibit the interaction of pRb with E2F allowing activation of E2F-dependent transcription. Phosphorylation of E2F by Cdns also promotes gene activation by stabilizing the interaction of E2F with transcriptional coactivators (Morris et al., 2000).

Though there is little sequence similarity between E2F-DP and SBF/MBF, the complexes perform analogous roles in regulating G₁/S transcription. The heterodimeric E2F complexes recognize a central c/gGCGCg/c sequence, which is similar to the central CGCG core sequences recognized by SBF, MBF and Res/Cdc10 complexes (Zheng et al., 1999). X-ray crystallographic studies have also revealed structural similarity within the DNA-binding domains of E2F, DP and Mbp1 (see section III A. and Taylor et al., 1997; Xu et al., 1997; Zheng et al., 1999). In summary, studies of SBF and E2F regulation are likely to be mutually informative regarding conserved mechanisms of cell cycle-regulated transcriptional control.

III. Anatomy of G₁-Transcription Factors: Swi4, Swi6 and Mbp1

The S. cerevisiae G₁-transcription factors SBF and MBF are members of a family of yeast G₁-transcription factors that share significant protein sequence similarities (reviewed in Breeden, 1996). As described above, the family includes S. cerevisiae Swi4, Swi6 and Mbp1 and S. pombe Res1, Res2 and Cdc10. Kluyveromyces lactis also has homologs to Mbp1 and Swi6, but their roles in cell cycle transcription have yet to be determined (Koch et al., 1993). The SBF/MBF family members are most conserved in three distinct regions: an N-terminal DNA-binding domain, a central ankyrin repeat domain implicated in protein-protein interactions and a C-terminal heterodimerization domain (Figure 1-4). Proteolytic mapping of the domains of Swi6
**Figure 1-4 The Yeast Family of Cell Cycle Transcription Factors.**

Schematic diagram of the yeast family of transcription factors. The Swi4/6 family members are most conserved in three distinct regions: the N-terminal DNA-binding domain (red striped box), the central ankyrin domain (blue bubble equals an ankyrin repeat) and the C-terminal heterodimerization domain (green checkered box). The DNA-binding domains of the Swi4/6 family members are highly homologous and share similarity with three other transcription factors in fungi, including Phd1, Sok2 and StuA (see Figure 1-5). Interestingly, the N-terminus of Cdc10 has low similarity to the DNA-binding domains of the Swi4 family, but Cdc10 does not bind DNA. All Swi4/6 family members have a central domain composed of four and one half ankyrin repeats. The most similar repeats are shown in darker blue, while the less similar repeats are shown in lighter blue. There is more similarity among the C-terminal heterodimerization domains of the DNA-binding proteins Swi4, Mbp1, Res1 and Res2 (see Figure 2-5) and between the C-terminal heterodimerization domains of Swi6 and Cdc10.
has confirmed that the three regions of sequence homology are indeed structurally distinct domains linked by flexible regions (Sedgwick et al., 1998). In this section, I describe in detail the structural domains of the Swi4/6 family of cell cycle transcription factors with a particular focus on SBF.

III. A. DNA-Binding Domain

Biochemical studies have revealed that Swi4 is the component of SBF that specifically binds SCB sequences (Andrews and Moore, 1992; Primig et al., 1992). Mobility shift assays using SCB elements and in vitro translated fragments of Swi4 have shown that amino acids 36 – 155 contain a DNA-binding activity specific for SCB elements (Primig et al., 1992). However, neither full-length in vitro translated Swi4 nor Swi4 from swi6Δ yeast extracts can form discrete complexes on DNA (Andrews and Herskowitz, 1989; Primig et al., 1992). These results suggested that, in the absence of Swi6, the Swi4 DNA-binding domain is inhibited from binding DNA. In contrast, purified Swi6 shows no DNA-binding specificity on its own (Koch et al., 1993; Sidorova and Breeden, 1993), and UV cross-linking studies have shown that Swi6 does not interact with the DNA, even in the context of SBF or MBF (Dirick et al., 1992).

The DNA-binding domain of Swi4 is highly homologous to the N-terminal domains of other members of the Swi4/6 family of transcription factors, including Mbp1 and the S. pombe cell cycle transcription factors Res1 and Res2 (Figure 1-5). Mbp1 has been shown to bind DNA through its first 124 amino acids (Dirick et al., 1992), and the N-terminal regions of both Res1 and Res2 are also sufficient for DNA-binding in vivo and in vitro (Ayte et al., 1995; Caliguri and Beach, 1993; Zhu et al., 1997). Like Swi4, the DNA-binding proteins Mbp1, Res1 and Res2 are also unable to bind DNA in the absence of Swi6 or Cdc10 (Ayte et al., 1995; Koch et al., 1993; Zhu et al., 1997). A few other transcription factors have also been identified in fungi whose
Figure 1-5 Alignment of the N-terminal DNA-binding Domains of Swi4 Family Members.

A sequence alignment of the DNA-binding domains of the seven fungal proteins which share homology with the DNA-binding domain of Swi4. The green boxes indicate regions of identity and similarity (I, V, L) (F, Y) (K, R) with either the Swi4 or Mbp1 DNA-binding domain. The asterisk marks the position of the E56K mutation of the res1 semi-dominant, Cdc10-independent allele. The crystal structure of the DNA-binding domain of Mbp1 has been solved and the locations of the β-sheets (red arrows) and α-helixes (yellow tubes) are indicated above the alignment. The core region of the structure folds into a “winged” helix-turn-helix (HTH) motif. The location of the HTH is indicated on the bottom of the alignment. It is predicted that helix α2 is the recognition helix involved in contacting the DNA.
DNA-binding domains are similar to those of the Swi4 family. These proteins do not have either ankyrin domains or Swi4/6 C-terminal heterodimerization domains and have all been implicated in regulating development. They include the *S. cerevisiae* proteins Phd1 (Gimeno and Fink, 1994) and Sok2 (Ward et al., 1995), which have both been implicated in regulation of pseudohyphal growth, and the *Aspergillus nidulans* protein StuA (Miller et al., 1992) that is required for normal development of conidiophores.

Though the primary structures of the DNA-binding domains of the Swi4 family are highly conserved, the sequences show no apparent similarity to those of other DNA-binding domains. The X-ray crystal structure of the DNA-binding domain of Mpbl has been solved and consists of a six-stranded β-barrel packed against a bundle of four α-helices (Taylor et al., 1997; Xu et al., 1997). The core region of the structure folds into a helix-turn-helix (HTH) motif with a short N-terminal β-strand and a C-terminal β-hairpin. The topology of this region is very similar to those of the “winged-HTH” DNA-binding domains (reviewed in Wintjens and Rooman, 1996) of catabolite gene activator protein (CAP) (Schultz et al., 1991), biotin repressor (BirA) (Wilson et al., 1992), the globular domain of the linker histone H5 (Clark et al., 1993), heat shock transcription factor (HSF) (Harrison et al., 1994) and ETS domains (Donaldson et al., 1996). Interestingly, though E2F and DP have no sequence similarity to the Swi4/6 family and both E2F and DP are required to make contact with DNA, the recently published crystal structure of an E2F-DP-DNA complex shows that both E2F and DP have a fold related to the “winged-HTH” (Zheng et al., 1999). The structural parallels between E2F-DP and the Swi4 DNA-binding family members emphasize the conservation between mammalian and yeast G1-transcription factors.
Though both crystal structures of the Mbp1 DNA-binding domain were solved in the absence of DNA, the structural homology to other HTH domains, including that of E2F-DP, predicts that basic helix α2 is the recognition helix involved in contacting the DNA. Interestingly, a semi-dominant allele of res1 has been isolated in which glutamic acid 56 of helix α2 is mutated to lysine (Caliguri and Beach, 1993) (Figure 1-5). Expression of res1E56K suppresses cdc10 mutants, suggesting that the mutant Res1 may have increased capacity to bind DNA in vivo in the absence of Cdc10. The position analogous to E56 in the Swi4/6 family of proteins is always acidic (E or D); however, in the developmental regulators Phdl, Sok2 and StuA, the equivalent site is a basic amino acid. Interestingly, the developmental regulators appear not to have regulatory partners, like Swi6, that facilitate their DNA-binding. These observations suggest that the acidic site in helix α2 in the Swi4/6 family of DNA-binding domains may play an important role in regulating DNA-binding of the full-length proteins (Breeden, 1996; Xu et al., 1997). In section IV A. and in Chapter II, I discuss in more depth the regulation of Swi4 DNA-binding.

III. B. Central Ankyrin Domain.

All the Swi4/6 family members possess four complete and one partial ankyrin repeats in their central regions (Fig. 1-4). Ankyrin repeats typically occur as four or more continuous copies of a 33-amino acid degenerate sequence which provides a β-hairpin-helix-loop-helix (β2α2) structural framework for sites of protein-protein interactions (reviewed in Sedgwick and Smerdon, 1999). The role of the central repeats in Swi4/6 family proteins is not fully known, but it is clear that they are essential for function in some family members. Point mutations have been identified in each of the ankyrin repeats of Swi6 that eliminate Swi6-dependent SCB-regulated transcription (Ewaskow et al., 1998; Sidorova and Breeden, 1993). Similarly, loss of
function mutations have been isolated in the ankyrin domains of Swi4 (M. Donoviel personal communications) and the Swi4/6 family member Cdc10 (Reymond et al., 1993). Though the ankyrin repeats are required for transcription, neither mutations in nor deletion of the entire ankyrin region of Swi6 or Swi4 impairs the interaction between Swi4 and Swi6 (Andrews and Moore, 1992; Ewaskow et al., 1998; Sidorova and Breeden, 1993). However, mutations in the ankyrin domain of Swi6 can modulate the DNA-binding activity of SBF. While very few mutations fully eliminate SBF binding to SCBs, mutations in the ankyrin repeats can cause a change in the mobility of the complex (Ewaskow et al., 1998). This result suggests that the ankyrin domains are integral to the structure of Swi6. Since Swi6 ankyrin mutants are competent to interact with Swi4 and bind DNA, the structural integrity of the ankyrin domains of Swi6 is likely important for transcriptional activation by SBF, possibly through interaction with other proteins.

Crystallographic studies of the Swi6 ankyrin repeat region predict that the Swi4/6 family members have a unique arrangement of secondary structure elements (Foord et al., 1999). The ankyrin domains of Swi6 form intramolecular interactions with adjacent transcriptional activation regions (TARs) in vivo (Foord et al., 1999; Sedgwick et al., 1998). Interestingly, many mutations identified in the Swi6 ankyrin domain are found in residues that are involved in the intramolecular interaction (Ewaskow et al., 1998). Interaction of the ankyrin domain with adjacent TARs of Swi6 may antagonize the activity of TARs, suggesting that the central ankyrin motifs may play a central role in regulating transcriptional activity.

The ankyrin motifs of Swi6 are also required for the interaction of Swi6 with Stb1, a regulator of the timing of Start transcription (Ho et al., 1999, and see section IV B.(ii a)). However, it is not known whether Stb1 interacts directly with the ankyrin motifs or if disruption
of the ankyrin motifs causes structural changes to other parts of the protein that directly interact with Stb1. The B-type cyclin Clb2 directly interacts with the ankyrin motifs of Swi4, and has been implicated in regulating Swi4 dissociation from SCBs (section IV C. and Siegmund and Nasmyth, 1996). Though there is significant homology in the ankyrin repeats of the Swi4/6 family members, Clb2 does not interact with the ankyrin repeats of Mbp1 or Swi6. Thus, the ankyrin repeats of the Swi4/6 family are functionally divergent.

III. C. C-terminal heterodimerization domain

The third region of homology between Swi4/6 family members is the C-terminal region (CTR) that directs interaction between the DNA-binding proteins and the regulator proteins. Co-immunoprecipitation experiments show a direct interaction between Swi4 and Swi6 through their C-terminal regions (Andrews and Moore, 1992; Primig et al., 1992; Sidorova and Breeden, 1993; Siegmund and Nasmyth, 1996). Though detailed mapping of the interaction has yet to be completed, crude deletion analysis has determined that the C-terminal 78 amino acids of Swi4 (Siegmund and Nasmyth, 1996) and amino acids 653 to 772 of Swi6 (Sedgwick et al., 1998) are required for interactions between these proteins. Likewise, the C-terminal 183 amino acids of Mbp1 are sufficient to interact with Swi6 (Siegmund and Nasmyth, 1996) and Res1 and Res2 interact with Cdc10 through their C-terminal regions (Ayte et al., 1995; Zhu et al., 1997). The C-terminal region is highly conserved and is predicted to contain alpha helices with amphipathic character (see Figure 2-5 and Breeden, 1996; Siegmund and Nasmyth, 1996). Interestingly, there is more similarity between the CTRs of the regulatory proteins, Swi6, kSwi6 and Cdc10, and between the CTRs of the DNA-binding proteins Swi4, Res1, Res2, Mbp1 and kMbp1 (Breeden, 1996). So far, there is no evidence that Swi4 and Mbp1 can interact, and the specific features of the CTRs may direct interactions between regulator and DNA-binding proteins and
The timing of SBF-mediated gene expression during the cell cycle is tightly controlled and requires multiple levels of regulation of SBF activity: the binding of SBF to SCBs in early $G_1$, the activation of SBF at Start, and the dissociation of SBF from SCBs during S phase. These sequential levels in regulation may aid in promoting the unidirectional progression of the cell cycle.
preclude interactions between two DNA-binding proteins. Another yeast transcription factor, Xbp1, is required in meiosis (see section V) and has a region homologous to the CTR of the Swi4/6 family (Mai and Breeden, 2000). However, it is not known whether Xbp1 can interact with any of the Swi4/6 family members. In Chapter II, I present experiments showing that the CTR of Swi4 is not just required for interaction with Swi6, but also plays a central role in the inhibition of Swi4 DNA-binding.

IV. REGULATION OF SBF

The timing of SBF-mediated gene expression during the cell cycle is tightly controlled and requires multiple levels of regulation of SBF activity (see Figure 1-6). In this section, I describe the mechanism of regulation of SBF at three levels: the binding of SBF to SCBs in early G1, the activation of SBF at Start, and the dissociation of SBF from SCBs after S phase.

IV A) Regulation of SBF DNA-binding

In vivo footprinting studies with both an SCB reporter plasmid and the CLN2 promoter as well as chromatin immunoprecipitation experiments show that SBF binding to SCBs is limited to late M and G1 phases (Cosma et al., 1999; Harrington and Andrews, 1996; Koch et al., 1996). This strict regulation of SBF binding to SCBs through the cell cycle is accomplished through changes in the subcellular localization of Swi6, the intrinsic properties of Swi4, and access of SBF to the chromatin-bound promoters.

i) Subcellular Localization of Swi6

SBF activity may be regulated by changes in the subcellular localization of Swi6. Swi6 is largely cytoplasmic during S, G2 and early M phases and is predominantly nuclear during late M and G1 phases (Figure 1-7 and Sidorova et al., 1995) and during α-factor treatment.
Figure 1-7 Subcellular Localization of Swi6 Throughout the Cell Cycle.

The subcellular localization of Swi6 changes throughout the cell cycle. Swi6 is largely cytoplasmic (red) during S, G2 and M phase and Swi6 is predominantly nuclear during late M and G1 phases. Localization of Swi6 is dependent on phosphorylation of ser-160, located next to a NLS. When ser-160 of Swi6 is phosphorylated, Swi6 is predominantly cytoplasmic. In vivo footprinting studies and ChIP experiments have determined that SBF binds SCBs (purple) during periods when Swi6 is nuclear.
Taba et al., 1991). Localization of Swi6 is dependent on phosphorylation of serine-160, which is located next to a nuclear localization signal (NLS). Serine-160 is phosphorylated during late G₁, S and M phases and may "hide" the NLS, preventing nuclear localization of Swi6 (Sidorova et al., 1995). Although Cdc28 plays a role in the timing of ser-160 phosphorylation, it is not the kinase directly responsible for ser-160 phosphorylation (Sidorova et al., 1995). Presently, the kinase and the phosphatase responsible for regulating ser-160 phosphorylation remain unknown.

The relocalization of Swi6 to the nucleus is coincident with the in vivo footprinting of SCBs in late M phase and G₁ phase (Harrington and Andrews, 1996; Koch et al., 1996). A mutant of Swi6 in which ser-160 is substituted to aspartic acid is constitutively localized to the cytoplasm, but this mislocalization has no effect on the periodicity of HO, CLN1 and SWI4 G₁/S expression. However, the peak transcript levels of these genes are drastically reduced in strains expressing the S160D swi6 mutant. These results suggest that limiting the amount of Swi6 in the nucleus is an important mechanism for regulating SBF-dependent transcription. In chapter II, I present Swi4 localization studies that show that, in contrast to Swi6, Swi4 remains nuclear throughout the cell cycle.

ii) Inhibition of Swi4 DNA-binding

In vivo footprinting of SCBs in late M and G₁ phases is coincident with localization of Swi6 to the nucleus, suggesting that Swi4 binding to SCBs occurs only when Swi6 is nuclear. Indeed, there is no evidence that full-length Swi4 can bind SCBs independently of Swi6. In swi6Δ strains, SCB-driven expression of CLN1 and CLN2 is severely reduced and expression of HO is eliminated despite the fact that Swi4 protein is present and stable in a swi6Δ mutant (Andrews and Herskowitz, 1989; Breeden and Nasmyth, 1987; Nasmyth and Dirick, 1991; Ogas et al., 1991). Further, in vivo footprinting studies have shown that, in the absence of Swi6,
protection of SCBs cannot be detected (Harrington and Andrews, 1996; Koch et al., 1996). Similarly, chromatin immunoprecipitation (ChIP) studies have shown that Swi4 localization to the HO promoter is undetectable in a swi6Δ strain (Cosma et al., 1999). While endogenous levels of Swi4 in the absence of Swi6 cannot activate SCB-reporter genes, over-expression of C-terminal truncations of Swi4 in vivo can promote Swi6-independent transcription from SCB elements (Andrews and Moore, 1992; Sidorova and Breeden, 1993). Ectopic expression of wild-type Swi4 also allows some activation of SBF-dependent gene expression, but this activation has been attributed to C-terminal degradation of Swi4 due to overexpression (Sidorova and Breeden, 1993). Together, these observations suggest a model in which the DNA-binding domain of Swi4 is inaccessible in the full-length protein when not complexed with Swi6. In chapter II, I explore this model through a series of in vivo and in vitro experiments.

ii) Chromatin Remodeling

Transcriptional activation in eukaryotes requires that transcription factors gain access to their target promoters in a chromatin environment (reviewed in Gregory and Horz, 1998; Workman and Kingston, 1998). Recent work has elegantly shown that SBF binding to the SCBs of the SBF-dependent gene HO is dependent on chromatin remodeling (Cosma et al., 1999; Krebs et al., 1999). HO expression is tightly regulated, with expression occurring only in haploid mother cells in late G1 (for review see Nasmyth, 1993). HO expression is dependent on multiple trans-acting factors, including two chromatin remodelers, the ATP-dependent remodeling complex SWI/SNF and the histone acetyltransferase SAGA (Spt-Ada-Gcn5-acetyltransferase)(Breeden and Nasmyth, 1987; Perez-Martin and Johnson, 1998; Pollard and Peterson, 1997). HO expression is also entirely dependent on the transcription factors Swi5 and
Figure 1-8 Ordered Recruitment of *Trans*-Acting Factors to the *HO* promoter.

The association of Swi5, SWI/SNF, SAGA and SBF with the *HO* promoter is cell cycle regulated. In late anaphase Swi5 localizes to the promoter of *HO*. In mother cells, Swi5 then recruits the SWI/SNF complex. The localization of Swi5 to the *HO* promoter is transient, and Swi5 dissociates from the *HO* promoter after recruitment of the SWI/SNF complex. However, in daughter cells the daughter cell-specific transcriptional repressor Ash1 interacts with Swi5, preventing recruitment of SWI/SNF. The chromatin remodeling activity of SWI/SNF on the *HO* promoter appears necessary for recruitment of SAGA and acetylation of histones on the SCB elements. Only after the SAGA complex has been recruited to the promoter is SBF allowed to bind the SCB elements. Once SBF is localized to the *HO* promoter, transcription can only occur when SBF is activated at the G1/S boundary. The schematic is modified from Figure 9 in (Cosma et al., 1999).
SBF (Andrews and Herskowitz, 1989; Breeden and Nasmyth, 1987) and on the transcriptional repressor Ash1 (Sil and Herskowitz, 1996).

ChIP experiments show that \textit{HO} expression is regulated by the ordered recruitment of the \textit{trans}-acting factors to the \textit{HO} promoter (see Figure 1-8 and Cosma et al., 1999; Krebs et al., 1999). In late anaphase the Zn-finger transcription factor Swi5 localizes to the promoter of \textit{HO}. In mother cells, Swi5 then recruits the SWI/SNF complex. The localization of Swi5 to the \textit{HO} promoter is transient, and after recruitment of the SWI/SNF complex Swi5 dissociates from the \textit{HO} promoter. However, in daughter cells the daughter cell-specific transcriptional repressor Ash1 interacts with Swi5 preventing recruitment of SWI/SNF. The chromatin remodeling activity of SWI/SNF on the \textit{HO} promoter appears necessary for recruitment of SAGA and acetylation of histones on the SCB elements. Only after the SAGA complex has been recruited to the promoter is SBF allowed to bind SCBs. Once SBF is localized to the \textit{HO} promoter, transcription can only occur when SBF is activated at the G₁/S boundary (see next section). The inability of SBF to localize to the \textit{HO} promoter until after chromatin remodeling has occurred suggests that chromatin remodeling may be a key regulatory feature of SBF-dependent transcription. However it is questionable whether Swi5, SAGA and SWI/SNF are participants in the regulation of all SBF-dependent genes. The main role of Swi5 during the cell cycle appears to involve the regulation of many genes in the late M/early G₁ cluster, including \textit{SIC1}, \textit{PCL9}, \textit{CDC6} and \textit{ASH1} (McBride et al., 1999). Further, Swi5-binding sites are predominantly found in the promoters of late M/early G₁ genes (Spellman et al., 1998), and the role of Swi5 in regulating late G₁-genes other than \textit{HO} remains unknown. DNA microarray studies have shown that mutation of SWI/SNF or SAGA does not affect the log phase levels of most G₁-specific transcripts (Holstege et al., 1998). However, it is possible that SWI/SNF or SAGA may regulate
the cell cycle-dependent expression of $G_1$-genes. It will be interesting to determine whether activation of other $G_1$-specific SBF-dependent genes involves the ordered recruitment of trans-acting factors before SBF is allowed to bind DNA.

IV. B. Regulation of SBF Transcriptional Activation

The binding of SCBs by SBF is not coincident with activation of SBF-dependent transcription; a secondary event must occur in order to activate SBF (Cosma et al., 1999, and see Chapter IV; Harrington and Andrews, 1996; Koch et al., 1996). Activation of SBF-dependent transcription during the cell cycle is largely dependent on $CLN3$ activity; however, deletion of $CLN3$ only delays Start, and does not arrest the cells in $G_1$. This observation suggests that other factors also play a role in activating SBF. In this section, I discuss both Cln3-dependent and Cln3-independent activation of SBF during the cell cycle.

i) $CLN3$-Dependent Activation of SBF

Passage through Start and activation of SBF and MBF require Cdc28 activity (Breeden and Nasmyth, 1987; Cross and Tinkleberg, 1991; Dirick and Nasymth, 1991). Expression of any one of the three $CLN$ genes can restore SCB- and MCB-driven gene expression to a strain lacking $cln$ function (Cross and Tinkleberg, 1991; Dirick and Nasymth, 1991). However, $CLN3$ appears to be the main activator of SBF and MBF. In contrast to $CLN1$ and $CLN2$, whose expression peaks in late $G_1$ and remains low during the rest of the cell cycle, $CLN3$ transcript levels are relatively constant during the cell cycle with a small peak of expression in late M phase (Cho et al., 1998; Spellman et al., 1998; Tyers et al., 1993). Further, although Cln3-Cdc28 kinase has a lower specific activity compared to Cln1-Cdc28 or Cln2-Cdc28 kinases, it is a much more potent activator of SBF- and MBF-dependent transcription (Levine et al., 1996; Tyers et al., 1993). Elegant experiments have confirmed that $CLN3$ is essential for the timely activation
of CLN1 and CLN2 gene expression, while CLN1 and CLN2 are important for proper execution of other Start-related events such as DNA-replication and budding (Dirick et al., 1995; Levine et al., 1996; Stuart and Wittenberg, 1995). DNA microarray experiments confirm that overexpression of CLN3 induces the expression of G1-genes (Spellman et al., 1998). This result clearly demonstrates the potency of Cln3 as an activator of the transcriptional program at Start.

As mentioned previously, cells will only pass through Start after they have reached a critical cell size and if there are available nutrients. Several lines of evidence suggest that Cln3 may be required for integrating these signals and coordinating cell size and nutrients with Start transcription. cln3Δ strains have delayed entry into Start and have a larger cell size, while an increased dosage of Cln3 leads to earlier entry into Start with a smaller cell size (Cross and Blake, 1993; Tyers et al., 1993; Dirick et al., 1995; Nash et al., 1988; Stuart and Wittenberg, 1995). Further, Cln3 protein and Cln3-Cdc28 kinase activity are considerably higher in cells growing in glucose compared to cells growing in non-fermentable carbon sources (Hall et al., 1998). Presently, the coordination between Cln3 activity and cell size is not fully understood, but, recent work suggests that the Ras-cAMP pathway and the translational efficiency of CLN3 mRNA may be key in coupling cell division to cell growth (Hall et al., 1998; and reviewed in Polymenis and Schmidt, 1999; Polymenis and Shmidt, 1997). The Ras-cAMP pathway’s major role is in the sensing of nutrient conditions (reviewed in Thevelein and deWinde, 1999). Glucose activates the Ras-cAMP pathway to cause increased cAMP production. Inactivation of the Ras-cAMP pathway leads to a G1 cell cycle arrest, which can be suppressed by overexpression of CLN3 (Hall et al., 1998). Interestingly, the Ras-cAMP pathway does not regulate the levels of CLN3 mRNA, but regulates the protein synthetic rate of Cln3 (Hall et al., 1998). The 5' leader of CLN3 mRNA contains a translational control element that makes CLN3
translation efficiency extremely sensitive to changes in cellular ribosome content and protein synthetic rates that correlate with growth rates in yeast (Polymenis and Shmidt, 1997). CLN3 mRNA is translated well when ribosome content of the cell is high, but is disproportionately inhibited as the cell’s ribosome content decreases. Decreases in cellular cAMP levels, by inactivation of the Ras-cAMP pathway, produce a decrease in total protein synthesis and a decrease in Cln3 protein levels and Cln3-Cdc28 kinase activity (Hall et al., 1998). Nutrient signals and Cln3 translation are also linked by the yeast phosphatidylinositol 3-kinase homologues Tor1 and Tor2 (reviewed in Thomas and Hall, 1997). Presently, it is unknown whether the TOR pathway and the Ras-cAMP pathway act together or separately in regulating Cln3 activity. This work suggests that the sensitivity of Cln3 protein synthesis is a key mechanism in coordinating cell growth with the initiation of cell division.

Other external signals also impinge on the activity of Cln3. Pheromone treatment of haploid yeast cells arrests cell division in the G1 phase. One mechanism contributing to mating-factor-dependent G1 arrest is the inhibition of Cln3-Cdc28 kinase activity by the cyclin-dependent kinase inhibitor Far1 (Jeoung et al., 1998; and reviewed in Mendenhall and Hodge, 1998). Understanding how multiple external signals affect Cln3 activity will be important in delineating how cells regulate passage through Start.

How Cln3-Cdc28 activates SBF at Start is not understood. Since Cdc28 activity is necessary for Start and since both Swi4 and Swi6 are phosphoproteins in vivo (Amon et al., 1993; Madden et al., 1997; Sidorova et al., 1995; Taba et al., 1991), phosphorylation of SBF by Cdc28 has been proposed as the mechanism of SBF-activation (Breeden, 1996). Since Swi6 has been implicated in both positive and negative regulation of SBF target genes (Dirick et al., 1992; Lowndes et al., 1992), most studies have focused on the role of Swi6 phosphorylation. However,
Cdc28 complexes do not appear to be responsible for direct phosphorylation of Swi6, and phosphorylation site mutants of Swi6 are not compromised in their ability to activate transcription at the appropriate time in G1 phase (Sidorova et al., 1995). These studies suggest that, if Cln3-Cdc28 activation of SBF is through direct phosphorylation, Swi4 may be the true biological target of Cln3-Cdc28. Alternatively, it has been suggested that SBF acts as a scaffold to localize Cln3-Cdc28 to the promoter where it functions to activate other components (Breeden, 1996). However, biochemical interaction between SBF and Cln3 has not been reported. Another possibility is that Cln3-Cdc28-dependent activation of SBF may be through other proteins that have yet to be identified.

ii) CLN3-Independent Activation of SBF

The CLN3 gene is normally dispensable, and cln3Δ strains still undergo SBF- and MBF-dependent transcription, albeit at a larger cell size. This result suggests that there are alternative pathways for activation of SBF. So far, three genes or pathways have been implicated in the alternative activation of SBF: BCK2, STB1 and the Protein Kinase C1 (PKC1)-MAPK pathway. Below I briefly outline the research implicating these genes in activating transcription at Start.

a) BCK2

BCK2 (bypass of C-kinase mutation) was originally isolated as a gene that, when overexpressed, can suppress the temperature-sensitive cell lysis defect of strains carrying mutations in the protein kinase C pathway (Lees et al., 1993). However, BCK2 also has significant links to Start. Expression of BCK2 from a low copy plasmid can suppress the cell cycle arrest phenotype of a cln1cln2cln3 strain (Epstein and Cross, 1994). More significantly, deletion of either BCK2 or CLN3 results in a large cell size phenotype but deletion of both genes causes either cell death or a slow growth phenotype depending on the background (DiComo et
al., 1995; Epstein and Cross, 1994). The effects of deleting both CLN3 and BCK2 suggest that Cln3 and Bck2 are in different pathways which both regulate progression through Start by activating G1-gene transcription. Consistent with this genetic result, deletion or overexpression of BCK2 does affect the transcription of G1-genes (DiComo et al., 1995; Wijnen and Futcher, 1999). bck2Δ strains have a modest decrease in the rate and levels of CLN2 and PCL1 transcription, and overexpression of BCK2 leads to increased expression of CLN1, CLN2, CLB5 and PCL1. However, the induction of G1-transcription by BCK2 is not fully dependent on either SBF or MBF function (DiComo et al., 1995; Wijnen and Futcher, 1999). Nonetheless, overexpression of BCK2 does not suppress the lethality of swi4mbp1 or swi4swi6 strains, suggesting that, like CLN3, the main role of BCK2 at Start is activation of transcription through SBF and MBF. As in the case with CLN3, the mechanism of BCK2-dependent activation of SBF and MBF is unknown.

b) STB1

Stb1 was isolated from yeast by affinity chromatography using a recombinant Swi6 ligand (Ho et al., 1999). stb1Δ strains have no detectable defects in terms of growth, cell cycle progression or cell-cycle transcription of SBF- or MBF-dependent genes. However, as is the case with bck2Δ strains, a stb1Δ strain in a cln3Δ background shows a significant growth defect. The cln3Δstb1Δ double-mutant grows slowly with a significant G1 delay that results in a large cell size phenotype. In the absence of cln3, deletion of stb1 exacerbates the delay in G1 transcription that is seen in the cln3Δ strain. Strains lacking both Stb1 and Bck2 do not have any additional phenotypes, suggesting that Stb1 and Bck2 may be in the same pathway (M. Costanzo, personal communication). Since Stb1 interacts directly with Swi6, STB1 may be
involved in transducing $BCK2$-dependent activation of SBF, but a direct link between Bck2 and Stb1 has not been established.

c) **PKCI-MAP Kinase Pathway**

The *PKCI*-MAP kinase pathway in yeast may also have a role in activating cell cycle progression. The *PKCI*-MAP kinase pathway is required to maintain cellular integrity during periods of polarized growth such as bud emergence (see section VII for more detail). Like $bck2$ mutants, strains mutated for genes encoding *PKCI*-MAP kinase pathway components are inviable when Cdc28 activity is decreased (Marini et al., 1996; Mazzoni et al., 1993). Further, overexpression of full-length *SLT2*, the terminal MAP kinase of the *PKCI*-MAP kinase cascade (see Figure 1-9), suppresses the temperature-sensitive phenotype of *cdc28* mutants (Mazzoni et al., 1993). Cdc28 is also required for polarized growth at Start, and the synthetic lethality between the two pathways may be due to deletion of two pathways required for budding. However, bud formation requires *de novo* protein synthesis, and cells cannot progress through Start without activation of $G_1$ transcription. Therefore, it has been suggested that the MAP kinase *SLT2* may be able to activate transcription at Start in the absence of Cdc28 activity. In sections VII to XII, I describe the *PKCI*-MAP Kinase pathway in detail, including its regulation and the possible connections between SBF regulation and the cell wall integrity pathway.

