PROTEINS PHYSICALLY INTERACTING WITH THE Swi6
CELL CYCLE REGULATORY TRANSCRIPTION FACTOR

by

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A thesis submitted in conformity with the requirements for
the degree of Doctor of Philosophy,
Graduate Department of Molecular and Medical Genetics
University of Toronto

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ABSTRACT

Entry into the mitotic cell cycle is a highly regulated event which integrates both extra- and intracellular signal inputs. In the budding yeast, *Saccharomyces cerevisiae*, entry and commitment into the mitotic cell cycle is marked by the Start transition point in late G1. It is likely that entry into the cell cycle at Start is catalyzed by a subset of genes that are transcriptionally induced at the Start transition. In budding yeast, two transcription factor complexes are known to regulate transcription at Start: the SBF and MBF complexes. SBF and MBF are heterodimers that respectively contain the Swi4 and Mbp1 DNA binding subunits. Swi6 is a common subunit in both complexes and likely plays a regulatory role. In this thesis, I describe my approach to studying the regulation of transcription at Start through studying the Swi6 subunit. The presence of protein-protein interaction motifs in Swi6, with no attributable function, prompted me to study

In Chapter 2, I describe the interaction between the Hrr25 and Swi6 proteins. I show that this interaction may reflect a role for Hrr25 in phosphorylating Swi6. I describe experiments which suggest that Hrr25 may regulate Swi6 to induce repair genes in response to DNA damage.

In Chapter 3, I describe the interaction between Stb1 and Swi6. I show evidence to suggest that this interaction reflects a role for STB1 in transcriptional induction at Start. Stb1 is a substrate for the Cdk, Cdc28, associated with the Cln G1 cyclin regulatory subunits. I suggest that phosphorylation of Stb1 by Cln-associated kinases activates STB1-dependent transcription at Start.
ACKNOWLEDGEMENTS

This thesis is dedicated to Brenda, who stuck with the project the numerous times it had been abandoned, and who stuck with me when it counted most.

Thank you to my parents and grandparents who cared far too much than I deserved.

Thank you to my friends, who made the past five and four-sixths years in grad school by far the richest years in my life (sic). Special thanks to Jeff and Ian for feeding and looking after me during rough times.

And thank you, Jen, for making me happy.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>Δ</td>
<td>deletion (i.e. symbol for gene deletion)</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 pair(s)</td>
</tr>
<tr>
<td>BCK</td>
<td>bypass of C-kinase mutation</td>
</tr>
<tr>
<td>CAK</td>
<td>Cdk-activating kinase</td>
</tr>
<tr>
<td>CDC (cdc)</td>
<td>cell division cycle</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>Cdk</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CTD</td>
<td>carboxyl terminal domain</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′, 6-diamidino-2′-phenylindole</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ECB</td>
<td>early cell cycle box</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HRR</td>
<td>HO, radiation resistance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropylthio-β-D-galactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pair(s)</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen-activated protein (kinase)</td>
</tr>
<tr>
<td>MBF</td>
<td>MCB-binding factor</td>
</tr>
<tr>
<td>MCB</td>
<td>MluI cell cycle box</td>
</tr>
<tr>
<td>min.</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MPF</td>
<td>maturation promoting factor</td>
</tr>
<tr>
<td>ORC</td>
<td>origin recognition complex</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>SBF</td>
<td>SCB-binding factor</td>
</tr>
<tr>
<td>SCB</td>
<td>Swi4/6 cell cycle box</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>S. pombe</td>
<td>Schizosaccharomyces pombe</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>STB</td>
<td>Sin3 binding protein</td>
</tr>
<tr>
<td>SWT</td>
<td>switching gene</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
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</table>
ts  temperature-sensitive
Wt  wild type
CHAPTER I

INTRODUCTION
The budding yeast, *Saccharomyces cerevisiae*, is used as a model eukaryotic organism due mainly to its accessibility for genetic analysis. Its short life cycle and ability to reproduce as either a haploid or diploid cell make it a powerful genetic tool. Although budding yeast is a unicellular fungus, it nonetheless possesses key characteristics of higher eukaryotic cells, such as nuclei and other membrane-bound organelles, introns, centromeres and telomeres. These features make yeast a useful model for studying most basic biological processes.

This thesis focuses on aspects of cell cycle regulation in *S. cerevisiae*. The budding morphology of budding yeast makes it an attractive system to study the regulation of the cell cycle. The budding yeast life cycle (Figure 1-1) begins after mitosis with an unbudded cell. At the time of DNA synthesis, a small exvagination of the membrane begins and gradually grows in size. This so-called bud separates from the 'mother' cell after mitosis. One advantage of the budding cycle is that bud morphology and size act as markers for cell cycle position. In fact, the budding morphology of *S. cerevisiae* was used to isolate a number of *cdc* (cell division cycle) mutants which showed a uniform cell cycle arrest at the non-permissive temperature (Hartwell, 1971; Hartwell et al., 1970; Hartwell et al., 1974). Subsequent characterization of this collection of *cdc* mutants has laid the foundation for the study of cell cycle control in budding yeast and other organisms.

As I discuss later, the mechanisms governing cell cycle progression are highly conserved between yeast and human cells. Perhaps the most striking example of
functional conservation was the identification of the genes encoding two major human cell cycle regulators by the complementation of fission and budding yeast mutants (Elledge et al., 1992; Lee and Nurse, 1987). The conservation between genes encoding yeast and human cell cycle regulators supports the use of budding yeast as a model system for understanding cell cycle control in human cells. Since cell cycle control mechanisms are often perturbed in human cancers (Harper and Elledge, 1996; Hartwell and Kastan, 1994), yeast may prove to be a useful model for studying the molecular mechanisms contributing to the development of human cancers.

1. OVERVIEW OF THE S. cerevisiae CELL CYCLE

In most eukaryotic cell cycles, the two major events, S-phase (marked by chromosome replication) and mitosis (M-phase) are separated by two gap phases, G1 and G2 (Figure 1-1). In budding yeast, G1 is the phase of the cell cycle during which small cells accumulate cell mass. During G2 phase in yeast and most eukaryotic cells, regulatory events occur that prepare the cell for chromosome segregation and/or cytokinesis.

A major regulatory event in G1 phase of the yeast cell cycle is the ‘Start’ transition which is analogous to the ‘restriction point’ in mammalian cells (Hartwell et al., 1974; Pardee, 1989). In both cases, it is a point in the cell cycle where the cell commits itself to completion of the mitotic cell cycle and becomes insensitive to any anti-mitogenic signals—at least until completion of the present cell cycle. In early G1, conditions such as the presence of an anti-mitogenic hormone or the absence of nutrients
Figure 1-1: Schematic Overview of the *Saccharomyces cerevisiae* Mitotic Cell Cycle.

The two major cellular events in the cell cycle, DNA synthesis (S phase) and mitosis (M phase), are separated by two gap phases, G1 and G2. In G1, yeast cells accumulate cell mass. Entry into the mitotic cycle occurs in late G1 at Start. Three cellular processes mark passage through Start: DNA replication, bud formation and spindle pole body duplication. The execution of these three events is a result of the transcriptional activation of a subset of genes at Start that catalyze these processes.
or serum can cause cells to exit the mitotic cycle. However, cells that have passed Start or the restriction point are committed to continue and complete the cell cycle before they can exit the cell cycle or differentiate.

As shown in Figure 1-1, budding yeast are ‘born’ as small, round cells. These small cells accumulate mass until they reach a critical cell size, at which the Start transition occurs (the general biology of the budding yeast cell cycle is reviewed in Lew, 1997). The molecular basis for Start is further discussed in section 2c and in Chapter 3. Following Start, the cell forms a membrane exvagination called a bud. The bud is the site of polarized secretion, causing the bud or ‘daughter cell’ to grow in size as the cell approaches mitosis. The daughter cell bud then separates from its mother cell during cytokinesis.

The spindle pole bodies formed after Start act as the microtubule organizing centres for mitotic spindle assembly. Unlike other eukaryotic cells which begin spindle assembly in late G2/mitosis, budding yeasts begin spindle assemble shortly after Start. The mitotic spindles form in G2 and elongate during mitosis to segregate the chromosomes.

A characteristic peculiar to budding yeast is that they do not undergo nuclear breakdown during mitosis. Rather, the nucleus remains intact and is dragged into the daughter cell during chromosome segregation. Nuclear division occurs just before cytokinesis. The end result is a large mother cell and a smaller daughter cell. In the subsequent cell cycle, the smaller daughter cell takes longer than the ‘mother’ cell to acquire the necessary cell size to enter Start.
2. OVERVIEW OF THE MOLECULAR MODEL FOR 'Start'

The current model for the molecular basis of Start centres upon the activation of a subset of genes whose products catalyze post-Start events. Once these gene products are produced, they begin a catalytic program that irreversibly commits the cell to the mitotic cycle. Figure-1-4 shows a schematic model for Start. Before I discuss this model in detail, I will describe the key regulators of Start and other eukaryotic cell cycle transitions, namely, cyclins and cyclin-dependent kinases (Cdk).

2a. Cyclins and Cyclin-Dependent Kinases (Cdk)

A conserved regulator of cell cycle progression in all eukaryotic cells is the cyclin-Cdk (cyclin-dependent kinase) complex. The Cdk acts as the catalytic kinase subunit, whereas the cyclin is believed to target the Cdk to specific substrates during the cell cycle. Cyclin binding is also required for Cdk kinase activity. As will be described below, both the cyclin and the Cdk are subject to multiple regulatory signals.

2a. i. The Cdk subunit

Numerous Cdks have been discovered in a variety of eukaryotic cells; there are five in budding yeast and at least nine in humans (Morgan, 1997). Most of the Cdks in human cells have roles in cell cycle regulation while in budding yeast, CDC28 is the major Cdk responsible for cell cycle progression. Another budding yeast Cdk, PHO85, has been implicated in cell cycle progression through regulation of the actin cytoskeleton, but the details have not been worked out (Lee et al., 1998). In addition to cell cycle regulation, Cdks have been implicated in general transcriptional regulation and metabolic control (reviewed in Huang et al., 1998; Morgan, 1997; Toh-e et al., 1988).
**CDC28** is homologous to Cdc2 in higher eukaryotes and cdc2+ in fission yeast. Whereas human Cdc2 has only been implicated in functioning at mitosis, budding yeast **CDC28**, like cdc2+ in fission yeast, catalyzes mitosis, Start and other cell cycle transitions. Indeed, mutant alleles of **CDC28** have been isolated that arrest cells in either G1 or G2 phase (Hartwell et al., 1974; Piggott et al., 1982). The function of **CDC28** during different phases of the cell cycle is determined by its cyclin subunit (Figure 1-2). The numerous Cdk species in higher eukaryotes may reflect the divergence of a fundamental Cdk into numerous isoforms to facilitate the intricate regulation of the cell cycle in multicellular eukaryotes.

2a. ii. The cyclin subunit

Cyclins serve as activating subunits for Cdns and are required for Cdk kinase activity. Cyclins derive their name historically from the circumstance of their discovery; they were originally observed in sea urchin embryos as proteins whose concentrations fluctuated dramatically throughout the cell cycle (Hunt, 1991). Subsequent work on meiotic maturation in frog oocytes showed that cyclins are the regulatory subunits of cyclin-Cdk complexes (Lohka et al., 1988). Since then, numerous cyclin genes have been identified in yeasts, fruit flies, humans and other mammals. Cyclins share similarity within a 100 amino acid stretch called the 'cyclin box' (Morgan, 1997). Some cyclins have been identified based on the two criteria of being a Cdk subunit and having significant homology across the cyclin box, but do not meet the classical criterion of fluctuating in abundance during the cell cycle (Measday et al., 1997; Tyers et al., 1992). The p35 'cyclin' has no cyclin box homology, and its levels are constant in non-cycling neuronal cells, but it can nonetheless be classified as a cyclin based on its ability to
activate Cdk5 (Tsai et al., 1994). Based on the criteria of cyclin box homology and/or ability to activate a Cdk, the family of cyclins then includes cyclins whose protein levels fluctuate only slightly and cyclins whose transcript and protein levels remain constant throughout the cell cycle. Although most cyclins have roles in cell cycle control, some have also been implicated in general transcription (Svejstrup et al., 1996), cellular metabolism (Huang et al., 1998) and actin regulation (Lee et al., 1998).

In budding yeast, there are nine cyclins subunits that activate Cdc28. The G1 cyclins CLNI, CLN2 and CLN3 have roles at around Start (Tyers et al., 1993). CLN3 functions before Start, and CLNI and CLN2 have roles post-Start in budding and DNA synthesis (Dirick et al., 1995; Stuart and Wittenberg, 1995). The CLB (cyclin B-like) cyclins function in S-phase and in G2/M. CLB5 and CLB6 are known as the S-phase cyclins because they are required for timely S-phase entry (Schwob and Nasmyth, 1993). CLB1-4 are known as mitotic cyclins and have roles in G2 and in promoting mitosis (Arnon et al., 1993; Fitch et al., 1992; Richardson et al., 1992). As will be discussed in section 2a.iv., the timely induction of cyclin activity throughout the cell cycle is an important regulatory mechanism that acts to specialize the Cdc28 kinase for different tasks during different phases of the cell cycle (see Figure 1-2). Like Cdc28, the Pho85 Cdk is activated by multiple Pcls (Pho85 cyclin). Three of these cyclins, Pcl1, Pcl2 and Pcl9 are G1 cyclins whose expression is activated at or before Start. These Pho85-cyclin complexes may play a role in polarized cell growth to the incipient bud (Lee et al., 1998; Tennyson et al., 1998).
2a. iii. Regulation of the Cdk subunit

Both the cyclin and the Cdk subunit are subject to various levels of regulation. The crystal structures of Cdk2 and cyclin A-Cdk2 complexes have revealed intimate details of Cdk regulation (Brown et al., 1995; De Bondt et al., 1993; Jeffrey et al., 1995). First, cyclin binding is a major requirement for Cdk activation. Cyclin binding causes two conformational changes in the Cdk that help to activate the kinase: (i) cyclin binding allows substrate binding to the Cdk by relieving steric inhibition by the Cdk T-loop (a structural loop so named because of a phosphorylatable threonine), and (ii) binding re-orients catalytic amino acids in the kinase domain for maximum activity.