**IV. C. Regulation of SBF Repression/Dissociation**

Clb-Cdc28 kinase activity is required for repression of SBF-dependent transcription in $G_2$ and M phases. In a $clb^{12}$ strain that is devoid of all Clb-Cdc28 activity, *CLN2*, *CLN1* and *PCL1* transcript levels remain high in $G_2$ phase (Amon et al., 1993). Further, even in strains with high *CLN* activity, overexpression of *CLB2* represses SBF-dependent transcription (Amon et al., 1993). This result suggests that repression of SBF-regulated genes by Clb kinases is dominant
over activation of SBF by Cln kinases. This regulation may aid in promoting the unidirectional progression of the cell cycle. Further, DNA microarray analysis has shown that overexpression of \textit{CLB2} causes the repression of most \textit{CLN3}-induced SBF-dependent \textit{G}_1-genes (Spellman et al., 1998).

Of the four B-type mitotic cyclins (Clb1-4), Clb2 seems to be the most important in that \textit{clb1Δclb3Δclb4Δ} strains show no detectable phenotype, whereas deletion of Clb2 alone greatly retards mitosis (Fitch et al., 1992; Surana et al., 1991). Co-immunoprecipitation experiments show that Swi4 and Clb2 interact in mitotically arrested cells (Amon et al., 1993). Further, \textit{in vitro} kinase assays on Clb2 immunoprecipitates from yeast lysates show that Swi4 is phosphorylated in the immunoprecipitates in a Cdc28-dependent manner (Amon et al., 1993). Although these experiments suggest that Swi4 is a substrate of Clb2-Cdc28 \textit{in vivo}, there is no direct evidence for this hypothesis. The Clb2-Swi4 interaction requires the ankyrin domains of Swi4 and is independent of Swi6 (Siegmund and Nasmyth, 1996). \textit{In vivo} footprinting studies show that SBF does not bind the SCBs of \textit{CLN2} when Clb2 kinase activity is high (Koch et al., 1996). These results suggest that \textit{CLB2}-dependent repression of SBF may involve phosphorylation of Swi4 by Clb2-Cdc28, which causes dissociation of SBF from the promoters of SBF-dependent genes. Interestingly though, the interaction between Swi4 and Clb2-Cdc28 appears specific: no interaction is detected between Mbp1 and Clb2-Cdc28. However, overexpression of \textit{CLB2} down-regulates the expression of MBF-dependent genes, suggesting that Clb2 also regulates MBF activity (Spellman et al., 1998).

\textbf{Roles of SBF Outside of the Cell Cycle}

Since strains lacking SBF arrest in \textit{G}_1 (Ogas et al., 1991) and since many \textit{G}_1-genes have at least one SCB or MCB element in their promoters (Spellman et al., 1998), the main function
of SBF is likely instigating the burst of transcription in late G₁ that is synonymous with Start progression. However, there are fewer than 300 genes whose transcription peaks at Start, but more than 1155 genes whose promoters contain matches to the SCB consensus elements (SPCD, 2000). As mentioned in section II B, Swi4 sequence specificity is probably not limited to consensus SCBs, suggesting there may be a large number of genes regulated by Swi4 that do not contain SCBs in their upstream regulatory sequences. A comparison of the number of SCB sites upstream of G₁ 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 are binding sites for other cell cycle transcription factors such as MCM/SFF (Spellman et al., 1998). This analysis suggests that SCB elements and SBF may regulate the transcription of many genes other than those induced at Start. In this section I describe three cellular roles for SBF outside of Start: in meiosis, the DNA damage response and as a target of the Protein Kinase C (PKC₁) pathway.

V. SBF and Meiosis

The mitotic G₁ cyclins may also play a role at the onset of meiosis (Colomina et al., 1999; Mai and Breeden, 2000). Though cln1Δ and cln2Δ strains can form spores (Dirick et al., 1998), CLNI may play a distinct role during meiosis. In contrast to cln2Δ strains, cln1Δ cells display a significant delay in sporulation with a 25% reduction in sporulation efficiency (Mai and Breeden, 2000), and overexpression of CLNI prevents meiosis (Colomina et al., 1999). The seemingly contradictory effects of deletion and overexpression of CLNI on meiosis can be explained by the hypothesis that CLNI activity is required for events early in meiosis and it is detrimental to invoke these events during the late stages of the meiotic program. This hypothesis is consistent with the transient induction of SWI4 and SWI6 early in the meiotic program (Leem
et al., 1998; Mai and Breeden, 2000). Also, deletion of either Swi4 or Swi6 results in reduced spore viability and recombination (Leem et al., 1998; Mai and Breeden, 2000). Presumably, the transient induction of SWI4 and SWI6 contributes to the transcription of CLN1 and other SBF-target genes required for meiosis. However, the genes induced by SBF in the early stages of meiosis have not been fully determined. It is assumed that SBF-dependent transcription during early meiosis must be tightly regulated, since SBF normally drives entry into the cell cycle that is antagonistic to meiosis and spore formation. Indeed, overexpression of CLN3 prevents meiosis, presumably because it activates SBF-dependent G1 transcription (Colomina et al., 1999).

Together, these observations suggest that the role of SBF during meiosis must be distinct from the role of SBF at Start. One possibility is that the specificity of SBF changes during meiosis so that SBF only activates a subset of genes necessary for meiosis. Another possibility is that, during meiosis, the cell may actively repress transcription of some SBF-target genes. Indeed, it has been shown that transcription of both CLN1 and CLN3 is actively repressed during late meiosis by the distant Swi4 family member Xbp1 (Mai and Breeden, 2000). It will be interesting to determine whether Xbp1 represses all SBF-dependent meiotic genes. Swi6 has also been shown to be necessary for the meiotic induction of the recombination genes RAD51 and RAD54, which have MCB elements in their promoters and may be regulated by MBF (Leem et al., 1998).

The S. pombe Swi4 family member Res2 has also been implicated in regulating the meiotic cell cycle (Miyamoto et al., 1994).

VI. SBF and DNA Damage

In S. cerevisiae and other eukaryotic cells DNA damage invokes both checkpoint responses and the transcription of a large group of DNA repair genes (see the review by Lowndes and Murguia, 2000). swi4Δ and swi6Δ strains but not mhp1Δ strains are sensitive to
the DNA damaging agent MMS and the DNA synthesis inhibitor hydroxyurea (HU) (Ho et al., 1997). This result suggests that SBF, but not MBF, is required for the DNA damage response. Moreover, SBF has been linked to two kinases involved in the DNA damage response: Rad53 (Sidorova and Breeden, 1997) and Hrr25 (Ho et al., 1997).

Rad53 is a dual specificity kinase that is required for the DNA-damage checkpoint pathway (reviewed in Elledge, 1996; Lowndes and Murguia, 2000). Activation of the checkpoint by DNA damage initiates a cell cycle delay, which allows time for repair and improves survival in the presence of damaging agents. When G1 cells are treated with the DNA-alkylating agent MMS, \textit{CLN1} and \textit{CLN2} mRNA levels are reversibly down-regulated (Sidorova and Breeden, 1997). Sidorova \textit{et al.} showed that Rad53 and Swi6 are not required for the initial down-regulation of \textit{CLN1} and \textit{CLN2}, but are both required to maintain repression of \textit{CLN} expression and delay G1 entry. The MMS-G1 delay is suppressed by overexpression of a Swi6-independent, truncated form of Swi4, suggesting that \textit{CLN} repression may be through Swi6. Consistent with this hypothesis, Swi6 becomes phosphorylated upon MMS treatment \textit{in vivo} in a Rad53-dependent manner. However, it has yet to be established whether phosphorylation of Swi6 is directly by Rad53 or a Rad53-associated kinase. It is also unknown whether phosphorylation of Swi6 upon Rad53 activation is required for repression of Swi6-dependent transcription.

When cells are exposed to DNA damage, many genes involved in DNA repair are transcriptionally induced, including the G1-specific genes encoding ribonucleotide reductase (\textit{RNR1, RNR2}, and \textit{RNR3}) (Elledge and Davis, 1990). \textit{hrr25, swi6} and \textit{swi4} mutants, but not \textit{mbp1} mutants, are defective in the induction of \textit{RNR} gene expression in response to HU \textit{in vivo}. Swi6 interacts with and is phosphorylated by Hrr25, a casein kinase I isoform. Strains lacking
Hrr25 are hypersensitive to double-strand DNA breaks and are extremely slow-growing (Ho et al., 1997). SBF may be a downstream target of Hrr25 because overexpression of SWI4 can suppress both the MMS and HU sensitivity and restore induction of the RNR genes in an hrr25Δ strain (Ho et al., 1997). Interestingly, the RNR genes have multiple MCB sites in their promoters, and the cell cycle-regulated G₁-induction of RNR1 is largely regulated by Mbp1 not Swi4 (Dirick et al., 1992; Elledge and Davis, 1990). This result suggests that, upon DNA damage, Hrr25 may modulate the activity of SBF to regulate genes it normally does not transcribe in the cell cycle. The mechanism of Hrr25 modulation of SBF activity is not known.

This work illustrates that SBF plays a role outside of the cell cycle upon DNA damage. SBF activity is modulated to repress the expression of CLN1 and CLN2 genes while activating the expression of genes involved in DNA repair. The failure to induce RNR genes in rad53 mutants suggests that Hrr25 and Rad53 may function in the same pathway (Huang and Elledge, 1997). However, a direct link between Rad53 and Hrr25 function has not been established and it is not known whether Hrr25 is required for repression of the CLNs during DNA damage.

VII. The PKC1-MAP Kinase Pathway and SBF

The PKC1-MAP kinase pathway is one of five MAP kinase pathways in yeast (reviewed in Gustin et al., 1998; Heinisch et al., 1999). The PKC1-MAPK pathway mediates maintenance of the cytoskeleton and cell wall integrity in response to numerous stresses, including heat shock, cell wall challenges, changes in external osmolarity and mating pheromone. As outlined briefly in section IV B. (ii, c), the PKC1-MAPK pathway has also been implicated in Start progression. Though it is thought that the PKC1-MAPK pathway mediates polarized growth through phosphorylation of target cytoskeletal proteins, it has recently been shown that activation of PKC1 also alters gene expression. In this section, I outline in detail the PKC1-MAPK pathway,
activation of the pathway, cell cycle roles of the pathway and, finally, the connections between SBF and the PKCI-MAP kinase pathway.

VII. A. PKCI in S. cerevisiae

The PKC superfamily of genes plays key roles in many organisms in the regulation of diverse cellular processes, including proliferation and regulation of the cytoskeleton (reviewed in Keenan and Kelleher, 1998; Livneh and Fishman, 1997). In mammalian cells, twelve distinct PKC isoforms have been isolated which share both sequence and functional characteristics, with each member activated by different stimuli (Mellor and Parker, 1998). Phosphatidylserine (PS) and diacylglycerol (DAG) activate classical mammalian PKC family members in a Ca^{2+}-dependent manner. However, not all members of the PKC family are regulated in this fashion. Other members are Ca^{2+}-insensitive, but are still activated by DAG in the presence of PS. The atypical members, PRK1 and PRK2, do not appear to respond to Ca^{2+}, DAG or PS, but are activated by the small GTPase RhoA. Regulatory domains in PKC have been identified for each specific signal, and each family member has a unique combination of regulatory domains that dictates whether the member is responsive to certain signals (for review, see Mellor and Parker, 1998).

The PKCI gene encodes the only S. cerevisiae homolog of the PKC superfamily of genes, and Pkcl is an essential serine/threonine-specific protein kinase (Antonsson et al., 1994; Levin et al., 1990; Watanabe et al., 1994). Yeast PKCI is a unique member the PKC superfamily in that it contains regions homologous to all known regulatory domains of mammalian PKCs (for review, see Mellor and Parker, 1998). Although this modular structure suggests that PKCI may be able to respond to all of the physiological activators of mammalian PKCs, purified Pkcl is not activated by combinations of PS, DAG or Ca^{2+} in vitro (Antonsson et
al., 1994). However, Pkcl is activated in response to the small GTPase Rho1. Rho1 is an essential protein of the Rho subfamily of Ras-related proteins and is required for cell growth (reviewed in Cabib et al., 1998, and subsequent sections). The GTP-bound form of Rho1 can interact with and is necessary for activation of Pkcl (Kamada et al., 1996; Nonaka et al., 1995). The exact mechanism of Rho1 activation of Pkcl remains uncertain. In vitro assays using recombinant proteins have shown that Rho-GTP does not stimulate Pkcl kinase activity but allows Pkcl to be stimulated by PS (Kamada et al., 1996). These results suggest that interaction of Pkcl with Rho-GTP in vivo might make Pkcl responsive to activation by PS or DAG, traditional activators of the mammalian PKC superfamily of signal transducers.

VII. B. The PKCI-MAPK Pathway

Pkcl regulates the mitogen-activated protein (MAP) kinase cascade depicted in Figure 1-9. The pathway consists of: the MAP kinase kinase kinase (MEKK) Bckl (Costigan et al., 1992; Irie et al., 1993; Lee and Levin, 1992), the redundant MAP kinase kinases (MEKs) Mkk1 and Mkk2 (Irie et al., 1993), and the MAP kinase Slt2 (Lee et al., 1993; Torres et al., 1991). Though Pkcl has not been shown to interact with Bckl in vivo, epistasis experiments have placed BCK1 directly downstream of PKCI (Costigan et al., 1992; Irie et al., 1993; Lee and Levin, 1992), and Pkcl can selectively phosphorylate Bck1 in vitro (Levin et al., 1994). Consistent with the activation of other MAP kinase cascades, activation of Bck1 leads to the sequential phosphorylation and activation of the downstream components Mkk1/Mkk2 (Irie et al., 1993; Kamada et al., 1995). In turn, the dual-specificity MKKI and MKK2 kinases are responsible for the phosphorylation of threonine 190 and tyrosine 192 in the activation loop of Slt2 (Lee et al., 1993). Phosphorylation of tyrosine 192 is directly correlated with kinase activity in vivo (Zarzov et al., 1996).
Figure 1-9 Schematic of the *PKCl*-MAPK Pathway.

Schematic representation of the main components of the *PKCl*-MAPK pathway. The components of the MAPK cascade are indicated in green, while the downstream targets of Slt2 are indicated in red. Please see text for more details on the individual proteins. The upstream activators of Pkc1 are discussed in sections IX and X and downstream targets are discussed in section XII. Dotted arrows represent the postulated branches of the pathway where detailed mechanisms of signal transmission are not fully known yet.
pkcl mutants have markedly thinner cell walls (Levin et al., 1994; Roemer et al., 1994) than wild-type cells and are sensitive to a variety of cell wall stresses such as heat shock. Cell wall phenotypes of pkcl mutants can be suppressed by inclusion of the osmotic stabilizer sorbitol in the growth medium (Levin and Bartlett-Heubusch, 1992; Paravicini et al., 1992). Strains carrying deletions of BCK1, SLT2 or both MKK1 and MKK2 are sensitive to temperature but not inviable like PKCl mutants (Lee and Levin, 1992). This result suggests that PKCl must have other roles besides activation of the Slt2-MAP kinase pathway. Interestingly, it has recently been shown that PKCl is required for both the depolarization and the repolarization of the actin cytoskeleton upon cell wall stress (Delley and Hall, 1999). However, components of the PKCl-MAPK pathway module are only required for repolarization (Delley and Hall, 1999). Consistent with this, both slt2 and bck1 mutants exhibit phenotypes associated with depolarization of the actin cytoskeleton, including delocalization of actin cortical spots, accumulation of secretory vesicles and defects in polarized cell growth (Costigan et al., 1992; Delley and Hall, 1999; Mazzoni et al., 1993). These studies suggest that the PKCl-MAPK pathway is required for cell polarization and for maintenance of cell wall integrity while an as yet uncharacterized PKCl-dependent pathway is required for depolarization. Below, I outline the numerous conditions that activate the PKCl-MAPK pathway.

VIII. Activation of the PKCl-MAP Kinase Pathway

VIII. A. Heat Stress Activation

Heat shock treatment of cells by shifting them from 23°C to 39°C induces the tyrosine phosphorylation of Slt2 and increases its kinase activity (Kamada et al., 1995; Zarzov et al., 1996). The phosphorylation and activation of Slt2 peaks at roughly 30 minutes after heat shock (Kamada et al., 1995). Mutants in the PKCl-MAP kinase pathway are temperature-sensitive and
lyse due to defects in the cell wall, suggesting that activation of Slt2 is required for integrity of the cell wall at higher temperatures (Kamada et al., 1995; Lee and Levin, 1992; Levin et al., 1990).

**VIII. B. Cell Wall Stability Challenges**

Mutants lacking components of the \textit{PKCl-MAPK} pathway are also sensitive to chemicals that challenge cell wall stability, such as SDS and calcoflour white (CFW) (Costigan et al., 1992; Igual et al., 1996). It is believed that these compounds have a disordering effect on cell wall structure (Klis, 1994). \textit{PKCl-MAPK} pathway mutants are also sensitive to caffeine and vanadate, however it is not known how these compounds effect the cell wall (Martin et al., 1996). Similar to heat stress sensitivity, the addition of the osmotic stabilizer sorbitol abolishes sensitivity to these compounds. It has been shown that Slt2 phosphorylation peaks 20 minutes after exposure to CFW, caffeine and vanadate (Ketela et al., 1999; Martin et al., 2000). The similar timing of activation of Slt2 by heat shock and cell wall stress suggests a common mechanism for activating the \textit{PKCl-MAPK} pathway.

**VIII. C. Hypotonic Stress Activation**

The \textit{PKCl-MAPK} pathway is also required for growth in low-osmolarity conditions (Levin and Bartlett-Heubusch, 1992). Organisms with cell walls, like yeast and plants, maintain an osmotic gradient across their plasma membranes to drive uptake of water for growth (Blomberg and Alder, 1992). It is predicted that decreasing external osmolarity increases the osmotic gradient, creating stress on the plasma membrane and cell wall. Thus, decreased osmolarity of the growth medium results in activation of the \textit{PKCl-MAPK} pathway (Davenport et al., 1995; Kamada et al., 1995). In contrast to both heat shock and chemical stress (see above), hypotonic stress induces phosphorylation of Slt2 within seconds (Davenport et al., 1995). The
speedy response to osmotic shock suggests that distinct regulators may activate *PKCI* in response to osmotic stress. Alternatively, activation may proceed through the same pathway, i.e., cell wall mechanosensors (see section IX); however, hypotonic stress may cause a quicker disorganization of the cell wall than other activators of the *PKCI-MAPK* pathway.

**VIII. D. Cell-Cycle Activation**

As stated in section IV B. (ii,c), the *PKCI-MAPK* pathway may play an important role in the cell cycle. Mutations in the *PKCI-MAPK* pathway show synthetic lethality when combined with G1-specific *cdc28* mutations (Marini et al., 1996; Mazzoni et al., 1993), and overexpression of *SLT2* can rescue some defects of a *cdc28* strain, allowing for passage through Start (Mazzoni et al., 1993). Tyrosine phosphorylation of Slt2 and Slt2 kinase activity are partially linked to Cdc28 activity (Marini et al., 1996; Zarzov et al., 1996), and Slt2 kinase activity is stimulated at the G1-S boundary in parallel with bud emergence (Zarzov et al., 1996). The mechanism that activates the *PKCI-MAPK* pathway at Start is unknown. One possibility is that Cdc28-dependent progression through Start initiates bud development and the stress on the cell wall from new bud growth activates Pkc1. Cln-Cdc28 activity induces DAG production at Start (Marini et al., 1996). However, as discussed in section VII A, attempts to show regulation of Pkc1 by DAG or related phorbol esters have been unsuccessful both *in vitro* and *in vivo* (Antonsson et al., 1994; Davenport et al., 1995; Watanabe et al., 1994).

**VIII. E. Mating Pheromone Activation**

*PKCI-MAPK* pathway mutants are defective in pheromone-induced shmoo formation and lyse after prolonged exposure to pheromone (Costigan et al., 1992; Errede et al., 1995; Madden et al., 1997). Pheromone treatment results in phosphorylation of Slt2 within 15 to 30 minutes (Buehrer and Errede, 1997; Errede et al., 1995; Ketela et al., 1999; Zarzov et al., 1996),
and prolonged α-factor exposure induces the transcription of genes encoding PKCl-MAPK pathway components (Roberts et al., 2000). These results indicate that the PKCl-MAPK pathway is required for polarized growth during mating. It is presently not known how pheromone treatment or mating activates the PKCl-MAPK pathway; however, there may be multiple mechanisms. Many genes have been implicated in regulating the phosphorylation and activation of Slt2 during α-factor treatment, including the mechanosensor Mid2 (see section IX A) and the cell polarity genes Spa2 and Bni1 (see sections X and XI). There also appears to be significant cross-talk between the mating factor pathway (see Figure 1-10) and the PKCl-MAPK pathway (Buehrer and Errede, 1997; Errede et al., 1995; Roberts et al., 2000; Zarzov et al., 1996). However, evidence suggesting pathway cross-talk is both conflicting and controversial and more work is needed to elucidate pathway interactions during α-factor response.

IX. Molecular Mechanisms of PKCl-MAPK Pathway Activation

The multiple stimuli that activate the PKCl-MAPK pathway and the different rates of phosphorylation of Slt2 suggest that there may be multiple mechanisms for PKCl activation. Indeed, many genes that act upstream of PKCl have been identified. However, complete elucidation of these pathways remains unknown. Below I outline the present state of knowledge of the various upstream activators of the PKCl-MAPK pathway from cell wall stress sensors to regulation of RHO1.

IX. A. Cell Wall Stress Sensors

The activation events listed above all cause some form of cell wall or plasma membrane stress. The state of integrity of the cell wall must be sensed and transduced to Pkc1 and transduced down the MAP kinase cascade. Numerous predicted single-pass transmembrane proteins with extracellular domains rich in serine and threonine have been implicated in
activating the \textit{PKCl}-MAPK cascade. These putative cell wall sensors, sometimes called mechanosensors, include Slg1 (also described as Hcs77 or Wsc1) and the related proteins Wsc2, Wsc3, Wsc4 (Gray et al., 1997; Jacoby et al., 1998; Vema et al., 1997) and Mid2 and the related protein Mtl2 (Ketela et al., 1999; Rajavel et al., 1999).

Interestingly, it appears that the putative cell wall sensors may have evolved to respond to different Pkc1 activating conditions. In some strain backgrounds, \textit{slg1}\textDelta mutants have phenotypes similar to \textit{PKCl}-pathway mutants; they have reduced growth-rates and they are sensitive to cell wall stresses including heat, SDS, CFW and caffeine, and these cell wall defects can be suppressed by sorbitol (Delley and Hall, 1999; Gray et al., 1997; Jacoby et al., 1998; Vema et al., 1997). Unlike \textit{slg1}\textDelta mutants, strains deleted for \textit{wsc2}, \textit{wsc3} or \textit{wsc4} do not have dramatic cell wall integrity phenotypes (Gray et al., 1997; Vema et al., 1997). However, deletion of three Wsc members (\textit{slg1}\textDelta\textit{wsc2}\textDelta\textit{wsc3}\textDelta) causes a stronger cell integrity defect than the single \textit{slg1} mutant, suggesting that the Wsc family members may be functionally redundant (Vema et al., 1997). Double mutants of \textit{slg1}\textDelta or \textit{slg1}\textDelta\textit{wsc2}\textDelta\textit{wsc3}\textDelta with members of the \textit{PKCl} pathway do not show additional defects, and epistasis experiments place \textit{PKCl} downstream of the Slg1 family of proteins (Gray et al., 1997; Vema et al., 1997). Consistent with the genetics, heat stress-induced activation of Slt2 is greatly reduced in strains lacking the Slg1 family of proteins (Gray et al., 1997; Jacoby et al., 1998; Vema et al., 1997).

Overexpression of \textit{MID2} can suppress the cell lysis defects of a \textit{slg1} mutant (Ketela et al., 1999; Rajavel et al., 1999). In contrast to the vegetative growth defects of \textit{slg1}\textDelta cells, under normal growth conditions \textit{mid2}\textDelta cells do not have any growth defects and appear to have normal cell walls (Ketela et al., 1999). Also in contrast to \textit{slg1}\textDelta strains, \textit{mid2}\textDelta cells are extremely sensitive to \textalpha-factor, are mildly sensitive to caffeine, are not temperature sensitive and have
increased CFW resistance (Ketela et al., 1999; Ono et al., 1994; Rajavel et al., 1999). The pheromone sensitivity of a mid2 mutant can be partially suppressed by overexpression of MTL1, a homolog of MID2 (Ketela et al., 1999; Rajavel et al., 1999). However, unlike mid2Δ strains, mtl1Δ strains display no obvious phenotypes but mid2Δmtl1Δ strains are temperature- and caffeine-sensitive; both phenotypes can be suppressed by sorbitol (Ketela et al., 1999; Rajavel et al., 1999). Epistasis experiments have placed Mid2 upstream of Pkcl and, upon treatment with α-factor, CFW, and heat shock, Mid2 function is required for full induction of Slt2 tyrosine phosphorylation and activation (Ketela et al., 1999; Lodder et al., 1999; Martin et al., 2000; Rajavel et al., 1999).

mid2Δslg1Δ double mutants are synthetic lethal in the absence of osmotic support, suggesting the two cell wall sensors may have overlapping roles in maintaining cell wall integrity. (Ketela et al., 1999; Rajavel et al., 1999). The mechanisms by which Slg1 and Wsc family members sense cell wall integrity remain to be determined. The predicted extracellular domains of both Mid2 and Slg1 are highly O-mannosylated (Ketela et al., 1999; Lodder et al., 1999; Rajavel et al., 1999), and glycosylation of these proteins may promote contact with the cell wall and function as molecular probes to detect cell wall stress. Presently, only the cellular localization of Mid2 and Slg1 have been determined. Slg1 localizes to the plasma membrane specifically at sites of cell wall growth or polarization (Delley and Hall, 1999). Mid2 has been localized to the plasma membrane, but it is not clear that Mid2 can localize to areas of polarized growth (Ketela et al., 1999; Rajavel et al., 1999). It is also not known how the sensors have been adapted to sense different cell wall disturbances (ie mating factor vs. heat shock), nor is it known how the signal is conveyed to activate Pkcl in vivo. Both slg1 and mid2 mutant phenotypes can be suppressed by overexpression of RHO1 or PKC1 (Gray et al., 1997; Jacoby et al., 1998;
Ketela et al., 1999; Rajavel et al., 1999; Verna et al., 1997). This result suggests that both families of cell wall stress proteins might activate the PKCl-MAPK pathway through RHO1. The Rho1 guanine nucleotide exchange factor Rom2 (see next section) is required for Rho1 activation after both heat shock and SDS treatment (Bickle et al., 1998). This result has led to the hypothesis that the cell wall mechanosensors may activate Rho1 through Rom2 (Delley and Hall, 1999). Remarkably, intracellular domains of the two families are not similar, suggesting that the components linking the mechanosensors to RHO1 may be different.

IX. B. RHO1-Dependent Activation of PKCl-MAPK Pathway

As previously discussed in section VII A, Rho1 is a small GTP-binding protein required for the activation of Pkc1, and many rho1 mutants have impaired Slt2 phosphorylation and kinase activity (Kamada et al., 1996; Martin et al., 2000; Nonaka et al., 1995). Rho1 is localized to regions of active cell growth such as the bud tip and neck regions between mother and daughter cells (Yamochi et al., 1994). Although, Rho1 may function to localize Pkc1 to areas of growth, Pkc1 localization studies have yet to be conducted. Rho-dependent localization of PKC has been demonstrated in mammalian cells (Mellor and Parker, 1998).

RHO1 is an essential gene and has other cellular roles besides activation of PKCl. Rho1 is an essential regulatory subunit of the beta-1,3-glucan synthase (GS) (Drgonova et al., 1996; Qadota et al., 1996), an enzyme that synthesizes beta-1,3-glucan, the major structural component of the yeast cell wall (reviewed in Smits et al., 1999). There are two highly related glucan synthase proteins in S. cerevisiae, the integral membrane proteins Fks1 and Fks2 (Mazur et al., 1995). FKS1 is the predominantly expressed gene during optimal growth conditions, while FKS2 is induced upon treatment with α-factor, CaCl2 or growth on poor carbon sources (Mazur et al., 1995). Rho1 interacts with both Fks1 and Fks2 and the GTP bound form of Rho1 is
required for GS activity (Drgonova et al., 1996; Qadota et al., 1996). Rho1 also regulates the localization of Fks1, directing glucan synthesis to sites of cell wall growth (Delley and Hall, 1999). Interestingly, RHO1 also controls transcription of the glucan synthase genes (Igual et al., 1996; Zhao et al., 1998). Rho1 may also control the actin cytoskeleton through interaction with Bni1, a formin-like protein that binds profilin (Pfy1) (Evangelista et al., 1997). Finally, Rho1 also interacts with Bem4, a Rho-type GTPase interacting protein (Mack et al., 1996), and Skn7, a two component signaling protein (Alberts et al., 1998; and reviewed in Gustin et al., 1998), but the biological roles of these interactions remains unknown. The multiple roles of Rho1 necessitate tight regulation of Rho1 activity.

The activity of Rho1 is upregulated by the guanine nucleotide exchange factor (GEF) Rom2 (Ozaki et al., 1996) and is downregulated by the GTPase-activating proteins (GAPs) Bem2 and Sac7 (Peterson et al., 1994; Schmidt et al., 1997). ROM2 was identified as a multicopy suppressor of a dominant negative rho1 mutant and rom2Δ cells have a temperature sensitive growth phenotype that can be suppressed by sorbitol or overexpression of RHO1 (Ozaki et al., 1996). Overexpression of a highly homologous GEF, ROM1, can also suppress rom2Δ strains, and rom1Δrom2Δ double deletion strains are inviable (Ozaki et al., 1996).

However, rom1Δ strains do not present any cell-wall integrity defects typical of PKCI-MAPK pathway mutants. This result suggests that Rom2 plays a major role in Pkcl1 activation. The temperature-sensitive phenotype of rom2Δ mutants can also be suppressed by deletion of SAC7 but not by deletion of BEM2 (Schmidt et al., 1997), suggesting that Sac7 is the main Rho1 GAP. However, both sac7Δ and bem2Δ strains have constitutively active Slt2 (Martin et al., 2000). It is presently not known how the GAP activities are regulated or whether they are differentially regulated.
Going farther upstream, Tor2 is a phosphatidylinositol kinase homologue that activates Rom2 (Schmidt et al., 1997). **TOR2** is an essential gene that appears to have two functions in G1. One is the redundant with the gene **TOR1** and controls translation initiation in early G1 (for review, see Thomas and Hall, 1997). The other function is unique to **TOR2** and promotes the organization of the actin cytoskeleton (Schmidt et al., 1997). The temperature-sensitive G1 arrest phenotype of tor2 mutants is suppressed by growth on sorbitol and by overexpression of **ROM2** and downstream **PKC1-MAPK** pathway components (Helliwell et al., 1998; Helliwell et al., 1998; Schmidt et al., 1997). These results suggest that Tor2 is signaling through Rom2, but the mechanism of Rom2 activation is unknown. Rom2 can be activated by cell wall stress independently of Tor2, suggesting that there are both Tor2-dependent and Tor2-independent pathways for activation of Rom2 (Bickle et al., 1998). Indeed, it appears that Slg1, the putative cell wall mechanosensor, does not require Tor2 for Rom2 activation (Delley and Hall, 1999). It is not clear whether all the Pkc1-activating cell wall sensors can activate Pkc1 independently of Tor2. The role of Tor2 in G1 suggests that Tor2 may be needed for the cell cycle-regulated activation of the **PKC1**-MAPK pathway in late G1.

**X. Localization of the **PKC1**-MAPK Pathway**

As mentioned previously, mammalian Pkc isoforms are localized to regions of polarized growth through interaction with Rho GTPases (Mellor and Parker, 1998). The subcellular localization of Pkc1 in yeast has yet to be determined, although evidence suggests that components of the **PKC1**-MAPK cascade may be localized to areas of polarized growth through interactions with the cell polarity protein Spa2. As is the case with **PKC1**-MAPK pathway mutants, spa2Δ cells are defective in polarized morphogenesis, and Spa2 has been localized to actin patches at growth sites such as the incipient bud, bud tips, bud necks during cytokinesis and
Figure 1-10  The Polarisome and Cross-Talk Between the *PKC1*-MAPK and the Mating Pheromone Pathway.

Schematic of the proteins involved in localization of the *PKC1*-MAPK pathway and "cross-talk" between the *PKC1*-MAPK Pathway and the Mating Pheromone Pathway. The components of the Mating Pheromone Pathway are depicted in red, the components of the polarisome are depicted in yellow and the components of the *PKC1*-MAPK pathway are depicted in green. Bni1 interacts with both components of the Mating Pheromone Pathway and the *PKC1*-MAPK pathway, providing a physical link between the two pathways. Spa2 also interacts with components of both pathways. The dotted arrows represent detected two-hybrid interactions of Spa2 with Ste11, Ste7, Mkk1 and Mkk2. Please see text for more details on the individual components.
Mating Pheromone Pathway

PKC1-MAPK Pathway
the projection tip of mating cells (Gehrung and Snyder, 1990; Snyder, 1989). The localization of Spa2 to the actin patches is mediated through the actin-binding protein Bud6 (Sheu et al., 1998). Biochemical experiments have determined that Spa2 can be found in a large protein complex that includes the formin-like protein Bni1, Bud6 and the Spa2 homolog Pea2 (Fujiwara et al., 1998; Sheu et al., 1998). This complex appears transient in nature, suggesting the association of the proteins may be temporally regulated, perhaps during polarized growth. All these proteins have been implicated in regulating the actin cytoskeleton, and the complex has been aptly named the “Polarisome” (Sheu et al., 1998). Interestingly, Bud6 and Spa2 do not require an intact actin cytoskeleton for proper localization to growth sites (Ayscough et al., 1997), which has led to the hypothesis that the “Polarisome” may mark sites of and initiate polarized growth (Sheu, 1998 and for a review see Gustin et al., 1998). Indeed, Spa2 is required for proper localization of Bni1 which, in turn, interacts with profilin (Pfy1), and profilin is required for actin polarization (Fujiwara et al., 1998).

Two-hybrid assays have shown that Spa2 can interact with the MEKs Mkk1 and Mkk2, suggesting that Mkk1 and Mkk2 are localized to regions of cell growth (Sheu et al., 1998) (see Figure1-10). Interestingly, Slt2 is hyperactive in spa2A strains compared to wild type cells, but spa2A cells have severe defects in further activating Slt2 upon pheromone treatment (Buehrer and Errede, 1997; Sheu et al., 1998). This result suggests that Spa2 may have a role in both repressing and activating Slt2. Deletions of SPA2 cause synthetic lethality with both bck1Δ and slt2Δ, indicating that Spa2 has at least one role outside of regulating the PKCl-MAPK pathway (Costigan et al., 1992; Costigan et al., 1994). Limited indirect immunofluorescence localization studies using Ha-Slt2 have determined that Slt2 can be localized to the cell wall upon heat shock.
(Kamada et al., 1995). It has not been determined whether Slt2 localizes to areas of polarized growth or whether Slt2 localization requires Mkk1, Mkk2 or Spa2.

XI. "Cross-Talk" Between the PKCl and Mating Pheromone MAPK Pathways

Haploid budding yeast of opposite mating types (a or α) can mate to form an a/α diploid cell. This mating process requires the coordination of numerous cellular events, including polarized growth toward mating partners, cell cycle arrest in G1 and increased expression of genes required for cell adhesion and cell fusion. These cellular events are regulated by the Mating Pheromone MAPK Pathway, which consists of the PAK kinase Ste20, the MEKK Ste11, the MEK Ste7 and the MAPK Fus3 (depicted in Figure 1-10 and reviewed in Gustin et al., 1998). One of the main functions of the pathway is the activation of the transcription factor Ste12, which is required for the transcriptional induction of genes needed for mating (reviewed in Gustin et al., 1998).

As discussed in section VIII E, the PKCl-MAPK pathway is also required for polarized growth during mating. How the PKCl-MAPK pathway is activated during mating is not fully understood. The mechanosensor Mid2 (see section IX A) is partially required for the full activation of Slt2 upon α-factor exposure (Ketela et al., 1999). However there may also be a direct physical interaction between the two MAPK pathways at the level of the “polarisome”. Bni1 interacts directly with the GTPase proteins Rho1 of the PKCl-MAPK Pathway and Cdc42 of the Mating Pheromone Pathway (Evangelista et al., 1997; and reviewed in Gustin et al., 1998). Genetic studies indicate that Bni1 and Spa2 function in the same pathway, and a bni1Δ strain requires both BCK1 and SLT2 for viability (Buehrer and Errede, 1997; Fujiwara et al., 1998). Like spa2Δ strains, bni1Δ strains are delayed in the activation of Slt2 upon α-factor treatment and are also delayed in the formation of the mating projection (Buehrer and Errede,
These results suggest that Spa2 and Bni1 may be involved in the "link" between the two MAPK-Pathways. Interestingly, Slt2 activation appears completely blocked in ste20Δ strains (Zarzov et al., 1996) and ste4Δ strains (Buehrer and Errede, 1997). However, there are contradictory results as to whether components downstream of Ste20 are required for Slt2 activation. One group found that ste11Δ and ste12Δ strains were not compromised for Slt2 activation (Zarzov et al., 1996), while a second group reported that activation of Slt2 was compromised in a ste12Δ strain and that de novo protein synthesis was necessary for Slt2 activation (Buehrer and Errede, 1997). The second group performed their experiments in a cdc28-compromised background, which might contribute to the differences in results. Alternatively, both upstream and downstream components of the Mating Pheromone Pathway may be required for activation of the PKC1-MAPK pathway. The physical connections between the two cascades through the Spa2-Bni1 multiprotein complex and the defects in spa2 and bni1 cells in activation of Slt2 upon α-factor treatment suggest that the "polarisome" may be one link. Interestingly, bck1Δ strains can still partially activate Slt2 upon α-factor treatment, but Bck1 is absolutely required for heat shock activation of Slt2 (Buehrer and Errede, 1997; Zarzov et al., 1996). Two-hybrid studies have also shown that Spa2 can interact with Ste11 and Ste7, but it is not known whether Spa2 is required for proper signal transduction through the Mating Pheromone Pathway. It has been proposed that Spa2 acts as a scaffold to bring Ste11 in close proximity to Mkk1 or Mkk2 during mating, resulting in their activation (Sheu et al., 1998). It will be interesting to determine the exact role of the "polarisome" in "cross-talk" between the two cascades.

As mentioned above, de novo protein synthesis and the transcription factor Ste12 have also been implicated in activation of Slt2 (Buehrer and Errede, 1997). Recent DNA microarray
studies have determined that the *PKCl*-MAPK pathway is required for the transcriptional induction of 90 genes after prolonged (60 to 120 minutes) exposure to pheromone (Roberts et al., 2000). This late response is dependent on an intact Mating Pheromone Pathway and on Bni1 (Roberts et al., 2000). Our present state of knowledge on the “cross-talk” between the pathways makes it difficult to assimilate the seemingly conflicting results of Ste12-independent activation of Slt2 and Ste12- and *PKCl*-dependent transcriptional response into one model. α-factor treatment induces the activation of Slt2 within 20 to 30 minutes (Buehrer and Errede, 1997; Errede et al., 1995; Ketela et al., 1999; Zarzov et al., 1996), but transcriptional activation occurs much later at 1 to 2 hours after α-factor treatment (Spellman et al., 1998). The differences may represent the involvement of the *PKCl*-MAPK pathway in two distinct responses, one involving an early role for Slt2 and the other a later role for Slt2. Future work will no doubt delineate the complex events of “cross-talk”.

**XII. Downstream Targets of the *PKCl*-MAPK Pathway**

The requirement of the *PKCl*-MAPK pathway in maintaining cell integrity in numerous situations suggests that the pathway must function through many downstream targets. The localization of some components of the MAPK cascade to areas of polarized growth or the plasma membrane (see previous sections) suggests that components of the cytoskeleton may be important targets of Slt2; to date, no direct cytoskeletal targets have been identified. In many systems, the major targets of MAPK cascades are transcription factors, and activation of the cascade leads to altered gene expression (reviewed in Schenk and Snarr-Jagalska, 1999; Treisman, 1996). Indeed, recent DNA microarray work showed transcriptional modulation of 90 genes in a strain expressing an activated allele of *PKCl* (Roberts et al., 2000). One target of Slt2 is the transcription factor *Rlm1*. However, *rlm1Δ* strains have only a subset of *PKCl*-MAPK
pathway mutant phenotypes, so there are likely other transcriptional targets of Slt2. In this section, I outline what is known about the known and putative downstream transcriptional targets of Slt2.

XII. A. The MADS Box Protein Rlm1

*RLM1* is a member of the MADS box family of transcription factors (Shore and Sharrocks, 1995). Many MADS box proteins are known targets of MAPK in mammalian cells (Treisman, 1996). Mutation of *RLM1* suppresses the lethality of a constitutively activated allele of *MKKI*<sup>S386P</sup> (Watanabe et al., 1995), and *rlm1Δ* strains are also resistant to growth inhibition by overexpression of an activated allele of *RHO1* (Dodou and Treisman, 1997). These results suggest that Rlm1 acts downstream of the *PKCI*-MAPK pathway. Indeed, Rlm1 is a substrate of Slt2 *in vitro* and is phosphorylated *in vivo* following heat shock in a Slt2-dependent manner (Watanabe et al., 1997). Rlm1 can also interact with Slt2 in a two hybrid system (Watanabe et al., 1995). Further, studies using a lexA-*RLM1* fusion protein and a reporter gene with a lexA-binding site showed that Rlm1 transcriptional activity is dependent on Slt2 (Dodou and Treisman, 1997; Watanabe et al., 1995). Interestingly, *RLM1* mRNA levels are co-regulated by *RLM1* and *SLT2* (Dodou and Treisman, 1997).

Recently, DNA microarray analysis identified 25 genes whose expression was altered after expression of a constitutively active *MKKI*<sup>S386P</sup> allele for four hours (Jung and Levin, 1999). Twenty-four of the *MKKI*<sup>S386P</sup>-induced genes were partially dependent on *RLM1*. This pattern suggests that, after 4 hours of Slt2 activation, Rlm1 may mediate the majority of the Slt2-effects. However, the phenotypes of *rlm1Δ* strains are much less severe than *PKCI*-MAPK pathway mutants (Watanabe et al., 1995). Similarly, overexpression of *RLM1* can only suppress the caffeine-sensitivity but not the temperature-sensitivity of a *bck1Δ* mutant (Watanabe et al., 1995).
1995). This work suggests that Rlm1 may be required for the Slt2-dependent transcriptional response of only a subset of genes.

XII. B. The HMG-Like Proteins Nhp6A and Nhp6B

Both *NHP6A* and *NHP6B* were isolated as high-copy suppressors of the lethality of a *bck2Δspa2Δ* strain and, when overexpressed, can suppress the temperature- and caffeine-sensitivity of a *slt2Δ* strain (Costigan et al., 1994). Nhp6A/B are related HMG (high-mobility-group)-like chromatin-associated proteins. Cells lacking both *NHP6A* and *NHP6B* have a phenotype similar to that of other *PKC1*-MAPK pathway mutants, and deletion of *SLT2* in a *nhp6AΔnhp6bΔ* strain causes no additional phenotypes, suggesting that the HMG-like proteins act in the same pathway as Slt2 (Costigan et al., 1994). Attempts to co-immunoprecipitate Slt2 with either Nhp6A or Nhp6B failed, and Nhp6A or Nhp6B do not appear to be phosphoproteins *in vivo*, suggesting that they are not direct targets of Slt2. Nhp6A/B are non-sequence-specific DNA-binding proteins that are implicated in the transcriptional induction of numerous genes both *in vivo* and *in vitro* (Paul1 et al., 1996). Overexpression of *NHP6A* or *NHP6B* also suppresses the transcriptional defects in strains expressing *swi6-ank* alleles and the caffeine-sensitivity of the *swi6-405* mutant (Sidorova and Breeden, 1999). *NHP6A/B* may have a specific role in the cell integrity pathway. However, the wide transcriptional induction of genes caused by overexpression of *NHP6A/B* and the lack of specific DNA-binding by Nhp6A/B suggest that *NHP6A/B* may be global regulators of transcription.

XII. C. SBF as a Potential Downstream Target of the *PKC1*-MAPK Pathway

As discussed in section VIII D, the *PKC1*-MAPK pathway plays a critical role during the G1/S transition. Overexpression of *SLT2* can rescue *cdc28* mutants, allowing for passage through Start. Since both Slt2 and Cdc28 are S/T kinases required for polarized growth, it has
been postulated that the ability of Slt2 to suppress cdc28 mutants is through Slt2-dependent phosphorylation of critical Cdc28 substrates regulating polarized growth. In agreement with this idea, overexpression of only the kinase domain of Slt2 can suppress cdc28 alleles (Mazzoni et al., 1993). However, passage through Start and successful completion of the cell cycle requires de novo protein synthesis that is partially accomplished by the transcriptional burst of G1-genes that are regulated by the SBF and MBF. Activation of SBF, and presumably MBF, requires Cln3-Cdc28 kinase activity (see section IV B). This suggests that one of the promiscuous targets of overexpressed Slt2 kinase in cdc28 strains may be SBF or proteins that activate SBF.

SBF or SBF-activating proteins may be true substrates of Slt2. Indeed, swi4Δ and swi6Δ strains share phenotypes with PKCl-MAPK pathway mutants. Some swi4Δ strains show a temperature-sensitive growth defect that is suppressed by sorbitol (Igual et al., 1996; Madden et al., 1997) and both swi4Δ and swi6Δ strains are sensitive to cell wall stresses such as SDS and CFW (Igual et al., 1996). It has also been shown that some swi6Δ strains are also sensitive to caffeine (Sidorova and Breeden, 1999); swi4Δ sensitivity to caffeine has yet to be tested. Interestingly, mbplΔ strains do not show cell wall defects (Igual et al., 1996), suggesting that these defects are specific to SBF and do not simply reflect defects in G1-transcription in general. Further, as is the case with PKCl-MAPK pathway mutants, swi4 mutants also exhibit polarized growth defects in both bud emergence and projection formation (Costigan et al., 1992; Costigan et al., 1994; Errede et al., 1995; Gray et al., 1997; Mazzoni et al., 1993; Ogas et al., 1991). Also, a genetic screen determined that, like other mutants in the PKCl-MAPK pathway, a swi4-100 mutant is synthetically lethal with spa2Δ (Flescher et al., 1993). This evidence suggests that SBF and the PKCl-MAPK pathway may function cooperatively to regulate polarized growth. In
chapter II, I present a series of experiments that show that SBF is a direct target of Slt2, linking the \textit{PKC1-MAPK} pathway directly with the cell cycle transcription factor SBF.

**XIII. Thesis Summary**

The goal of my thesis work was to elucidate novel mechanisms of regulating the G\textsubscript{1} transcription factor SBF. Specifically, I have determined that the inhibition of Swi4 DNA-binding is intrinsic to the protein and that the MAPK Slt2 can modulate SBF activity.

In chapter II, I present a series of biochemical and genetic experiments that examine the regulation of Swi4 DNA-binding. I determined that, in the absence of Swi6, full-length Swi4 is inhibited from binding DNA. This inhibition is intrinsic to Swi4 and mediated through the C-terminal region of Swi4. I show that the C-terminal region of Swi4 can interact \textit{in vitro} with the N-terminal DNA-binding domain of Swi4. My data suggest that intramolecular interactions with the C-terminal region of Swi4 physically prevent the DNA-binding domain from binding SCBs. This intrinsic inhibition of Swi4 DNA-binding may be an important mechanism regulating SBF activity, because I show that, in contrast to Swi6, Swi4 remains nuclear throughout the cell cycle.

In chapter III, I present a series of experiments performed in collaboration with Kevin Madden which physically link the SBF with the \textit{PKC1-MAPK} pathway. We determined that Swi4 is directly downstream of Slt2 and that Slt2 physically interacts with and phosphorylates both Swi4 and Swi6. Further, I found that activation of Slt2 by heat shock leads to the transcriptional induction of the SBF-dependent genes \textit{PCL1} and \textit{PCL2} but not \textit{CLN1} and \textit{CLN2}.

In chapter IV, I explore the mechanism by which Slt2 modulates SBF activity. I present evidence that heat shock activation of Slt2 can modulate the activity of Swi4 to regulate the transcription of non-cell cycle-regulated genes. Significantly, this work confirms that Swi4 has important roles outside of the cell cycle in maintaining the integrity of the yeast cell.
CHAPTER II

Regulation of Cell Cycle Transcription Factor Swi4 through Auto-Inhibition of DNA-Binding

Chapter 2 is a modified version of a published paper:


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I performed all the experiments described in this Chapter.
In *Saccharomyces cerevisiae*, two transcription factors, SBF (SCB binding factor) and MBF (MCB binding factor), promote induction of gene expression at the G$_1$/S phase transition of the mitotic cell cycle. Swi4 and Mbp1 are the DNA-binding components of SBF and MBF, respectively. The Swi6 protein is a common subunit of both transcription factors and is presumed to play a regulatory role. SBF-binding to its target sequence, the SCBs, is a highly regulated event and requires the association of Swi4 with Swi6 through their C-terminal domains. Swi4 binding to SCBs is restricted to late M and G$_1$ phases when Swi6 is localized to the nucleus. I show that, in contrast to Swi6, Swi4 remains nuclear throughout the cell cycle. This result suggests that the DNA-binding domain of Swi4 is inaccessible in the full-length protein when not complexed with Swi6. To explore this hypothesis, I expressed Swi4 and Swi6 in insect cells using the baculovirus system. I determined that partially purified Swi4 cannot bind SCBs in the absence of Swi6. However, Swi4 derivatives, carrying point mutations or alterations in the extreme C-terminus, were able to bind DNA in the absence of Swi6, and the C-terminus of Swi4 inhibited Swi4 derivatives from binding DNA *in trans*. Full-length Swi4 was determined to be monomeric in solution, suggesting an intramolecular mechanism for auto-inhibition of binding to DNA by Swi4. I detected a direct *in vitro* interaction between a C-terminal fragment (CTR) of Swi4 and the N-terminal 197 amino acids of Swi4, which contain the DNA-binding domain. Together, my data suggest that intramolecular interactions involving the C-terminal region of Swi4 physically prevent the DNA-binding domain from binding SCBs. The interaction of the CTR of Swi4 with Swi6 alleviates this inhibition, allowing Swi4 to bind DNA.
The timing of SBF-mediated gene expression is tightly controlled and requires multiple levels of regulation of SBF activity: the binding of SBF to SCBs in early G\textsubscript{1}, the activation of SBF at Start, and the dissociation of SBF from SCBs after S phase. \textit{In vivo} footprinting studies with both an SCB reporter plasmid and the \textit{CLN2} promoter, as well as chromatin immunoprecipitation experiments, show that SBF is bound to SCBs in late M and G\textsubscript{1} phases (Cosma \textit{et al.}, 1999; Harrington and Andrews, 1996; Koch \textit{et al.}, 1996). Interestingly, the binding of SCBs by SBF is not coincident with SBF-mediated transcription; a secondary event must occur in order to activate SBF-dependent transcription. Activation of SBF is dependent on the activity of Cln3/Cdc28 kinase at Start (Dirick \textit{et al.}, 1995; Stuart and Wittenberg, 1995). However, the mechanism of Cln3-dependent activation of SBF remains a mystery, and direct interaction of Cln3 with SBF has not been reported. In G\textsubscript{2} phase, the Clb1-4/Cdc28 kinases become active and are required for repression of SBF-dependent transcription (Amon \textit{et al.}, 1993; Koch \textit{et al.}, 1996). Repression of SBF by Clb kinases may involve the interaction of Clb2 with Swi4 and/or the phosphorylation of Swi4 (Amon \textit{et al.}, 1993). Clb2 interacts with the central ankyrin domain of Swi4 \textit{in vitro} (Siegmund and Nasmyth, 1996). It has been postulated that, upon exit from mitosis, the rapid proteolysis of the B-type cyclins allows SBF to once again bind SCBs (Koch \textit{et al.}, 1996).

SBF activity is also regulated by changes in the subcellular localization of Swi6. Swi6 is largely cytoplasmic during S, G\textsubscript{2} and early M-phases, and is predominantly nuclear during late M and G\textsubscript{1} phases (Sidorova \textit{et al.}, 1995). Localization of Swi6 is dependent on the phosphorylation of serine-160, which is located next to a nuclear localization signal (NLS). Serine-160 is phosphorylated during late G\textsubscript{1}, S and M phases and may "hide" the NLS.

\textbf{INTRODUCTION}

The timing of SBF-mediated gene expression is tightly controlled and requires multiple levels of regulation of SBF activity: the binding of SBF to SCBs in early G\textsubscript{1}, the activation of SBF at Start, and the dissociation of SBF from SCBs after S phase. \textit{In vivo} footprinting studies with both an SCB reporter plasmid and the \textit{CLN2} promoter, as well as chromatin immunoprecipitation experiments, show that SBF is bound to SCBs in late M and G\textsubscript{1} phases (Cosma \textit{et al.}, 1999; Harrington and Andrews, 1996; Koch \textit{et al.}, 1996). Interestingly, the binding of SCBs by SBF is not coincident with SBF-mediated transcription; a secondary event must occur in order to activate SBF-dependent transcription. Activation of SBF is dependent on the activity of Cln3/Cdc28 kinase at Start (Dirick \textit{et al.}, 1995; Stuart and Wittenberg, 1995). However, the mechanism of Cln3-dependent activation of SBF remains a mystery, and direct interaction of Cln3 with SBF has not been reported. In G\textsubscript{2} phase, the Clb1-4/Cdc28 kinases become active and are required for repression of SBF-dependent transcription (Amon \textit{et al.}, 1993; Koch \textit{et al.}, 1996). Repression of SBF by Clb kinases may involve the interaction of Clb2 with Swi4 and/or the phosphorylation of Swi4 (Amon \textit{et al.}, 1993). Clb2 interacts with the central ankyrin domain of Swi4 \textit{in vitro} (Siegmund and Nasmyth, 1996). It has been postulated that, upon exit from mitosis, the rapid proteolysis of the B-type cyclins allows SBF to once again bind SCBs (Koch \textit{et al.}, 1996).

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preventing nuclear localization of Swi6. The relocalization of Swi6 to the nucleus is coincident with the in vivo footprinting of SCBs in late M phase (Harrington and Andrews, 1996; Koch et al., 1996).

Although, until this study, the subcellular localization of Swi4 was unknown, several lines of evidence suggested that an additional control over SBF activity occurred through regulation of DNA-binding by Swi4. There is no evidence that full-length Swi4 can bind SCBs independently of Swi6. In swi6Δ strains, SCB-driven expression of CLN1 and CLN2 is severely reduced and expression of HO is eliminated despite the fact that Swi4 protein is present and stable in a swi6Δ mutant (Andrews and Herskowitz, 1989; Breeden and Nasmyth, 1987; Nasmyth and Dirick, 1991; Ogas et al., 1991). Further, in vivo footprinting studies have shown that protection of SCBs cannot be detected in the absence of Swi6 (Harrington and Andrews, 1996; Koch et al., 1996). While endogenous levels of Swi4 in the absence of Swi6 cannot activate SCB reporter genes, overexpression of C-terminal truncations of Swi4 in vivo can promote Swi6-independent transcription from SCB elements (Andrews and Moore, 1992; Sidorova and Breeden, 1993). Ectopic expression of wild type Swi4 also allows some activation of SBF-dependent gene expression but this has been attributed to C-terminal degradation of Swi4 due to overexpression (Sidorova and Breeden, 1993). Together, these observations suggest a model in which the DNA-binding domain of Swi4 is inaccessible in the full-length protein when not complexed with Swi6. In this chapter I explore this model through a series of in vivo and in vitro experiments.

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MATERIALS AND METHODS

Strains and plasmids. Standard methods for yeast culture and transformation were followed (Guthrie and Fink, 1991). Standard rich medium (YPD) and supplemented minimal medium (SD) were used (Kaiser et al., 1994). Yeast strains are shown in Table 2.1.

To construct a full-length clone of Swi6 with convenient restriction enzyme sites at the 5’ end, an NcoI site was introduced at the ATG of the SWI6 open reading frame by PCR amplification from a SWI6 template using the following primers: 5’CCGGCCATGGCGTTGGAAGAAGTGG-3’ and 5’CCGTCTCATTGTCATCAGTGCC-3’. The 630bp PCR product was digested with NcoI and Apal and cloned into NcoI-Apal-digested pSL1180 (Pharmacia) to create plasmid BA786. An Apal-BgII fragment carrying the remainder of the SWI6 gene was then cloned into Apal-BgII-digested pBA786 to reconstitute the entire gene (pBA788). The BgII-SalI genomic fragment containing SWI4 was cloned into the BamHI/SalI site of a modified pUC18 in which the BgII polylinker was incorporated at the EcoRI/HindIII site. The vector expressing a fusion of glutathione S-transferase (GST) to the C-terminal 144 amino acids of Swi4 was generated using PCR to amplify the 3’ end of Swi4. The primers used were 5’EcoRISwi4 (5’GTGCAGATCTTCGATATCAGAT’3) and 3’EndSwi4 (5’GACTGTCGACCATGGTTATGCCTTGCCCTC’3). The PCR product was digested with EcoRI and SalI and cloned into the EcoRI/SalI sites of vector pGEX-4T-2 (Pharmacia) to create pBA1248. The integrity of all PCR products was confirmed by sequence analysis. To construct a vector for expression of Swi4 from the constitutive GPD promoter, a BgII-SalI fragment containing the SWI4 gene was cloned from vector pBA476 into the BamHI/SalI sites of vector p424 GPD (ATCC # 87357) to create pBA1262. Plasmids used for in vitro transcription/translation of Swi4 and Swi6 have been previously described (pBA462 for full
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or Reference</th>
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<tr>
<td>BY263</td>
<td>MATa trp1Δ63 GAL2+ ura3-52 lys2-801 ade2-107 his3Δ200 leu2-1</td>
<td>(Measday et al., 1994)</td>
</tr>
<tr>
<td>BY107*</td>
<td>MATα swi6ΔHIS3</td>
<td>(Ogas et al., 1991)</td>
</tr>
<tr>
<td>BY108</td>
<td>MATα swi4ΔHIS3</td>
<td>(Ogas et al., 1991)</td>
</tr>
<tr>
<td>BY184</td>
<td>MATα swi4ΔHIS3 SCBlacZ</td>
<td>J. Ogas</td>
</tr>
<tr>
<td>BY185</td>
<td>MATα swi6ΔHIS3 SCBlacZ</td>
<td>J. Ogas</td>
</tr>
<tr>
<td>BY289</td>
<td>MATα TRP+</td>
<td>this study</td>
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* This strain and all following strains are isogeneic to BY263 with the exceptions noted.
length Swi4, pBA513 for Swi6, and pBA586, a derivative of Swi4 which has an internal deletion of amino acids 198-745 (Andrews and Moore, 1992).

**Immunofluorescence.** For indirect immunofluorescence with Swi4 antiserum, a polyclonal Swi4 antibody was affinity-purified and preabsorbed to a 1:1 mixture of fixed yeast cells and spheroplasts of the yeast strain BY184 essentially as described (YGAC website http://ycmi.med.yale.edu/YGAC/antibodycleanup.html, Gehring and Snyder, 1990; Ogas et al., 1991; Pringle et al., 1991). 200 mL of strain BY184 were grown to log phase in YPD at 30°C, harvested and fixed by the addition of formaldehyde to 3.7% for 1 hour at 30°C with shaking. The fixed cells were washed twice with 1.2 M sorbitol, 50 mM KH₂PO₄ pH 7.5 and resuspended in 4 mL of 1.2 M sorbitol, 50 mM KH₂PO₄ pH 7.5. Spheroplasts were made from 2 mL of fixed cells by the addition of β-mercaptoethanol to 0.1% and zymolyase (20000T) to 0.25 mg/mL, followed by incubation for 1 hour at 30°C. The spheroplasts were washed twice with 1.2 M sorbitol, 50 mM KH₂PO₄ pH 7.5 and added to the remaining 2 mL of whole fixed yeast cells. The cell mixture was washed and resuspended in 4 mL of phosphate-buffered saline (PBS). 200 μL of the cell mixture was incubated with 200 μL of affinity-purified Swi4 antibodies at 4°C for 1 hour with shaking. Cells were pelleted, and the antibody supernatant was transferred to another tube containing 200 μL of fresh cell mixture. Antibody-cell incubation was repeated 7 times, including an overnight incubation, resulting in a 5-fold dilution of the pretreated affinity-purified Swi4 antibodies.