In addition to cyclin binding, protein phosphorylation also plays a role in Cdk regulation (reviewed in Morgan, 1997). There are two major sites of phosphorylation on the Cdk: a threonine residue in the T-loop region (Thr-160 in human Cdk2) and tyrosine-15 (for human Cdk5). Phosphorylation at threonine-160 further activates the Cdk by inducing additional changes in the T-loop (Russo et al., 1996). The kinase responsible for this phosphorylation is called CAK (Cdk-activating kinase). The CAK activity in human cells has been attributed to another cyclin-Cdk complex, Cdk7-cyclin H (Nigg, 1996), whereas the CAK activity in budding yeast, Cak1, is a monomer and shows no homology to Cdk5s (Espinoza et al., 1996; Kaldis et al., 1996; Thuret et al., 1996). Yeast Cak1 also differs from human CAK in that Cak1 phosphorylates both the monomeric Cdk and the cyclin-bound Cdk, whereas human CAK phosphorylates the Cdk only when in complex with a cyclin (Kaldis et al., 1998). CAKs do not seem to be regulated in any specific way, and the abundance of CAK activity in most cells has led to the speculation that CAK phosphorylation is not an important regulator of cyclin-Cdk activity (Nigg, 1996).
In contrast, phosphorylation at tyrosine-15 plays a crucial role in halting the cell cycle in cases where it is dangerous for the cell to enter mitosis (reviewed in Lew and Kornbluth, 1996; Morgan, 1997). In fission yeast, the kinases Wee1 and Mik1 phosphorylate cdc2 on tyrosine-15. This Tyr-15 phosphorylation is required to prevent mitosis in the presence of unreplicated or damaged DNA (Enoch and Nurse, 1990; Rhind et al., 1997). Phosphorylation on the analogous tyrosine on human Cdc2 has been reported to inhibit mitosis in the presence of damaged DNA (Jin et al., 1996). In budding yeast, however, Tyr-15 does not have a role in inhibition of mitosis in response to unreplicated or damaged DNA (Amon et al., 1992; Sorger and Murray, 1992). Instead, phosphorylation of Tyr-18 (analogous to Tyr-15) on budding yeast Cdc28 has a mitotic checkpoint function and prevents mitosis when budding or spindle assembly is compromised (Lew and Reed, 1995; Lim et al., 1996). Dephosphorylation of Tyr-15 is catalyzed by the Cdc25 phosphatase family. In humans, different forms of the Cdc25 phosphatase are activated during G1 and mitosis and link extracellular signal transduction pathways to Cdk activation (Berry and Gould, 1996; Draetta and Eckstein, 1997).

Protein inhibitors add a final layer to Cdk regulation. These so-called CKIs (cyclin-dependent kinase inhibitors) bind and inhibit Cdks with first order kinetics (reviewed in Elledge and Harper, 1994; Peter and Herskowitz, 1994). In humans, mutations in genes encoding Cdk inhibitors have been implicated in the development of many cancers (Harper and Elledge, 1996). Since cells in many tumours are mutated for the p16 CKI gene, p16 is thought to be involved in keeping cells in G0 (reviewed in Hussussian et al., 1994; Karnb et al., 1994; Sheaff and Roberts, 1995). The p21 inhibitor has a role in preventing entry into the cell cycle during DNA damage (Harper and
Elledge, 1996), while p27 and p15 play roles in TGF-β-mediated cell cycle arrest (Reynisdottir and Massague, 1997). Three Cdk inhibitors have been identified in budding yeast: Pho81, Far1 and Sic1. Far1 mediates G1 cell cycle arrest in response to mating pheromone (Peter and Herskowitz, 1994), Pho81 inhibits the Pho85 Cdk in the presence of high phosphate levels to repress expression of genes involved in phosphate metabolism (Hirst et al., 1994; Schneider et al., 1994), and Sic1 inhibits Clb5/6-Cdc28 complexes to prevent DNA replication until Start is traversed (Mendenhall, 1993; Schwob et al., 1994).

2a. iv. Cyclin regulation

As mentioned earlier, the levels of most cyclins fluctuate dramatically throughout cell cycle. As such, the major mechanism for regulation of cyclins lies in controlling their abundance at different times in the cell cycle. Two regulatory mechanisms act to limit the time frame of cyclin abundance. The first is the transcriptional regulation of the cyclin gene. In most cases, cyclin gene expression is tightly limited to a specific time in the cell cycle (Figure 1-2 and Nasmyth, 1993). The mechanisms in budding yeast for activating cyclin transcription at Start are the main focus of my thesis and will be discussed further in section 2c and Chapter 3.

Transcriptional activation of cyclin genes can account for the rapid appearance of cyclin protein. On the other side of the coin, the rapid disappearance of cyclin protein is facilitated by a dedicated protein degradation machinery (reviewed in King et al., 1996). Not only does degradation remove the cyclin protein so that it will not passively interfere with subsequent cell cycle stages, but in the case of mitotic cyclins, the cyclin degradation step per se is what triggers a cell cycle transition. In budding yeast, the
mitotic cyclins, Clb1-4 are degraded at the metaphase to anaphase transition in a ubiquitin-dependent manner by a multi-protein proteasome called the Anaphase Promoting Complex (APC, reviewed in Deschaies, 1997; King et al., 1996). Degradation of mitotic cyclins is required for progression through mitosis; mutations in APC components and overexpression of stable CLB1 and CLB2 alleles cause cells to arrest at mitosis (Ghiara et al., 1991; Irniger et al., 1995; Surana et al., 1993; Zachariae et al., 1996). The yeast G1 cyclins, Cln1-3, are targeted for degradation at the G1/S boundary. Degradation of the G1 cyclins is promoted by a ubiquitin-dependent proteasome that is distinct from the APC; that is, the fundamental, ubiquitin-mediated proteolysis is the same but the protein subunits involved are different (Deschaies, 1997; King et al., 1996). Studies with Cln2 suggest that phosphorylation by Cdc28 targets the Clns for degradation (Willems et al., 1996). The importance of controlling Cln levels for normal cell cycle progression is evident from two experiments that examined the consequences of CLN2 overexpression. One group reported that overexpression of CLN2 causes cell death (Amon et al., 1993), while another study showed that CLN2 overexpression causes abnormal, elongated buds (Lew and Reed, 1993). The instability of yeast cyclins is attributable to the so-called PEST amino acid sequences or to the destruction box which target the cyclins for degradation (Rogers et al., 1986); deletion of the PEST sequences in CLNs or the destruction box in mitotic cyclins stabilize the cyclin protein (reviewed in Morgan, 1997; Tyers et al., 1992).
Figure 1-2: Cell Cycle Progression Catalyzed By Cyclical Waves of Cyclin Activity.

An important component of Cdk regulation is the sequential activation of cyclin gene expression throughout the cell cycle. Cln3-Cdc28 (yellow) activates gene expression at Start; Cln1/2-, Pcl1/2- and Clb5/6-Cdc28 (blue) catalyze bud formation and entry into S phase. Clb1,2,3,4-Cdc28 (red and green) play roles during G2 and Mitosis.
The intricate regulation of both the Cdk and cyclin subunit, especially the temporal regulation of cyclin abundance, explains how cyclin-Cdks can act as the main catalysts or 'workhorses' for cell cycle progression. Cyclical waves of cyclin activity may specialize the Cdk to perform different, required tasks throughout the cell cycle (Figure 1-2). Although the cyclical waves of cyclin activity may account largely for Cdk-mediated regulation of the cell cycle, the relatively large number of Cdks—and the regulation of the Cdk subunit as described above—likely serve to further specialize the roles of the cyclin-Cdk complex according to the cell’s or organism’s needs (for example, p35/Cdk5 in neuronal differentiation, Nikolic et al., 1996).

2b. Gene Products Expressed at Start

As mentioned above, the commitment at Start to completion of the mitotic cell cycle is likely due to the rapid transcriptional activation of genes whose products catalyze an irreversible entry into the cell cycle. In this section, I summarize some of the genes that are specifically expressed at Start.

2b. i. The CLN1 and CLN2 G1 cyclins.

CLN1 and CLN2 are genetically redundant in that either cyclin can complement a cln1Δcln2Δcln3Δ mutant which arrests before Start (Nasmyth and Dirick, 1991; Wittenberg et al., 1990). However, the CLNs are likely to have distinct roles in vivo since each cyclin-Cdk complex, when immunoprecipitated from yeast extracts, co-precipitates with a different profile of phosphorylated substrates (Tyers et al., 1993). Nonetheless,
there are two roles implicated for both CLN1 and CLN2: driving S-phase entry and bud formation (reviewed in Nasmyth, 1996).

CLN1 and CLN2 drive the onset of S-phase by phosphorylating the Cdk inhibitor Sic1, thereby targeting it for degradation (Schwob et al., 1994). Sic1 inhibits the Clb5- and Clb6-associated Cdc28 kinase. Since Clb5 and Clb6 are responsible for driving S-phase (discussed in the next subsection), Sic1 acts indirectly as an inhibitor of S-phase. Thus, Cln1/2 promote S-phase entry by removing this inhibition (see Figure 1-3).

The role of CLNI/2 in pre-bud site assembly was originally suggested in experiments with overexpressed CLN1 or CLN2. Overexpression of CLNI,2 causes hyperpolarization of actin patches to the bud tip (Lew and Reed, 1993). Moreover, a cln1Δcln2Δ double mutation is coletual with a third mutation in certain genes that are involved in budding (Benton et al., 1993; Cvrckova and Nasmyth, 1993). These genes include BUD2 (GTPase for BUD1, a Ras-like G-protein), CLA4 (STE20 kinase homologue involved in cytokinesis) and CDC12 (component of the septin ring at the bud neck).

2b. ii. The S phase cyclins Clb5 and Clb6

Clb5 and Clb6 are referred to as S phase cyclins because genetic experiments have implicated them in promoting DNA synthesis. The clb5Δclb6Δ double mutant exhibits delayed S-phase entry, and ectopic CLB5 expression can advance the onset of DNA synthesis (Schwob and Nasmyth, 1993). Because in the absence of CLB5/6, S phase is delayed but not abolished, the other B-type cyclins, CLB1-4, likely drive S phase in the clb5Δclb6Δ mutant. Indeed, a strain lacking all six CLB genes (clb1-6Δ) arrests before S phase (Schwob et al., 1994). In a normal cell cycle, however, CLB5/6 likely act
Figure 1-3: **Entry into S phase Catalyzed by Cln1/2- and Clb5/6-Cdc28**

Clb5/6-Cdc28 catalyze entry into S phase, likely through phosphorylation of subunit(s) of the protein complex bound to origins of DNA replication. In G1, Clb5/6 activity is held in check by the Sic1 Cdk inhibitor. Cln1 and Cln2 are produced during the transcriptional burst at Start, and Cln1,2-Cdc28 phosphorylate Sic1 to target it for degradation. Now free from Sic1 inhibition, Clb5,6-Cdc28 can induce entry into S phase.
as the cyclins for driving S phase since CLB5/6 are transcribed before S phase whereas CLB1-4 are transcribed after S phase (reviewed in Nasmyth, 1993).

CLB5/6 likely effect S-phase by phosphorylating substrates in the protein complexes bound to origins of DNA replication. A likely candidate for a Clb5/6 substrate is Cdc6, a protein which associates with replication origins specifically in the G1 window of the cell cycle (Donovan et al., 1997). Cdc6 copurifies with Cdc28 and can associate with Clb5 in vitro (Elsasser et al., 1996; Piatti et al., 1996). Moreover, Clb5-Cdc28 can phosphorylate purified Cdc6 in vitro.

2b. iii. PCL1 and PCL2 of the PCL cyclin family

As noted earlier, the Pcl cyclins act as the cyclin subunits for the Pho85 Cdk. Pho85 and its cyclin subunits seem to have roles in regulation of cell metabolism and actin localization, but not in cell cycle regulation per se (Huang et al., 1996; Lee et al., 1998; Schneider et al., 1994). Of the ten PCLs identified so far, only PCL1 and PCL2 are transcriptionally induced at Start. Their induction at Start likely reflects a role for the PCLs and Pho85 in actin regulation (Lee et al., 1998); mutants defective in PHO85 and in a subset of PCLs display aberrant actin localization. Since actin is involved in the transport of cytoplasm to the incipient bud, the induction of PCL1/2 at Start likely contributes to the establishment and/or maintenance of polarized cell growth. The cyclins CLN1/2, as will be discussed later, function more in bud site formation than in actin-mediated transport (Lew and Reed, 1995). The finding that the CLNs and the PCLs function in two different aspects of budding is consistent with the lethality that results in abrogating both of these pathways in the cln1Δ cln2Δ pcl1Δ pcl2Δ quadruple mutant (Measday et al., 1994).
2b. iv Gene products with direct roles in DNA synthesis

A number of genes with direct roles in DNA synthesis are transcriptionally induced at Start. These genes include the *CDC9* DNA ligase (Barker et al., 1985; Peterson et al., 1985), the *POL1* DNA polymerase (Johnston et al., 1987) and the *RNRI/2* subunits of ribonucleotide reductase (Elledge and Davis, 1987; Elledge and Davis, 1990). With the exception of Cdc6, a protein which binds to replication origins, most of the proteins in this category are stable proteins (Zhou and Jong, 1993). As such, their cell-cycle regulated transcription is unlikely to play a major role in the regulation of their activity since the proteins can persist throughout the cell cycle (Falconi et al., 1993).

2b. v Gene products involved in cell wall biosynthesis

A recent database search for genes with known Start-specific promoter elements (see section 2c) identified many genes involved in the synthesis of cell wall components (Igual et al., 1996). These genes include *FKSI* (subunit for (1-3)-β-glucan synthase), *MNN1* (involved in mannosylation of proteins) and *CSD2* (chitin synthase III). The expression of these genes at the Start transition likely serves to provide an ample supply of cell wall components to facilitate bud emergence and expansion. The *SWE1* protein kinase is also transcribed during the G1/S transition and can loosely be included in this category of cell wall biosynthesis genes; Swel acts to inhibit mitosis in the event of improper bud formation (Sia et al., 1996).