Wild type cells (BY263) and swi4Δ cells (BY184) were grown to early log phase and fixed in 3.6% formaldehyde at 30°C for 2 hours. Cells were washed twice with 100 mM KH₂PO₄ pH 7.4, resuspended in 100 mM KH₂PO₄ pH 7.4, 0.1% β-mercaptoethanol, 0.25mg/mL zymolyase (20 000T) and incubated at 30°C for 30 minutes to digest the cell wall. The cells
were then washed twice in PBS containing 1.2 M sorbitol. Cells were incubated with PBS plus 2% Bovine Serum Albumin (BSA, Sigma) for 30 minutes at room temperature. The cells were then pelleted, washed once in PBS/0.2%BSA and incubated for 2 days at 4°C in a 1/20 dilution of affinity-purified and preabsorbed anti-Swi4 antibody in PBS/0.2%BSA. The cells were then washed three times, for 10 minutes each, with PBS/0.2% BSA. A 1:20 dilution of preabsorbed sheep anti-rabbit CY3-conjugated secondary antibody (a gift from M. Snyder) in PBS/0.2%BSA was added and incubated for two hours at room temperature. The cells were then washed again as outlined above, followed by a final wash with PBS containing 0.05 μg/mL 4',6-diamidino-2-phenylindole (DAPI). One drop of the cell suspension was deposited on a polylysine-coated slide (Flow Labs). Once the cells had settled, the adhered cells were rinsed with PBS/0.1%BSA and mounted in 90% glycerol in PBS containing 0.1% p-phenylamine diamine. Cells were observed at 630X magnification using a Leica DM-LB microscope using Nomarski optics and a Princeton CCD camera. Swi4 staining was visualized with rhodamine fluorescence optics. 800 wild type cells were scored for nuclear staining and position in the cell cycle as judged by bud size.

Construction of baculoviral vectors. We used the Bac-N-Blue Baculovirus system to express both Swi6 and Swi4 derivatives in insect cells (Invitrogen). To construct a baculovirus expressing Swi6, the NcoI-HindIII fragment from pBA788 containing full-length SWI6 was cloned into the NcoI/HindIII sites of the baculovirus transfer vector pBlueBacIII (Invitrogen). To construct a Swi4 expression vector, a BglII fragment from pBA476 containing full-length SWI4 was cloned into the BamHI site of pBlueBacIII (Invitrogen). The SWI4ΔAnkyrin motif (Swi4ΔAA) transfer vector was constructed by digestion of pBA476 with NsiI followed by religation to create a Swi4 derivative with a 1047bp internal deletion. The deleted SWI4
fragment was then cloned into the BamHI site of the baculovirus transfer vector pBACHISA (Invitrogen). The Swi4Δ144 transfer vector was constructed by cloning the BglII/EcoRI fragment from BA476 into the BamHI/EcoRI site of baculovirus transfer vector pBlueBac4.5. The transfer vectors were co-transfected with baculovirus genomic DNA into Sf9 insect cells and recombinant baculoviruses were isolated and plaque purified as outlined in the Invitrogen Bac-N-Blue Transfection Kit Manual.

Expression and purification of Swi6 from insect cells. Monolayers of High Five cells (Invitrogen) (1x10^7 cells in 150 cm^2 flask) were infected at a multiplicity of infection (M.O.I.) of five plaque-forming units (p.f.u) per cell. 45 hours after infection, the cells were harvested by centrifugation at 1000 rpm for 5 minutes, washed with cold water and resuspended in 1 mL/flask of cold lysis buffer (50 mM Tris-HCl[pH 7.4], 0.5 mM EDTA, 50 mM NaCl, 1 mM phenylmethyl-sulfonyl fluoride [PMSF], 1 µg/mL leupeptin, 1 µg/mL pepstatin). Cells were lysed by drawing the resuspended cells eight times through a 27.5 gauge needle, and the lysate was clarified by centrifugation at 13 000 x g for 20 minutes. Lysates typically contained 10 mg total protein/mL. Swi6 was purified from the insect lysates as described for Swi6 expressed in bacteria (Sidorova and Breeden, 1993). Proteins were precipitated with 20-35% ammonium sulfate and the precipitate was resuspended and dialyzed overnight into lysis buffer, and further purified over a 1 mL DEAE sepharose column (Pharmacia) and eluted by a salt gradient. Peak fractions were dialyzed overnight into lysis buffer with the addition of 20% glycerol and stored at -80°C. Swi6 protein was estimated to be at least 80% pure as judged by Coomassie blue staining of fractions resolved by SDS-PAGE.

Expression and partial purification of SBF and Swi4 derivatives from insect cells. For the production of SBF (Swi6 and Swi4) in insect cells, monolayers of High Five cells (five 150cm^2
flasks of 1x10^7 cells each) were infected with both full-length Swi4- and Swi6-containing baculovirus at an M.O.I. of five p.f.u. per cell of each virus. 45 hours after infection the cells were harvested and lysed as outlined above for Swi6 infected cells. SBF lysates typically contained 10 mg/mL total protein. The lysate was loaded on a 5 ml HiTrap Heparin Column (Pharmacia) pre-equilibrated with Buffer A (50 mM Tris-HCl [pH 7.4] 0.5 mM EDTA, 100 mM NaCl). The column was washed with 15 mL of Buffer A and the bound proteins were eluted with a 40 mL linear salt gradient (0.1 - 1.0 M NaCl) and collected in 2 mL fractions. SBF peak elution was in fractions 12 and 13, which contained 450 mM-550 mM salt as determined by conductivity. The peak fractions were pooled, glycerol was added to 20% and stored at -80°C. Peak fractions typically contained 0.5 mg/mL protein.

For the production of full-length Swi4, Swi4Δ144 or Swi4ΔAA in insect cells, monolayers of High Five cells (five 150 cm^2 flasks of 1x 10^7 cells each) were infected by the baculovirus at a M.O.I of five p.f.u. per cell. The purification procedure for the Swi4, Swi4ΔAA or Swi4Δ144 was identical to that described above for SBF, with peak elution in fraction 12. However, the peak for Swi4ΔAA was broader. The production of Swi4 and the Swi4 derivatives in insect cells was significantly reduced in the absence of co-expression with Swi6. Using western blot and gel shift assays, I estimate that this procedure caused an 8-fold enrichment of Swi4 and significantly decreased insect cell proteins that bound non-specifically to SCB-containing probes (see Results).

Gel retardation assay. Yeast extracts were prepared and gel shift assays were performed using an SCB-containing probe as previously described (Andrews and Herskowitz, 1989). Binding reactions were performed in 20 μL of assay buffer (25 mM Tris-HCl [pH 7.4], 10% glycerol, 3 mM MgCl₂, 0.2 mM EDTA). Poly(dI.dC)-poly(dI.dC) (Pharmacia) was added at 5 μg/reaction
mixture for crude yeast and crude insect cell extracts and at 1 µg/reaction mixture for partially purified protein samples. Unlabeled wild type and mutant SCB competitor were prepared from annealed oligonucleotides as described previously and added to the binding reaction as indicated (Andrews and Moore, 1992). For competition experiments with the Swi4 C-terminus, a recombinant protein composed of the last 144 amino acids of Swi4 fused to GST (GST-4CTR) was purified from E.coli harboring the appropriate expression plasmid as previously described (Measday et al., 1997). The GST-4CTR protein was incubated with thrombin while still bound to Glutathione-S-Sepharose beads (Pharmacia). The C-terminal fragment of Swi4 was eluted from the bound GST and dialyzed into Buffer A. The final concentration of the C-terminal fragment of Swi4 (CTR) was 0.2µg/µl. Gel retardation assays using the CTR fragment were performed as described above, except the reactions were incubated at 4°C for 20 minutes prior to the addition of the probe, and for 10 minutes at room temperature after the addition of the probe.

**Screen for Swi4 CTR mutants.** To generate random mutations in the SWI4 C-terminal region the last 432 nucleotides of Swi4 were amplified by PCR using Taq DNA polymerase, the primers 5’EcoRI (5’GTGCAGATC TTGCATATCAGAT3’) and 3’MutSalI (5’CCTAGACTTCAGGTTGTCTT3’) and the SWI4 gene as a template. The PCR product was digested with EcoRI and SalI and cloned into vector pBA1262 that had been digested with EcoRI and SalI. The resulting pool of mutagenized SWI4 plasmids was used to transform BY185 (swi6A SCB::lacZ) (Gietz et al., 1992). The colonies were transferred to nitrocellulose filters and assayed for β-galactosidase activity as described (Breeden and Nasmyth, 1985). Transformants that turned blue before the BY185 transformant containing vector alone were selected. Mutant plasmids were then isolated from the yeast strains, passaged through E. coli and used to retransform BY185 to confirm that the increase in β-galactosidase activity was due to the
plasmid-borne SWI4 gene (Rose and Botstein, 1983). Once increased β-galactosidase activity was confirmed, the mutated SWI4 genes were sequenced. To determine whether the Swi4 proteins encoded by the mutated SWI4 genes could still interact with Swi6 in vivo, the mutant SWI4 genes were used to transform BY184 (swi4Δ SCB::lacZ), yeast extracts were prepared and gel shift assays were performed using an SCB-containing probe as described above.

**Glycerol gradients.** Sedimentation in glycerol gradients was performed essentially as described (Davey and Funnell, 1994). Glycerol gradients (4 mL of 40-10% v/v glycerol in Buffer A) were poured in seven steps of 500 μL and allowed to equilibrate for 1 hour at room temperature followed by 1 hour at 4°C. 50 μg of partially purified Swi4, Swi4Δ144 or SBF preps were layered on top of the gradient along with 100 μg of an internal control protein (catalase, from the Pharmacia Gel Filtration High Molecular Weight Calibration Kit). A control gradient with 100 μl of protein markers containing 100 μg each of catalase, aldolase and albumin (Pharmacia) was run in parallel. The gradients were centrifuged in a Sw60.1 rotor at 55K for 13 hours. Two-drop fractions (90-100 μl) were collected from the bottom of the tube using a syringe needle. Bradford assay was used to detect molecular weight standards and the internal control protein catalase. To assay fractions containing Swi4 or Swi4 derivatives, 50 μl of each fraction were analyzed by immunoblotting using anti-Swi4 antibodies and visualized by chemiluminescence.

**In Vitro Transcription and Translation of Swi4 and Swi6.** To produce full length Swi4, full-length Swi6 and an internal deletion of Swi4 the plasmid templates pBA462, pBA513 and pBA586 were used, respectively, as recommended in the T7 "TnT" Coupled Reticulocyte Lysate System (Promega). To produce Swi4Δ421 and Swi4Δ896, pBA462 was linearized with either XbaI or NsiI and used as a template in the TnT System.
Batch affinity chromatography. GST and GST fused to the last 144 amino acids of Swi4 (GST-4CTR) were purified from E. coli harboring the appropriate expression plasmid as previously described (Measday et al., 1997). For affinity chromatography utilizing insect cell derived Swi4 and Swi6, GST and GST-4CTR were bound to Glutathione S-Sepharose 4B (Pharmacia) at a concentration of 1 \( \mu \)g/\( \mu \)L beads. 10 \( \mu \)L of either GST or GST-4CTR beads were incubated with 10 \( \mu \)g of partially purified Swi4, Swi4\( \Delta \)144 or Swi6 for 45 minutes at 4°C. The beads were harvested and the unbound supernatant was collected. The beads were washed 4 times in 1 mL of lysis buffer (50 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 50 mM NaCl) for 5 minutes each followed by 4 washes for 5 minutes each in RIPA-500 buffer (50 mM Tris-HCl pH 7.5, 0.1% SDS, 0.5% deoxycholate, 500 mM NaCl, 1% Triton X-100). After the final wash, the beads were resuspended in 25 \( \mu \)L of 1 X SDS-polyacrylamide gel loading dye and boiled. The bound and one half of the unbound supernatant fractions were separated on a 6% SDS PAGE. The proteins were transferred to nitrocellulose and the Swi4 derivatives were detected by Western blotting as outlined above. For affinity chromatography utilizing in vitro translated and transcribed Swi4 and Swi6, 15 \( \mu \)L of either GST or GST-4CTR beads were incubated with 30 \( \mu \)L PBS and 7 \( \mu \)L of either in vitro translated Swi4, Swi6, Swi4\( \Delta \)421, Swi4\( \Delta \)Anks or Swi4\( \Delta \)896 for 2 hours at 4°C. The beads were harvested and the unbound supernatant was collected. The beads incubated with Swi4, Swi6, Swi4\( \Delta \)421 and Swi4\( \Delta \)Anks were washed 3 times for 2 minutes each in 100\( \mu \)L RIPA-500 buffer followed by a wash in PBS buffer. The beads incubated with Swi4\( \Delta \)896 were washed 3 times for 2 minutes each in 100\( \mu \)L Buffer A followed by a wash in PBS buffer. After the final wash, the beads were resuspended in 30\( \mu \)L of a 1X SDS-polyacrylamide gel loading dye and boiled. The bound and one half of the unbound supernatant
fractions were separated on a 10% SDS-PAGE gel. The gels were fixed, treated with Amplify (Amersham), dried, and exposed to X-ray film.
RESULTS

Nuclear localization of Swi4 throughout the cell cycle. In vivo footprinting analysis showed that SCBs are protected by SBF throughout G1 phase, indicating that Swi4 and Swi6 must be nuclear at least in G1 (Harrington and Andrews, 1996; Koch et al., 1996). Indeed, Swi6 localization studies have confirmed that the majority of Swi6 is nuclear throughout late M and G1 phases but is largely cytoplasmic during the rest of the cell cycle (Sidorova et al., 1995). The nuclear localization of Swi6 is coincident with binding of SBF to SCBs, implying that Swi4 must be present in the nucleus at the time of Swi6 localization. To investigate the subcellular localization of Swi4 throughout the cell cycle, I developed an indirect immunofluorescence assay using my Swi4 antibodies on wild type (wt) and swi4Δ log phase cells. No distinct staining was seen when swi4Δ cells were stained with Swi4 antiserum (Figure 2-1, swi4Δ panels). To assay cells for Swi4 staining, 800 wild type cells were scored for nuclear staining (using DAPI) and position in the cell cycle by assessing bud morphology. I found that 60% of all cells scored had a distinct Swi4 staining signal, suggesting that my protocol or antibodies were not optimized to achieve 100% staining. However, of the 60% of cells that were stained, nuclear staining was seen in cells at all stages of the cell cycle, both in unbudded and budded cells. I conclude that, unlike Swi6 whose localization changes during the cell cycle, Swi4 remains nuclear throughout the cell cycle.

The Swi4-Swi6 complex from insect cells can bind SCBs in vitro. Since Swi4 is nuclear in S, G2 and M phases but fails to bind SCBs, I next sought to investigate the mechanism regulating Swi4 binding to DNA. To generate reagents useful for my studies, I constructed vectors for expressing Swi4 and Swi6 in insect cells. While Swi6 can be purified from E. coli, attempts at
Figure 2-1 Subcellular Localization of Swi4.

Swi4 localization was assayed using indirect immunofluorescence with Swi4 antiserum and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Wild type (Wt) and swi4Δ cells were photographed at 630X magnification using an imaging system (see Materials and Methods). Photographs of the same fields of cells viewed with Nomarski optics (panels labeled ‘Nomarski’) and stained with DAPI (4’,6-diamidino-2-phenylindole) to visualize cell nuclei are shown.
Figure 2-2 Reconstitution of SBF in Insect Cells.

A gel retardation assay using an SCB-containing probe and either crude yeast or insect cell lysates is shown. The labeled probe contains 3 SCB sequences from the upstream region of the HO gene (see Andrews and Herskowitz, 1989). The following extracts were used in the binding assays: Lane 1, probe alone; lanes 2-4, 10 μg of crude yeast extract from swi4Δ, swi6Δ or wild type strains as indicated at the top; lane 5, 10 μg of crude cell lysate from uninfected insect cells; lane 6, 10 μg of crude cell lysate from insect cells coinfecte\n with Swi4 and Swi6-expressing baculoviral vectors. The positions of migration of the SBF complex and the unbound probe are indicated to the right.
expressing Swi4 in bacteria have met with limited success (Sidorova and Breeden, 1993; Taba et al., 1991). To determine whether Swi4 and Swi6 produced in insect cells formed a functional SBF complex, I performed gel retardation analysis using an SCB-containing probe. Incubation of the probe with crude yeast extracts from wild type, swi4Δ and swi6Δ cells showed the formation of SBF in the wild type extracts (Figure 2-2, lanes 2 and 4) as previously described (Andrews and Herskowitz, 1989; Andrews and Moore, 1992; Nasmyth and Dirick, 1991; Ogas et al., 1991; Sidorova and Breeden, 1993; Taba et al., 1991). Incubation of the probe with crude insect cell lysates from cells co-infected with baculoviral vectors expressing Swi4 and Swi6 led to formation of a major complex that co-migrated with SBF from yeast extracts (Figure 2-2, lane 6). This complex was not seen when extracts from uninfected insect cells were used in the assay (Figure 2-2, lane 5). Since the SBF complex formed from insect cells and yeast extracts migrated at the same position, SBF is likely composed of only Swi4 and Swi6 proteins. Although I cannot exclude the possibility that other proteins are in this complex, any additional protein would need to be present in both yeast and insect cells. I conclude that SBF can be functionally reconstituted by expression of Swi4 and Swi6 in insect cells.

Inhibition of Swi4 binding to DNA in the absence of Swi6. Although it is clear that Swi4 binds DNA in the context of SBF, there is no evidence that Swi4 can bind DNA on its own. Two observations led me to hypothesize that the DNA-binding domain of Swi4 is inaccessible in the full-length protein when not complexed with Swi6. First, SCB-driven gene expression is reduced or eliminated in a swi6Δ strain even though Swi4 is present and nuclear through the cell cycle (this paper, Andrews and Herskowitz, 1989; Breeden and Nasmyth, 1987; Nasmyth and Dirick, 1991; Ogas et al., 1991). Second, in vivo footprinting studies have shown that binding of Swi4 to SCB sequences cannot be detected in the absence of Swi6 (Harrington and Andrews,
1996; Koch et al., 1996). In order to rigorously test the hypothesis that the DNA-binding domain of Swi4 is inaccessible in the full-length protein, I partially purified both Swi4 and Swi6 from insect cells. I adapted previously established procedures to purify Swi6 produced in insect cells (Figure 2-3A, lane 3, Sidorova and Breeden, 1993; Taba et al., 1991). I used a heparin column to greatly enrich both Swi4 and SBF expressed in insect cells (Figure 2-3B & C). Co-expression of Swi4- with Swi6-containing baculoviruses significantly increased the production of Swi4, suggesting that the Swi4-Swi6 interaction may stabilize Swi4 in insect cells. By contrast, Swi4 was poorly produced when expressed in the absence of Swi6 in insect cells. However, by using heparin-agarose chromatography, I was able to obtain an 8-fold purification of Swi4 as assessed by densitometry of Swi4 Western blots and by measuring the specific activity of the Swi4 proteins in a gel shift assay (Figure 2-3C and data not shown). Further, the purification procedure reduced non-specific DNA-binding proteins in the insect cell extracts. Problems with insolubility were encountered when the Swi4 heparin fractions were subjected to additional purification steps.

I used my purified Swi6 and Swi4 preparations in gel retardation assays to assess binding of full-length Swi4 to SCBs in the absence of Swi6. First, I confirmed that my partially purified SBF fractions supported SBF-complex formation in vitro (Figure 2-3D, lane 2). Binding was specific for SCB DNA since the SBF-complex was competed with wild type SCB oligonucleotide competitor and not mutant SCB oligonucleotide competitor, confirming that the purification procedure did not alter the SCB binding ability of SBF produced in insect cells (Figure 2-3D, lanes 3 - 4). Incubation of partially purified Swi4 with purified Swi6 allowed for efficient reconstitution of the SBF complex in vitro (Figure 2-3D, lane 7). Incubation of the C-terminal half of Swi6 fused to GST with partially purified Swi4 also produced an SBF complex.
Figure 2-3. SCB-Binding Activity of Partially Purified Swi4, Swi6 and SBF.

(A) Purification of Swi6 protein expressed in insect cells. Swi6-containing fractions obtained during purification were analyzed by 6% SDS-PAGE followed by Coomassie Blue staining. Lane 1, crude lysate from insect cells expressing Swi6 protein; lane 2, 20-35% ammonium sulfate precipitation; lane 3, DEAE-Sepharose fraction. A 10 μL aliquot of each fraction was loaded per lane. (B) Purification of SBF expressed in insect cells. SBF-containing fractions obtained during purification were analyzed as described in A) for Swi6. Lane 1, crude lysate from insect cells infected with both SWI4 and SWI6-expressing baculoviruses; lane 2, heparin agarose fraction. A 10 μL aliquot of the crude and partially purified fraction was loaded. (C) Enrichment of Swi4 expressed in insect cells. Swi4-containing fractions obtained during purification were separated by 6% SDS-PAGE and analyzed by Western blotting with affinity-purified Swi4 antiserum. Lane 1, crude lysate from insect cells infected with a SWI4 baculoviral vector (10 μg); lane 2, heparin agarose fraction (10 μg). For Panels A) through C), the positions of migration of molecular weight markers are indicated to the left. (D) Gel retardation assay with partially purified SBF, Swi4 and Swi6. A labeled SCB-containing probe (see legend to Figure 2-2) was incubated with the following protein preparations as indicated above the lanes: lane 1, no extract; lanes 2-4, SBF heparin agarose fraction (1 μg); lane 5, Swi6 DEAE Sepharose fraction (3 μg); lane 6, Swi4 heparin agarose fraction (5 μg); lane 7-9, both the partially purified Swi4 and Swi6-containing fractions. Where indicated above the lanes, a 100-fold molar excess of either wild type SCB competitor DNA (Wt) or mutated SCB competitor DNA (Mut) was added. The position of migration of SBF is shown to the right. The bar to the right of the figure marks the position of migration of complexes composed of the SCB probe and C-terminally-degraded Swi4 in the preparation. The asterisk (*) in lane 6 marks the position of migration of a
complex composed of the SCB probe and either full-length Swi4 protein or a small C-terminal truncation of Swi4.
(data not shown). The reconstituted SBF complex was competed by wild type SCB-containing DNA and not by a mutated competitor (Figure 2-3D, lanes 8 - 9). By contrast, incubation of full-length Swi4 with the SCB probe led to formation of a series of faster migrating complexes (indicated by a bar in Figure 2-3D). These complexes likely represent SCB-binding by C-terminal truncations of Swi4 since they were competed by wild type SCB-containing DNA and were unaffected by the addition of Swi6. One minor complex, indicated by an asterisk (*) on Figure 2-3D, may reflect binding of the SCB probe by full-length Swi4 protein. I used phosphorimager analysis to compare the amount of the (*) complex to the amount of SBF-complex formed upon addition of excess Swi6. My analysis suggested that less than 5% of the Swi4 protein in the assay was participating in the (*) complex (data not shown). Therefore, if the (*) product indeed contains full-length Swi4, the ability of intact Swi4 to bind SCBs in the absence of Swi6 must be severely compromised. Since I am using partially purified components, derived from a heterologous system, my results suggest that full-length Swi4 cannot bind DNA efficiently in the absence of Swi6. No experiments with recombinant full-length Swi4 have been previously reported. The mechanism of inhibition appears intrinsic to Swi4 and is alleviated upon the interaction of Swi6 with Swi4 through the CTR of Swi4.

**Domains required for auto-inhibition of Swi4 binding to DNA.** As outlined earlier, several lines of evidence suggested that the C-terminus of Swi4 is necessary for the regulation of Swi4 binding to DNA. To confirm this hypothesis, I expressed a truncated version of Swi4, Swi4Δ144, lacking the C-terminal 144 amino acids, in insect cells. Previously, our lab used gel retardation assays to show that Swi4Δ144 in crude yeast extracts formed a Swi6-independent complex with SCB-containing DNA (Andrews and Moore, 1992). A similar result was obtained using an *in vitro*-translated truncation of Swi4 (Primig et al., 1992). I used Swi4Δ144 that had been
Figure 2-4. Analysis of SCB-Binding by Deletion Derivatives of Swi4.

(A) Schematic of the Swi4 derivatives expressed in insect cells. The relative positions of the N-terminal DNA-binding domain, the multiple ankyrin repeats and the C-terminal Swi6-interaction domain are indicated. ‘His’ indicates the presence of a N-terminal poly-histidine tag. (B) Gel retardation assay with partially purified SBF or C-terminally-truncated Swi4 (Swi4Δ144). A labeled SCB-containing probe (see legend to Figure 2-2) was incubated with the following protein preparations as indicated above the lanes: lane 1, no extract; lane 2, SBF heparin agarose fraction (1 µg); lane 3, 3 µg of purified Swi6; lanes 4-7, 5 µg partially-purified Swi4Δ144. Lane 5 also contains 3 µg of a Swi6 DEAE-Sepharose fraction. In lanes 6 and 7, a 100-fold molar excess of either wild type SCB competitor DNA (Wt) or mutated SCB competitor DNA (Mut) was added as indicated above the lanes. (C) Gel retardation assay with partially purified SBF or an internally-deleted Swi4 derivative (Swi4ΔAA). The labeled SCB probe was incubated with the following protein preparations as shown above the lanes: lane 1, no extract; lane 2, SBF heparin agarose fraction (1 µg); lane 3, 3 µg of purified Swi6; lanes 4, 5 µg partially purified Swi4ΔAA; lanes 5-7, 5 µg partially purified Swi4ΔAA and 3 µg purified Swi6. In lanes 6 and 7, a 100-fold molar excess of either wild type SCB competitor DNA (Wt) or mutated SCB competitor DNA (Mut) was added as indicated above the lanes.
partially purified from insect cells in a gel shift assay with an SCB-containing probe. Swi4Δ144 formed a specific complex with DNA that was efficiently competed by wild type but not by mutated SCB-containing DNA (Figure 2-4B lanes 4, 6 - 7). Since Swi6 interacts with Swi4 through the last 78 amino acids of Swi4, the addition of Swi6 did not affect Swi4Δ144 DNA-binding (Figure 2-4B, lane 5; Siegmund and Nasmyth, 1996). A larger Swi4 truncation, Swi4Δ421, behaved in a similar manner (data not shown). My results confirm the hypothesis that the C-terminal 144 amino acids of Swi4 are involved in the inhibition of Swi4 binding to DNA.

Ankyrin motifs have been implicated in the auto-inhibition of numerous transcription factors, including NF-kB (reviewed in Ghosh et al., 1998). I next used convenient restriction sites in the Swi4 gene to construct a baculoviral Swi4 derivative, Swi4ΔAA, with an internal deletion of 349 amino acids. This deletion disrupts the first ankyrin domain of Swi4 along with a significant region between the DNA-binding domain and the ankyrin domain. The first and fourth ankyrin domains of Swi4 were first identified due to their similarity to other ankyrin domains. Upon closer inspection, it became apparent that Swi4 has three other degenerate ankyrin repeats (Bork, 1993). Single amino acid changes in the ankyrin repeats of both Swi6 and Swi4 result in proteins that are temperature-sensitive for function (M. Donoviel personal communication, Ewaskow et al., 1998; Sidorova and Breeden, 1993). This result suggests that deletion of the first ankyrin repeat in Swi4ΔAA should greatly reduce, if not abolish, the function of the ankyrin domains. Unlike Swi4Δ144, Swi4ΔAA had only limited ability to bind DNA in the absence of Swi6 (Figure 2-4C, lane 4). Full binding was restored upon the addition of Swi6 (Figure 2-4C, lanes 5 - 7). I conclude that the inhibition of Swi4 binding to DNA does not involve amino acids 199-547 of Swi4.
Point mutants in the Swi4 CTR can alleviate Swi4 DNA-binding auto-inhibition. My in vitro experiments showed that the inhibition of Swi4 binding to DNA was relieved by deletion of the C-terminal 144 amino acids of Swi4. The C-terminal 78 amino acids of Swi4 are required for interaction with Swi6 (Siegmund and Nasmyth, 1996). This region of Swi4 is conserved in other members of the Swi4 family of transcription factors and is predicted to be highly α-helical in structure (Figure 2-5A). To ask whether the region of the Swi4 C-terminus involved in inhibition of Swi4 DNA-binding was separable from the Swi6-interaction domain, I undertook a screen for point mutations in the CTR-encoding region of SWI4 that alleviate the DNA-binding inhibition of Swi4. I used PCR-mediated mutagenesis to introduce random mutations into the region encoding the C-terminal 144 amino acids of Swi4. The mutagenized SWI4 CTR fragments were cloned into a SWI4 gene on a 2μ plasmid to allow expression from the constitutive GPD promoter. I transformed the pool of CTR mutants into a swi6Δ strain carrying an integrated SCB::lacZ reporter gene. Using a β-galactosidase filter test, I identified Swi4 mutants which, in the absence of Swi6, allowed a higher level of SCB::lacZ expression than the wild type SWI4 gene expressed from the same vector.

Using this screen, I identified four new SWI4 mutants (Table 2-2). Two mutants, Swi4-9.1 and Swi4-9.2, were the result of improper ligation of the PCR product into the SWI4-containing vector. Mutant Swi4-9.1 had an addition of 12 amino acids onto the C-terminus of Swi4 (A-N-F-N-K-I-L-T-L-T-I-S). This addition resulted in a 4-fold increase of SCB-dependent expression in the absence of Swi6. Mutant Swi4-9.2 was a large C-terminal truncation at amino acid 803 that allowed for a 3-fold activation of SCB::lacZ expression in the absence of Swi6. These results support our in vitro data demonstrating that the C-terminus of Swi4 inhibits Swi4 DNA-binding. I also identified a smaller C-terminal truncation in our screen. In Swi4-3.3, a 1bp
**Table 2-2. β-galactosidase activity of Swi4 mutants**

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>β-galactosidase activity (Millers units)*</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>p424 GPD</td>
<td>0.1</td>
<td>vector control</td>
</tr>
<tr>
<td>p424 GPD-Swi4</td>
<td>11.4</td>
<td>wild type Swi4</td>
</tr>
<tr>
<td>p424 GPD-Swi4-9.1</td>
<td>41.3</td>
<td>addition of 12 amino acids to the C-termini of Swi4</td>
</tr>
<tr>
<td>p424 GPD-Swi4-9.2</td>
<td>33.8</td>
<td>C-terminal truncation at amino acid 803</td>
</tr>
<tr>
<td>p424 GPD-Swi4-3.3</td>
<td>15.6</td>
<td>1 bp deletion at amino acid 1081:12 amino acid truncation + 3 new amino acids</td>
</tr>
<tr>
<td>p424 GPD-Swi4-GY</td>
<td>22.4</td>
<td>E1076G, N1092Y</td>
</tr>
</tbody>
</table>

* See Materials and Methods (Rose and Botstein, 1983); average of three independent transformants.
deletion in the codon for L1085 results in the truncation of 12 amino acids from the C-terminus of Swi4, and the addition of 4 amino acids (N-W-T-I). This smaller alteration results in a modest but reproducible induction of $SCB::lacZ$ expression (Table 2-2). Finally, a fourth mutant was isolated, Swi4-C26, which had three point mutations: N995H, E1076G and N1092Y. By separating the N995H mutation from the E1076G and N1092Y mutations, I was able to determine that the 2-fold induction in $SCB::lacZ$ activity was due to the two most C-terminal mutations. The mutant carrying these two mutations was named Swi4-GY.

Since the C-terminal region of Swi4 is also required for interaction with Swi6, I next asked if our new Swi4 mutants could still interact with Swi6. To answer this question, I performed DNA-binding assays using an SCB-containing probe and crude yeast extracts from a swi4Δ strain transformed with plasmids encoding the Swi4 mutants. Western blot analysis using Swi4 antibody showed that all the Swi4 mutant proteins were expressed (Figure 2-5C). Crude lysates from cells expressing mutant Swi4-9.2, which lacks the Swi6-interaction domain, did not support an SBF-complex; however, a distinct faster migrating species was formed (Figure 2-5B lane 5). In contrast, crude lysates from cells expressing either wild type Swi4 or the Swi4 mutants, Swi4-9.1, Swi4-3.3 and Swi4-GY, supported SBF-complex formation (Figure 2-5B, lanes 4, 6 - 7). The mutants Swi4-3.3 and Swi4-GY appeared to form the SBF-DNA complex less efficiently, which correlated with decreased expression levels as determined by Western blot (Figure 2-5C, lanes 5 and 6). Although I saw SBF complex formation by the Swi4 CTR mutants in the presence of Swi6, I did not see a lower molecular weight complex that might correspond to binding of the Swi4 CTR mutant proteins to SCBs in the absence of Swi6 (data not shown). I presume that the increased ability of the Swi4 CTR mutants to bind SCBs in the absence of Swi6 is sufficient to yield increased SCB-dependent transcription but may be undetectable using my
Figure 2-5. Gel Retardation Analysis of Wild Type *SWI4* and Mutant *swi4* Alleles Using Yeast Cell Extracts.