2c. Start-Specific Transcription Factors

Start is marked by a sharp burst of transcription from a subset of genes that catalyze the G1/S transition. As noted above, these genes include the G1 and S-phase
Figure 1-4: The SBF and MBF Transcription Factor Complexes.

Gene expression at Start transition in budding yeast is dependent upon two transcription factor complexes: the SBF (SCB-binding factor) and the MBF (MCB-binding factor) complexes. SBF binds to the SCB (ＳＷＴ4/6 cell cycle box) elements, while MBF binds to the MCB (Ｍｕｌ cell cycle box) elements in the promoter regions of cell cycle-regulated genes. The Swi4 and Mbp1 proteins are respectively the DNA-binding subunit for the SBF and MBF complexes. Swi6 by itself does not bind DNA specifically and is believed to play a regulatory role in these complexes. In general, SCB elements are found in the upstream regions of most G1 cyclin genes and cell wall biosynthesis genes, while MCB elements are found in the promoters of genes required for DNA synthesis. The timed expression of these genes acts to catalyze the Start transition.
cyclins and other genes required for DNA replication and bud formation. In this section, I describe the transcription factors that are responsible for this burst of gene expression at Start. I follow by discussing the mechanism(s) for activating these transcription factors. My work in chapter 3 focuses on the mechanisms that activate transcription at Start.

2c. i. The SBF and MBF transcription factor complexes

As shown in Figure 1-4, the two transcription factor complexes known to activate transcription at Start are the SBF and MBF complexes (reviewed in Andrews and Mason, 1993; Breeden, 1995). The SBF (SCB-binding factor) complex contains the Swi4 and Swi6 proteins and activates transcription mainly through a cis-acting sequence element called the SCB (Swi4/6-dependent cell cycle box; consensus sequence = CACGAAA). Genes activated by SBF include the G1 cyclins (\textit{CLN1, CLN2, PCL1, PCL2}, Ogas et al., 1991), the \textit{HO} endonuclease gene (Nasmyth, 1985), the gene encoding the \textit{SWE1} protein kinase (Sia et al., 1996) and a number of genes required for cell wall biosynthesis (Igual et al., 1996). The Swi4 subunit in SBF is responsible for specific binding to SCB sequences, while the other subunit, Swi6, does not bind DNA specifically. In \textit{swi4A} or \textit{swi6A} mutants, \textit{HO} is not expressed, and the expression of G1 cyclins and cell wall biosynthetic genes is reduced (Andrews and Herskowitz, 1989; Breeden and Nasmyth, 1987; Igual et al., 1996; Ogas et al., 1991).

Swi6 interacts with another DNA-binding protein, Mbp1, to form the MBF transcription factor complex (MCB-binding factor, also known as DSC1 Koch et al., 1993). MBF binds to a DNA consensus sequence, ACGCGTNA, known as the MCB element (\textit{Mlu1 cell cycle box}). Genes activated by MBF include the S-phase cyclins
(CLB5, CLB6), the SWI4 gene (Foster et al., 1993) and many genes involved in DNA synthesis (such as the CDC9 ligase and the POL1 DNA polymerase).

While SWI4 (in most backgrounds), SWI6 and MBP1 are not, by themselves, essential for cell viability, certain double mutant combinations, such as swi4Δmbp1Δ and swi4Δswi6Δ mutants are inviable (Koch et al., 1993; Nasmyth and Dirick, 1991; Ogas et al., 1991). These double mutants arrest in late G1, prior to DNA synthesis. The ability of ectopic CLN2 expression to rescue these double mutants suggests that they die because of insufficient levels of cyclin expression. The viability of swi4Δ and mbp1Δ single mutants suggest interplay between SBF and MBF in vivo. In in vitro experiments, SBF and MBF are each able to bind both SCB and MCB elements (Dirick et al., 1992; Koch et al., 1993), and studies have shown that on the CLNL promoter, SBF can act through the MCB elements (Partridge et al., 1997). However, this crosstalk between SCB- and MCB-binding by SBF/MBF has not been observed in in vivo footprinting experiments (Harrington and Andrews, 1996; Koch et al., 1996).

Since Swi6 does not bind DNA and is a common subunit in both SBF and MBF, various authors have speculated that it is the regulatory subunit in both complexes. At some promoters, mutation of SWI6 causes deregulated gene expression with levels that are intermediate between repressed and activated transcription (Dirick et al., 1992; Foster et al., 1993; Lowndes et al., 1992). This observation led to the hypothesis that SWI6 acts as both a transcriptional activator and repressor.

SBF and MBF are presently the only transcription factors in S. cerevisiae known to drive cell cycle-regulated transcription at Start. However, promoter analyses on the CLNL, CLN2 and SWI4 promoters made it clear that transcriptional activation of genes at
Start is a complicated process that may involve more players than currently identified (Cross et al., 1994; Foster et al., 1993; Partridge et al., 1997; Stuart and Wittenberg, 1994). First, studies on the SWI4, CLN1 and CLN2 promoters suggest at least one unidentified cell cycle-regulated DNA element. Furthermore, two studies report evidence for three independent pathways that contribute to the transcriptional regulation of CLN1 and CLN2 (Breeden and Mikesell, 1994; DiComo et. al., 1995, and discussed in the next section).

The protein structures of Swi4, Swi6 and Mbp1 are discussed in section 3.

2c. ii. Activation of SBF and MBF in late G1

The current model for Start (Figure 1-5) puts the cyclin-Cdk complex, Cln3-Cdc28, genetically upstream of SBF/MBF activation. Although earlier experiments showed that CDC28, with any one of CLN1, CLN2 or CLN3, is sufficient to drive Start, more careful experiments later showed that Cln3-Cdc28 is responsible for activating Start in the normal cell cycle. CLN3, as the sole G1 cyclin, is sufficient to activate Start transcription at the correct cell size, but cells with only CLN1/CLN2 and lacking CLN3 activate Start transcription at a larger cell size than in wild type cells (Dirick et al., 1995; Stuart and Wittenberg, 1995). Whereas Cln3-Cdc28 is specialized for activating transcription at Start, the major role of Cln1,2-Cdc28 likely lies in activating post-Start events such as DNA synthesis and bud formation (Levin et al., 1996). The temporal regulation of the Cln3 protein supports its role in acting upstream of SBF/MBF activation; Cln3 is present throughout the cell cycle but CLN3 expression is induced early in the cell cycle in M/G1 (McInerny et al., 1997).
Figure 1-5: Activation of Gene Expression at Start.

The levels of the Cln3 G1 cyclin likely act as an indicator of nutrient conditions and the cellular mRNA translation rate. Sufficient levels of Cln3 can then effect Start transcriptional activation through the SBF and MBF transcription factor complexes. Bck2 has also been implicated in the activation of Start gene expression, and may link other cellular signals, such as cell size, to Start transcription. There is genetic evidence for at least one more pathway that activates transcription at Start (denoted by question mark and dark nebula). The gene products made at Start then catalyze the Start transition through catalyzing cellular events such as spindle pole body duplication, DNA synthesis, and bud formation.
The experiments mentioned above show that CLN3 is the only one of the CLN G1 cyclins that is required and sufficient for activating Start-dependent transcription in a normal cell cycle. However, CLN3 is not the sole activator of Start. There are likely other pathways or mechanisms for activating Start since cln3Δ cells still show activation of Start transcription, albeit at a larger cell size. The BCK2 gene (bypass of C-kinase) has also been implicated in activating transcription at Start. Strains lacking both BCK2 and CLN3 are very slow-growing with delayed G1 progression, and the delay in Start-specific gene expression in this double mutant strain is exacerbated relative to the cln3 single mutant (DiComo et al., 1995; Epstein and Cross, 1994). Moreover, overexpression of BCK2 can drive cells to enter Start early. BCK2 appears to play a smaller role than CLN3 in the normal cell cycle since bck2Δ single mutants show practically no defect except a marginally larger mean cell size. The importance of BCK2 function becomes manifest only in the absence of CLN3 as judged by the exacerbated slow growth and delay in Start-dependent gene expression seen in the bck2Δcln3Δ double mutant.

BCK2 has been associated with the yeast Protein Kinase C (PKC) pathway. Overexpression of BCK2 can suppress the lethality caused by deletion of PKC1 and the high-temperature lethality caused by deletion SLT2 (or MPK1), the terminal MAP kinase of the yeast PKC pathway (Lee et al., 1993). Further evidence associate the PKC pathway with activation of Start transcription; Slt2 was shown to phosphorylate Swi6, and overexpression of two SBF target genes suppressed slt2Δ mutant phenotypes (Madden et al., 1997). In Chapter 4, I discuss unpublished data from other labs that further implicate SLT2 as an activator of Start transcription.
Since the bck2Δ cln3Δ double mutant is sick but still alive, and since Start transcription is delayed but not abolished, at least one more pathway, in addition to the BCK2- and CLN3-dependent pathways, can activate SBF/MBF. In Figure 1-5, BCK2 and CLN3 are shown operating independently on SBF with at least one more pathway that can operate in the absence of these two genes. An independent study that approached the activation of SBF from a different perspective also concluded that there are at least three independent mechanisms for activating CLN1/2 transcription at Start; in cells mutated for SWI6 and where SWI4 activity is deregulated, there is still a two-fold cell cycle regulation in CLN1/2 transcription (Breeden and Mikesell, 1994). Whether the three pathways suggested in this latter study reflect the multiple pathways implicated by the BCK2 studies is still a matter of speculation. In Chapter 3, I describe data that implicate a novel protein, Stb1, as an activator of transcription at Start.

The dependence of SBF/MBF activation on CLN-kinases and the fact that CLN1 and CLN2 are SBF target genes led to the proposal of the ‘positive feedback model’ (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Ogas et al., 1991). According to this model, Cln1 and Cln2 protein synthesized early at Start act on SBF and MBF to further induce their own gene expression, leading to a positive feedback loop that might explain the irreversibility of Start. This model was later refuted when cells deleted for CLN1 and CLN2 were seen to activate Start transcription with the same kinetics and at the same cell size as wild-type cells (Dirick et al., 1995; Stuart and Wittenberg, 1995). This observation subsequently led to the view that CLN3 is the only CLN cyclin necessary for Start-specific transcription. This new view, however, does not explain previous genetic observations, that cln1Δcln2Δcln3Δ triple mutants and cdc28 mutants
arrest in G1 with low levels of Start transcripts (Cross, 1990; Hartwell et al., 1974; Richardson et al., 1989). If CLN3 were the sole CLN cyclin required for Start transcription, and in its absence, another CLN-independent pathway activated Start transcription, then the cln1Δcln2Δcln3Δ mutant should not arrest with low levels of Start transcripts. Rather, the CLN-independent pathway would activate transcription, and the cells would probably arrest after Start because CLN1/CLN2-dependent activation of DNA synthesis and/or bud formation is compromised. However, this is not observed; rather, we see a G1 arrest that occurs before Start transcription. The same reasoning holds for the cdc28 arrest. These observations implicate CLN1,2 as necessary for activation of Start transcription in the absence of CLN3. Perhaps, a positive feedback mechanism does take place, but it is masked by CLN3 activation such that it is apparent only in cln3Δ cells. Moreover, after Cdc28 function at Start, de novo protein synthesis is not required for Start transcription; this observation further suggests that CLN1/CLN2 positive feedback is unimportant in the presence of CLN3 which is transcribed early in the cell cycle before Start (Marini and Reed, 1992).

Although Marini and Reed did not see a requirement for protein synthesis after Cdc28 function for Start transcription, an in-depth examination of the CLN2 promoter revealed a promoter element that did require protein synthesis after Cdc28 function for expression. This fragment of the CLN2 promoter (designated UAS2) contains no SCBs or MCBs, but is nonetheless SWI4-dependent, is able to confer cell cycle-regulated transcription. This expression is dependent on both the presence of Clns and de novo protein synthesis after Cdc28 function (Cross et al., 1994; Stuart and Wittenberg, 1994). These observations are consistent with a role for Cln2 synthesized at Start in inducing its
own gene expression. In Chapter 3, I will present further evidence implicating CLN1/2 in activating Start transcription in cln3A cells.

2c. iii. Molecular mechanism for SBF/MBF activation

The requirement for CDC28 and CLN3 to initiate Start transcription with proper timing and the observation that cdc28 mutants arrest just before Start have led to the proposal that Cln3-Cdc28 may act directly upstream of SBF/MBF. The observation mentioned above that after Cdc28 function, protein synthesis is not required for Start transcription suggests that Cdc28 functions immediately before SBF/MBF activation (Marini and Reed, 1992). However, there is no biochemical evidence for Cln3-Cdc28 acting directly to activate SBF/MBF. The obvious hypothesis is that Cln3-Cdc28 phosphorylates one or both components of SBF/MBF to activate the complexes. However, a study of SWI6 derivatives that were mutated at consensus Cdk phosphorylation sites and at a serine residue that is phosphorylated in a cell cycle-dependent manner did not show a requirement for Cdk phosphorylation for cell cycle-regulated expression of an SBF-dependent gene (Sidorova et al., 1995). An alternative hypothesis is that SBF/MBF may act as protein scaffolds on DNA to recruit Cln3/Cdc28 onto promoters where the kinase can directly regulate components of the general transcription machinery (Koch and Nasmyth, 1994). This model predicts an observable interaction between Cln3-Cdc28 and SBF/MBF, but there is no published evidence for such an interaction. Moreover, Cln3 does not confer cell cycle-regulated transcription to a heterologous promoter when recruited by the Gal4 DNA-binding domain (Yuen Ho, unpublished data).
Regardless of the dearth of biochemical data on SBF/MBF activation, Cln3-Cdc28 clearly acts upstream of SBF/MBF. The inability to biochemically link Cln3-Cdc28 with SBF/MBF may indicate the existence of unidentified, adaptor proteins that communicate the Cln3-Cdc28 regulation to SBF/MBF. In Chapter 3, I describe a possible adaptor protein, Stb1, which binds Swi6 and is a substrate for Cln-associated kinases. In addition to SBF/MBF, Cln3 may activate Start transcription through SBF/MBF-independent pathways (Breeden and Mikesell, 1994; Cross et al., 1994; Foster et al., 1993; Stuart and Wittenberg, 1994). This would obscure any effects of mutating the Cdk phosphorylation sites on Swi4 and Swi6. Moreover, as discussed above, there are clearly \textit{CLN3}-independent mechanisms for activating Start transcription (discussed in section 2b. ii. and Chapter 3).