(A) Alignment of the extreme C-terminal regions of Swi4 family members. Residues identical to Swi4, or a conservative substitution are boxed. Putative α-helices are indicated by the arrows as predicted by the PHD protein structure algorithm. The filled-in box shows the amino acids that were deleted in Swi4-3.3. The asterisks (*) indicate the positions of the point mutations in Swi4-GY: E1076G, N1092Y. (B) A labeled SCB probe was incubated with 10 μg of crude extract from a swi4Δ yeast strain (BY184) transformed with the following plasmids: lane 1, no extract; lane 2, empty vector, p424GPD; lane 3, pGPD-*SWI4* (wild type *SWI4*); lane 4, pGPD-*SWI4*-9.1; lane 5, pGPD-*SWI4*-9.2, lane 6, pGPD-*SWI4*-3.3 and lane 7, pGPD-*SWI4*-GY as indicated. The *SWI4* mutations in the various plasmids are described in Table 2 and in the text. The positions of migration of SBF and a complex of Swi4-9.2 and the SCB probe are indicated to the right. (C) Western blot analysis of extracts used in binding assay shown in B). 50 μg of the crude yeast lysates used in the gel retardation analysis were separated on a 6% SDS-PAGE and the Swi4 protein in the extracts was visualized with Swi4 antiserum. The Swi4 protein present in each extract is indicated above the lanes (see Panel B above).
biochemical assay. I conclude that small alterations in the extreme C-terminus of Swi4 can alleviate DNA-binding inhibition but do not affect interaction with Swi6.

**A C-terminal fragment of Swi4 can inhibit Swi4 DNA-binding in trans.** I determined that both truncations and point mutations in the C-terminus of Swi4 appear to alleviate the inhibition of Swi4 DNA-binding. My results suggest that the C-terminus of Swi4 may inhibit DNA-binding of a C-terminal truncation of Swi4. To test this model I performed gel retardation assays using an SCB-containing probe, partially purified Swi4Δ144 and the C-terminal 144 amino acids of Swi4 (CTR, Figure 2-6). Incubation of Swi4Δ144 with increasing amounts of the CTR, prior to the addition of the SCB probe, resulted in an inhibition of Swi4Δ144 binding to the SCB probe (Figure 2-6, lane 7). At low concentrations of CTR, the amount of Swi4Δ144-SCB complex increases. This increase may be due to the small amounts of GST which were present in the CTR prep; I found that addition of GST alone also increased the amount of Swi4Δ144-SCB complex (Figure 2-6, lane 4). My gel shift assay shows that the CTR of Swi4 can functionally interact with an N-terminal region of Swi4 to inhibit Swi4 binding to DNA.

**Analysis of Swi4 complexes on glycerol gradients.** Since my *in vitro* studies were performed with partially purified reagents expressed in insect cells, it is unlikely that Swi4 auto-inhibition depends on another protein binding the CTR of Swi4. Rather, my results suggest that the CTR of Swi4 may be involved in either an inter- or intramolecular interaction with another region or molecule of Swi4 to inhibit DNA-binding. To determine whether Swi4 forms dimers or multimers in solution, I analyzed protein size by glycerol gradient sedimentation. Swi4 ran at approximately 67 kDa, a mass which is drastically smaller than its predicted mass of 123 kDa (Figure 2-7). This suggests that, in solution, Swi4 must be highly asymmetric in shape and monomeric in nature. Truncating the C-terminal 144 amino acids of Swi4 did not significantly
**Figure 2-6. Inhibition of Swi4Δ144 –SCB Complex Formation by the CTR of Swi4.**

A gel retardation assay with an SCB-containing probe is shown (see legend to Figure 2-2). The probe was incubated with the following protein preparations as indicated above the lanes: lane 1, no extract; lane 2, SBF, heparin agarose fraction (1 μg); lanes 3-7, 2 μg of partially-purified Swi4Δ144. The binding reaction shown in lane 4 also contained 1 μg of GST, and the reactions shown in lanes 5-7, contained increasing amounts of purified Swi4 CTR (C-terminal 144 amino acids of Swi4) as indicated above the lanes (in μgs).
Figure 2-7. Glycerol Gradient Sedimentation of Swi4, Swi4Δ144 and SBF.

50 μg of partially purified Swi4, Swi4Δ144 and SBF were analyzed by glycerol gradient sedimentation. Glycerol gradients (4 mL of 10-40% (v/v) glycerol) were centrifuged for 13 hours at 55,000 rpm. Fractions from the gradients were analyzed on Western blots using anti-Swi4 antibody. The peak fractions of size standards (MW), run in parallel gradients, are indicated by C (catalase, 232kDa), A (aldolase, 158kDa) and B (bovine albumin, 67kDa). Fraction numbers are shown at the top of the figure with the bottom of the gradients on the left.
change the sedimentation of Swi4. By contrast, the addition of Swi6 to Swi4 greatly increased the sedimentation of Swi4 (Figure 2-7, top panel). SBF ran at 180 kDa which is close to the predicted size of a heterodimer of Swi4 (123 kDa) and Swi6 (91 kDa).

**Interaction of the CTR of Swi4 with N-terminal domains.** My glycerol gradient assays revealed that Swi4 was monomeric in solution, suggesting that the inhibition of Swi4 binding to DNA involves an intramolecular interaction. In order to determine whether the C-terminal 144 amino acids of Swi4 were capable of forming protein-protein interactions within Swi4, I fused the C-terminal 144 amino acids of Swi4 to GST (GST-4CTR; Figure 2-8A) and performed batch affinity chromatography assays with either insect cell-produced Swi4 derivatives or *in vitro* translated Swi4 derivatives. GST or GST-4CTR was incubated with full-length Swi4, Swi4Δ144 or Swi6 produced from insect cells (Figure 2-8B). The GST-4CTR fusion bound Swi6 (Figure 2-8B, lane 12) as previously demonstrated (Sidorova and Breeden, 1993). The GST-4CTR fusion also bound full-length Swi4 and Swi4Δ144 (Figure 2-8B, lanes 4 and 8), showing that the C-terminal 144 amino acids of Swi4 can interact *in vitro* with the first 949 amino acids of Swi4. This result is consistent with my gel shift analysis showing a functional inhibition of Swi4Δ144 binding to SCBs by the CTR. To further define the N-terminal interaction domain of Swi4, affinity chromatography experiments were conducted with a series of *in vitro* transcribed and translated Swi4 derivatives (Swi4, Swi4Δ421, Swi4ΔANKs and Swi4Δ896, Figure 2-8A). Like the insect cell-produced proteins, the C-terminal 144 amino acids of Swi4 interacted with *in vitro* translated full-length Swi4 protein (Figure 2-8C, lane 4). The GST-4CTR fusion also bound both Swi4Δ421 and Swi4ΔAnks proteins, indicating that the interaction does not require the Ankyrin motifs or the C-terminus of Swi4 (Figure 2-8C, lanes 12 and 16). Further, the GST-4CTR fusion interacted directly with the N-terminal 197 amino acids...
Figure 2-8. Binding of the C-terminal 144 Amino Acids of Swi4 to Swi6 and N-terminal Regions of Swi4 \textit{in vitro}.

(A) Schematic of the Swi4 proteins used in the assay. The relative positions of the DNA-binding domain, ankyrin motifs and C-terminal domain (Swi6-interaction domain) are depicted. (B) 10 μg of partially purified Swi4, Swi4Δ144 or Swi6, derived from insect cell extracts (see Figure 2), were incubated with either GST or GST fused to the C-terminal 144 amino acids of Swi4 (GST-4CTR) immobilized on glutathione beads. The unbound (U) and bound (B) fractions were separated on a 6% SDS-PAGE. The gels were then blotted and incubated with Swi4 antiserum (lanes 1-8) or Swi6 antiserum (lanes 9-12) to identify the Swi4 or Swi6 proteins. The positions of migration of molecular weight markers are indicated to the left. (C) 7μL of \textit{in vitro} translated Swi4, Swi6, Swi4Δ421, Swi4ΔAnks and Swi4Δ896 were incubated with either GST or GST-4CTR immobilized on glutathione beads. The unbound (U) and bound (B) fractions were separated on a 10% SDS-PAGE. The positions of migration of molecular weight markers are indicated to the left.
of Swi4, which contains the DNA-binding domain of Swi4 (Figure 2-8C, lane 20). This interaction appeared weaker than interactions between the 4CTR and larger N-terminal fragments of Swi4, suggesting that there may be other parts of Swi4 which provide structural support for this interaction. Together with my evidence that Swi4 is monomeric in solution, my data suggest that the C-terminus of Swi4 is involved in an intramolecular interaction with an N-terminal DNA-binding region of Swi4 and that this interaction causes inhibition of Swi4 DNA-binding in the absence of Swi6.
DISCUSSION

I found that Swi4 is nuclear throughout the cell cycle and yet is incapable of promoting transcription in the absence of Swi6. I reconstituted active SBF \textit{in vitro} from Swi4 and Swi6 expressed using the baculoviral system in insect cells. Partially purified full-length Swi4 could not bind SCBs in the absence of Swi6; however, Swi4 derivatives truncated at the C-terminus or carrying point mutations in the extreme C-terminus of Swi4 were able to bind DNA in the absence of Swi6. Further, the binding of a C-terminally truncated Swi4 protein to SCBs was inhibited by addition of a C-terminal fragment of Swi4 \textit{in trans}. Full-length Swi4 was monomeric in solution, suggesting an intramolecular mechanism for auto-inhibition of binding to DNA by Swi4. I detected a direct interaction between a C-terminal fragment of Swi4 and the N-terminal 197 amino acids of Swi4, which contain the DNA-binding domain of Swi4. My data suggest that the interaction of the CTR of Swi4 with the N-terminal DNA-binding domain of Swi4 physically inhibits the DNA-binding domain from binding SCBs. Interaction of the CTR of Swi4 with Swi6 alleviates this inhibition, allowing Swi4 to bind DNA.

The C-terminus of Swi4 inhibits the binding of Swi4 to SCBs. My experiments implicate the extreme C-terminus of Swi4 in the auto-inhibition of Swi4 binding to DNA. The C-terminus of Swi4 also contains the Swi6 interaction domain, which has been localized to the last 78 amino acids of Swi4 (Siegmund and Nasmyth, 1996). As depicted in Figure 2-5 A, this region of Swi4 is very similar to the comparable region in other members of the Swi4 family, and it has been predicted to contain alpha helices with amphipathic character (Breeden, 1996; Sidorova and Breeden, 1993; Siegmund and Nasmyth, 1996). While my screen for mutations in the CTR of Swi4 was not saturating, I isolated a large truncation of Swi4, as well as three isolates with different mutations that affect the extreme C-terminus of Swi4. One mutant, Swi4-GY, carried
Figure 2-9. Model of the Auto-inhibition of Swi4 Binding to DNA.

An intramolecular interaction involving the extreme C-terminus of Swi4 (CTR, small shaded lobe) and a more N-terminal region of the protein is depicted. My data suggest that when Swi4 is not bound to Swi6 (as shown on the left), the CTR of Swi4 is free to form an intramolecular interaction with the DNA-binding domain of Swi4, which prevents Swi4 from binding to SCBs (CACGAAA). Binding of Swi6 to the CTR of Swi4 disrupts the intramolecular interaction of Swi4, allowing Swi4 to bind SCBs (as diagrammed on the right).
CACGAAA

DNA Binding Domain of Swi4

CTR of Swi4
two mutations in the extreme C-terminus of Swi4, E1076G and N1092Y. While I have yet to separate the mutations, it is interesting to note that both map to conserved residues (Figure 2-5A). In another mutant, Swi4-3.3, a predicted α-helix is deleted from the Swi4 C-terminus. Notably, both of these latter mutants could still bind Swi6 to form SBF in vitro. This result suggests that the domains or residues responsible for the Swi4-Swi6 interaction may be distinct from those necessary for Swi4 auto-inhibition. Alternatively, the domains or residues required for the Swi4 auto-inhibitory function and the Swi4-Swi6 interaction function may be shared and my SBF assay was not sufficiently sensitive to detect a decrease in the Swi4-Swi6 interaction. It will be interesting to determine whether the Swi4-Swi6 and Swi4-Swi4 interaction domains are fully separable. I suspect that the Swi6 interaction domain may localize to the conserved residues or putative α-helices N-terminal to the more C-terminal region that my data implicate in Swi4 auto-inhibition.

Model for Swi4 DNA-binding inhibition. I found that Swi4 was largely monomeric in solution and that the CTR of Swi4 could interact with the N-terminal 197 amino acids containing the DNA-binding domain of Swi4 in vitro. Further, the addition of the CTR of Swi4 to a binding reaction containing a C-terminally truncated Swi4 protein (Swi4Δ144) inhibited DNA-binding by Swi4Δ144. Together, my data suggest an intramolecular model for inhibition of Swi4 binding to DNA. Interestingly, intramolecular interactions within the Swi6 protein have recently been reported (Sedgwick et al., 1998). The internal ankyrin domains of Swi6 form a stabilized central structure with which adjacent transcriptional activation domains interact. I propose that the intramolecular interaction of the CTR of Swi4 with the N-terminal region of Swi4 results in the DNA-binding domain of Swi4 becoming inaccessible or incapable of binding DNA in the absence of Swi6 (Figure 2-9). Upon addition of Swi6, the Swi4-Swi4 interaction is disrupted,
alleviating the inhibition. To test this model, it will be important to determine whether the interaction of the Swi4-CTR with the Swi4 N-terminal region is disrupted upon the addition of Swi6. Presently we do not know the relative-binding affinities of Swi4-Swi4 and Swi4-Swi6 interactions, however the model predicts that the relative-binding affinity of Swi4-Swi6 will be greater than the relative-binding affinity between Swi4-Swi4.

My batch affinity chromatography showed that the CTR can interact directly with the N-terminal 197 amino acids of Swi4. This result suggests that the inhibition of Swi4 DNA-binding by the CTR may be due to a direct masking of the DNA-binding domain. Alternatively, the CTR of Swi4 may interact with amino acids outside of the core DNA-binding domain causing a conformational change in the DNA-binding domain. Swi4 proteins containing a fusion of the minimal DNA-binding domain (amino acids 36-170) to the C-terminal 65 or 146 amino acids of Swi4 were reported to form a complex with DNA in the absence of Swi6 (Primig et al., 1992). If an intramolecular interaction is the mechanism for Swi4 auto-inhibition, then the CTR of Swi4 may be interacting either with the first 36 amino acids of Swi4, which are N-terminal to the DNA-binding domain, or with amino acids 170-197 that lie C-terminal to the DNA-binding domain. Alternatively, direct fusion of the CTR with the DNA-binding domain may cause a conformational rigidity that prevents the intramolecular interaction. Indeed, it has been suggested that both Swi4 and Swi6 have inherent modularity and flexibility that are crucial for their in vivo function (Sedgwick et al., 1998). Hydrodynamic analysis and proteolytic cleavage studies of Swi6 have determined that the N-terminal 15 kDa domain of Swi6 is connected to the central ankyrin region of Swi6 by a long, extended and potentially flexible linker region. This 15 kDa domain appears to have no function and it is thought to represent a non-functional remnant of a DNA-binding domain from a common ancestor which has remained active in some family
members (reviewed in Breeden, 1996). Members of the Swi4/Swi6 family of transcription factors share a similar domain structure, and the flexible arm adjoining the N-terminal domain of Swi6 with the central core ankyrin domain may be a conserved feature of the entire family. This domain may provide the flexibility needed for the N-terminal DNA-binding domain of Swi4 to interact with the C-terminal domain.

Intramolecular interactions have been implicated in the regulation of DNA-binding for many transcription factors, including numerous Ets family members, such as Ets-1 and GABP\(\alpha\) (reviewed in Bassuk, 1997). The ability of Ets-1 to bind DNA is negatively regulated by at least two domains: a N-terminal region and a C-terminal region (Hagman and Grosschedl, 1992; Lim et al., 1992). Recent studies revealed that a direct interaction between the N-terminal Ets-1 inhibitory sequence and both the Ets domain and the C-terminal inhibitory sequences are responsible for the intramolecular inhibition of Ets-1 DNA-binding activity (Jonsen et al., 1996; Petersen et al., 1995; Skalicky et al., 1996). In the full-length protein, the two inhibitory domains interact allosterically, stressing the Ets domain and destabilizing DNA contacts. Loss of coupling between the two domains leads to an altered conformation in the N-terminal inhibitory region, allowing the Ets domain to make stable contacts with the DNA. However, this relaxed conformation is transient and reestablishment of the interaction between inhibitory regions causes repression of DNA-binding. Stable DNA-Ets-1 interactions are established through several mechanisms which disrupt the Ets-1 intramolecular interaction, including phosphorylation of the N-terminal inhibitory region and direct protein-protein interaction with the N-terminal inhibitory region (Giese et al., 1995; Rabault and Ghysdael, 1994). A similar method of DNA-binding inhibition has also been established for GABP\(\alpha\), whose interaction with the ankyrin containing protein GABP\(\beta\) allows GABP\(\alpha\) to bind DNA (Brown and McKnight,
Though there is little sequence homology between Swi4 and the Ets family of proteins, the recent crystal structure of the DNA-binding domain of the Swi4 family member Mbp1 revealed that they do share a common fold in their core DNA-binding domains. The core consists of a short strand N-terminal to the HTH and a β-hairpin C-terminal to the HTH (Taylor et al., 1997; Xu et al., 1997). Although the structure of Mbp1 outside the core diverged from that of the Ets proteins, similar allosteric forces may contribute to the DNA-binding inhibition of the Swi4 family of transcription factors.

**Role of Swi4 DNA-binding Inhibition in Regulation of SBF.** Ctb/Cdc28 activity is necessary for dissociation of SBF from the CLN2 promoter in G2 phase and mitosis (Harrington and Andrews, 1996; Koch et al., 1996). Ctb2 immunoprecipitation experiments have shown that Swi4 can interact with Ctb2 during M phase and that Swi4 is phosphorylated in vivo (Amon et al., 1993; Siegmund and Nasmyth, 1996). Interestingly, the interaction of Ctb2 with Swi4 appears independent of Swi6. These observations have led to the suggestion that Ctb interaction with Swi4 is necessary for preventing Swi4 from binding DNA. My data suggest that the inhibition of Swi4 DNA-binding is intrinsic to Swi4, and does not require any other proteins. The role of CtbS may not be to inhibit Swi4 DNA-binding but rather to promote dissociation of SBF from SCBs. Ctb-dependent regulation of SBF may occur through disruption of the Swi4-Swi6 interaction. Once Swi4 and Swi6 are dissociated from each other, Swi6 is transported out of the nucleus, allowing the CTR of Swi4 to form an intramolecular interaction with the DNA-binding domain of Swi4, which inhibits Swi4 DNA-binding. Interestingly, gel retardation assays with whole cell extracts of synchronized cells arrested at different stages of the cell cycle show that the SBF complex can form at all stages of the cell cycle (Taba et al., 1991). This result suggests that the inhibition of Swi4 DNA-binding is immediately relieved upon the addition of
Swi6. To test this model, it will be important to determine the relative affinities of Swi4-Swi4 and Swi4-Swi6 interactions.

It is unlikely that Swi6 is fully excluded from the nucleus throughout M and G2 phases, although SBF footprinting is not detected. Possibly, the formation of an SBF-DNA complex is undetectable in M and G2 phases because Clb-Cdc28 is continually disrupting the SBF complex and Swi6 is actively transported out of the nucleus. Alternatively, in addition to the auto-inhibition of Swi4 DNA-binding, there may be another mechanism regulating SBF-DNA complex formation during G2 and M phases. Both the role and sites of Swi4 phosphorylation by Clb2-Cdc28 have yet to be established. One possibility is that phosphorylation of Swi4 may alter the affinity of the Swi4-Swi6 interaction or the stability of the Swi4 auto-inhibition. There is one consensus Cdc28 phosphorylation site in the CTR of Swi4 (S1007) and numerous other potential sites in the CTR and the N-terminal DNA-binding domain. It will be interesting to determine whether Clb/Cdc28 phosphorylates these sites, and whether phosphorylation contributes to the regulation of Swi4 DNA-binding.
CHAPTER III

SBF Cell Cycle Regulator as a Target of the Yeast PKC1-MAP Kinase Pathway

Chapter 3 is a modified version of a published paper. Figures 3-1, 3-3 and 3-4 are reprinted with permission from Science 275 (1997), K. Madden, Y. Sheu, K. Baetz, B. Andrews, and M. Snyder, SBF Cell Cycle Regulator as a Target of the Yeast PKC-MAP Kinase Pathway.

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In this collaborative effort, Kevin Madden performed the epistasis experiments depicted in Figure 3-1 and analysis of in vivo Swi6 phosphoforms depicted in Figure 3-3. The in vitro kinase assay depicted in Figure 3-4 was performed using purified SBF that I provided. I performed the Northern analysis depicted in Figure 3-2.
ABSTRACT

Protein kinase C signaling is highly conserved among eukaryotes and has been implicated in the regulation of cellular processes such as cell proliferation and growth. In the budding yeast, \textit{PKC1} functions to activate the \textit{SLT2 (MPK1)} mitogen-activated protein kinase cascade, which is required for the maintenance of cell integrity during periods of polarized growth. Genetic studies and analysis of protein phosphorylation \textit{in vivo} and \textit{in vitro} indicated that the SBF transcription factor, an important regulator of gene expression at the G\textsubscript{1} to S phase cell cycle transition, is a target of the Slt2 MAP kinase. I found that Slt2 was required for the transcriptional induction of the SBF target genes \textit{PCL1} and \textit{PCL2}, but not \textit{CLN1} and \textit{CLN2}, in response to heat shock. These studies provide evidence for a direct role of the \textit{PKC1} pathway in the regulation of the yeast cell cycle and cell growth and indicate that conserved signaling pathways can act to control key regulators of cell division.
INTRODUCTION

The coordination of polarized growth and cell proliferation is a critical process in many cell types. In yeast, the PKCl-MAPK pathway functions to maintain the integrity of the cytoskeleton and cell wall and is a candidate regulatory system for coupling cell growth and proliferation (reviewed in Chapter 1, sections VII to XII, and Heinisch et al., 1999). The PKCl-MAPK pathway contains Pkc1, which activates a MAP kinase cascade that consists of sequentially activated protein kinases: Bck1, a MEKK, the redundant Mkk1 and Mkk2 MEKs, and Slt2, a MAP kinase (Costigan et al., 1992; Irie et al., 1993; Lee and Levin, 1992; Lee et al., 1993; Torres et al., 1991). Genetic evidence has implicated this pathway in the regulation of cell cycle progression (see chapter I sections IV B and VIII D, and DiComo et al., 1995; Marini et al., 1996; Mazzoni et al., 1993; Morgan et al., 1995). In addition, the Slt2 MAP kinase is specifically activated during bud emergence and mating projection formation, periods of the cell cycle during which cell growth is highly polarized (Errede et al., 1995; Zarzov et al., 1996).

The SBF transcription factor also regulates both the yeast cell cycle and polarized growth (reviewed in Chapter I, and Breeden, 1996). SBF regulates the transition from G1 into S phase by activating the expression of the G1 cyclins CLN1, CLN2, PCL1 and PCL2 (Espinoza et al., 1994; Measday et al., 1994; Nasmyth and Dirick, 1991; Ogas et al., 1991). Cln and Pcl association with the Cdc28 (Richardson et al., 1989) and Pho85 Cdns (Espinoza et al., 1994; Measday et al., 1994), respectively, promotes entry into S phase and also promotes polarized growth (Lee et al., 1998; Lew and Reed, 1993). Thus, both the transcription factor SBF and the PKCl-pathway function to mediate cell cycle progression and polarized growth.

Intriguingly, swi4 and swi6 mutant strains share similar phenotypes with PKCl-MAPK pathway mutants; these phenotypes include temperature-sensitive growth defects that are
suppressed by sorbitol (Igual et al., 1996; Madden et al., 1997), and sensitivity to cell wall stresses such as SDS, CFW and caffeine (Igual et al., 1996; Sidorova and Breeden, 1999). Also, \textit{swi4Δ} mutants exhibit a defect in projection formation upon exposure to mating pheromone, as do \textit{PKC1}-MAPK pathway mutants (Costigan et al., 1992; Costigan et al., 1994; Errede et al., 1995; Mazzoni et al., 1993). Finally, a \textit{swi4-100} mutant strain requires the \textit{PKC1}-pathway scaffold protein \textit{SPA2} for viability (Flescher et al., 1993). Together, this evidence suggests that SBF and the \textit{PKC1}-MAPK pathway may function cooperatively in regulating polarized growth.

In this chapter, I present experiments conducted in collaboration with Kevin Madden from Mike Snyder's laboratory at Yale University, in which we show that SBF is a direct target of Slt2, linking the \textit{PKC1}-cell wall integrity pathway directly with cell cycle transcription factor SBF.
MATERIALS AND METHODS

Strains and plasmids. Standard methods for yeast culture and transformation were followed (Guthrie and Fink, 1991). Standard rich medium (YPD) and supplemented minimal medium (SD) were used (Kaiser et al., 1994). Strains used in this chapter all are congenic to wild-type S288C background (Y792) and have been previously described: bck1Δ::TRP1 (Y782)(Costigan et al., 1994), slt2Δ::LEU2 (Y783)(Costigan et al., 1994), and swi4Δ::HIS3 (YK74)(Ogas et al., 1991).

High copy plasmids containing the yeast 2μ origin of replication and SLT2 (Costigan et al., 1994) or PCL1 (Ogas et al., 1991) have been previously described. To construct a high copy plasmid expressing PCL2, a HindIII – SalI fragment from a genomic library containing the full-length PCL2 gene was subcloned into pRS426 to create pBA623. A clone containing full-length PHO85 was isolated from a pYE24 genomic library and named pBK55. To construct a high copy plasmid expressing SWI4, an 8-kb SphI genomic fragment containing full-length SWI4 was subcloned into pYE352 to create pBK327. To construct a high copy plasmid expressing MBP1, PCR was utilized to amplify MBP1 and flanking sequences. The PCR product was sequenced to checked for fidelity and subcloned into pYE24 to create pBK72. The high copy vector expressing a hemagglutinin (HA) epitope tagged Slt2 (pBA1019) and the inactive kinase allele, slt2K54R have been previously described (Zarzov et al., 1996).

Growth Assays. To check for cell wall temperature-sensitivity defects, strains were streaked out onto either rich YPD plates or rich YPD plates containing 1M sorbitol and were incubated for three days at 37°C. To check for suppression of temperature-sensitive lytic phenotypes, slt2Δ (Y783) and bck2Δ (Y782) strains were transformed with high copy plasmids containing SLT2,
Transformants were streaked on plates containing synthetic media lacking uracil and incubated for three days at 37°C.

**Northern blot analysis.** To examine *PCLI*, *PCL2*, *CLNI* and *CLN2* gene expression in response to heat shock, wild type (Y792) and *slt2A* (Y783) strains were grown at 30°C in YPD medium to an optical density at 600nm of 0.4. Half the sample was shifted to 39°C for 3 hours to elicit a heat shock response. Immediately after heat shock, both the heat shocked cells and control cells cultured at 30°C were pelleted at 3000g in a 4°C centrifuge for 3 minutes. The supernatant was poured off and the cells were quickly frozen in liquid nitrogen. RNA was isolated and Northern blotting was done as described previously (Measday et al., 1997).

The probes used for the Northern blot analysis were a 600-bp *EcoRI-HindIII* fragment of the *ACT1* gene (Measday et al., 1994); a 864-bp PCR product containing the *PCLI* coding sequence (Measday et al., 1997); a 1.3 kb PCR product containing the *PCL2* gene (Measday et al., 1997); a 1.3kb XhoI-NcoI fragment of *CLN2* (Harrington and Andrews, 1996); and a 2.5kb *EcoR1* fragment of *CLNI* (Hadwiger et al., 1989). Probes were labeled using random-primed synthesis with Klenow DNA polymerase in the presence of α-32P-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).

**Immunoblot analysis of Swi6.** Extract preparation was performed essentially as previously described (Kamada et al., 1995). Cells were grown to mid log phase in YPD media at 30°C or shifted to 39°C for 1 hour before preparation of extracts. Pheromone-treated cells were grown to mid-log phase in YPD media and treated with 5μM α-factor (Sigma) for either 1hr or 2hrs before extract preparation. Pheromone block and release cells were grown to mid-log phase in YPD media, treated with 5μM α factor (Sigma) for 2hrs before being centrifuged, washed with YPD
and released into fresh YPD for 15 minutes before extract preparation. The cell response was terminated by further diluting the culture (1:1) with ice-cold stop mix (0.9% NaCl, 1mM NaN₃, 10mM EDTA and 50mM NaF). Cells were harvested by centrifugation at 3000g for 5min and washed once with cold stop mix. The cell pellet was suspended in 0.4 mL of ice-cold lysis buffer [50mM Tris-HCl (pH 7.5), 150mM NaCl, 5mM EDTA, 5mM EGTA, 0.2mM Na₃VO₄, 50mM KF, 30mM sodium pyrophosphate, 15mM p-nitrophenylphosphate, 20μg/mL leupeptin, 20 μg/mL of benzamidine, 10 μg/mL of pepstatin A, 40 μg/mL of aprotinin, and 1mM PMSF]. An equal volume of glass beads was added to this suspension and cells were broken by vortexing 6X 1 minutes with 1 minute incubations on ice between each burst. The beads and cellular debris were removed by centrifugation at 13,000g at 4°C, and the supernatant was further clarified by one additional 10 minute centrifugation. Glycerol was added to the lysate to a final concentration of 30%. Protein concentration of cell extracts was measured by Bio-Rad protein assay. 5μg of total yeast protein from each sample were separated on a 10% SDS-PAGE, blotted onto Immobilon-P (Millipore), and probed with affinity-purified anti-Swi6 antibodies as previously described (Ho et al., 1997).

For calf intestinal alkaline phosphatase (CIP) treatment experiments, affinity-purified anti-Swi6 antibodies were used to immunoprecipitate Swi6 from 1mg of total protein isolated from cells that had been incubated at 39°C for 1hr. Immunoprecipitates were resuspended in 45μL of NEB (New England Biolabs) Buffer 3 with 1% SDS. After 5 minutes of incubation at 37°C, the supernatant was removed and diluted to 0.1% SDS in NEB Buffer 3 with either 30U of CIP (NEB) or 30U of CIP together with 4mM sodium molybdate (CIP + inhib). Phosphatase reactions were incubated for 30 minutes at 37°C, and then Swi6 was re-immunoprecipitated from the phosphatase reactions. Each sample was then separated on a 10% SDS-PAGE, blotted onto
Immobilon-P (Millipore), and probed with affinity-purified anti-Swi6 antibodies as previously described (Ho et al., 1997).