2c. iv. \textit{CLN3} links nutrient status and transcription at Start

As mentioned earlier, Cln3 protein is present throughout the cell cycle (Tyers et al., 1993), but consistent with its role for activating Start, \textit{CLN3} expression is induced early in the cell cycle at M/G1 (McInerny et al., 1997). In the past year, studies on the transcriptional and translational regulation of \textit{CLN3} have offered insight into how \textit{CLN3} links the growth conditions and status of the cell to transcriptional activation at Start. First, \textit{CLN3} transcription is positively regulated by glucose and nitrogen sources; the glucose regulation of \textit{CLN3} transcription is through multiple AAGAAAAA repeats in the \textit{CLN3} promoter (Parviz and Heideman, 1998; Parviz et al., 1998). The regulation of Cln3 translation adds a second layer of regulation; translation of \textit{CLN3} mRNA is repressed under nitrogen deprivation (Gallego et al., 1997). The translational regulation of \textit{CLN3} mRNA is likely brought about by an upstream open reading frame in the 5' leader of
CLN3 mRNA. Translation of the CLN3 open reading frame requires leaky scanning from ribosomes that bypass this upstream ORF (Polymenis and Schmidt, 1997). Under fast growth conditions, the increase in cellular ribosomes would increase the chances of bypassing the upstream ORF. Such a translational control mechanism likely serves to make Cln3 synthesis extremely sensitive to the cellular translational rate. Since deletion of the upstream ORF in CLN3 causes an acceleration of Start, translational regulation of Cln3 may be responsible for setting Cln3 protein at threshold levels such that a change in Cln3 translation would lead to a change in the timing of Start.

In addition to a decrease in the translation of CLN3 mRNA, ubiquitin-mediated degradation of Cln3 protein is upregulated in low nitrogen (Gallego et al., 1997). Thus, a picture emerges where CLN3 acts as a molecular link between entry into the mitotic cell cycle at Start and growth conditions such as translational rate, nitrogen and glucose levels.

Although the work on CLN3 regulation is starting to illuminate how nutrient conditions affect entry into Start, our understanding of how Start is activated in the cln3Δ mutant is very limited. Does BCK2 act as the link between nutrient conditions and Start in the cln3Δ mutant? An alternative hypothesis would be that BCK2 acts to link not nutrient status but cell size to the activation of Start. These issues are discussed further in Chapter 3 and are the main focus of Chapter 4 (Future Directions).
3. THE PROTEIN STRUCTURES OF Swi4, Swi6, Mbp1

Swi4, Swi6 and Mbp1 belong to a family of yeast cell cycle transcription factors that share both functional and sequence homology (reviewed in Breeden, 1995). In this section, I describe the conserved structural features of Swi4, Swi6 and Mbp1. These structural modules, the DNA-binding domain, the central ankyrin repeat region and the C-terminal heterodimerization domain, are also shared by the homologues of SWI4/6 and MBP1 in other yeasts (Figure 1-6 and section 4 below).

3a. The DNA binding Domain of Swi4 and Mbp1

Swi4 and Mbp1 respectively serve as the DNA binding subunits of SBF and MBF. Swi6, on the other hand, does not bind specifically to DNA (Koch et al., 1993; Sidorova and Breeden, 1993) but likely acts as the regulatory subunit or to enhance the DNA-binding affinity of these complexes. The DNA-binding domains of Swi4 and Mbp1 lie at their N-termini. For Swi4, the DNA-binding domain has been localized to amino acids 36-155 (Primig et al., 1992) and for Mbp1, it has been localized to its N-terminal 124 amino acids (Dirick et al., 1992). The DNA-binding domains of Swi4 and Mbp1 are highly related (81% identity), and structural data for Mbp1 reveal that this domain constitutes a novel helix-turn-helix motif (Taylor et al., 1997). The homology between the DNA-binding domains of Swi4 and Mbp1 is also shared by the cell-cycle transcription factors in fission yeast (Figure 1-6). There are other transcription
Figure 1-6: The Yeast Family of Cell Cycle Transcription Factors.

(A) The cell cycle transcription factor complexes in S. cerevisiae and S. pombe. (B) Schematic diagram of the yeast family of cell cycle transcription factors. The putative DNA-binding domain in Cdc10 (yellow) is not as similar to the DNA-binding domains of Swi4, Mbp1, Res1, Res2 and K. lactis Mbp1 (reddish orange) as theirs are to one another. The C-terminal association domain in Swi4, Mbp1, Res1, Res2 and K. lactis Mbp1 (reddish blue) are distinct from the C-terminal association domains of Swi6, Cdc10 and K. lactis Swi6 (bluish orange). The four and one half ankyrin repeats are highly conserved in all these proteins (see Figure 1-7). The most similar repeats are in dark blue, and the less similar repeats are in lighter blue.
factors that share homology with this DNA-binding domain, such as Xbp1 (response to cellular stress Mai and Breeden, 1997), Phd1 (pseudohyphal growth Gimeno et al., 1992) and Sok2 (suppressor of a protein kinase A defect, Ward and Garrett, 1994) in *S. cerevisiae*, as well as Efg1 in *Candida albicans* (Breeden, 1995) and StuA in *Aspergillus nidulans* (Miller et al., 1991). However, these five proteins do not share the other structural features that are unique to the yeast family of cell cycle transcription factors (see section 4).

3b. The Ankyrin (Swi6/Cdc10) Repeat Domain in Swi4, Swi6 and Mbp1

The ankyrin repeat domain is found in the central regions of Swi4, Swi6 and Mbp1. The ankyrin repeat is predominantly an α-helical protein domain that is found in numerous and functionally diverse proteins such as membrane signalling proteins, transcription factors, ankyrin and Cdk inhibitors (Tevelev et al., 1996; Gorina and Pavletich, 1996). Ankyrin repeats are found in tandem array repeats of 4 in the case of the yeast cell cycle transcription factors and up to 26 for ankyrin (reviewed in Michaely and Bennett, 1992). Despite the diversity of proteins containing this domain, the ankyrin repeats are known to perform only a single function, that is to mediate protein-protein interactions.

In the yeast cell cycle transcription factor family, each member contains two repeats with strong similarity to the ankyrin consensus (Andrews and Herskowitz, 1989; Breeden and Nasmyth, 1987; Koch et al., 1993). Using a more degenerate consensus obtained from the alignment of numerous proteins, two more, less similar repeats were found between the original two repeats in addition to an N-terminal half of a fifth repeat (Bork, 1993). The importance of the ankyrin repeats in the yeast cell
Figure 1-7: Alignment of the Central Ankyrin Repeat Region

The central ankyrin repeat regions in members of the yeast family of cell cycle transcription factors are aligned with each repeat underlined below and numbered (from N-term. to C-term.) on top, with repeat 5 designating the half repeat. The repeats with high similarity to the ankyrin consensus are underlined with a solid line, and the repeats with low similarity to the consensus are underlined with a dashed line. Residues that are identical across at least five proteins or conserved (F,Y; E, D; Q, N; I, L, V, A; S, T) amongst all the proteins shown are boxed in yellow. Note that the similarity between these proteins across this domain extends to residues beyond and between ankyrin consensus residues. (modified from Koch et al., 1993)
cycle transcription factor family was suggested by the isolation of point mutations in the ankyrin repeats of cdc10+, SWI6 and SWI4 that resulted in temperature-sensitivity for these genes (Ewaskow et al., 1998; Reymond et al. 1992; Michael Donoviel and Yuen Ho, unpublished data). Mutational and modelling analysis of the Swi6 ankyrin repeats suggest that the consensus residues in the ankyrin repeat form a three-dimensional molecular scaffold from which to 'present' the non-conserved residues or the residues between the repeats. These non-conserved amino acids are proposed to be the residues responsible for mediating specific protein-protein interactions (Ewaskow et al., 1998). This can explain why, unlike the case with SH2 and SH3 domains, there has been no consensus motif found for ankyrin repeat-binding proteins.

In Swi4, the ankyrin repeats can bind the mitotic cyclin, Clb2, and this interaction may be involved in turning off SBF-mediated transcription (Amon et al., 1993; Siegmund and Nasmyth, 1996). As yet, the function or interacting protein(s) for the ankyrin repeats in other family members have not been identified. Nevertheless, the conservation of this protein-protein interaction motif across cell cycle regulators in such evolutionarily distant yeasts suggests that protein-protein interactions play an important role in cell cycle transcriptional regulation. In Chapter 3, I present data indicating that a novel protein, Stb1 binds Swi6, in manner dependent on the Swi6 ankyrin repeats.

3c. The C-terminal Region (CTR) of Swi4, Swi6 and Mbp1

In addition to forming a complex on DNA, Swi4 can physically interact with Swi6 in vitro in the absence of other yeast proteins and DNA, suggesting that the interaction is direct and independent of DNA-binding (Andrews and Moore, 1992, Yuen Ho, unpublished). The region involved in the Swi4-Swi6 heterodimerization has been
localized to the C-terminus of each protein. For Swi4, the C-terminal 40 residues are sufficient for interaction with Swi6 (Siegmund and Nasmyth, 1996), and for Swi6, deletion of 89 amino acids from the C-terminus of the protein abolishes interaction with Swi4 (Andrews and Moore, 1992).

Mbp1 forms the MBF/DSC1 complex with Swi6 on MCB elements in vitro, and the MBF complex can be purified from yeast extracts as an intact complex of Mbp1 and Swi6 (Dirick et al., 1992; Koch et al., 1993; Lowndes et al., 1992). At its C-terminus, Mbp1 has 44% identity to the C-terminal region of Swi4, and this C-terminal domain is responsible for heterodimerization with Swi6 (Siegmund and Nasmyth, 1996).

The C-terminal region of Swi6 does not have strong similarity with Swi4/Mbp1 but shares similarity with Swi6 homologues in fission yeast and _K. lactis_ (discussed in section 6).

### 3d. Nuclear Localization of the Swi6 Protein

Although Swi6 is a predominantly acidic protein, there is a cluster of basic amino acid residues, KLKK, that lies about 150 amino acids N-terminal to the ankyrin repeat region. This basic cluster is necessary for the nuclear import of the Swi6 protein, and it is sufficient to confer nuclear localization when fused to a heterologous protein (Sidorova et al., 1995). This regulation may be specific to Swi6 since the cluster lies in a region where there is no homology to the Swi6 homologues in fission yeast and _K. lactis_ nor to _SWI4/MBP1_ and its homologues.

The regulation of Swi6 nuclear entry is through the phosphorylation or dephosphorylation of serine-160 which is just 3 amino acids N-terminal to the basic cluster mentioned above (Sidorova et al., 1995). Swi6 is seen to be predominantly
Figure 1-8: Cellular Localization of the Swi6 Protein Throughout the Cell Cycle

The Swi6 protein is predominantly nuclear in G1, G2/M and M phase cells, while in S and G2 phase cells, Swi6 is predominantly cytoplasmic. The cellular localization of Swi6 is dependent on phosphorylation on serine-160. Phosphorylation of serine-160 is correlated with cytoplasmic Swi6. The localization of Swi6 in the nucleus coincides with the binding of SCB elements in vivo.
nuclear throughout G1 and in late mitosis; in G2 cells, Swi6 is mainly cytoplasmic. This cellular localization of Swi6 is regulated through the phosphorylation of serine-160. The phosphorylation of serine-160 is correlated with cytoplasmic Swi6. Mutation of serine-160 to a non-phosphorylatable alanine causes Swi6 to be constitutively nuclear, while mutation to a phosphoserine-mimetic aspartate residue causes Swi6 to remain cytoplasmic (Figure 1-8). Cdc28 plays a role in the phosphorylation of Ser-160, but it is not absolutely required (Sidorova et al., 1995).

The significance of Swi6 cellular localization is unclear. The serine-160 mutant that is constitutively cytoplasmic, and hence unable to access chromatin, shows a slight decrease in the transcript levels of the HO gene, but the cell cycle regulation, which in the case of HO is absolutely dependent on SWI6, remains intact (Sidorova et al., 1995). Perhaps a few molecules of Swi6 are able to enter the nucleus despite the regulation by serine-160. Nonetheless, the accessibility of Swi6 to chromatin does not seem to be the major regulatory mechanism behind activation of transcription at Start. Nuclear Swi6 correlates with SBF binding throughout early to late G1 (Harrington and Andrews, 1996), but Start transcription does not occur until late G1.

3e. The Putative Leucine Zipper in the Swi6 C-terminus

The Swi6 protein, as well as its homologues in K. lactis and S. pombe, have potential leucine zipper motifs at their C-termini (Breeden, 1995; Sidorova and Breeden, 1993). The leucine zipper is a protein-protein interaction domain that mediates dimerization of many transcription factors (Landschulz et al., 1988). Structurally, the leucine zipper is an amphipathic α-helix with heptad repeats of leucine residues such that in the folded helix, the leucines are presented on one side of the helix. When two
proteins with leucine zippers interact via this domain, the leucines on either protein interlock in a ‘zipper-like’ fashion. The presence of this motif in Swi6, Cdc10 or the K. lactis Swi6 has not been confirmed structurally. It has only been inferred from the heptad repeats of leucine residues in a region that has been predicted to fold as an amphipathic helix. A Swi6 protein with the leucine zipper region deleted retains the ability to bind Swi4, but the complex is incapable of binding DNA (Sidorova and Breeden, 1993). As yet, no protein(s) have been found that bind to the putative leucine zipper in Swi6.

4. THE FAMILY OF YEAST CELL CYCLE TRANSCRIPTION FACTORS

As mentioned above, Swi4, Swi6 and Mbp1 all belong to a family of yeast cell cycle transcription factors that share both functional and amino acid sequence similarity. In the fission yeast, *Schizosaccharomyces pombe*, Cdc10 acts as the Swi6 analogue and forms a complex with Res1 (Caligiuri and Beach, 1993; Lowndes et al., 1992; Tanaka et al., 1992) or Res2 (Miyamoto et al., 1994). Res1 and Res2 are homologous to Swi4/Mbp1 and confer DNA binding specificity. Homologues of SWI6 and MBP1 have also been found in the yeast *Kluveromyces lactis* by low stringency hybridization (Koch et al. 1993). As yet, no functional studies have been done on the K. lactis homologues to confirm that they are indeed functionally homologous to their budding and fission yeast counterparts. The most striking similarities between members of this family lie in their structural homology based on amino acid sequence (see Figure 1-7). Almost all the homologues share the same basic module of an N-terminal DNA binding domain, a central ankyrin repeat region followed by a C-terminal heterodimerization domain. Limited proteolytic analysis of Swi6 fragments confirmed that these three domain
modules, as predicted by amino acid sequence, do exist in the protein (Sedgwick et al., 1998). These three domain modules show sequence similarity across the family members (as described above). The highest similarity, however, is across the ankyrin repeat region. Whereas most ankyrin repeat-containing proteins differ in their number of repeats, the members of this family all have four and one-half repeats, with the first and third repeat having the strongest similarity to the ankyrin repeat consensus sequence. Moreover, the amino acid sequence similarity in these proteins extends beyond the ankyrin consensus; that is, they have similar sequences that are not shared by other ankyrin-repeat proteins. The similarity in function, the conservation of the three-domain module and the amino acid sequence similarity across these domains has led to the speculation that the yeast family of cell cycle transcription factors arose from the gene duplication of a primordial transcription factor (Koch et al., 1993). This idea becomes quite significant when one considers that budding yeast are as evolutionarily separated from fission yeast as they are from human cells.

Because the K. lactis family members have not been well studied, I only discuss the \textit{S. pombe} homologues below.

\textbf{4a. Cdc10 in \textit{S. pombe}}

Cdc10 is the fission yeast homologue of Swi6 and binds, in a complex with Res1 or Res2, to MCB-like elements in \textit{S. pombe} (Lowndes et al., 1992). Like Swi6 as noted above, Cdc10 has 2 known interacting partners, Res1 and Res2, which act as the DNA-binding subunits. Cdc10 is required for gene expression at Start, and like Swi6, it seems also to have a role as a transcriptional repressor (Tahara et al., 1998). Unlike Swi6 however, Cdc10 is an essential gene; \textit{cdc10} cells arrest in G1 (Nurse and Bissett, 1981).
This difference may reflect distinct functional mechanisms of Swi6 and Cdc10: Cdc10 appears to be absolutely necessary for expression of target genes, whereas swi6Δ mutants show a deregulated expression that is intermediate between the peaks and troughs of cell cycle-regulated transcription (Koch et al., 1993; Lowndes et al., 1992).

Swi6 and Cdc10 share 30% amino acid sequence identity between the C-terminal two-thirds of the protein. However, there are blocks of stronger similarity within this region such as the ankyrin repeats and the heterodimerization domain (Breeden and Nasmyth, 1987). The N-termini of the proteins show some divergence; unlike Swi6, Cdc10 contains a region with homology to the DNA-binding domains in Swi4/Mbp1 (Primig et al., 1992). Although in vitro translated Cdc10 cannot bind DNA (Zhu et al., 1994), the presence of this DNA binding domain may reflect an ability of Cdc10 to bind DNA under special circumstances (discussed in the next section).

4b. Res1 and Res2 in S. pombe

Res1 and Res2 are structurally homologous to Swi4/Mbp1. They show strong similarity across the ankyrin repeat domain, the C-terminal region and in the DNA-binding domain. The Res2 DNA-binding domain is more similar to Res1 than it is to Swi4/Mbp1. Similar to Swi4/6 and Mbp1, Res1 and Res2 form heterodimers with a regulatory subunit, Cdc10. Res1 can bind to the S. pombe MCB elements when complexed with Cdc10 (Ayte et al., 1995; Caligiuri and Beach, 1993). Others have reported that Cdc10, Res1 and Res2 bind MCB elements as a heterotrimer in vitro and in vivo (Zhu et al., 1997). Whereas Res1 seems to play a role in the mitotic cell cycle, Res2 appears to be involved primarily in regulating meiosis (Miyamoto et al., 1994; Zhu et al., 1994). However, both Res1 and Res2 appear functionally similar in that they can partly
substitute for one another's functions. Interestingly, *SWI4* and *MBP1* also appear to be functionally redundant (reviewed in Andrews and Mason, 1993; Koch et al., 1993). The parallel between the two yeast systems can be further extended because, like Res2, *SWI6* and *SWI4* have recently been implicated in regulating *S. cerevisiae* meiosis (Leem et al., 1998).

4c. Rep2, a Transcriptional Co-Activator Subunit for Res2-Cdc10

Rep2 is a zinc finger-containing protein that is believed to function as a transcriptional coactivator subunit for the Res2-Cdc10 complex. Rep2 binds Res2 *in vitro* and is found in Res2-Cdc10 complexes *in vivo* (Nakashima et al., 1995). Mutation of *rep2* causes a partial reduction in the expression of genes regulated by Res2-Cdc10 complexes, and overexpression of *rep2* can suppress the lethality of a *cdc10* mutation and the growth defect of *res1* mutants. Using an artificial budding yeast one- and two-hybrid system, Tahara et al. found that Res2-Cdc10 complexes without Rep2 can bind to MCB sequences but cannot activate transcription; recruitment of Rep2 into the complex is required for transcriptional activation (Tahara et al., 1998).

In budding yeast, no other activator subunits have been reported for SBF or MBF. In Chapter 3, I describe evidence implicating a novel protein, Stbl, as a possible activator subunit in SBF and/or MBF.

5. ANALOGY BETWEEN BUDDING YEAST SBF/MBF AND E2F/DP1 IN HIGHER EUKARYOTES

As alluded to earlier, despite *SWI4/6* and *MBP1* having conserved, functional and sequence homologues in distantly related yeasts, they have no identified homologues in
higher eukaryotes. However, the E2F/DP1 family of transcription factors may be considered to be functionally analogous to SBF/MBF (reviewed in Johnson and Schneider-Broussard, 1998; La Thangue, 1994). Despite the lack of homology between these proteins, the E2F/DP1 binding site resembles an MCB and an SCB element joined end-to-end. Like MCB and SCB elements, the E2F/DP1 binding site is found upstream of genes that are expressed at the restriction point and are involved in cell proliferation—genes such as dihydrofolate reductase, cyclin A and Myc (Mean et al., 1992; Merrill et al., 1992). The inactivation of E2F after the restriction point resembles Swi4 inactivation after Start in that both are mediated by cyclin binding—cyclin A in the case of E2F (Sanchez and Dynlacht, 1996) and Clb2 for Swi4 (Amon et al., 1993). Whereas E2F/DP1 resembles Swi4 and Mbp1 functionally, the retinoblastoma gene, Rb, functionally resembles Swi6. Rb is a tumour suppressor gene that is mutated in numerous tumours; its molecular function is to repress transcriptional activation by E2F during early G1 (reviewed in Hatakeyama and Weinberg, 1995; Sanchez and Dynlacht, 1996). In late G1, Rb is phosphorylated by cyclin D- and E-associated Cdks which causes dissociation of Rb thereby activating E2F-dependent gene expression. The parallel between Rb and Swi6 lies in the fact that Swi6 has also been proposed to act as repressor in SBF/MBF in addition to its role as an activator of transcription (Dirick et al., 1992; Foster et al., 1993; Lowndes et al., 1992). Moreover, both Rb and Swi6 are phosphorylated at around the time of S-phase (Sidorova et al., 1995).
6. THESIS SUMMARY

In my thesis work, I used Swi6 as a starting point to study the regulation of SBF and MBF. I chose to study Swi6 in particular because it is believed to act as the regulatory subunit in SBF and MBF—both as a positive and negative regulator (reviewed in Breeden, 1995)—so it is likely to lie at the nexus for regulatory signals. The presence of protein-protein interaction motifs in Swi6 with no known function prompted me to study the regulation of Swi6, and hence SBF/MBF, through finding proteins that physically interacted with Swi6. After checking the efficacy of several methods, both biochemical and genetic, to find interacting proteins, I decided upon Swi6 protein affinity chromatography. Using this method I have identified two proteins whose interaction with Swi6 was previously unknown. These two proteins are the protein kinase Hrr25 and a novel protein, Stb1.

In Chapter 2, I describe the significance of the Hrr25-Swi6 interaction. I show that Swi6 is a substrate for the Hrr25 protein kinase. I provide evidence that Hrr25 and SBF are involved in the transcriptional induction of DNA repair genes in response to the DNA-alkylating agent, MMS and to the DNA synthesis inhibitor, hydroxyurea. In support of a role for SBF in inducing DNA repair genes, the Swi6 protein, which is cytoplasmic in G2, was seen to be predominantly nuclear in MMS-treated, G2 cells.

In Chapter 3, I describe the significance of the Stb1-Swi6 interaction. I provide evidence that Stb1 is a substrate for Cln-Cdc28 kinase complexes. I provide evidence to suggest that STB1 is involved in activating SBF/MBF. I suggest that Cln-Cdc28 phosphorylation of Stb1 activates STB1-dependent activation of Start transcription.
Chapter II

Role for the Casein Kinase I Isoform, Hrr25, and the Cell Cycle-Regulatory Transcription Factor, SBF, in the Transcriptional Response to DNA Damage in *Saccharomyces cerevisiae*


I did the experiments described in this Chapter, except Ryuj Kobayashi performed microsequencing of the p54 peptides shown in Table 2-1.
ABSTRACT

In budding yeast, DNA damage or depletion of the ribonucleotide pool results in the transcriptional induction of a set of genes with known or putative roles in DNA repair. This transcriptional response is dependent upon RAD9, the ATM-like kinase, MECl and the serine/threonine kinases, RAD53 and DUN1. I report evidence that another protein kinase, Hrr25 is also involved in the transcriptional response to DNA damage, specifically through its interaction with the transcription factor, Swi6. I show that Swi6 associates with Hrr25 and is an in vitro substrate for the Hrr25 kinase. I found that swi4Δ, swi6Δ, hrr25Δ, but not mbp1Δ mutants are sensitive to the DNA synthesis inhibitor hydroxyurea (HU) and the DNA-alkylating agent methyl methanesulfonate (MMS) and are defective in the transcriptional induction of a subset of DNA damage-inducible genes. Moreover, the sensitivity of swi6Δ mutants to hydroxyurea/MMS and the transcriptional induction defect of the hrr25Δ mutant are rescued by overexpression of SWI4, implicating the SBF complex in the HRR25-dependent response to DNA damage.
INTRODUCTION

In this chapter, I describe experiments that implicate SBF and a Swi6-interacting protein, Hrr25, in inducing the expression of DNA repair genes after exposure to DNA damage or in response to the inhibition of DNA synthesis. Below, I provide a brief review of the DNA damage response in budding yeast.

1. THE RESPONSE TO DNA DAMAGE IN S. cerevisiae

In response to DNA damage, eukaryotic cells undergo both a checkpoint response and a DNA repair response. The checkpoint response delays cell division to allow repair of damaged DNA before proceeding through the cell cycle, and in this way, serves to maintain the integrity of the genome (reviewed in Siede, 1995). DNA damage-responsive checkpoints have been observed that (i) prevent chromosome segregation in the presence of DNA strand breaks or other alterations (G2/M checkpoint); (ii) prevent the replication of errors in DNA accumulated by pre-S phase cells (G1/S checkpoint), and (iii) prevent mitosis when DNA replication is incomplete (S-phase checkpoint). To repair DNA during the checkpoint-induced cell cycle arrest, some genes involved in DNA repair are transcriptionally induced (the transcriptional response, reviewed in Bachant and Elledge, 1996). Cells that are defective in either the checkpoint response or the DNA repair response are hypersensitive to DNA damaging agents. The importance of an intact DNA damage response has been revealed by mutations in DNA-damage-response genes that have been implicated in many cancers (Hartwell and Kastan, 1994).
Below, I outline the checkpoint arrest and the transcriptional response to damaged DNA or inhibition of DNA synthesis in budding yeast. I also briefly review the signal transduction pathway and downstream effectors for these two responses to DNA damage.

1a. Cell Cycle Arrest in Response to DNA Damage

In budding yeast, cell cycle delays have been observed in G1/S, G2/M and S phase in response to DNA damage or inhibition of DNA replication. The G2/M checkpoint was the first identified and has subsequently been the most extensively studied DNA damage checkpoint (Hartwell and Weinert, 1989). The first reported observation of the checkpoint phenomenon in any organism was made using the budding yeast rad9 mutant.

The G2 checkpoint response to DNA damage acts to prevent mitosis in the presence of broken or damaged chromosomes (reviewed in Siede, 1995). The G2 checkpoint can be triggered by external DNA damaging agents such as methylmethane-sulfonate (MMS, a DNA alkylating agent), ultraviolet light (UV) and X-ray irradiation, as well as by internally-created damage such as thermo-inactivation of DNA ligase which causes accumulation of unligated DNA fragments (Weinert and Hartwell, 1993).

Like the G2/M checkpoint, a cell cycle delay can be induced at G1/S in response to MMS, UV and X-ray irradiation (Siede et al., 1993). The discovery of the G1/S checkpoint lagged behind the discovery of the G2/M checkpoint (Weinert and Hartwell, 1988) because the G2/M checkpoint is more pronounced than the G1/S checkpoint in two ways. First, the G2 checkpoint-induced delay can last up to 10 hours while the G1/S delay is only 30-60 minutes long. Second, the G2/M checkpoint is more sensitive than
the G1/S checkpoint, requiring a lower threshold of damage for activation (Weinert and Lydall, 1993)

The S-phase checkpoint imposes a cell cycle delay in G2 phase when DNA replication is incomplete (Navas et al., 1995). The S phase checkpoint can be induced by treatment of cells with hydroxyurea or other agents that inhibit DNA synthesis. The cell cycle delay imposed by the S phase checkpoint is thought to prevent chromosome segregation when DNA replication is incomplete. However, a recent report suggests that an important role of the S phase checkpoint is to maintain the DNA synthetic capability of the cell when DNA replication is stressed (Desany et al., 1998). The anaphase arrest induced when DNA synthesis is blocked may be a secondary consequence of the essential function of the S phase checkpoint.