*In vitro Kinase Assays.* Kinase assays were performed essentially as previously described (Tyers et al., 1992) except for the following modifications. Lysates were made from wild-type cells transformed with pBA1019. For each kinase reaction 500 μg of total yeast protein was used for the Slt2-Ha IP. 2.5 μg of purified SBF was used per kinase reaction (Baetz and Andrews, 1999). Half of each reaction was separated on a 10% SDS-PAGE gel, dried and used for autoradiography.
RESULTS

Suppression of Slt2 cell wall defects by overexpression of SWI4, PCL1 and PCL2. The phenotypic similarities between \( slt2\Delta \) and \( swi4\Delta \) mutants indicated that \( SLT2 \) and SBF may function in the same pathway. Because Swi4 and Swi6 are phosphoproteins (Amon et al., 1993; Sidorova et al., 1995) and the regulation of transcription factors through MAP kinase signaling has been shown in other systems (Su and Karin, 1996; Thomson et al., 1999; Treisman, 1996), my collaborator, Kevin Madden, decided to investigate whether SBF might be activated by Slt2. Plasmids containing either \( SWI4, SWI6, MBP1, PHO85 \) or \( G_1 \) cyclin genes were transformed into both \( slt2\Delta \) and \( bck1\Delta \) strains, and transformants were assayed for suppression of the \( 37^\circ C \) temperature-sensitive growth defect (Figure 3-1). Overexpression of \( SWI4, PCL1 \) or \( PCL2 \) suppressed the \( 37^\circ C \) growth defect (Figure 3-1). Overexpression of \( MBP1, PHO85 \) (Figure 3-1), \( CLN1, CLN2, CLN3 \) or \( SWI6 \) (data not shown) failed to rescue the growth defects of \( bck1\Delta \) or \( slt2\Delta \) mutants at \( 37^\circ C \). High-copy \( SLT2 \) plasmids were also transformed into \( swi4\Delta \) mutants; these plasmids did not suppress the temperature-sensitive growth of \( swi4\Delta \) strains at \( 37^\circ C \).

Taken together, these observations support the hypothesis that SBF functions downstream of \( SLT2 \). We next determined that overexpression of \( SWI4 \) and the \( G_1 \) \( PCL \) cyclins could also suppress the lysis or projection formation defects of \( slt2\Delta \) mutants exposed to mating pheromone (Madden et al., 1997). In summary, we found that overexpression of \( SWI4, PCL1 \) or \( PCL2 \) genes can suppress multiple phenotypes of \( SLT2 \) MAP kinase pathway mutants, but overexpression of \( SLT2 \) cannot rescue \( swi4\Delta \) defects, suggesting that \( SWI4 \) acts downstream of \( SLT2 \).

Slt2 activity is necessary for the heat shock induction of \( PCL1 \) and \( PCL2 \). One of the many ways MAPK kinase pathways modify cellular events is through regulating transcriptional events
Figure 3-1 Growth phenotypes of PKC1-MAP pathway and swi4Δ mutants and suppression of the slt2Δ and bck1Δ mutants by cell cycle regulatory components.

A) Growth of WT, bck1Δ, slt2Δ, and swi4Δ strains at 37°C on either rich YPD plates (left) or YPD plates containing 1 M sorbitol (right).  B) Assay for the suppression of slt2Δ (left) and bck1Δ (right) strain growth defects at 37°C by either YEp24 vector alone or high-copy number plasmids containing either SLT2, SWI4, PCL1, PCL2, PHO85 or MBP1.
Figure 3-2 Slit2-dependent induction of PCL1 and PCL2 in response to heat shock.

A) Northern blot analysis. mRNA was isolated from log phase cultures of wild type (Wt) and slit2Δ (slt2Δ) strains after growth at 30°C or after growth at 39°C for 3 hours. The expression levels of PCL1, PCL2, CLN1, CLN2 and ACT1 were determined by Northern blot analysis.

B) The Northern blot shown in (A) was quantitated using a Phosphorimager as described in Materials and Methods. Levels of PCL1, PCL2, CLN1 or CLN2 transcripts normalized to ACT1 transcript levels are graphed. Results shown are representative of three independent experiments.
(reviewed in Schenk and Snarr-Jagalska, 1999; Treisman, 1996). Since we showed that overexpression of SWI4, and two SBF-dependent genes, PCL1 or PCL2, could suppress the lysis defects of PKCl-MAPK pathway mutants, I next asked whether Slt2 might function as an activator of SBF-dependent gene expression. To investigate the role of Slt2 in SBF-dependent gene expression, I performed Northern blot analysis to examine expression of four SBF-dependent genes, CLN1, CLN2, PCL1 and PCL2, in wild-type and slt2A strains. No differences in expression levels were detected for any of the genes in strains grown at either 25°C or 30°C. However, when strains were incubated for 3 hours at 39°C, the steady state levels of PCL1 and PCL2 mRNA were induced 2-3 fold (Figure 3-2). The induction of PCL1 and PCL2 gene expression in response to heat shock was reduced by 50 – 75% in a slt2A strain. This level of Slt2 dependence is similar to that seen for several SBF-regulated genes involved in cell wall biosynthesis in response to heat shock (Igual et al., 1997). No difference was detected in the induction of the CLN1 or CLN2 mRNA in a slt2A strain. This result is consistent with the result that overexpression of CLN1 or CLN2 cannot suppress the cell wall lysis defects of PKCl-MAP Kinase pathway mutants, while overexpression of PCL1 and PCL2 can suppress the cell wall lysis phenotypes. Thus, SLT2 is required for the heat shock-dependent induction of a subset of SBF-dependent genes.

**Slt2-dependent phosphorylation of Swi6 in vivo.** Our genetic experiments and gene expression analysis suggested that SBF acted downstream of Slt2 and that Slt2 modulated the expression of SBF-dependent genes. Given these results, we next asked whether SBF is a target of the PKCl-MAPK Slt2. Since Swi6 has been implicated in modulating, both positively and negatively, the expression of SBF target genes (Breeden, 1996; Dirick et al., 1992; Lowndes et al., 1992) and Swi6 has been determined to be a phosphoprotein in vivo (Sidorova et al., 1995), we asked
**Figure 3-3 In vivo evidence for Slt2-dependent phosphorylation of Swi6.**

Immunoblot analysis of Swi6 was performed on 5µg of total yeast protein extracts prepared from the following strains: lanes 1, 3, and 7-12, wild type strain Y792 (SLT2); lane 2, swi6Δ strain YK175 (swi6Δ); lane 4, wild type strain that overexpresses SLT2 Y792 + 2µ SLT2 (2µ SLT2); lane 5, slt2Δ strain Y783 (slt2Δ); and lane 6, a slt2Δ strain that overexpresses a kinase-inactive allele of SLT2, Y783 + 2u slt2k54R (slt2k54R). Strains were either grown at 30°C (lanes 1 and 9), shifted to 39°C for 1 hour (lanes 2-8) or treated with α-factor for the indicated times (lanes 10 – 12) before preparation of the extracts. To confirm that the slower migrating form of Swi6 was due to phosphorylation, extracts were treated with CIP (lane 7) or CIP plus phosphatase inhibitor (lane 8).
whether the PKCI- MAP kinase pathway influenced the phosphorylation state of Swi6. To address this, immunoblot analysis with affinity-purified antibodies to Swi6 was performed using whole cell extracts from various strains. The analysis revealed two major isoforms of Swi6 in the lysates from log phase cells (Figure 3-3 lane 1). Phosphatase treatment of Swi6 immunoprecipitates resulted in loss of the slowly migrating species and an increase in the faster migrating isoform (Figure 3-3, lane 7); the presence of the phosphatase inhibitor sodium molybdate prevented this conversion (Figure 3-3, lane 8). Thus, the slowly migrating species is a phosphorylated form of Swi6. A strain overexpressing SLT2 accumulated the slowly migrating phosphorylated Swi6 isoform (Figure 3-3, lanes 4 and 5). However, expression of a stable but inactive kinase allele, slt2K54R (Zarzov et al., 1996), in a slt2Δ strain did not increase accumulation of the slowly migrating isoform. This indicates that the presence of phosphorylated Swi6 requires the kinase activity of Slt2.

Slt2 activity is stimulated by heat shock or by treatment of cells with mating pheromone (Errede et al., 1995; Kamada et al., 1995; Zarzov et al., 1996). In cells grown at 30°C, the faster migrating form was the major Swi6 isoform detected (Figure 3-3, lane 1 and 9), whereas incubation of cells at 39°C for one hour (lane 3) or exposure to α-factor (lanes 10 and 11) resulted in the increase of the phosphorylated slower migrating form of Swi6. The presence of intermediate species of Swi6 may indicate that several residues on Swi6 are phosphorylated by Slt2 or that other kinases also phosphorylate Swi6. Thus, for each of these different growth conditions, phosphorylation of Swi6 correlates with the level of Slt2 activity.

Direct phosphorylation of Swi4 and Swi6 by Slt2 in vitro. Co-immunoprecipitation assays determined that Slt2 could be found in a complex with both Swi4 and Swi6 (Madden et al., 1997), suggesting that SBF might be a direct target of Slt2 in vivo. To determine whether SBF
Figure 3-4 *In vitro* phosphorylation of Swi4 by Slt2.

Kinase reactions were done without immune complexes (lane 1), with immune complexes isolated from yeast extracts containing Slt2-HA (lanes 3, 5 and 7), or with immune complexes isolated from yeast extracts containing untagged Slt2 (lanes 2, 4 and 6). Either partially purified SBF from insect cell extracts (lanes 4 and 5) or a preparation in which Swi4 was immunodepleted from the insect cell extracts (lanes 6 and 7) was added to the reactions. The identities of the phosphoproteins are indicated to the right of the kinase assay.
was a direct target of Slt2, we performed \textit{in vitro} kinase assays with Slt2-Ha (Zarzov et al., 1996) immunoprecipitated from yeast extracts. Slt2-Ha immunoprecipitated from yeast extracts exhibited phosphorylation of the immunoglobulin heavy chain, Slt2-Ha itself, and a third phosphoprotein of 100kDa (Figure 3-4, lane 3). Next, my partially purified SBF derived from insect cells (see Chapter II and Baetz and Andrews, 1999) was added to the kinase reaction resulting in the phosphorylation of a protein that migrated at the expected position of Swi4 (Figure 3-4 lane 5). Immunodepletion of Swi4 from the kinase reaction, using Swi4-antibodies, confirmed that the slow migrating phosphoprotein was Swi4 (Figure 3-4 lane 7). Phosphorylation of Swi6 in this reaction was obscured by the 100kDa phosphoprotein. However, phosphorylation of Swi6 by Slt2 was confirmed by 2-D gel analysis (data not shown; Madden et al., 1997). We conclude that both Swi6 and Swi4 are substrates of Slt2-HA \textit{in vitro}. 
DISCUSSION

One of the many ways MAP kinase signaling pathways modify cellular events is through regulating transcriptional events. For example, recent DNA microarray analysis determined that the expression of roughly 90 genes can be modulated by an activated allele of *PKCl* in budding yeast (Roberts et al., 2000). This result suggests that transcription factors may be the major targets of the *PKCl* pathway. In a collaboration with Kevin Madden and Mike Snyder from Yale University, we show through genetic studies, immunoblot assays and kinase assays that SBF is a target of the MAP kinase Slt2. Further, I determined that Slt2 activity is required for the full heat shock induction of a subset of SBF-dependent genes.

**Slit2-dependent induction of a subset of SBF-dependent genes.** Our discovery that overexpression of *SWT4* and the Swi4-regulated genes *PCL1* and *PCL2* suppressed the lysis defects of *PKCl*-MAPK pathway mutants (Figure 3-1) and that SBF is an substrate of Slt2 (Figures 3-3 and 3-4), suggests that SBF may be an important target of Slt2 *in vivo*. I used Northern blot analysis to show that Slt2 is required for the full heat shock induction of the G1 cyclins *PCL1* and *PCL2* (Figure 3-2). Interestingly, Slt2 activity does not affect the expression levels of the G1 cyclins *CLN1* and *CLN2* (Figure 3-2 and Gray et al., 1997). This result suggests that *PCL1* and *PCL2* may have an important role in maintaining cell wall integrity that is not shared with the *CLNs*. In agreement with this, C. Igual et al., identified a group of cell wall biosynthetic genes that are transcriptionally regulated by both Slt2 and SBF (Igual et al., 1996). The cell wall biosynthetic genes identified, *FKSI*, *MNN1*, *CSD2*, *KRE6* and *GAS1*, all have important functions in maintaining cell wall integrity (reviewed in Klis, 1994). Further, overexpression of *PKCl* has no effect on expression of a *HO::lacZ* reporter gene (Gray et al., 1997) and DNA microarray analysis using an activated allele of *PKCl* did not result in the
transcriptional induction of all G1 genes (Roberts et al., 2000). These results suggest that Slt2 may act to modulate SBF to induce transcription of only a subset of SBF-dependent genes whose functions are necessary for maintenance of cell wall integrity.

**Role of PKCl-MAPK pathway at Start.** Previous work has implicated the PKCl pathway in cell cycle progression and polarized growth at Start (see Chapter I sections IV B and VIII D). Indeed, there is evidence that PKCl may have a role specifically at Start. Slt2 kinase activity is stimulated at the G1-S boundary in parallel with bud emergence (Zarzov et al., 1996), and the activation of Pkc1 appears to be partially dependent on Cdc28 activity (Marini et al., 1996; Zarzov et al., 1996). Further, overexpression of SLT2 can rescue some defects of a cdc28 mutant strain, including defects in the activation of SBF-dependent transcription at Start (Mazzoni et al., 1993). However, though our data indicates that Slt2 activates SBF, the observation that swi4 and pkcl mutants, as well as swi4 and slt2 mutants, are coletal (Gray et al., 1997; Igual et al., 1996; Madden et al., 1997) predicts that SBF and Slt2 have some independent functions.

Consistent with this interpretation, strains lacking SBF activity arrest predominantly in G1 (Nasmyth and Dirick, 1991; Ogas et al., 1991), whereas slt2Δ strains grown at 25°C, 30°C, or shifted to 37°C did not reveal an increase in the proportion of G1 cells (data not shown). This suggests that Slt2 activation of SBF may not play a significant role at Start. Indeed, Pkc1 is not required for the cell cycle-regulated transcription of MNN1, FKS1 or GAS1, cell wall biosynthetic genes whose heat shock induction is dependent on Slt2 (Igual et al., 1996). Activation of SBF-dependent transcription at Start is dependent on the activity of Cln3/Cdc28 kinase (Dirick et al., 1995; Stuart and Wittenberg, 1995). Comprehensive DNA microarray hybridization experiments have determined that nearly all the genes whose cell cycle expression peaks in late G1 can be induced by overexpression of CLN3 (Spellman et al., 1998). This result
suggests that, though Slt2 is essential in the absence of Cdc28 activity at Start, Slt2-activation of SBF at Start may not be required for SBF-dependent cell cycle transcription. Rather, Slt2 may be required for regulation of other biological targets necessary for cell cycle progression. Alternatively, Slt2 may be required in coordination with Cln3 to activate SBF-dependent transcription during a normal cell cycle. It will be interesting to determine whether overexpression of CLN3 in a slt2Δ strain allows for the induction of all G1 genes.

New roles for SBF outside the cell cycle. Since strains lacking SBF arrest in G1 (Ogas et al., 1991) and many G1 genes have at least one copy of the SCB element in their promoters (Spellman et al., 1998), it appears likely that the main role of SBF is in late G1. However, there are fewer than 300 genes whose transcription peaks at Start, but more than 1155 genes whose promoters contain matches to the SCB consensus elements (SCPD, 2000). A comparison of the number of SCB sites upstream of G1 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 or MCM/SFF sites (Spellman et al., 1998). This analysis suggests that SCB elements and SBF may regulate the transcription of many genes other than those induced at Start. Our work suggests that one pathway that may regulate SBF outside of Start is the PKCl-MAPK pathway. In Chapter IV, I investigate the role of SBF and the PKCl-MAPK pathway outside of G1 phase.
CHAPTER IV

Regulation of cell cycle transcription factor SBF by the cell integrity MAP kinase Slt2

I performed all of the experiments described in this Chapter with the following exceptions:
The PCL1 and PCL2 reporter gene assays shown in Figures 4-1 and 4-3 were performed by Michael Chang, a fourth year undergraduate student whom I co-supervised. The DNA microarray experiments (Figure 4-6 and Table 4-2) were performed in collaboration with Jason Moffat.
ABSTRACT

In *Saccharomyces cerevisiae*, the heterodimeric transcription factor SBF (SCB binding factor) promotes induction of gene expression at the G1/S-phase transition of the mitotic cell cycle. Mitotic commitment is associated not only with major alterations in gene expression but also with highly polarized cell growth. The MAP kinase Slt2 is required to maintain cell wall integrity during periods of polarized growth and cell wall stress. Previous work linked the cell wall integrity MAPK pathway with the cell cycle and established SBF as a downstream target of Slt2 (Chapter III). I describe a series of experiments aimed at defining the regulatory pathway involving the cell cycle transcription factor SBF and the Slt2-MAP kinase. Under conditions in which the Slt2-MAPK pathway is activated, expression of two G1 cyclin genes, *PCL1* and *PCL2*, was dependent on Swi4, the DNA-binding subunit of SBF, and on Slt2. Moreover, chromatin immunoprecipitation experiments using Swi4 and Swi6 antibodies showed Slt2-dependent recruitment of SBF to the promoters of *PCL1* and *PCL2* under conditions where the Slt2-MAPK pathway is activated. DNA microarray analysis was performed and a subset of 28 genes was identified that required both *SLT2* and *SWI4* for expression during heat stress. Some of the Swi4/Slt2-dependent genes are not cell cycle-regulated nor do their promoters contain consensus SCB elements. Nonetheless, SBF was localized to the promoters of these genes. This study suggests that activation of Slt2 may regulate SBF to allow SBF function independent of its established role in cell cycle transcription.
INTRODUCTION

In many systems, the major targets of MAP kinase cascades are transcription factors; indeed, activated alleles of *PKCI* lead to the transcriptional modulation of 90 genes, presumably through activation of the downstream Slt2 MAP kinase (Roberts et al., 2000). However, only two transcription factors have been identified as targets of Slt2, the MADS-box transcription factor Rlm1 (Watanabe et al., 1997; Dodou et al., 1997) and SBF (Chapter III; Madden et al., 1997). DNA microarray analyses show that Rlm1 is only required for regulating 25 of the *PKCI*-dependent genes (Jung and Levin, 1999). Also, *RLM1* mutants do not exhibit any of the phenotypes of *PKCI* pathway mutants (Watanabe et al., 1995), suggesting that Rlm1 is not the only important transcriptional target of Slt2. By contrast, *swi4* and *swi6* mutant strains share similar phenotypes to strains mutated for *PKCI* pathway genes. *swi4A* strains show a temperature-sensitive growth defect that is suppressed by sorbitol (Igual et al., 1996; Madden et al., 1997) and both *swi4* and *swi6* mutant strains are sensitive to cell wall stressors such as SDS, CFW (Igual et al., 1996) and caffeine (Sidorova and Breeden, 1999). *swi4* mutants also exhibit defects in projection formation upon exposure to mating pheromone, as do *PKCI* pathway mutants (Madden et al., 1997). These phenotypic similarities suggested that SBF may be a major transcriptional target of Slt2. Indeed, genetic studies, coimmunoprecipitation experiments and kinase assays have established SBF as a target of Slt2 kinase (Chapter III and Madden et al., 1997).

Interestingly, it appears that Slt2 may be able to modulate the activity of SBF. Slt2 activity is required for the maximum heat shock induction of only a subset of SBF-dependent G₁ genes, including *PCL1* and *PCL2* (Chapter III and Madden et al., 1997), and numerous cell wall biosynthetic genes (Igual et al., 1996). Consistent with this, overexpression of the G₁ cyclins
PCL1 and PCL2 but not CLN1 or CLN2 suppresses the cell lysis defects of a slt2Δ strain (see Chapter III and Madden et al., 1997). Though there is evidence that the PKC1-MAPK pathway has a specific role at Start (see Chapter I, sections IV B and VIII D), Slt2 activation of SBF may not play a significant role at Start (see Chapter III). Instead, activation of the PKC1-MAPK pathway may modulate SBF to regulate the transcription of a subset of genes required outside of G1 phase for maintenance of cell integrity.

In this chapter, I set out to test the hypothesis that Slt2 may act to modulate SBF to induce transcription of only a subset of SBF-dependent genes. I began by exploring the regulation of the SBF- and Slt2-dependent PCL1 and PCL2 genes. I used chromatin immunoprecipitation assays (ChIPs) to show that, upon activation of the PKC1-MAPK pathway by either heat shock or α-factor treatment, SBF specifically localized to the promoters of PCL1 and PCL2 and that this localization was dependent on Slt2. In collaboration with Jason Moffat, I performed a series of DNA microarray experiments, which suggest that Slt2-dependent alteration of SBF facilitates the utilization of SBF in a non-cell cycle capacity to regulate the transcription of 28 genes. These experiments establish a new role for SBF outside of the cell cycle.
MATERIALS AND METHODS

Plasmid constructs. The plasmids Wt-Swi6 and SA4-Swi6 are 2μ-based plasmids previously described as plasmids pBd176 and pBd1756 respectively (Sidorova et al., 1995). The PCL1 promoter-lacZ reporter construct, prPCL1.751-146 (pBA537), was previously described as pΔSS-HCS26 (Ogas et al., 1991). To construct the reporter plasmid prPCL1.363-146 (pBA548), a 217 bp SspI – StuI fragment containing the SCBs of the PCL1 promoter was excised from plasmid pBA532 (Ogas et al., 1991) and cloned into BgIII-XhoI-digested pΔSSBglII (Andrews and Moore, 1992). To construct reporter plasmid prPCL1.751-363 (pBA569a), a 388bp EcoRV – SspI fragment of the PCL1 upstream sequences was cloned into the blunted XhoI-digested pΔSSBglII. To construct a PCL2 promoter-lacZ reporter construct, the PCL2 promoter from -982 to -82 bp from the start site was amplified using the following primers: 5’-AACGCGTCGACCCTATTCTATCGATGGACC-3’ and 5’-AACGCGTCGACCGAGAATTATAAAGTG-3’. The PCR product was digested with SalI and cloned into SalI-digested pΔSSBglII to create plasmid prPCL2.982-82 (pBA1306). prPCL2.982-82 was sequenced to assure fidelity of the sequence after PCR amplification.

Strains and medium. Yeast strains used in this study are listed in Table 2-1. Standard methods for yeast growth and transformation were followed (Guthrie and Fink, 1991). Synthetic minimal medium (SD) and rich medium (YPD) were used (Kaiser et al., 1994). Most of the yeast strains used in this study are isogenic derivatives of BY263 (an S288C derivative; Measday et al., 1997). Disruption and epitope tagging of SLT2 was achieved by homologous recombination at its chromosomal locus using a PCR-based method (Longtine et al., 1998). The slt2Δ strain was verified by PCR and phenotypic assays.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or Reference</th>
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<tbody>
<tr>
<td>BY263</td>
<td>MATA trp1Δ63 ura3-52 lys2-801 ade2-107 his3Δ200 leu2-1</td>
<td>(Measday et al., 1994)</td>
</tr>
<tr>
<td>BY107</td>
<td>MATA swi6ΔHIS3</td>
<td>(Ogas et al., 1991)</td>
</tr>
<tr>
<td>BY108</td>
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<td>(Ogas et al., 1991)</td>
</tr>
<tr>
<td>BY1321</td>
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<td>this study</td>
</tr>
<tr>
<td>BY1342</td>
<td>MATA slt2ΔkanMX6</td>
<td>this study</td>
</tr>
<tr>
<td>BY551</td>
<td>MATA mbp1ΔTRP1</td>
<td>(Ho et al., 1997)</td>
</tr>
<tr>
<td>BY332</td>
<td>MATA ste12ΔLEU2 his4 leu2 trp1 ura3 can1</td>
<td>I. Sadowski</td>
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* All strains listed are isogenic to the parent strain BY263 with the exception of BY332.
**In vivo assays for Swi6 phosphorylation.** To assess the ability of Wt-Swi6 and SA4-Swi6 to suppress the cell wall sensitivity of a swi6Δ strain, plasmids expressing the two SWI6 alleles (pBd176 and pBd1756) and a plasmid control (pBA70) were transformed into strain BY107. To analyze Swi6 phosphoisofoms in vivo, the transformants were grown in YPD medium at 30°C or grown at 30°C followed by heat shock at 39°C for 1 hour. Yeast extracts were prepared and Western blot analysis was performed as previously described (Chapter III and Madden et al., 1997). The transformants BY107 + pBA70, BA107 + pBd176 and BA107 + pBd1756, along with control strains BY263 and BY1342, were grown in YPD medium overnight at 30°C. Cell density in the cultures was determined and serial dilutions were performed to allow spotting of 1000, 100, 10 and 1 cells onto either YPD plates or YPD plates containing 0.03% SDS. The plates were incubated at 30°C for 3 days.

**β-galactosidase assays.** To assay activity of CYC::lacZ reporter genes in log phase cultures, yeast transformants were grown in 5 mL of SD-URA medium to an OD₆₀₀ of 0.6 at 30°C. For heat shock experiments, cultures were grown to an OD₆₀₀ of 0.6 at 30°C, then transferred to test tubes that had been preincubated at 39°C and cultures were incubated at 39°C for 30min. To arrest cells with α-factor, cells were grown in minimal medium supplemented with 1M sorbitol. Once the cultures reached an OD₆₀₀ of 0.3, α-factor (Procyon Biopharma) was added to the cultures to a final concentration of 5 μM for 2 hours before harvesting. The cells were harvested by centrifugation at 3,000 x g for 5 minutes, 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 previously described (Rose and Botstein, 1983). Data are presented as the mean values from triplicate experiments.
Cell cycle synchronization, heat shock and chromatin immunoprecipitation. To examine Swi4/Swi6 localization to promoters in vivo upon heat shock or pheromone treatment, chromatin immunoprecipitation experiments were performed. For heat shock experiments, 50 mL cultures of cells were grown to an OD₆₀₀ of 0.6. One culture was held at 30°C while two other cultures were shifted to 39°C for either 30 or 60 minutes. Samples of cells were cross-linked with 1% formaldehyde for 15 minutes 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 OD₆₀₀ 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 hours until at least 95% of the cells were arrested in G₁ phase as determined by microscopy. Cells were pelleted, then resuspended in fresh YPD medium. Fifty mL samples were taken before the arrest, before the release and every 10 minutes after release and cross-linked with 1% formaldehyde for 15 minutes at 30°C. In both cases, cross-linking was quenched by the addition of glycine to 125 mM. Cells were pelleted at 3,000 x g for 5 minutes, 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 previously described (Strahl-Bolsinger et al., 1997). Cell lysis was performed in 400 µL lysis buffer (50 mM Hepes-KOH pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% Na-deoxycholate plus one EDTA-Free Protease pellet for every 50 mL [Boehringer Mannheim]) and 400 µL glass beads. The suspension was sonicated four times for 10 sec each (resulting in an average fragment size of 0.5 kb) and clarified by centrifugation for 15 minutes at 14,000 g. Protein concentration for each sample was determined by Bradford Assay (Biorad). 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 minutes each in 1 mL lysis buffer, and twice for 10 minutes each in 1 mL TBS. Finally, the samples were processed for DNA purification as previously described (Strahl-Bolsinger et al., 1997).

**PCR analysis of immunoprecipitated DNA.** PCR reactions were carried out in 25 μL volumes. Serial dilutions of the immunoprecipitated material and the input material for the whole cell extracts (WCE) were performed to assure the PCR reactions were performed in the linear range. Typically PCR reactions were performed with 1/2000 of the immunoprecipitated material, and 1/20000 of the input material for whole cell extracts (WCE). PCR was performed using Platinum Taq polymerase (GibcoBRL) with 25 picomoles of each primer and 0.5 μCi of P\(^{32}\)-α-dATP. For multiplex PCR with the PCL2, PCL1, PHOS and CLNI promoter primer pairs, 2.0 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 minutes denaturation at 95°C followed by 25 cycles with 30 seconds at 95°C, 30 seconds at 53°C and 60 seconds at 70°C, and a final extension step of 5 minutes at 70°C. The gene-specific primers were designed as 20mers with roughly 50% GC content. The PCR primer sequences used for the amplification of promoter regions in PHOS, PCL1, PCL2, CLNI, RLM1, YLR110c, YNL300w, HHFI, OXAI, SBPI and SRL1/YOR248c are available upon request. The PCR products were separated on a 5% polyacrylamide gel, dried and exposed to Kodak BIOMAX-MR film.

**Microarray hybridization and analysis.** Microarrays containing all yeast ORFs or predicted ORFs were purchased from the MicroArray Centre at the Ontario Cancer Institute. Detailed protocols for the construction of the microarrays, labeling, hybridization, and scanning can be obtained from [http://www oci.utoronto.ca/services/microarray](http://www.oci.utoronto.ca/services/microarray). Image acquisition was with a
GSI Lumonics ScanArray® 4000 and ScanArray® software v1.2. Image analysis, including detection of more than 12000 spots per array, was with QuantArray® software v3. Data exported from QuantArray® was refined to adjust for differences in scanning intensities between Cy3 and Cy5 and reproducibility between duplicate spots. Differences in scanning intensities between Cy3 and Cy5 signals was corrected for by normalization of the Cy3 or Cy5 signal intensity of each spot to the Cy3 or Cy5 average signal intensity for the entire array (eg Spot X Cy3 Normalization = Spot X Cy3 signal/Average Cy3 signal for the array). Uneven spot hybridization within a sub-region was tracked manually by visual inspection of the arrays and noted prior to normalization. In cases where uneven hybridization occurred the spot signals were normalized against the average intensity of its subarray, not average of the full array. In general, we have found that normalization of the data globally (full-array) or by sub-array does not significantly alter the results on high quality chips. However, uneven hybridization on a single chip is better corrected for by normalization of the data by sub-array. Because sub-array median normalization precludes us from seeing large changes in gene expression patterns, we have tried to limit our use of sub-array normalization and derive correction factors from entire data sets. In cases where hybridization to one of the duplicate spots was uneven (spots not within 2-fold of each other), the gene was rejected from further analysis. Duplicate spots increase the level of confidence in a given intensity value and are a good indication of hybridization fluctuations for a given gene. Any partially overlapping spots were also excluded from the data set. Intensity ratios (Cy3/Cy5) were derived from normalized Cy3 and Cy5 intensity values.
RESULTS

Heat shock induction of *PCL1* reporter gene expression is dependent on Slt2 and Swi4 but independent of Swi6. Upon heat shock Slt2 is required for the 2-3 fold transcriptional induction of numerous cell cycle-regulated genes, including the G1 cyclins *PCL1* and *PCL2* (Igual et al., 1996; Madden et al., 1997). To characterize the molecular mechanism of the Slt2-dependent transcriptional response, I turned to an *in vivo* assay to analyze the roles of Swi6 and Swi4 in the Slt2-dependent transcription of *PCL1*. To facilitate this analysis, a series of *PCL1* reporter genes was created. Sequences 5' to the *PCL1* translational start site, spanning from −751 to −146 relative to the *PCL1* ATG, were placed upstream of a *CYC1::lacZ* reporter gene on a yeast vector to create the reporter plasmid *prPCL1*-751-146 (Ogas et al., 1991) (Figure 4-1A). This region of the *PCL1* promoter contains four consensus SCB elements beginning at −327, -303, -294 and -265 and one consensus MCB site at −313. To assay the contribution of SBF and Slt2 to the UAS activity of the *prPCL1*-751-146 reporter, we transformed wild type, swi4, swi6, *mbpl* and *slt2* mutant strains with the *prPCL1*-751-146 plasmid and the control plasmid *CYC1::lacZ*. None of the strains containing the control reporter gene produced β-galactosidase activity (data not shown). A wild-type strain containing the *prPCL1*-751-146 reporter gene had significant β-galactosidase activity at 30⁰; upon heat shock, the β-galactosidase activity was induced 2 fold (Figure 4-1B). Thus, the behavior of the *PCL1* reporter gene in response to heat shock mirrors that of the endogenous *PCL1* gene (Chapter III, Madden et al., 1997). As previously shown, the β-galactosidase activity from the *prPCL1*-751-146 reporter gene was completely dependent on Swi4 (Figure 4-1B) (Ogas et al., 1991). Surprisingly, we discovered that the UAS activity from the promoter of *prPCL1*-751-146 was independent of Swi6 (Figure 4-1B); this is unique behavior
Figure 4-1 Slt2/Swi4-dependent and Swi6-independent activation of PCL1-lacZ reporter genes.