Another checkpoint has been observed to operate during S phase; S phase cells that are subjected to DNA alkylation by MMS undergo a prolonged S phase, presumably to permit repair of the damaged DNA (Paulovich and Hartwell, 1995). This slowing of the ongoing S phase is dependent upon the known checkpoint genes, \textit{RAD53} and \textit{MECI} (see section-2 below).

\textbf{1b. The Transcriptional Response to DNA Damage}

During DNA damage-induced arrest, the cell begins to actively repair the damaged DNA. To facilitate the DNA repair process, a subset of repair genes are transcriptionally induced (reviewed in Bachant and Elledge, 1996). In budding yeast, these genes include \textit{RNR1}, \textit{RNR2}, \textit{RNR3} (subunits of ribonucleotide reductase, Elledge and Davis, 1987; Elledge and Davis, 1990), \textit{RAD54} (recombinational repair, Johnston
and Johnson, 1995), POLI (DNA Polymerase I, Johnston et al., 1987) and CDC9 (DNA ligase, Peterson et al., 1985). The importance of the transcriptional activation of repair genes became evident with the isolation of a mutant, dun1 (Zhou and Elledge, 1993), which is defective for DNA damage-induced transcription and is hypersensitive to DNA damaging agents such as methylmethanesulfonate (MMS) and UV-irradiation (UV).

2. SIGNAL TRANSDUCTION FOR THE DNA DAMAGE RESPONSE

Recent studies have begun to delineate a pathway by which the damage signal is transduced to the checkpoint and transcriptional response apparatus (Figure 2-1). When DNA damage or incomplete replication is sensed, the related kinases, Mec1 and/or Tel1 phosphorylate Rad9 to activate the G1 or G2 checkpoint. Phosphorylation of an as yet unidentified Rad9 analogue has been proposed for activation of the S phase checkpoint. DNA polymerase ε is a good candidate for this RAD9 analogue since it is required only for the S phase checkpoint, and it is believed to act as the sensor for stalled replication (Navas et al., 1995). Sun et al. (1998) found that phosphorylated Rad9 associates with the C-terminal FHA (forkhead-associated) domain (FHA2) in the Rad53 serine/threonine kinase. Analysis of rad53 mutants suggests that the C-terminal FHA2 domain mediates the G1 and G2 checkpoint responses, while an FHA domain at the N-terminus (FHA1) is believed to bind an unidentified Rad9 analogue to mediate the S phase response. Rad9 may bind Rad53 to mediate Mec1/Tel1 phos-phorylation of Rad53 since phosphorylation of Rad53 is dependent on RAD9 as well as MEC1/TEL1 (Navas et al., 1996; Sanchez et al., 1996; Sun et al., 1996). RAD9, and the kinases MEC1/TEL1 and RAD53, are required
Figure 2-1: Signal Transduction Pathway for DNA Damage in *S. cerevisiae*.

DNA damage in G1 or G2 phase of the cell cycle causes phosphorylation on Rad9 that is dependent on the ATM-like kinases, Mec1 and Tel1. Phosphorylation on Rad9 promotes its binding to the FHA2 domain (forkhead associated) on the Rad53 serine/threonine kinase. The signal responsive to DNA damage during S phase or to inhibition of DNA synthesis has been proposed to be transduced through an unidentified Rad9 analogue that binds the FHA1 domain in the C-terminus of Rad53. The binding of Rad9 to Rad53 may promote Mec1/Tel1 phosphorylation of Rad53. Rad53 may mediate G1 arrest through phosphorylation of Swi6 to down-regulate expression of G1 cyclins. Pds1 is an inhibitor of the metaphase-to-anaphase transition and may be the effector for G2 arrest following DNA damage. Dun1 is a serine/threonine kinase that is required only for the transcriptional response. It may phosphorylate the Crt1 transcriptional repressor to inactivate its repression of *RNR2* and *RNR3* transcription. There is no evidence placing *PDS1* or *DUN1* directly downstream of *RAD53*. Rather, *PDS1* has been suggested to be in a parallel pathway with *RAD53* (see text).
for both the checkpoint and transcriptional responses (Allen et al., 1994; Weinert and Hartwell, 1993; Weinert et al., 1994). Rad53 is responsible for phosphorylating the downstream effector(s) for at least some aspects of the checkpoint response. One study reported that Rad53 may phosphorylate Swi6 to delay G1 progression by downregulating CLNI and CLN2 transcription (Sidorova and Breeden, 1997).

The transcriptional response shares much of the same upstream signal transduction pathway as the checkpoint pathway. The divergence between the checkpoint and transcriptional response pathways is at the level of the serine/threonine kinase, DUN1, which is required only for transcriptional induction of repair genes and not for checkpoint delays, and has therefore been placed genetically downstream of RAD53. The observation that damage-induced Dun1 autophosphorylation is dependent on RAD53 supports the genetic data placing DUN1 downstream of RAD53 (Zhou and Elledge, 1993, see Figure 2-1).

3. EFFECTORS FOR CHECKPOINT-MEDIATED CELL CYCLE ARREST

There appear to be multiple mechanisms for effecting DNA damage-induced cell cycle arrest at different phases of the cell cycle. Although a lot is known about the signal transduction pathway in budding yeast, our understanding of the effector mechanisms in S. cerevisiae for cell cycle arrest lags behind our understanding of the fission yeast and human models.
3a. Inhibition of Mitosis in Response to DNA Damage by Pds1

Until recently, the effectors for cell cycle arrest in budding yeast have remained elusive. Work with the anaphase promoting complex (APC) has suggested that inhibition of the transition from metaphase to anaphase may be responsible for DNA damage-induced mitotic arrest in budding yeast. The Pds1 protein acts as an inhibitor of sister chromatid separation and is degraded by the APC at the metaphase-to-anaphase transition (Ciosk et al., 1998). Mutants defective in PDS1 exhibit normal mitotic arrest during DNA replication blocks but are defective for the arrest in response to DNA damage (Yamamoto et al., 1996). These data suggest that Pds1 acts as the effector for mitotic arrest in response to DNA damage but not to blocks in DNA replication. Molecular analysis of Pds1 revealed that the protein is phosphorylated after DNA damage but not in response to inhibition of DNA replication; this damage-dependent phosphorylation is dependent on MEC1 and RAD9 but not RAD53 (Cohen-Fix and Koshland, 1997). These studies suggest that PDS1 is an effector for mitotic arrest during DNA damage, and that it lies downstream of MEC1/RAD9, but on a parallel pathway to RAD53.

3b. Effectors for Cell Cycle Arrest in Humans and Fission Yeast

Our understanding of the effectors for cell cycle arrest is more advanced in human cells and fission yeast. The study of human cells has yielded a model for G1 arrest and two mechanisms for G2 arrest in response to DNA damage. Studies in S. pombe have uncovered mechanisms for mitotic arrest in response to both DNA damage and inhibition of DNA synthesis.
3b. i. **G1 cell cycle arrest in humans**

In human cells, DNA damage in G1 causes the stabilization of the p53 protein by the Mec1/Tel1-related ATM kinase (Kastan et al., 1992). The p53 transcription factor then activates expression of the p21 gene and GADD45, which together act to prevent the onset of S-phase. The p21 protein inhibits G1 Cdns, and both p21 and GADD45 inhibit DNA synthesis through inactivation of PCNA (Harper et al., 1993; Kastan et al., 1992; Smith et al., 1994). The p53 and ATM genes are often mutated in cancerous cells, stressing the importance of the checkpoint response in tumorigenesis (Dulic et al., 1994; Hartwell and Kastan, 1994; Savitsky et al., 1995).

3b. ii. **The role of Tyr-15 in human and *S. pombe* Cdns in mediating cell cycle arrest**

In both fission yeast and human cells, cell cycle arrest in G2 is mediated by inhibition of the Cdc2 kinase by phosphorylation on tyrosine-15. As mentioned in Chapter 1, phosphorylation of Tyr-15 on Cdns leads to inhibition of kinase activity. In fission yeast, inhibition of cdc2 by Tyr-15 phosphorylation mediates mitotic arrest in response to both DNA damage and replication blocks (Enoch and Nurse, 1990; Rhind et al., 1997) In humans, phosphorylation of CDC2 has only been demonstrated to mediate the G2 DNA damage checkpoint arrest (Jin et al., 1996).

Recently, inhibition of the Cdc25 phosphatase was shown to be important for the G2 DNA damage checkpoint arrest in fission yeast and human cells. As mentioned briefly in Chapter 1, Cdc25 is a dual-specificity phosphatase that removes the inhibitory phosphate on Tyr-15 on Cdns, thereby leading to their activation. In response to DNA damage in *S. pombe* and human cells, the protein kinase Chk1 phosphorylates and inhibits the Cdc25 phosphatase, thereby preventing activation of Cdc2 (Furnari et al., 1997; Sanchez et al., 1997).
3c. The Role of CDC28 in *S. cerevisiae* Checkpoint Arrest

Although Tyr-15 phosphorylation on Cdc2 clearly plays a role in checkpoint-mediated cell cycle arrest in fission yeast and human cells, mutation of the equivalent tyrosine-19 residue on Cdc28 does not abolish checkpoint-mediated arrest caused by DNA damage (Amon et al., 1992; Sorger and Murray, 1992). Rather, tyrosine-19 phosphorylation plays a role in the budding morphogenesis checkpoint (Lew and Reed, 1995). Moreover, deletion of the Cdc28 inhibitor *SIC1* does not confer sensitivity to DNA damaging agents or to replication blocks (Nugroho and Mendenhall, 1994), and cyclin overproduction does not interfere with checkpoint function (Stueland et al., 1993). Two studies further suggest that inhibition of Cdc28 is not important for cell cycle arrest. First, Cdc28-associated H1 kinase activity remains high during the checkpoint delay (Nugroho and Mendenhall, 1994). Second, study of the *cdc28-5M* allele suggested a novel role for CDC28 in the budding yeast DNA damage checkpoint (Li and Cai, 1997). This study found that the *cdc28-5M*, and other *cdc28* temperature sensitive alleles, were defective for checkpoint function at the non-permissive temperature. What was surprising was that the *cdc28-5M* mutant strain had reduced Cdc28 kinase activity at the non-permissive temperature. Also, the *cdc28-5M* allele was recessive. This is contrary to the situation expected if inhibition of Cdc28 were required for checkpoint function; in such a case, one would expect high Cdc28 kinase activity in a checkpoint-defective mutant at the restrictive temperature, and one would expect that the *cdc28* mutation would be dominant, not recessive. Rather, these data suggest that CDC28 may actually play an active role in mediating the cell cycle arrest.
4. EFFECTORS FOR THE TRANSCRIPTIONAL RESPONSE
TO DNA DAMAGE

A number of studies have uncovered promoter elements that either confer or are required for DNA damage-induced gene expression (reviewed in Bachant and Elledge, 1996). The regulation of the various damage-inducible promoters does not appear to be related, suggesting that there are numerous DNA elements and numerous DNA-binding regulators that mediate the transcriptional response at different promoters. Except for the Crt1 repressor of RNR2/3 transcription, most of these promoter studies do not tie into the signal transduction pathway outlined in Figure 2-1.

Analysis of the RAD2 (exonuclease I) promoter revealed two promoter sequences that were necessary for the transcriptional induction of RAD2 expression after treatment with UV (Siede et al., 1989). The two related sequences, DRE1 and DRE2 (damage responsive element), are bound by a protein or protein complex in uninduced cells, but the relative amounts of protein-bound DNA increases after exposure to DNA damage (Siede and Friedberg, 1992).

A 29 base pair element was identified in the RAD54 (recombinational repair) promoter that was required for full gene induction in response to damage by UV, X-rays and MMS (Cole and Mortimer, 1989). Unlike the DREs in the RAD2 promoter, this element did not affect vegetative expression, suggesting that it specifically plays a role in DNA damage induction.

In the PHRI (photolyase) promoter, a repressor, PRP (photolyase regulatory protein), acts to repress gene expression in vegetative cells, but after UV treatment, Prp DNA-binding activity quickly disappears, and PHRI expression is induced (Sebastian
and Sancar, 1991). The 39 base pair Prp-binding site is sufficient to confer repression and damage-inducibility on a heterologous promoter.

For the RNR2 gene (encodes subunit for ribonucleotide reductase), both activation and relief of repression account for its damage-dependent induction (Elledge and Davis, 1989). Promoter analysis on the RNR2 gene uncovered a 42 base pair DRE (damage responsive element) that contains both URS sequences (upstream regulatory sequences that mediate gene repression) and UAS sequences (upstream activating sequences that activate gene expression). Although the RNR2 DRE can confer damage inducibility on a heterologous promoter, it gave a lower induction than the whole, native promoter, suggesting that there are additional damage-responsive elements outside the DRE.

The transcriptional repressor Crt1 operates through promoter elements in the RNR2 and RNR3 promoters (Huang et al., 1998; Zhou and Elledge, 1992). Crt1 is a DNA-binding protein which recruits the Ssn6-Tup1 corepressor complex. The Crt1 protein is phosphorylated in response to DNA damage, and the phosphorylated protein no longer binds to DNA. This suggests that phosphorylation of Crt1 by an upstream kinase, likely Dun1, activates transcription of target genes by inactivating the Crt1 repressor. Although numerous promoter elements and transcription factors regulate the expression of various damage-induced genes in S. cerevisiae, only Crt1 has been linked to an upstream kinase (ie. DUN1) in the signal transduction pathway (Figure 2-1).

5. THE HRR25 KINASE AND DNA DAMAGE

Cells mutated for the gene encoding the Hrr25 kinase are hyper-sensitive to double-stranded DNA breaks induced by endonuclease expression, X-irradiation or
continuous exposure to MMS (Hoekstra et al., 1991). Hrr25 is a casein kinase 1 isoform that has dual-specific protein kinase activity in vitro (Hoekstra et al., 1994). Like other casein kinase isoforms, Hrr25 clearly has multiple roles in vivo. In addition to the apparent defects in DNA double-strand break repair, hrr25 mutant cells sporulate poorly, are very slow growing and show a G2 cell cycle delay among other phenotypes (Hoekstra et al., 1991). Moreover, kinase assays on Hrr25 immunoprecipitates from yeast extracts show phosphorylation of many co-immunoprecipitated proteins, suggesting that Hrr25 may have multiple substrates in vivo (DeMaggio et al., 1992). Prior to my study, a role for HRR25 in the transcriptional or checkpoint response to DNA damage had not been reported.

I report that Hrr25 interacts with and phosphorylates the Swi6 protein in vitro. I find that hrr25Δ mutants are defective in the transcriptional induction of the RNR2 and RNR3 genes in response to inhibition of DNA replication and to DNA damage. In addition to defining a biochemical interaction between Hrr25 and Swi6, I report that, like cells deleted for HRR25, both swi6 and swi4 mutants are sensitive to DNA-damaging agents and show defective damage-induced transcription of RNR2 and RNR3. My observations suggest a novel role for the SBF complex (Swi4/Swi6), through its interaction with the Hrr25 protein kinase, in the transcriptional response to DNA damage.
MATERIALS AND METHODS

Yeast Strains

All yeast strains used for plating assays and northern blot analysis were isogenic to strain JO34 (MATa, ura3-52, lys2-801a, ade2-1070, his3Δ200, leu2-Δ1, SCB-LacZ) with the exceptions noted. The swi4Δ strain (swi4ΔHIS3) and the swi6Δ strain (swi6ΔHIS3) have been described previously (Andrews and Moore, 1992; Ogas, 1992). The mbp1Δ (mbp1ΔTRP1) allele is a complete deletion of the MBP1 coding sequences and was constructed by amplification of MBP1 flanking sequences from an MBP1 genomic clone by inverse polymerase chain reaction using primers designed to anneal to nucleotides -20 to -1 and 2477-2497 of MBP1 (numbering relative to the A in the ATG initiator codon). The blunted PCR fragment was ligated to a SmaI-StuI restriction fragment carrying the TRP1 gene (Jones and Prakash, 1990) to create plasmid pBA884. The 2.8 kB mbp1ΔTRP1 disruption cassette was obtained from pBA884 by digestion with NotI and SalI and used to transform strain BY263 (trp1Δ63, GAL2+, otherwise isogenic to JO34). The hrr25Δ deletion strain was made by transformation of a diploid derivative of strain BY263 with an hrr25Δhis::URA3 disruption allele (Hoekstra et al., 1991). The diploid was sporulated and meiotic progeny deleted for HRR25 recovered by tetrad dissection (Sherman and Hicks, 1991). For plating assays and northern blot experiments, yeast strains were transformed with either vector Yep24 (URA3-2-micron) or with a high copy SWI4 plasmid, pBA314. The SWI4 plasmid was constructed by cloning a XhoI-SalI fragment containing SWI4 and flanking sequences from clone B3.2.
(Andrews and Herskowitz, 1989) into the *SalI* site of pUC18Bgl2 (a derivative of pUC18 with *BglII* linkers inserted at the *EcoR1* and *HinDIII* sites) to create plasmid pBA313. The *SWI4* gene was then isolated on a *BglII* fragment and cloned into BamHI-digested Yep24 to create plasmid pBA314. Other yeast strains are described in the relevant sections below.

**Expression and purification of recombinant Swi6**

To construct a bacterial Swi6 expression vector, 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′CCGCCATGCGTTGG-3′ and 5′CCGTCTCATTGTCATCAGTGCC. The 630 bp PCR product was digested with *NcoI* and *ApaI* and cloned into *NcoI-ApaI*-digested pSL1180 (Pharmacia) to create plasmid pBA786. An *ApaI-BglII* fragment carrying the remainder of the *SWI6* gene was then cloned into *ApaI-BglII*-digested pBA786 to reconstitute the entire gene (pBA788). The *NcoI-BglII* fragment containing the *SWI6* gene from pBA788 was then cloned into *NcoI-BamHI* digested pET19b (Novagen) to create pBA789. Swi6 protein was produced from pBA789 in *E. coli* strain BL21(DE3) [from Novagen] and purified as described (Sidorova and Breeden, 1993) except that expression was induced with 0.5 mM IPTG for 6 hours (30°C) and the final fractionation was over DEAE-Sephacel resin. The purified protein was dialyzed into SB buffer (20mM HEPES pH 7.2, 10% glycerol, 0.1mM DTT, 0.1mM PMSF) with 80mM CaCl₂ for coupling onto AffiGel-10 resin as described below. For kinase assays, Swi6 was dialyzed into 15 mM HEPES pH7.5, 100 mM NaCl, 10 mM MgCl₂, 5% glycerol, 0.1 mM PMSF, 1 mM dithiothreitol.
Protein Affinity Chromatography

Purified Swi6 protein was coupled to AffiGel-10 resin (Bio-Rad) according to the manufacturer's recommendations. Based on the protein concentration of the protein solution before and after coupling, the concentration of coupled protein on the resin was calculated to be 40 μM. For the preparation of yeast extracts, yeast cells (strain BJ2168, a ura3-52 leu2 trp1 prb1-122 pep4-3 prcl-407) were grown to mid-log phase in YPD medium (Kaiser et al., 1994). The cells were then lysed in Lysis Buffer (100mM Tris-HCL pH 8.0, 100mM NaCl, 10mM MgCl2, 1mM EDTA, 10% glycerol, 1mM dithiothreitol, 20mM NaF, 50mM β-glycerophosphate, 2mM benzamidine, 2mg/mL aprotinin, 2mg/mL leupeptin, 1mg/mL pepstatin, 1mM PMSF) using agitation in the presence of glass beads. For small-scale experiments, cells from 0.5-1L cultures were either vortexed with glass beads in 15 mL Sarstedt tubes or lysed in a BioSpec mini-bead-beater. For preparative-scale chromatography, 1.6 g of protein extract were prepared from 30 g of wet cell pellet by lysing with 10 X 20 second bursts in the mid-sized chamber of a Bio-Spec Beadbeater. After lysis, extracts were centrifuged at 100 000g for one hour and passed over Swi6 affinity columns. In a typical analytical experiment, approximately 4 mg of protein extract were loaded onto 20μL micro-columns. For preparative chromatography, the clarified supernatant was loaded onto a 0.5mL column of the Swi6-coupled resin that had been sequentially washed and equilibrated in SB with 1M NaCl (SB-1000) and 100mM NaCl (SB-100). The column was then washed in 10 column volumes of SB-100, and eluted with SB-1000. Analytical affinity chromatography experiments with hrr25 deletion strains were done with strain 7D (hrr25Δ) and an isogenic wild type strain (W303, DeMaggio et al., 1992).
Microsequencing of p54(Hrr25)

p54 purified by Swi6 protein affinity chromatography was prepared for microsequencing as described (Collins et al., 1995). In brief, p54 was excised from a preparative SDS-polyacrylamide gel after soaking the gel slice for 1 hour in water. The gel slice was cut into small pieces and washed with 50% methanol for 20 minutes, decanted and dried briefly under vacuum. Proteins were digested with 500ng of Achromobacter protease I in 0.1 M Tris-HCl (pH 9.0), 0.1% Tween-20 for 24 hours at 30°C. Peptides were extracted from the gel with 50% acetonitrile, 0.06% trifluoroacetic acid (TFA) and separated with HPLC using a Vydac C18 column (2.1mm X 250 mm) and eluted with a gradient of acetonitrile:isopropanol (3:1) in 0.09% TFA. Peptides were sequenced using an automated protein sequencer (Applied Biosystems 470, 473 and 477).

Kinase Assays with affinity column eluates

Hrr25 kinase assays with the column eluates were done in a reaction buffer containing 15mM HEPES pH7.5, 200 mM NaCl, 25mM MgCl2, 1mM dithiothreitol, 1 μM ATP and 10 μCi of γ-32P ATP (Dupont). 1/50 of the eluate from a micro-column was added per reaction. Kinase reactions also contained 100ng Swi6 protein (see above), myelin basic protein (Sigma), Histone H1 (Boehringer Mannheim) or casein (Sigma) as indicated. Reactions were stopped after 15 minutes at 30°C with SDS sample buffer and boiled before electrophoresis on SDS-polyacrylamide gels. The gels were dried and exposed to XAR-5 film (Kodak).

HA-Hrr25 IP kinase assay

Immunoprecipitation-kinase assays with HA-tagged Hrr25 were done essentially as described (Tyers et al., 1992) with modifications. Strain JO34 (wildtype) was
transformed with either HA-tagged Hrr25 (gift from Merl Hoekstra) or vector pRS316 (Sikorski and Hieter, 1989) and grown in selective medium (Kaiser et al., 1994) to maintain the plasmid. Cells were harvested in early log phase and lysed in IPK buffer (50mM Tris-HCl pH 7.5, 1% NP-40, 0.05% SDS, 0.05% sodium deoxycholate, 5mM EDTA, 5mM DTT, 100mM NaCl with protease/phosphatase inhibitors as in Lysis Buffer). HA-Hrr25 was immunoprecipitated from the extracts with monoclonal antibody 12CA5 (Wilson et al., 1984), washed twice in IPK buffer, twice in IPK buffer with 1M NaCl without inhibitors and twice in kinase buffer (Tyers et al., 1992). Where indicated, Swi6 and casein were added to 100ng per kinase reaction.

**Plating assay for sensitivity to DNA-damaging agents**

For viability assays, cells were grown to early log phase in minimal medium. The cells were harvested, washed twice and resuspended in 100mM KH2PO4 pH 7.5. The cell suspension was then briefly sonicated and counted using a hemacytometer. Cells were plated at densities of 100, 500, 5000, 50 000 and 500 000 (for hrr25A mutants) per plate onto SD minimal plates (Kaiser et al., 1994) containing either no drugs, 100mM hydroxyurea (Sigma), 200mM hydroxyurea, 0.01% MMS (Sigma) or 0.02% MMS. The plates were incubated at 30°C until full-size colonies appeared on the lowest dilution plates. The percent viability (Table 2) was calculated as the percentage of viable colonies on drug-containing plates versus non-drug containing plates. In calculating viability, plates containing at least 200 colonies were used in the calculations where possible.

**S phase checkpoint assay**

The S-phase checkpoint assay was done basically as described (Navas et al., 1995). The wild type and swi4A strains were synchronized by arresting the cells in late
G1 with the mating pheromone, α-factor, followed by release at 25°C into YPD medium or YPD with 200mM hydroxyurea. Cultures were harvested at 30, 60, 75 and 90 minutes after release from α-factor for anti-tubulin immunofluorescence (described below). The WT strain in the absence of hydroxyurea underwent spindle elongation at 60 min, and the swi4Δ strain underwent spindle elongation at 90 min. Figure 2-4 shows the data from the 60 min. sample for wild type and the 90 min. sample for the swi4Δ sample.

**Swi6 cellular localization after MMS treatment**

Wild type JO34 cells overexpressing Swi6 from the 2µ high copy plasmid, YEpSWI6 (Sidorova and Breeden, 1993) were grown to early log phase in minimal media without leucine to maintain the plasmid. MMS was added to 0.05%. Samples were collected at 15 min. time-points for α-Swi6 immunofluorescence (see below). Figure 2-7 is from cells treated with MMS for 60 minutes. MMS-treated cells were washed and resuspended in 100 mM KPO4 pH 7.4 before fixing in formaldehyde as described below.

**Pre-adsorbing antibodies for immunofluorescence**

The α-Swi6 polyclonal antibody (prepared as in Sidorova and Breeden, 1993) was pre-adsorbed against JO42 (swi6Δ) cells; the secondary goat-α-rabbit and the tertiary sheep-α-goat, CY3 conjugated antibody (the pre-adsorbed secondary and tertiary antibodies were gifts from Kevin Madden c/o M. Snyder lab) were pre-adsorbed against S288C (WT) cells. 500mL of culture was grown in YPD to mid-log phase. The cells were then fixed, washed and digested with zymolase as outlined below (indirect immunofluorescence). The digested spheroplasts were washed twice in PBS, 1.2M sorbitol, and an equal volume of undigested, whole cells was added. 200µL of antibody was added to an equal volume of the spheroplast/whole cell mixture and nutated at 4°C for 1 hour. The
antibody supernatant was recovered by centrifugation. This process was repeated 4 times with fresh spheroplast/whole cell mixture.

**Indirect immunofluorescence**

Immunofluorescence of Swi6 was done as follows: cells were prepared for immunofluorescence essentially as described (Gehrung and Snyder, 1990; Pringle et al., 1991) with modifications. Harvested cells were fixed by addition of formaldehyde to 3.7%, followed by incubation for 60 min. at 30°C with shaking. The fixed cells were washed with 100mM KH₂PO₄ pH 7.4 and resuspended in 100mM KH₂PO₄ pH 7.4, 0.1% β-mercaptoethanol, 0.25mg/mL zymolyase 20 000T, followed by incubation at 30°C for 30 min. to digest the cell wall. The cells were then washed gently and resuspended in phosphate-buffered saline (PBS) containing 1.2 M sorbitol. One drop of the cell suspension was placed in a well of a polylysine-coated slide (Flow Labs). Once cells had settled, the bound cells were rinsed once with PBS/0.1% bovine serum albumin (BSA), once with PBS/0.1% BSA/0.1% NP-40 and once with PBS/0.1% BSA. Cells were blocked for ½ hour in PBS/2% BSA, then incubated overnight at 4°C in 1/500 dilution of affinity purified and preadsorbed α-Swi6 antibody (described above). The cells were then washed once with PBS/0.1% BSA, once with PBS/0.1% BSA/0.1% NP-40 and once with PBS/0.1% BSA. A 1:10 dilution of pre-adsorbed goat-α-rabbit secondary antibody was added and incubated for 2 hours at room temperature. The cells were then washed in the same manner as after the primary antibody incubation. The washed cells were then incubated with a 1:100 dilution of the pre-adsorbed sheep-α-goat, CY3-conjugated tertiary antibody for 1 hour at room temperature. The cells were then washed once in PBS/0.1%BSA, twice in PBS/0.1%BSA/ and once in PBS/0.1% BSA. The stained cells
were mounted in 90% glycerol in PBS containing 0.1% p-phenylene diamine and 0.05 μg/mL diamidinophenylindole (DAPI). Cells were observed at 1000X magnification using Nomarski optics and a CCD camera mounted on a Leica DM-LB microscope. Images were captured on Kodak Tri-X 400 film and ‘push-processed’ as 800 film.