(Panel A) Schematic diagram of the PCL1 upstream regulatory regions used to create CYC::lacZ reporter constructs. Location of consensus SCB and MCB motifs is indicated. The indicated strains were transformed with either prPCL1.751-146 reporter plasmid (Panel B), the prPCL1.363-146 reporter construct (Panel C) or the prPCL1.751-363 reporter construct (Panel D). Transformants were grown at 30°C to log phase (open bars) or grown at 30°C to log phase and then heat shocked for 30 minutes at 39°C (filled bars). Cell lysates were made and the β-galactosidase activity (Miller units) was determined. Activity depicted was the mean of three experiments.
for a Swi4-dependent gene. Even though there is one consensus MCB element in the PCLI promoter, deletion of MPB1 did not affect prPCLI-751-146 reporter activity (Figure 4-1B). The heat shock-dependent induction of the PCLI reporter gene was completely eliminated in the slt2Δ strain (Figure 4-1B). Thus, the reporter gene analysis suggests that the expression of PCLI is dependent on both Slt2 and Swi4 but independent of Swi6. To confirm the PCLI reporter gene analysis, Northern blot analysis was performed to determine the expression of PCLI in wild type, swi4Δ, swi6Δ and slt2Δ strains. The Northern blots confirmed that the expression of PCLI is dependent on both Slt2 and Swi4, but independent of Swi6 (data not shown).

To identify the region of the PCLI promoter required for the Swi4/Slt2-dependent, Swi6-independent heat shock induction, two additional reporter genes were created. The prPCLI-363-146 gene contains a segment of the PCLI promoter that lies immediately upstream of the translation start site and contains the four SCB elements. The prPCLI-751-363 construct contains only the 5' region of the promoter fragment used in our first PCLI reporter construct (Figure 4-1A). Neither half of the PCLI upstream region showed UAS activity comparable to the intact PCLI, nor was the heat shock induction of either half of the PCLI upstream region as strong as the intact PCLI promoter. This implies that both the 5' and 3' regions of the promoter are necessary for proper transcriptional regulation. Unlike the prPCLI-751-146 UAS activity, the activity of the 3' PCLI promoter construct prPCLI-363-146 activity was dependent on both Swi4 and Swi6 (Figure 4-1C). By contrast, regulation of the 5' PCLI promoter construct (prPCLI-751-363) mimicked that of the full-length reporter gene; both the 5' PCLI reporter gene and the full length PCLI reporter gene were dependent on Slt2 and Swi4 and independent of Swi6 (Figure 4-1D). Remarkably, the 5' region of the PCLI promoter does not contain any consensus SCB
elements. However, mobility shift assays using the -751 to -363 region of the \textit{PCL1} promoter as a probe with purified SBF (Baetz and Andrews, 1999) showed that SBF can form a complex on this region of the promoter (data not shown). These results suggest that Swi4 may recognize a motif in the 5' region of the \textit{PCL1} promoter whose consensus is different from the standard SCB element. In summary, the reporter gene analysis shows that the region from -751 to -363 bp upstream of the \textit{PCL1} start site, which contains no consensus SCBs, is required for maximum reporter gene activity and mediates Swi4- and Slt2-dependence of \textit{PCL1} expression.

\textbf{Phosphorylation of Swi6 by Slt2 is not required to maintain cell wall integrity.} Activation of the \textit{PKCl}-pathway by heat shock or treatment with \(\alpha\)-factor (Figure 3-3, and Madden et al., 1997), SDS or CFW (data not shown), results in Slt2-dependent phosphorylation of Swi6. These results have led to the suggestion that Slt2 may regulate gene expression through phosphorylation of SBF (Chapter III, and Madden et al., 1997). However, our promoter analysis of \textit{PCL1} suggested that Swi6 may not be required for the Slt2-dependent heat shock response. To determine whether Slt2-dependent phosphorylation of Swi6 is required to maintain cell wall integrity, I used a swi6 phosphorylation site mutant. Slt2 is a serine/threonine-specific kinase (Hunter and Plowman, 1997). All of the serine and threonine residues on Swi6 that are phosphorylated \textit{in vivo} have been identified and mutated to alanine to create the Swi6 mutant named SA4-Swi6 (Figure 4-2A) (Sidorova et al., 1995). I first wanted to determine if the SA4-Swi6 mutant abolishes Slt2-dependent phosphorylation of Swi6. Slt2-dependent phosphorylation of Swi6 can be detected by electrophoretic mobility shift on SDS-PAGE gels (Figure 3-3, and Madden et al., 1997). I assayed Swi6 phosphoisoforms in yeast extracts from strains expressing the wild type \textit{SWI6} or the mutant \textit{SA4-SWI6} either grown at 30\(^\circ\)C or grown at 30\(^\circ\)C then heat
Figure 4-2. Slt2 phosphorylation of Swi6 is not required for cell wall integrity.

(A) Schematic diagram of the serine and threonine sites of Swi6 that are phosphorylated *in vivo* and which are mutated to alanine in the mutant *SA4-SWI6*. (B) The phosphorylation site mutant SA4-Swi6 is not phosphorylated *in vivo*. Western blot analysis using affinity-purified anti-Swi6 antibodies was used to detect Swi6 phosphoisoforms in yeast extracts from a *swi6Δ* strain transformed with a 2μ plasmid containing either wild type *SWI6* (Wt) or the Swi6 phosphorylation site mutant *SA4-SWI6* (SA4). (C) Phosphorylation site mutant *SA4-SWI6* can suppress SDS sensitivity of a *swi6Δ* strain. Serial dilutions of the strains indicated were spotted on YPD and YPD plus 0.03% SDS and photographed after three days at 30°C.
shocked at 39°C for one hour. Upon heat shock, I saw an increase in the phosphorylated form of Wt Swi6 as previously described (Chapter III and Madden et al., 1997). However, no detectable phosphorylation of Swi6 occurred in the heat shocked cells expressing the SA4 –SWI6 mutant gene (Figure 4-2B). I conclude that SA4-Swi6 mutant cannot be phosphorylated by the Slt2 kinase in vivo.

I next asked whether the SA4-Swi6 mutant was able to complement the cell wall defects of a swi6Δ strain. Though swi6Δ strains are not temperature sensitive, swi6Δ strains are sensitive to SDS, CFW and caffeine (Igual et al., 1996; Sidorova and Breeden, 1999). I plated wild type cells, slt2Δ, swi6Δ, swi6Δ + Wt-Swi6 and swi6Δ + SA4-Swi6 on YPD medium and on YPD medium containing 0.03% SDS. As previously shown, both the slt2Δ and swi6Δ strain failed to form colonies on the SDS-containing medium (Igual et al., 1996)(Figure 4-2C). The SDS sensitivity of the swi6Δ strain was suppressed by expression of either wild-type Swi6 or SA4-Swi6. Expression of SA4-Swi6 also complemented the CFW sensitivity of the swi6Δ strain (data not shown). My assays suggest that although Swi6 is clearly phosphorylated in a Slt2-dependent manner in vivo, phosphorylation of Swi6 by Slt2 is not essential for maintenance of cell wall integrity.

α-factor induction of PCL2 is partially dependent on Slt2 and SBF. Previous studies have shown that, as is seen with PCL1, overexpression of PCL2 can rescue the temperature-sensitive phenotype of slt2Δ strains (Madden et al., 1997). To begin to ask if PCL2 was regulated in a manner similar to PCL1, a PCL2 reporter gene was constructed in which sequences 5' to the PCL2 transcriptional start, spanning from -982 to –82, were placed upstream of the CYC::lacZ reporter gene (prPCL2-982-82, Figure 4-3A). This region of the PCL2 promoter contains two SCB elements at -370 and -393, three MCB elements at -378, -394 and -577 and one
Figure 4-3. Pheromone induction of PCL2 expression is partially dependent on Sit2 and SBF.

(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. (B) Alpha-factor induced expression of β-galactosidase from the prPCL2.989-82 reporter plasmid. The indicated 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 α-factor for 2 hours (filled bars). Cell lysates were made and the β-galactosidase activity (Miller units) of each strain was determined. Activity depicted was the mean of three experiments.
pheromone response element (PRE) at −520. The PRE is bound by the Ste12 transcription factor which is activated by the pheromone response pathway (reviewed in Madhani and Fink, 1998).

In wild type cells, the prPCL2.982-82 reporter failed to reiterate the heat shock induction of PCL2 that was previously seen (Chapter III and Madden et al., 1997). However, like the endogenous PCL2 gene, the reporter gene was responsive to the pheromone α-factor, which arrests sensitive cells in G1 phase in preparation for mating. PCL2 is the only member of the PHO85 cyclin family whose transcript is immediately induced in response to α-factor treatment and by STE12 overexpression (Measday et al., 1994; Measday et al., 1997; Roberts et al., 2000). Since overexpression of PCL1 or PCL2 can suppress the pheromone sensitivity phenotype (mating projection defects and cell lysis) of a slt2Δ strain (Chapter III and Madden et al., 1997), we used our PCL2 reporter gene to ask whether induction of PCL2 in response to α-factor induction was dependent on Slt2. Since PKCI-MAPK pathway mutants lyse after prolonged exposure to α-factor (Costigan et al., 1992; Errede et al., 1995; Madden et al., 1997), we performed our experiments in the presence of sorbitol to circumvent the lysis phenotype. The prPCL2.982-82 construct was used to transform wild type, swi4Δ, slt2Δ and ste12Δ strains. The PCL2 promoter activity was significantly increased one hour after α-factor treatment, indicating that the addition of sorbitol did not significantly alter the ability of the cells to respond to pheromone (Figure 4-3B). The α-factor-dependent induction of the PCL2 reporter gene was fully dependent on the PRE-binding transcription factor Ste12 (Figure 4-3B). Recent DNA microarray analysis suggests that the PKCI-MAPK pathway is required for the pheromone-induced Ste12-independent expression of genes after extended pheromone treatment (60 to 120 minutes) (Roberts et al., 2000). Since α-factor induction of PCL2 gene expression was Ste12-dependent, we were surprised to find that α-factor induction of the PCL2 promoter reporter was
also partially dependent on Slt2 (Figure 4-3B). Though roles for the Slt2 pathway during pheromone response have been described (Buehrer and Errede, 1997; Roberts et al., 2000; Sheu et al., 1998; Zarzov et al., 1996), this is the first example of the PKCl pathway being required for full transcriptional activation of a Ste12-dependent gene. Surprisingly, we also found that α-factor induction of the PCL2 reporter was partially dependent on Swi4 and fully dependent on Swi6. In summary, we have shown that Slt2 and Swi4 are both required for heat shock induction of PCL1 expression and α-factor induction of PCL2 expression.

Increased localization of Swi4 and Swi6 to the promoters of PCL1 and PCL2 upon heat-shock and α-factor treatment. PCL1 gene expression peaks in late G1 (Aerne et al., 1998; Measday et al., 1997; Spellman et al., 1998) and the promoter of PCL1 contains multiple SCB elements (Ogas et al., 1991). Therefore, I was surprised to find that the UAS activity of the PCL1 upstream region was independent of Swi6 (see above). All studies to date indicate that both Swi4 and Swi6 are required for SCB-driven gene expression (Andrews and Herskowitz, 1989; Breeden and Nasmyth, 1987; Nasmyth and Dirick, 1991); in the absence of Swi6, Swi4 is unable to bind SCBs both in vitro (Chapter II and Baetz and Andrews, 1999) and in vivo (Cosma et al., 1999; Harrington and Andrews, 1996; Koch et al., 1996).

To directly examine the association of Swi4 and Swi6 with the PCL1 and PCL2 promoters, I performed chromatin immunoprecipitation (ChIP) assays using affinity-purified Swi4 and Swi6 polyclonal antibodies. Cells were synchronized in early G1 with α-factor, and then released into fresh media to allow the cells to progress through a synchronous cell cycle. Samples were taken at 10 minute intervals following release from α-factor and were analyzed for position in the cell cycle by propidium iodide staining and FACS. Cells begin to traverse the G1/S boundary 20 minutes 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. I then measured the abundance of specific DNA sequences within the immunoprecipitates using the polymerase chain reaction (PCR) and appropriate primer pairs. Each reaction contained four sets of primers, which enabled me to simultaneously measure the relative abundance of Swi4 and Swi6 at the promoters of *PCL1*, *PCL2*, *CLN1* and *PHO5*. For *PCL1*, *PCL2* and *CLN1* 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 (SCP), its transcription is not cell cycle-regulated (Spellman et al., 1998) and there have been no reports of Swi4- or Swi6-dependent transcription of *PHO5*. In agreement with this, ChIP using either affinity purified Swi4- or Swi6-antibodies did not enrich for *PHO5* promoter DNA above that of the ChIP negative controls of protein A-sepharose alone (no antibody) or ChIP using non-crossed linked lysates (NX). Further, ChIP using *swi4Δ* or *swi6Δ* strains and affinity purified Swi4- or Swi6-antibodies respectively, did not enrich for *PCL1*, *PCL2* or *CLN1* promoter DNA (data not shown). This suggests that my ChIPs are specifically enriching for DNA that Swi4 and Swi6 are localizing to *in vivo*.

The localization of Swi4 and Swi6 to the *CLN1* promoter peaked in late G1, which is the time at which SBF-dependent cell cycle-regulated transcription is known to occur (Figure 4-4A, 20 minute time point). SBF also interacted with the promoter of *CLN2* at the same time (data not shown). Thus, my ChIP assays, using either Swi4 or Swi6 antibodies, provide a representative snapshot of what is occurring *in vivo*. The cell cycle expression of *PCL1* is similar to that of *CLN1* and *CLN2* (Aerne et al., 1998; Measday et al., 1997; Spellman et al., 1998), and I found that peak localization of Swi4 and Swi6 to the *PCL1* promoter also occurred at 20 minutes. Expression of *PCL2* peaks earlier than that of *PCL1* and *CLN1* and is partially
Figure 4-4. Swi4 and Swi6 localization to the promoters of \textit{PCL1} and \textit{PCL2} during heat shock and \(\alpha\)-factor treatment.

(A) Cell cycle Chromatin Immunoprecipitation (IP) using affinity-purified Swi4 antibodies (left panel) and Swi6 antibodies (right panel). A wild type strain (BY 263) was grown to mid log phase, then blocked in \(G_1\) with \(\alpha\)-factor and released into fresh medium and samples were taken every ten minutes. FACS analysis determined that cells were progressing through the \(G_1\)-S boundary at the 20 minute timepoint (not shown). Samples taken of the unsynchronized log phase culture (log), the \(G_1\) arrested cells (\(\alpha\)-factor) and at 10 minute time-points after release were cross-linked with formaldehyde. Whole cell extracts (WCE) were made and ChIP was done using protein A-sepharose alone (pA), or with affinity-purified Swi4 or Swi6 antibodies as indicated. In the top panels multiplex PCR was performed to amplify the promoter regions of \textit{PCL2}, \textit{PCL1}, \textit{PHOS} and \textit{CLNI} in each of the ChIP samples, in an immunoprecipitation using extract which was not cross-linked (NX), and in whole cell extracts (WCE). The graphs depict the results of phosphorimager analysis of each PCR product. The results are expressed as a percentage of the product in WCE.

(B) Heat shock Chromatin Immunoprecipitation. Cultures of a wild type strain (BY263) that was either grown at 30°C (white bars) or grown at 30°C and heat shocked at 39°C for 30 minutes (gray bars) or 60 minutes (black bars) were cross-linked with formaldehyde. Lysates were made and ChIP was performed using using protein A-sepharose alone (pA), or with affinity-purified Swi4 antibodies (\(\alpha\)-Swi4) or affinity-purified Swi6 antibodies (\(\alpha\)-Swi6). PCR was performed to amplify the promoters of \textit{PCL1}, \textit{PCL2} and \textit{PHOS} on 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.
A

Swi4-Chromatin IP

Swi6-Chromatin IP

% WCE IPed vs. Time after release (min)

B

PCL2

PCL1

PHO5

% WCE IPed

Antibody: pA α-Swi4 α-Swi6

Legend:

- Log
- 39°C 30 minutes
- 39°C 60 minutes
dependent on Swi5 (Aerne et al., 1998; Measday et al., 1997). My ChIP experiments did not detect a strong Swi4 or Swi6 association with the \textit{PCL2} promoter during the cell cycle. This result suggests that either our time points did not catch the peak binding of SBF to the \textit{PCL2} promoter or that SBF binding to the \textit{PCL2} promoter is not required for cell cycle regulation of \textit{PCL2}.

Upon $\alpha$-factor treatment the expression of \textit{CLN1}, \textit{CLN2} and \textit{PCL1} genes is repressed (Measday et al., 1997; Tyers et al., 1992). As mentioned above, \textit{PCL2} is the only G1 cyclin whose expression is induced by $\alpha$-factor treatment (Measday et al., 1994) and full $\alpha$-factor induction of \textit{PCL2} is SBF dependent (Figure 4-3). Upon $\alpha$-factor treatment Swi4 and Swi6 localization to either the \textit{CLN1} promoter (Figure 4-4A) or the \textit{CLN2} promoter (data not shown) remained low, while Swi4 and Swi6 localization to \textit{PCL2} promoter dramatically increased (Figure 4-4A). Remarkably, even though the \textit{PCL1} gene is not expressed during $\alpha$-factor treatment, Swi4 and Swi6 localization to the \textit{PCL1} promoter also dramatically increased upon $\alpha$-factor treatment. I conclude that the increased association of SBF to the \textit{PCL1} promoter during $\alpha$-factor treatment is not sufficient for \textit{PCL1} expression. My results suggest that there must be an activation event specific to the SBF on the promoter of \textit{PCL2} during the pheromone response that does not activate SBF on the promoter of \textit{PCL1}.

I next asked whether heat shock affected the localization of SBF to the promoters of \textit{PCL1} and \textit{PCL2}. I performed ChIP experiments on cross-linked cells that were grown at 30$^\circ$C or cells that were grown at 30$^\circ$C and heat shocked at 39$^\circ$C for either 30 or 60 minutes. I used phosphorimager analysis to compare the PCR product of the ChIP to that of the whole cell extract (WCE). Heat shock increased the localization of both Swi4 and Swi6 to the promoters of \textit{PCL1} and \textit{PCL2} (Figure 4-4B) but not the promoter of \textit{CLN1} (data not shown). In
Figure 4-5. Slt2 activity is partially required for SBF localization to the promoters of \( PCL1 \) and \( PCL2 \).

(A) PCR amplification of ChIP from wild type and \( slt2\Delta \) strains. Wild type (BY263) and \( slt2\Delta \) (BY1342) cultures grown at 30\(^\circ\)C (log), heat shocked for 1 hour at 39\(^\circ\)C (HS) or treated with \( \alpha \)-factor for 2hrs (\( \alpha \)) were cross-linked with formaldehyde. Lysates were made and ChIP was performed using protein A-sepharose alone (pA), Swi4 antibodies (Swi4-Ip) or Swi6 antibodies (Swi6-Ip). Multiplex PCR was performed to amplify the promoter regions of \( PCL2 \), \( PCL1 \), \( PHO5 \) and \( CLN1 \) from each of the immunoprecipitated chromatin and the whole cell extract (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 \( CLN1 \) are depicted as % of the WCE immunoprecipitated. Wildtype ChIPs are depicted in the light blue bars, \( slt2\Delta \) ChIPs are depicted in the dark blue bars. This is a representative of two separate experiments.
summary, under two conditions which activate the MAP kinase Slt2, heat shock and α-factor treatment, SBF localization to the promoters of \(PCL1\) and \(PCL2\) increases.

Localization of SBF to the promoters of \(PCL1\) and \(PCL2\) upon heat shock and pheromone treatment is partially dependent on Slt2. Since I saw increased binding of SBF to the promoters of \(PCL1\) and \(PCL2\) under two conditions which activate Slt2 (Kamada et al., 1995; Zarzov et al., 1996), I next tested the localization of SBF to \(PCL\) promoters in a \(slt2\Delta\) strain. I used Swi4 and Swi6 polyclonal antibodies to immunoprecipitate chromatin from formaldehyde cross-linked wild type and \(slt2\Delta\) cells that were either in log phase, heat shocked at 39°C for 60 minutes or treated with α-factor for 2 hours. Since both heat shock and α-factor treatment cause cell lysis in \(slt2\) mutant cells, both the wild type and \(slt2\Delta\) cells were grown in media containing sorbitol. Addition of sorbitol did not affect α-factor induction of \(PCL2\) promoter activity (above) 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 \(slt2\Delta\) cells (Figure 4-5). In wild type cells upon heat shock, the localization of Swi4 and Swi6 to both the \(PCL1\) and \(PCL2\) promoters increased. However, in \(slt2\Delta\) cells the localization of Swi4 and Swi6 to the promoters of \(PCL1\) and \(PCL2\) was reproducibly reduced.

α-factor treatment of wild type cells also results in increased localization of Swi4 and Swi6 to the \(PCL1\) and \(PCL2\) promoters, but the localization of SBF to the \(PCL1\) and \(PCL2\) promoters is reproducibly reduced in \(slt2\Delta\) cells. Notably, deletion of \(SLT2\) does not fully eliminate the induction of SBF localization to the \(PCL1\) and \(PCL2\) promoters. This indicates that Slt2 activity is only partially required for the increased localization of SBF to the promoters of \(PCL1\) and \(PCL2\) upon heat shock or α-factor treatment.
Figure 4-6  DNA microarray analysis of *slt2Δ* and *swi4Δ* strains following heat shock. (A) Signature plot for a wild-type strain (BY263) with reciprocally labelled (Cy3 and Cy5) cDNAs. Fluorescently labeled cDNAs derived from wt cells following a 45 minute temperature shift to 39°C were hybridized to two DNA microarrays, each carrying all of the predicted open reading frames of yeast in duplicate. Data from one such microarray is displayed as the Cy5/Cy3 ratio \[\log_{10}(\text{expression ratio})\] versus the average intensity \[\log_{10}(\text{intensity})\] for each probe. All dots are coloured blue and lie within a two-fold expression ratio cutoff as expected. (B) Correlation plot of a *swi4Δ* strain versus a wild-type strain following mild-heat shock. Fluorescently labelled cDNAs derived from *swi4Δ* and wild-type cultures treated by mild heat shock were analyzed on two microarrays. This process was repeated a second time. The four microarrays were analyzed in parallel and one representative dataset is displayed in correlation with a wild-type dataset. Grey dots indicate genes whose expression levels lie within the 2-fold cutoff ratio. Red dots indicate genes whose expression is induced more than 2-fold in a *swi4Δ* strain. Green dots indicate genes whose expression is reduced more than 2-fold in a *swi4Δ* strain. Orange, yellow, blue and light grey spots indicate genes that lie outside the 2-fold cutoff ratio in a reciprocally labelled wild-type strain. (C) Correlation plot of a *slt2Δ* strain versus a wild-type strain following mild-heat shock. Fluorescently labelled cDNAs derived from *slt2Δ* and wild-type cultures treated by mild heat shock were analyzed on two microarrays. This process was repeated a second time. The four microarrays were analyzed in parallel and one representative dataset is displayed in correlation with a wild-type dataset. The colour scheme of the dots is the same as in (B). (D) Correlation plot of a *slt2Δ* strain versus a *swi4Δ* strain. Representative *slt2Δ* and *swi4Δ* datasets were plotted against each other. Light green spots represent genes whose expression is reduced more than 2-fold in both *swi4Δ* and *sltΔ* strains. Orange spots represent
genes whose expression is induced more than 2-fold in both swi4Δ and slt2Δ strains. Yellow and blue spots represent genes whose expression is either reduced or increased more than 2-fold in a swi4Δ strain, respectively. Dark green and red spots represent genes whose expression is either reduced or increased more than 2-fold in a slt2Δ strain, respectively. The figure was made by Jason Moffat.
DNA microarray analysis of global gene expression in swi4Δ and slt2Δ mutants. SLT2 is required for the heat shock induction of the SBF-dependent genes PCL1, PCL2 and various cell wall biosynthetic genes (Chapter III, and Madden et al, 1997; Igual et al., 1996). The PCL1 reporter gene assays present above (Figure 4-1) have determined that the heat shock induction of PCL1 is dependent on Swi4, but might not be mediated through traditional SCB elements. Further, chromatin immunoprecipitation experiments presented above suggest that Slt2 activity may regulate the localization of SBF on the promoters of Slt2-responsive genes. These results suggest that there may be unique elements in the promoters of PCL1 and PCL2 that may specify SLT2-dependent localization of SBF. In view of this, Jason Moffat and I performed DNA microarray analysis to identify new genes that require both SLT2 and SWI4 for their heat shock induction. To characterize the genome-wide changes in transcription that accompany heat shock, DNA microarrays of the known and predicted genes of Saccharomyces cerevisiae were probed with differentially labeled (Cy3, Cy5) cDNA pools derived from cultures heat shocked for 45 minutes at 39°C.

Transcriptional profiles for swi4Δ and slt2Δ strains were compared with those of a wild-type strain treated identically. For our experiments, we defined genes as dependent on SWI4 and/or SLT2 if expression levels consistently changed more than 2-fold in the mutant strains relative to wild-type in four independent experiments (see Materials and Methods). SWI4 was required for the minimum 2-fold change of more than 300 genes (Figure 4-6B) while SLT2 was required for the minimum 2-fold change of more than 150 genes (Figure 4-6C). Comparison of the swi4Δ versus slt2Δ profile showed that the expression of 28 genes was dependent on both SWI4 and SLT2 following heat shock (Figure 4-6D and Table 1). I believe the DNA microarray analysis is representative of in vivo expression profiles for three reasons. First, as predicted,
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<td>HHF1</td>
<td>YBR009c</td>
<td>S</td>
<td>0</td>
<td>0</td>
<td>ATGCGAAAAA (-375)</td>
</tr>
<tr>
<td>HTB2</td>
<td>YBL002w</td>
<td>S</td>
<td>0</td>
<td>3 (-435, -363, -231)</td>
<td>CTGCGAAAA (-291)</td>
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<tr>
<td>TUP1</td>
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<tr>
<td>YOX1</td>
<td>YML027w</td>
<td>G1</td>
<td>1 (-210c)</td>
<td>3 (-497, -436, -231)</td>
<td></td>
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<td><strong>RNA remodeling genes</strong></td>
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<td>DBP2</td>
<td>YNL112w</td>
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<td>0</td>
<td>ACAAGAAA (-58c)</td>
</tr>
<tr>
<td>SBP1</td>
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<td>NCR</td>
<td>1 (-861c)</td>
<td>1 (-217)</td>
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<td><strong>Mitochondrial and metabolic genes</strong></td>
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<tr>
<td>OXA1</td>
<td>YER154w</td>
<td>NCR</td>
<td>1 (232c)</td>
<td>2 (-546, -262)</td>
<td></td>
</tr>
<tr>
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<td>YPR355w</td>
<td>G1</td>
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<td>NCR</td>
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<td>AACAAGAAA (-125c)</td>
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<td>SRL1*</td>
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<td>G1</td>
<td>2 (-586, -540)</td>
<td>2 (-587, -186)</td>
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<tr>
<td></td>
<td>YOR248c</td>
<td>G1</td>
<td>2 (-923, -877)</td>
<td>2 (-924, -523)</td>
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<tr>
<td><strong>Cyclins</strong></td>
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<tr>
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<td>YDL127w</td>
<td>G1</td>
<td>2 (-393, -370)</td>
<td>3 (-577, -394, -378)</td>
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<tr>
<td>PCLI</td>
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<td>G1</td>
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<td>2 (-865, -313)</td>
<td>CTGCGGAAT (-383)</td>
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<td>ATGCGGAAT (-484)</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>ACGCGGAAA (-219)</td>
</tr>
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</table>

Cell Cycle Expression is determined from (Cho et al., 1998; Spellman et al., 1998); NCR = Not Cell-Cycle Regulated

- *Note YOR247w and YOR248c share the same promoter
- **PCLI was not identified in the DNA microarray analysis
CLN1 and CLN2 expression was unaffected by heat shock in either a \textit{swi4\Delta} or \textit{slt2\Delta} strain (Chapter III and Madden et al., 1997). Second, expression of the Slt2-dependent gene \textit{RLM1} was reduced in a \textit{slt2\Delta} strain (Dodou and Treisman, 1997; Roberts et al., 2000) but was unaffected by deletion of \textit{SWI4}. Finally, expression levels of \textit{PCL2}, \textit{MNN1}, and \textit{FKS1}, three genes whose expression was known to be dependent on both Slt2 and Swi4 (Igual et al., 1996; Madden et al., 1997), were reduced more than 2-fold in expression in both the \textit{swi4\Delta} and \textit{slt2\Delta} strains. Though \textit{PCLI} expression was consistently reduced in both \textit{swi4\Delta} and \textit{slt2\Delta} strains, it was not identified in the microarray experiments because \textit{PCLI} expression was reduced by less than the cut-off of 2-fold. This result suggests that there may be other genes co-regulated by \textit{SWI4} and \textit{SLT2} that our screen has missed. Nonetheless, the microarray experiments identify a group of 28 genes that appear to be coordinately regulated by Slt2 and Swi4 (Table 4-2).

\textbf{Swi4 and Swi6 localize to the promoters of some newly identified Slt2/Swi4-dependent genes.} Several of the Slt2/Swi4-regulated genes that Jason Moffat and I identified in our DNA microarray experiments contain either consensus SCB elements or consensus MCB elements in their promoters (Table 4-2). However, 7 of the 28 genes that were dependent on both Slt2 and Swi4 contained neither consensus SCB nor MCB elements within 1000 base pairs upstream of the presumptive open reading frame (Table 4-2). To test whether some of the new genes that we identified in our microarray experiments were direct targets of SBF, I used my chromatin immunoprecipitation assay to ask whether SBF localized to the promoters of some of the genes. I designed primers to specifically amplify the promoter regions of some the Swi4/Slt2-dependent genes identified in our microarray experiment. I used these primers on the DNA isolated for the ChIP assay shown in Figure 4-5. As expected, neither Swi4 nor Swi6 immunoprecipitated the negative control promoter of \textit{PHOS} or the promoter of \textit{RLM1} (Figure 4-7) whose expression is
Figure 4-7. Swi4 and Swi6 localize to the promoters of Slt2/Swi4-dependent genes.

Wild type (BY263) cultures grown at 30°C (log) or grown at 30°C and heat shocked for 1 hour at 39°C (HS) were cross-linked with formaldehyde. Whole cell extracts (WCE) were made and ChIP was performed using protein A-sepharose alone (pA), Swi4 antibodies or Swi6 antibodies. As indicated PCR was performed on the ChIP samples and on a WCE to amplify the promoter regions of the genes listed to the right of the panel.
Antibody: Swi4 Swi6

Proteins:
- PHO5
- RLM1
- YLR110c
- YNL300w
- YOR247c
- HHF1

Conditions:
- PA
- log
- HS
- log
- HS
- WCE
dependent on the activation of the *PKCl*-pathway and independent of Swi4 (DNA microarray data above and Dodou and Treisman, 1997; Roberts et al., 2000). Likewise, I did not detect localization of SBF to the promoters of *OXAl* or *SBPl* (data not shown). However, I did detect SBF localization to the promoters of *YLR110c*, *YNL300w*, *HHFl* and the shared promoter of *SRL1* and *YOR248c*. Interestingly, as indicated in Table 1, *YLR110c* is not classified as a cell cycle-regulated gene by DNA microarray analysis (Cho et al., 1998; Spellman et al., 1998) and the promoter of *HHFl* does not contain consensus SCB elements. My ChIP experiments suggest that regulation of SBF by Slt2 may allow SBF to regulate non-cell cycle genes. Since I did not detect SBF on all the promoters, I concluded that the role of Slt2 and Swi4 in regulating some of the genes may be indirect.
DISCUSSION

I have used genetic and biochemical approaches along with DNA microarray analysis to describe a group of genes whose expression is dependent on both the cell cycle transcription factor Swi4 and the PKC1-activated MAP kinase Slt2. The analysis shows that genes that are sensitive to both Slt2 and Swi4 are uniquely regulated, which may reflect the ability of Slt2 to modulate the activity of Swi4. This modulation may allow Swi4-dependent transcription in the absence of Swi6. I propose that Slt2-dependent regulation of the G1 transcription factor Swi4 facilitates the utilization of Swi4 in the non-cell cycle role of regulating expression of a group of genes required for cell wall integrity.

Swi6 phosphorylation and the PKC1-pathway.

My studies show that although Swi6 phosphorylation is dramatically increased upon Slt2 activation (Chapter III and Madden et al., 1997), a Swi6 mutant that is not phosphorylated in vivo is capable of suppressing the cell wall integrity defects of a swi6Δ strain (Figure 4-2C). These results suggest that although Swi6 is necessary for proper cell wall maintenance (Igual et al., 1996; Sidorova and Breeden, 1999), phosphorylation of Swi6 by Slt2 may be coincident with Slt2-activation but not necessary for maintenance of cell wall integrity. Similarly, Swi6 phosphorylation upon Rad53 activation is coincident with repression of Swi6-dependent transcription; however it is not known whether the repression is dependent on Swi6 phosphorylation (Sidorova and Breeden, 1997). One possibility is that Swi4 is the relevant biological target of Slt2, and Swi6 becomes phosphorylated due to the proximity of Swi6 to Swi4 (see below for further discussion). Due to the role of Swi6 in regulating the peaks and troughs of cell cycle transcription, most studies to date have focused on the role of Swi6 phosphorylation. However, my results suggest that a renewed focus on the role of Swi4 phosphorylation may be
fruitful. Alternatively, phosphorylation of Swi6 by Slt2 may indeed have a cellular role that has yet to be identified.

**Swi4 and Slt2 regulate the heat shock induction of 28 genes.** In order to understand the transcriptional role of Swi4 and Slt2 in maintaining cell wall integrity, Jason Moffat and I performed DNA microarray analysis to identify genes that require both Slt2 and Swi4 for their expression during heat shock. We identified 28 genes whose transcriptional induction was dependent on both Swi4 and Slt2 (Figure 4-6D and Table 4-2). These genes can be grouped into 5 major classes: 1) cell wall genes or putative cell wall genes; 2) DNA interacting genes; 3) RNA remodeling genes; 4) mitochondrial and metabolic genes; and 5) genes of unknown function.

Previous studies have suggested that the *PKC1* pathway and Swi4 have separable effects on the expression of cell wall biosynthetic genes; it was proposed that Swi4 may regulate only cell cycle expression while the *PKC1* pathway regulated the basal expression and heat shock induction of the cell wall genes (Igual et al., 1996). However, our microarray experiments reveal 10 known or putative cell wall genes whose expression during heat shock was dependent on both Swi4 and Slt2. This group included two cell wall biosynthetic genes, *MNN1* and *FKSI*, previously identified as being co-regulated by Pkc1 and Swi4 (Igual et al., 1997). My work suggests that for at least the *MNN1* and *FKSI* genes the effects of Swi4 and Slt2 are not separable, as Swi4 is required for their heat shock induction. As expected, we also identified 8 other known or putative genes involved in cell wall maintenance. Cell wall defects similar to those seen in Slt2 and Swi4 mutants have been reported for yeast strains mutant for four of the genes we identified in our microarrays: the GDP-mannose pyrophosphorylase-encoding gene *PSAI1* (Gellissen and Hollenberg, 1997), the H+ symport-encoding gene *PMAI1* (McCusker et al., 1987), the aquaporin-encoding gene *AQY1* (Bonhivers et al., 1998) and the covalently linked cell
wall component-encoding gene *YLR110c* (Mrsa et al., 1999). Our array study suggests that these genes are targets of the *PKC1*-pathway. *SCW4* and *SUN4* have been identified as genes encoding soluble cell wall proteins (Cappellaro et al., 1998), while the *YOR383c* and *YNL300w* genes encode putative glycophasphatidylinositol (GPI)-anchor proteins which are predicted to be incorporated into the cell wall (Caro et al., 1997). It will be interesting to determine whether mutations in these putative cell wall genes also cause similar osmotic cell wall defects.

The next major set of genes identified in our microarrays contained genes involved in transcription or chromosome structure, including *HHF1*, *HTB2*, *TUP1*, *ARG80* and *YOXI*. Interestingly, four of these genes have been implicated in the repression of transcription. *HHF1* is required for silencing, and depletion of *HHF1* causes an increase the expression of 15% of all genes, most of which are located within 20kb of the telomeres (Wyrick et al., 1999). In fact, a large subset of induced genes were members of the COS family of subtelomerically-encoded proteins. *HTB2* can also repress transcription when directed to DNA, but its role in repression is not fully characterized (Recht et al., 1996). *TUP1* represses the transcription of a wide variety of genes including hexose transporters, alpha-glucosidases, mating-specific genes, DNA damage-inducible genes and genes involved in flocculation (DeRisi et al., 1997). *ARG80* encodes a MADS-box-containing protein with high similarity to Mcm1 and Rlm1 and has been implicated in the repression of at least 6 genes necessary for anabolic arginine biosynthesis. (Qiu et al., 1990). The Slt2/Swi4-dependent induction of these genes upon heat shock suggests that maintenance of cell wall integrity upon heat shock may require the repression of numerous genes. Though we identified 4 genes involved in repression of transcription through our DNA microarray analysis, we did not identify many genes whose expression was induced in both the *swi4Δ* and *slt2Δ* strain. We specifically chose to look at mRNA levels after 45 minutes of heat
shock to attempt to identify genes whose transcription was directly dependent on Slt2 and Swi4; therefore, it is likely this time period is too short to see effects of compromised repression.

Interestingly, we also identified numerous genes whose roles in cell wall maintenance remains a mystery. These include three genes which encode proteins that have been implicated in RNA processing, *DBP2*, *SBP1* and *YNR051c*, a gene encoding a transketolase which is needed for the biosynthesis of aromatic amino acids, *TKL1*, and a mitochondrial gene *OXAI*. We also identified several genes whose cellular role is undetermined. Roles for these genes in maintaining the integrity of the cell wall need to be explored.

Role of *PKC1*-pathway and SBF in pheromone response. In addition to regulating a subset of Swi4 targets during mitotic growth, my work suggests that Slt2 may also partner with SBF to regulate the pheromone-dependent induction of gene expression. DNA microarray analyses have examined genome-wide patterns of gene expression in response to pheromone treatment (Roberts et al., 2000). Interestingly, pheromone treatment induces several transcriptional responses; including the expression of early pheromone-induced genes dependent on the transcription factor Ste12 and the expression of late pheromone-induced genes dependent on *PKC1*. The late-induced genes are not enriched for 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. Interestingly, *PCL2* was identified as an early pheromone-induced gene, whose expression was dependent on *STE12*. My 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 the promoter (Figure 4-3). 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, I found that there was an enrichment of SBF on the promoter of PCL1 upon α-factor treatment (Figure 4-4). As described earlier, SBF localization to G1-specific promoters in early G1 phase is necessary but not sufficient for transcription activation (see Chapter I, section IV A and Cosma et al., 1999; Harrington and Andrews, 1996; Koch et al., 1996). My 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. Slt2 has some transactivation activity (Costigan et al., 1994; Soler et al., 1995); so far, 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 with the ChIP assays. Alternatively, SBF activation in response to Slt2 may require other proteins.

Sl2-dependent regulation of Swi4 activity. Through DNA microarray analysis, 28 genes were identified whose transcription was dependent on both Slt2 and Swi4. Interestingly, though these genes are dependent on Swi4, many are not cell-cycle regulated in late G1 or S phase when SBF-dependent transcription normally occurs, nor do all the promoters of Slt2/Swi4-dependent genes contain consensus SCB elements (Table 4-2). However, my ChIP experiments showed that Swi4 can localize to the promoters of many of these genes, including genes whose promoters are devoid of consensus SCB elements and genes whose expression is not induced in late G1 or S phase. Together, the DNA microarray analysis and ChIP experiments provide striking evidence
that the function of Swi4 is not limited to the cell cycle. I propose that Slt2-dependent regulation of Swi4 facilitate the utilization of Swi4 outside of the cell cycle.

Using a \textit{PCL1::lacZ} reporter gene, we found that the heat shock induction of \textit{PCL1} expression was dependent on Swi4 and Slt2 and independent of Swi6. The basal levels of \textit{PCL1} were also not affected in a \textit{swi6Δ}. The Swi6-independence was surprising since both Swi6 and Swi4 are required for the cell cycle-regulated expression of SBF target genes at Start (Breeden, 1996; Dirick et al., 1992; Lowndes et al., 1992). Swi6 regulates Swi4 DNA-binding (Baetz and Andrews, 1999; Harrington and Andrews, 1996; Koch et al., 1996) and also contains two trans-activating regions necessary for cell cycle-dependent transcription (Sedgwick et al., 1998). While a definitive test of the requirement of Swi6 for the cell cycle expression of \textit{PCL1} has not been done, my cell-cycle ChIP experiments show that Swi6 is localized to the promoter of \textit{PCL1} at the peak of G\textsubscript{1} transcription (Figure 4-4). The 3’ SCB-containing region of the \textit{PCL1} promoter was dependent on both Swi4 and Swi6, comparable to other SBF-dependent genes (Andrews and Herskowitz, 1989; Breeden and Nasmyth, 1987; Nasmyth and Dirick, 1991). I propose that the cell cycle regulation of \textit{PCL1} may require both Swi6 and Swi4 and is mediated through the 3’ SCB-containing region of the \textit{PCL1} promoter. In contrast, the heat shock induction of \textit{PCL1} requires Swi4 and Slt2 but not Swi6 and may be mediated by the more 5’ upstream sequences that is devoid of consensus SCBs. If the heat shock expression of the newly identified Slt2/Swi4-dependent genes is also Swi6-independent (see Chapter V, section II), heat shock activation of Slt2 may modulate the activity of Swi4 to function in the absence of Swi6.

I envision two models to explain the apparent Swi6-independent regulatory properties of Swi4. First, both \textit{in vitro} and \textit{in vivo} experiments show that Swi4 has no intrinsic ability to bind 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 (Chapter II, Baetz and Andrews, 1999). A simple model to explain the Swi6-independence of Swi4 is that phosphorylation of Swi4 by Slt2 may relieve the intramolecular interactions that prevent Swi4 from binding DNA in the absence of Swi6 (Chapter II, Baetz and Andrews, 1999). Consistent with this model, co-immunoprecipitation experiments and kinase assays show that Slt2 can interact with and phosphorylate both Swi4 and Swi6 in vitro (Chapter III, Madden et al., 1997). Phosphorylation of the Ets-1 transcription factor can modulate its DNA-binding inhibition (Cowley and Graves, 2000); likewise, phosphorylation of Swi4 might affect its auto-inhibition of DNA-binding or the sequence specificity of DNA-binding.

Second, Slt2 may regulate the interaction of Swi4 with another transcription factor, which can alleviate the auto-inhibition of Swi4 DNA-binding (Baetz and Andrews, 1999) 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 (Bouquin et al., 1999). Likewise, Swi4 may also have alternative partners that regulate the binding of Swi4 to DNA.

MAP kinases have been implicated in the regulation of nucleosomal and chromatin remodeling regulation (reviewed in Belotserkovskaya and Berger, 1999; Thomson et al., 1999). Recent dissection of the SBF-dependent gene HO has shown that directed chromatin remodeling of the locus is required before Swi4 can bind the promoter (Cosma et al., 1999; Krebs et al., 1999). This work suggests that the promoters of other Swi4-dependent genes may also have to be remodeled 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, my ChIP experiments indicate that Slt2 is partially responsible for localizing Swi4 and Swi6 to the promoters of \textit{PCL1} and \textit{PCL2} upon heat shock and \(\alpha\)-factor treatment.

If the role of Slt2 is to specifically modulate the chromatin structure of only Slt2/Swi4-dependent genes, the DNA sequence specificity of Swi4 might not have to be modulated. Consistent with this idea, promoter analysis of the newly identified subset of Slt2/Swi4-dependent genes did not reveal any conserved motifs that are unique to this subgroup of genes. Instead, many degenerate SCB-like elements were found in these promoters (Table 4-2). Though detailed analysis of Swi4 DNA sequence specificity has yet to be conducted, Swi4 may indeed bind these SCB-like sequences; SBF binding is not limited to consensus SCB elements (Cross et al., 1994; Partridge et al., 1997). Further, it has recently been proposed that SBF may bind the degenerate SCB consensus of ACRMSAAA (where R is A or G, M is A or C, and S is C or G) found in the promoters of G1-genes (Spellman et al., 1998). This might explain why we were unable to identify any difference between the SCB motifs of the G1-genes and the subset of Slt2/Swi4-dependent genes. Presently, only three \textit{in vivo} targets of Slt2 have been identified; a more complete view of \textit{in vivo} substrates of Slt2 will be required to better define the dual role of Slt2 and SBF in activating gene transcription (see Chapter V, section III).
CHAPTER V

Thesis Summary and
Future Directions
I Thesis Summary

In all eukaryotic cells, the commitment to enter the cell cycle at the "Restriction Point" or "Start" in late G1 is a highly regulated event characterized by a burst of gene transcription. In budding yeast, the heterodimeric transcription factor SBF is required for the induction of a large group of genes at Start. Once SBF is activated in late G1, the program of gene expression and other events that ensue drive cells irreversibly into the cell cycle. As important as SBF is in cell cycle regulation, SBF is also utilized outside of the cell cycle to evoke numerous transcriptional responses necessary to maintain cell integrity during meiosis, DNA damage and cell wall stresses. Understandably, budding yeast have evolved a complex set of mechanisms that regulate and modulate the activity of SBF. The focus of my thesis was to elucidate novel mechanisms that regulate the transcription factor SBF.

SBF binding to DNA is a highly regulated event (see Chapter I, section IV A). Both in vivo footprinting and ChIP experiments (Cosma et al., 1999; Harrington and Andrews, 1996; Koch et al., 1996) showed that Swi4 binding to SCB DNA elements is restricted to only late M and G1 phases, coincident with Swi6 localization in the nucleus (Sidorova et al., 1995). Further, in vivo footprinting on SCBs does not occur in the absence of Swi6 (Harrington and Andrews, 1996; Koch et al., 1996). These results led to the hypothesis that Swi4 is inhibited from binding DNA in the absence of Swi6 protein. However, the hypothesis remained untested due largely to a lack of appropriate Swi4 reagents. Therefore, as described in Chapter II, I embarked on expressing Swi4 in insect cells using the baculovirus system. I expressed and partially purified both Swi4 and Swi6 from insect cells, which provided me with a source of SBF from a heterologous system. The SBF produced in insect cells was competent to bind SCB-containing DNA, allowing me to perform a series of DNA-binding experiments. I determined that Swi4
could not bind SCBs in the absence of Swi6. This inhibition was intrinsic to Swi4 and mediated through the C-terminal region of Swi4. Further, through a series of biochemical and genetic experiments, I found that the C-terminal region of Swi4 could interact in vitro with the N-terminal DNA-binding domain of Swi4. My data suggest that intramolecular interactions with the C-terminal region of Swi4 physically prevent the DNA-binding domain from binding SCBs. Upon the addition of Swi6, the interaction of the CTR of Swi4 with Swi6 alleviates this inhibition, allowing Swi4 to bind DNA. The intrinsic inhibition of Swi4 DNA-binding may be an important mechanism regulating SBF activity because, in contrast to Swi6, Swi4 remains nuclear throughout the cell cycle.

SBF binding to SCBs is not sufficient for SBF-dependent transcription. Instead, an activation event must occur. As outlined in Chapter I (section IV B), during a normal cell cycle, the Cln3-Cdc28 Cdk complex is necessary for SBF activation. However, in the absence of Cln3, SBF-dependent transcription still occurs, which has led to the hypothesis that other cellular pathways must activate SBF: one such pathway is the PKC1-MAPK pathway. In Chapter II, I present the results of a collaborative study with Kevin Madden and Mike Snyder (Yale University) that provides direct evidence linking the PKC1-MAPK pathway with the G1 transcription factor SBF. We showed that Swi4 is directly downstream of Slt2 and that Slt2 physically interacts with and phosphorylates both Swi4 and Swi6. Further, I found that activation of Slt2 by heat shock leads to the transcriptional induction of the SBF-dependent genes PCL1 and PCL2 but not CLN1 and CLN2.

The work presented in Chapter III suggests that Slt2 can modulate the activity of SBF, allowing SBF to function outside of the cell cycle to transduce part of the PKC1-MAPK pathway transcriptional response. In Chapter IV, I present a series of experiments aimed at defining the
regulatory pathway involving the cell cycle transcription factor SBF and Slt2. I began by exploring the transcriptional regulation of the SBF and Slt2-dependent genes PCL1 and PCL2. Through a series of ChIP experiments, I found that upon activation of the PKC1-MAPK pathway by either heat shock or pheromone treatment, SBF localization to the promoters of PCL1 and PCL2 increases and that this localization is partially dependent on Slt2. Further, in collaboration with Jason Moffat, I performed a series of DNA microarray experiments that identified 28 genes whose expression was dependent on both Swi4 and Slt2. I determined that SBF can localize to the promoters of some of these genes, even genes that are not cell cycle regulated or genes that do not contain consensus SCB elements in their promoters. This work suggests that the PKC1-MAPK pathway may utilize Swi4 outside of the cell cycle to elicit part of its transcriptional response necessary for cell integrity. This work also provides preliminary data suggesting that the heat shock induction of Slt2/Swi4-dependent genes does not require factor Swi6 (see next section).

II. Future Directions

Is Swi6 necessary for the heat shock induction of Slt2/Swi4-dependent genes?

In Chapter IV, I began to explore the regulation of PCL1 heat shock expression by using a series of PCL1 reporter gene constructs. Surprisingly, the promoter sequences spanning from −751 to −146 from the transcriptional start site of PCL1 were dependent on Swi4 and Slt2 for their heat shock induction, but independent of Swi6. As discussed previously, the Swi6-independence is a unique behavior for a Swi4-dependent gene and no other SCB-containing reporter construct has displayed Swi6 independence (Andrews and Herskowitz, 1989; Breeden and Nasmyth, 1987; Nasmyth and Dirick, 1991). However, this unique behavior appears to require a region of the promoter that does not contain consensus SCB elements. One intriguing possibility is that Swi6
is not necessary for the heat shock induction of PCL1 or the newly identified Slt2/Swi4-dependent genes. Indeed, this may explain why swi4Δ strains, but not swi6Δ strains, are temperature-sensitive for growth at 37°C (Gray et al., 1997; Igual et al., 1996; Madden et al., 1997), and why overexpression of SWI4 but not SWI6 can rescue the temperature sensitivity of PKCI-MAPK pathway mutants (Igual et al., 1996; Madden et al., 1997).

Although Swi6 does not appear to be required to mediate the heat shock response of the PKCI-MAPK pathway, Swi6 does appear to be required for maintenance of cell wall integrity. Both swi6Δ and swi4Δ strains are sensitive to SDS, CFW (Figure 4-2 and Igual et al., 1996; Madden et al., 1997) and mating pheromone (personal communication with M.Chang). Swi6 is also required for the full α-factor induction of the PCL2 reporter gene (Figure 4-3). These results suggest that Swi6 is required for mediating the response of the PKCI-MAPK pathway. Since overexpression of MBPI, the other known DNA-binding partner of Swi6, does not rescue PKCI-MAPK pathway mutants (Igual et al., 1996; Madden et al., 1997) it is likely the cell wall defects of Swi6 mutants are due to inappropriate regulation of Swi4-dependent transcription. However, one can not exclude the possibility that other transcription factors are regulated by Swi6. In this section, I propose a series of experiments to clarify the role of Swi6 in the Slt2/Swi4-dependent heat shock induction of PCL1 and the newly identified genes (Table 4-2).

I propose to perform a simple series of experiments to explore the requirement for Swi6 in the transcriptional response of the PKCI-MAPK pathway to heat shock. I will use Northern blot analysis to assay the mRNA levels of PCL2 and some of the newly identified Slt2/Swi4-dependent genes, including YLR110c, YNL300w, YOR247c/SRL1 and HHFI, before and after heat shock in wild type, swi4Δ, slt2Δ and swi6Δ strains. I anticipate that Northern analysis will confirm, that like PCL1, Swi6 is not required for the expression of the newly identified genes.
Slt2/Swi4-dependent genes. Similarly, I propose to use ChIP experiments to ask whether Swi4 can still localize to the promoters of Slt2/Swi4-dependent genes upon heat shock in a swi6Δ strain. It may also be informative to repeat the DNA microarray experiments using a swi6Δ strain to determine a) whether Swi6 is required for heat shock induction of genes and b) whether the subset of Swi6-dependent genes identified is identical or different then the subset of the genes regulated by Slt2 and Swi4.

If the heat shock induction of Slt2/Swi4-dependent genes does not require Swi6, and Swi4 can localize to the promoters of these genes (Chapter IV), it would be interesting to investigate the mechanism of Swi4 DNA-binding to these promoters. With the Swi4 reagents I have generated and the in vitro techniques describe in Chapter II, along with functional, abundant and easily purified HA-Slt2 kinase from yeast cells, many questions regarding the mechanism of Swi4-DNA-binding may be explored. Are there unique sequences in the 5' region of the PCL1 promoter (SCB-less, Swi4-Slt2-dependent heat shock induction) that allow Swi4 to bind DNA in the absence of Swi6? Does phosphorylation of Swi4 by Slt2 change the DNA-binding activity of Swi4? Also, using crude yeast extracts it will be possible to determine whether any Slt2-dependent, Swi4-containing complexes form on the 5' region of the PCL1 promoter and, if so, whether Swi4 requires another protein to bind DNA in the absence of Swi6.

Is the Slt2/Swi4-dependent transcriptional response different for different PKC1-MAPK pathway activators?

As discussed in Chapter I (section VIII and IX), the PKC1-MAPK pathway is activated by multiple stimuli, including heat stress, cell wall stability challenges (SDS, CFW), hypotonic stress, cell cycle progression and mating pheromone activation. PKC1-MAPK pathway mutants have cell wall defects that make them sensitive to heat shock, SDS, CFW, hypotonic shock and
mating pheromone treatment. These mutants also have bud morphology defects associated with polarized growth and passage through the cell cycle. As described previously, swi4 and swi6 mutants also have cell wall defects and share similar phenotypes with PKC1-MAPK mutants. In Chapter III, I presented a series of experiments that directly linked SBF to Slt2. In Chapter IV, I presented a series of experiments showing that, upon heat shock, Swi4 and Slt2 co-regulate a subset of genes. Together, this work suggests that activation of the PKC1-MAPK pathway results in a Swi4-dependent transcriptional response. However, it is not yet established whether a) each PKC1-MAPK pathway activator elicits a transcriptional response, b) whether various activators induce the same set of genes or whether each activator induces a separate subset of genes or c) whether Swi4 and/or Swi6 is required for all PKC1-MAPK pathway transcriptional responses to every PKC1 activator.

To answer these questions, I propose to perform a series of DNA microarray experiments similar to those performed in Chapter IV. I will determine the genes that require Slt2 and/or Swi4 for their transcriptional response after treatment with pheromone, SDS, CFW and hypotonic shock. The Slt2/Swi4-dependent heat shock genes that were identified in Chapter IV do not overlap with the late-induced pheromone-responsive genes that are predicted to be PKC1-dependent (Roberts et al., 2000). Further, in contrast to heat shock conditions in which Slt2 is required for the induction of both PCL1 and PCL2 (Chapter III), Slt2 is only required for full α-factor induction of PCL2 (Chapter IV). These results suggest that different activators of PKC1 will elicit different transcriptional responses. The shared sensitivity of swi4, swi6 and slt2 mutants to SDS and CFW suggests that both Swi4 and Swi6 will be required for the transcriptional response to these activators of PKC1. Presently, it is not known whether swi4 or swi6 mutants are sensitive to hypotonic shock. Therefore, I propose to determine the hypotonic
sensitivity of swi4 and swi6 mutants. Further, I also propose to determine whether overexpression of SWI4 can rescue the slt2Δ sensitivity to SDS, CFW and hypotonic stress, which would indicate that SBF functions in all of these pathways.

The experiments I propose may reveal that Swi4, plus or minus Swi6, is required for the transcriptional response of each activator of PKCI, and that each stimulus results in a different transcriptional profile. In this case, the following question will arise: How are the activities of Slt2 and SBF modified by different stimuli? I determined that, upon α-factor treatment, SBF localization to the promoters of PCL1 and PCL2 was drastically increased (see Chapter IV). However, only PCL2 gene expression was induced upon α-factor treatment. One intriguing possibility is that Slt2 may not be modifying the activity of SBF, but may be regulating the access of SBF to Slt2-dependent promoters. Following SBF localization, a secondary activating event may then occur at only the genes which are required to respond to the stimulus (see Figure V-1). I propose to test this hypothesis using the ChIP assay. In Chapter IV, I showed that Swi4 is required for the transcription of YLR110c, YNL300w, YOR247c and HHF1 and that SBF localizes to their promoters. By microarray analysis, none of these genes appears to be induced by α-factor (Roberts et al., 2000; Spellman et al., 1998). I propose to determine whether, like PCL1, SBF localizes to the promoters of these genes in a Slt2-dependent manner upon α-factor treatment. A similar analysis could be conducted once/if activator-specific Slt2/Swi4-dependent genes are identified (see above). If Slt2 is regulating the access of SBF to promoters, the role of Slt2 may be to specifically modulate the chromatin structure of only Slt2/Swi4-dependent genes. Indeed, MAP kinases have been implicated in the regulation of chromatin remodeling (reviewed in Belotserkovskaya and Berger, 1999; Thomson et al., 1999). Therefore, to fully understand the Slt2-dependent mechanism of SBF activation, it will be important to determine the chromatin
Figure 5-1 A combinatorial model for the regulation of Slt2/Swi4-dependent transcription

This is a schematic diagram of one possible mechanism by which Slt2 might localize SBF to the promoters of Swi4/Slt2-dependent genes and activate certain genes in response to specific stimuli. Upon activation of Slt2, Slt2 is required for the nucleosomal remodeling on all Slt2/Swi4-dependent promoters. The remodeling allows for Swi4 to localize to all Swi4/Slt2-dependent promoters. However, to differentiate the SBF that is bound to all Swi4/Slt2-dependent genes from the SBF bound to promoters that are required for the heat shock response, a secondary event must occur. Here the red box represents an unknown protein(s) that specifically recognizes and activates only the SBF that is bound to the promoters of genes required for the PKCl-heat shock response.
Heat Shock

Chromatin Remodeling

Localization of SBF

Activation of a subset of SBF bound genes

Heat Shock Induced Swi4/Slt2-Dependent Genes

Pheromone Induced Swi4/Slt2-Dependent Genes

Heat Shock
remodeling complexes involved and the mechanism by which Slt2 directs or regulates their nucleosome remodeling activity.

**SBF and chromatin remodeling.**

*S. cerevisiae* contains numerous characterized and putative chromatin remodeling complexes/proteins (for review see Perez-Martin, 1999). Interestingly, the roles and biological targets of these complexes are largely unknown. The large number of chromatin remodeling proteins suggests that the budding yeast cell may have evolved to use different chromatin remodeling complexes as a mechanism to regulate subsets of genes. As discussed in Chapter I (section IV A), SWI/SNF- and SAGA-dependent chromatin remodeling must occur on the *HO* promoter in order for SBF to bind. This result suggests that chromatin remodeling may be a key regulatory feature of SBF-dependent transcription. Presently, it is not known whether SWI/SNF or SAGA is required to regulate the cell cycle-dependent expression of all SBF *G1* genes. I have accumulated evidence that one chromatin remodeling protein which may have a role in SBF-regulated gene transcription is Isw1.

*Isw1* encodes an ATP-dependent chromatin remodeling factor that is homologous to the *Drosophila* nucleosome remodeling factor *dIsw1* (Tsukiyma et al., 1999). In *Drosophila*, Isw1 is the common catalytic subunit of three distinct nucleosome-remodeling complexes, each with different biochemical remodeling properties (reviewed in Guschin and Wolffe, 1999). The *in vivo* roles and targets of these complexes are not yet known. In contrast to *Drosophila*, *S. cerevisiae* Isw1 is only found in one protein complex. Like dIsw1, the biological roles of the yeast Isw1 complex remain unknown. Isw1 is highly homologous to two other yeast proteins, Isw2 and Chd1. Interestingly, though single null mutants and all combinations of double null
mutants display no detectable mutant phenotypes, the triple mutant is temperature-sensitive (Tsukiyama et al., 1999), and significantly larger than wild type cells (data not shown).

In affinity chromatography experiments using the DNA-binding domain of Swi4 as ligand (Swi4-DNAB), I identified Isw1 as specifically binding the column. Further, I found that the interaction between the Swi4 DNA-binding domain and Isw1 from crude yeast extracts does not require DNA. However, purified Isw1 (gift from T. Tsukiyama) does not interact with Swi4, suggesting that other, yet unidentified proteins may mediate the interaction. It will be interesting to determine the biological significance of this interaction. One possibility is that Isw1 may be required for proper activation or repression of SBF-G1 genes. Alternatively, Isw1 may be involved in the regulation of SBF in the PKCl-MAPK pathway or in other roles of SBF outside of the cell cycle. To ask whether Isw1 is involved in the transcriptional regulation of SBF-dependent G1-genes, I propose to do a series of cell cycle Northern blots to compare the cell cycle-regulated transcription of SBF-and MBF-dependent genes in wild type and isw1Δ strains. The synthetic genetic interaction seen in the isw1isw2chd1 strain suggests that these genes may have redundant functions in the cell. Therefore, the analysis of cell cycle transcription should also be conducted in double mutant strains and the triple mutant deleted for ISWI, ISW2 and CHD1. Preliminary results suggest that the Isw1 family of genes is not required for the timing or magnitude of SBF-dependent G1-transcription, but rather for the timing of repression. In a isw1Δisw2Δ strain I saw a ten minute delay in repression of PCL1 and CLB5 but not RNR1. This preliminary result, along with the interaction of Isw1 with Swi4, strongly suggests that the ISW-family of proteins may be another key in the multi-faceted regulation of SBF transcription at Start. However, further characterization must be performed. It will be interesting to determine whether the ISWI-family of proteins is involved in the regulation of Slt2/Swi4-dependent gene
transcription. A detailed analysis of the role of chromatin remodeling complexes must be performed in order to complete our knowledge of SBF regulation.
REFERENCES