Immunofluorescence with the anti-tubulin was done as above for Swi6, except that no tertiary antibody was used. The anti-tubulin antibody (YOL 1/34) was from Accurate Chemical and Scientific Corp., and the goat-anti-mouse, FITC-conjugated secondary antibody was from Sigma. Both antibodies were used according to the manufacturer’s recommendations.

**Northern blot analysis**

Cultures for RNA extraction were grown in minimal media to early log phase. An aliquot of cells was taken from the culture (0 min. time point) and hydroxyurea was then added to a final concentration of 200mM. For each time point, 15 mL of cells were harvested and total RNA was prepared as described (Kaiser et al., 1994) with some modifications. In short, cell samples were resuspended in LETS buffer (100mM LiCl, 10mM EDTA, 10mM Tris pH7.4, 0.2% SDS, 0.1% diethyl-pyrocarbonate [buffered Tris HCl was added after overnight incubation of the solution with diethyl pyrocarbonate]) and lysed by agitation in the presence of glass beads and phenol. The aqueous layer was washed twice with phenol, precipitated with LiCl and ethanol and resuspended in DEPC-treated water. RNA was quantified by absorbance at 260nm, and 20-30μg was loaded onto a 1.4% agarose, 6% formaldehyde, 1X MOPS gel for electrophoresis. The RNA was transferred onto Hybond N+ membrane (Amersham) by capillary action. Pre-
hybridization and hybridizations were done in 1.5X SSPE, 10% PEG 8000, 7% SDS, 0.1mg/mL sheared salmon sperm DNA at 65°C. Blots were washed in 2X SSC, 0.1% SDS at room temperature, followed by a 0.2X SSC, 0.1% SDS wash at room temperature or at 42°C if needed. The blots were exposed to Kodak XAR-5 film for autoradiography and quantitated using a phosphorimager (Molecular Dynamics) and ImageQuant 3.33 software. The probes used for northern analysis have been described. RNR2 and UBI4 probes are described in Allen et al. (1994); RNR3 in Elledge and Davis (1990) and ACTI in Harrington and Andrews (1996).
RESULTS

Binding of Hrr25 to Swi6 Protein Affinity Columns.

To identify proteins that may regulate Swi6 activity, I used protein affinity chromatography to look for proteins in crude yeast extracts that physically associate with Swi6. To do this, I compared the profile of proteins from a crude yeast extract that were retained on a Swi6 affinity resin to those proteins bound by the resin alone. I detected a 54 kDa protein (p54) that bound specifically to the Swi6 column but not to the control column (Figure 2-2A). Microsequencing of purified p54 yielded two peptide sequences that showed a perfect or near perfect match with the published amino acid sequence of Hrr25, a dual-specificity casein kinase 1 (CK1) isoform (Table 2-1, Hoekstra et al., 1991). I confirmed that p54 was indeed Hrr25 in two ways. First, I found that p54 was absent in eluates from a Swi6-affinity column when the extracts were derived from an hrr25A strain (Figure 2-2B). Second, antibodies raised against Hrr25 (DeMaggio et al., 1992) recognized a 54 kDa band in Swi6 column eluates but not in control column eluates (data not shown). I conclude that Swi6 and Hrr25 are able to form a specific protein complex in vitro.

Swi6 is a substrate for the Hrr25 protein kinase in vitro.

Although HRR25 shows strong homology to known casein kinase 1 isoforms (68% identity and 86% similarity across kinase domain) and has been shown to have casein kinase activity in vitro (DeMaggio et al., 1992), biologically relevant substrates
Figure 2-2: Binding of p54(Hrr25) to Swi6 Protein Affinity Columns.

(A) Yeast extracts from a protease-deficient strain (BJ2168) were loaded onto either a control column (no coupled protein, lane 1) or a Swi6-coupled column (lane 2) and eluted with 1M NaCl. (B) Extracts made from either a wild-type (lanes 1 and 2) or an hrr25Δ strain (lanes 3 and 4) were loaded onto either a control column (lanes 1 and 3) or Swi6-coupled columns (lanes 2 and 4) and eluted with 1M NaCl. Protein molecular weight markers are indicated to the left of the gel photographs, and p54 (Hrr25) is indicated by the arrow on the right.
Table 2-1: Comparison of the Sequenced p54 Peptides to Published Sequences of *HRR25*

<table>
<thead>
<tr>
<th>Peptide 1</th>
<th>Peptide 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGSGSFGDIYHGTNLISGEEVAI</td>
<td>DLNANSNAAS?K</td>
</tr>
<tr>
<td><em>HRR25</em> (a.a.(^1) 15-37)</td>
<td><em>HRR25</em> (a.a.(^1) 312-323)</td>
</tr>
<tr>
<td>IGSGSFGDIYHGTNLISGEEVAI</td>
<td>DLNANSNAASAS</td>
</tr>
</tbody>
</table>

\(^1\)amino acids
Figure 2-3: *In Vitro* Phosphorylation of Swi6 by Hrr25.

(A) Kinase assays were done on eluates from Swi6 affinity columns or from control columns (no coupled protein). The presence of Swi6 on the column resin is indicated by a '+' above the lane while '-' denotes the control column with no coupled protein. The columns were loaded with extracts from either a wild-type (lanes 1-7) or hrr25Δ strain (lanes 8, 9) as indicated above the photograph ("extract applied on column"). Exogenous substrate (100 ng) was added to the kinase assays as indicated above the lanes (mbp=myelin basic protein, H1=Histone H1). (B) Kinase assays were done with 12CA5 (anti-HA) immunoprecipitates from yeast cells expressing an HA-Hrr25 fusion protein (lanes 1, 3) or cells transformed with an empty vector (lane 2). In lanes 2 and 3, 100ng of casein and Swi6 were added to the kinase reaction as indicated by (+) above the lanes. The positions of migration for phosphorylated Swi6, Hrr25 and casein are indicated on the right. Molecular weight markers are shown on the left.
have not been identified. I tested whether the interaction between Hrr25 and Swi6 might reflect the fact that Swi6 is a substrate for the Hrr25 kinase; I used Swi6 affinity column eluates as a source of the Hrr25 protein for in vitro kinase assays. I found that a kinase activity that was specific to the Swi6-column eluates could efficiently phosphorylate recombinant Swi6 protein in vitro (Figure 2-3A, lane 3). The kinase activity towards Swi6 was dramatically reduced in eluates from a resin-only control column and absent in column eluates derived from extracts prepared from an hrr25Δ strain (Figure 2-2A, lanes 2 and 8). The kinase activity seen in the Swi6-column eluates phosphorylated casein but not myelin basic protein (mbp) or Histone H1 (Figure 2-2A, lanes 5-7). The phosphorylation of both casein and Swi6 was inhibited by CK1-7, a specific inhibitor of human casein kinase I (data not shown, Chijiwa et al., 1989). Swi6 was also phosphorylated by Hrr25 kinase immunoprecipitated from yeast extracts with a hemagglutinin tag (Figure 2-2B). My data demonstrate that Swi6 is a substrate for the Hrr25 protein kinase in vitro.

Both SBF (swi4Δ, swi6Δ) and hrr25Δ Mutants Show Sensitivity to the DNA-Damaging Agent, Methylmethanesulfonate (MMS), and the DNA Synthesis Inhibitor, Hydroxyurea (HU)

Because deletion of HRR25 is known to cause sensitivity to DNA damaging agents (Hoekstra et al., 1991), I wondered whether the interaction between Hrr25 and Swi6 might reflect a role for Swi6 in the cellular response to DNA damage. To test this hypothesis, I assayed the sensitivity of swi6Δ, swi4Δ, mbp1Δ and hrr25Δ mutants to continuous
Table 2-2A: Sensitivity of \textit{swi4A}, \textit{swi6A} and \textit{mbp1A} Mutants to Hydroxyurea and MMS

<table>
<thead>
<tr>
<th>Mutant</th>
<th>100mM HU*</th>
<th>150mM HU*</th>
<th>0.01% MMS</th>
<th>0.02% MMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>69 %</td>
<td>64 %</td>
<td>63 %</td>
<td>6 %</td>
</tr>
<tr>
<td>\textit{swi4A}</td>
<td>0 %</td>
<td>0 %</td>
<td>0 %</td>
<td>0 %</td>
</tr>
<tr>
<td>\textit{swi6A}</td>
<td>0 %</td>
<td>0 %</td>
<td>4 %</td>
<td>0 %</td>
</tr>
<tr>
<td>\textit{mbp1A}</td>
<td>88 %</td>
<td>78 %</td>
<td>42 %</td>
<td>4 %</td>
</tr>
<tr>
<td>\textit{swi6A} \textit{2\mu SWI4}</td>
<td>53 %</td>
<td>36 %</td>
<td>22%</td>
<td>0%</td>
</tr>
</tbody>
</table>

The strains above were grown to early log phase in minimal media, harvested, washed and resuspended in phosphate buffer before plating onto minimal plates containing either hydroxyurea or MMS in the concentrations shown. The sensitivity of cells to drug-containing plates was calculated from the number of colonies that arose on HU- or MMS-containing medium divided by the number of colonies that arose from an equal number of cells plated on non-drug containing plates (* HU=hydroxyurea, %=percentage viability).

Table 2-2B: Sensitivity of \textit{hrr25A} Mutants to Hydroxyurea and MMS

<table>
<thead>
<tr>
<th>Mutant</th>
<th>100mM HU*</th>
<th>150mM HU*</th>
<th>0.01% MMS</th>
<th>0.02% MMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>94 %</td>
<td>92%</td>
<td>90%</td>
<td>3%</td>
</tr>
<tr>
<td>\textit{hrr25A}</td>
<td>0 %</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>\textit{hrr25A} \textit{2\mu SWI4}</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

The strains above were grown to early log phase in minimal media, harvested, washed and resuspended in phosphate buffer before plating onto minimal plates containing either hydroxyurea or MMS in the concentrations shown. The sensitivity of cells to drug-containing plates was calculated from the number of colonies that arose on HU- or MMS-containing medium divided by the number of colonies that arose from an equal number of cells plated on non-drug containing plates (* HU=hydroxyurea, %=percentage viability).
exposure to MMS or hydroxyurea in a plating assay. \( hrr25A \) mutant strains were previously shown to be sensitive to the continuous presence of MMS while \( swi6A \) strains have been shown to exhibit reduced viability after a transient exposure to MMS (Johnston and Johnson, 1995). Sensitivity of these mutants to hydroxyurea treatment had not been previously tested. MMS is a DNA-alkylating agent that is known to cause DNA strand breaks (Dhillon and Hoekstra, 1994) while hydroxyurea inhibits DNA synthesis through inhibition of ribonucleotide reductases (Harder and Follmann, 1990). Both agents are known to elicit transcriptional induction of DNA repair genes, most notably the \( RNR \) genes (ribonucleotide reductase genes, reviewed in Elledge et al., 1992). I found that both the \( swi6A \) and \( hrr25A \) strains show a marked decrease in viability compared to wild-type when plated onto media containing either MMS or hydroxyurea (Table 2-2). In addition, I found that \( swi4A \) but not \( mbp1A \) mutants were sensitive to growth in the presence of MMS and HU. The viability of the \( mbp1A \) mutant had also previously been observed to be unaffected after an exposure to MMS that was transient (Johnston and Johnson, 1995). The drug sensitivity of the \( swi4A \) and \( swi6A \) strains is not a secondary consequence of an unusual cell cycle distribution of cells in the culture since, in the strain background I used, neither mutant showed an abnormal proportion of cells with 1N and 2N DNA content in minimal media, as assayed by FACS analysis (fluorescence-activated cell sorter; data not shown). As described earlier, Swi6 binds to DNA through a DNA-binding subunit, forming either the SBF complex with Swi4 or the MBF complex with Mbp1. My data suggest that the SBF, but not the MBF complex, is involved in the sensitivity of \( swi6A \) strains to MMS and hydroxyurea.

Although Swi6 is normally essential for transcriptional activation through both
the SCB and the MCB elements, overproduction of Swi4 can bypass the Swi6 requirement for activation of an SCB reporter (Breeden and Nasmyth, 1987). Since my data implicated SBF (complex of Swi4/Swi6) in the sensitivity of the swi6Δ mutant to MMS and hydroxyurea, I tested the ability of Swi4 overproduction to rescue the drug-sensitivity of a swi6Δ mutant. I found that a high-copy plasmid containing the SWI4 gene rescued the sensitivity of a swi6Δ mutant to plating in the presence of hydroxyurea or MMS (Table 2-2). Swi4 overproduction did not suppress the MMS and hydroxyurea-sensitivity of an hrr25Δ mutant in this assay (Table 2-3).

**The MMS and HU-Sensitivity of hrr25Δ and SBF-Deficient Strains May Reflect a Defect in the Transcriptional Induction of DNA Repair Genes.**

To gain insight into the molecular mechanisms underlying the MMS and HU-sensitivity of hrr25Δ and SBF-deficient strains, I focused on the response to hydroxyurea because the cellular effects of HU are better understood. As mentioned earlier, hydroxyurea inhibits ribonucleotide reductase and, through this inhibition, indirectly inhibits DNA synthesis (Harder and Follmann, 1990). Two classes of hydroxyurea-sensitive mutants have been characterized: (i) mutants defective in the S-phase checkpoint that are unable to inhibit mitotic division in the presence of unreplicated DNA (Allen et al., 1994; Navas et al., 1996; Navas et al., 1995) and (ii) mutants defective in ribonucleotide reductase function or the transcriptional induction of RNR gene expression after hydroxyurea depletion (Navas et al., 1995; Zhou and Elledge, 1993). Neither swi6Δ nor hrr25Δ cells showed a defective S phase checkpoint response in viability assays following transient HU treatment (Merl Hoekstra,
Figure 2-4: S Phase Checkpoint in Wild Type and swi4Δ Cells as Assayed by Mitotic Spindle Elongation After Treatment With Hydroxyurea.

Wild type (J034) and swi4Δ (J057) cells were arrested in late G1 phase with α-factor mating pheromone and released into YPD medium with or without 200mM hydroxyurea. Sample aliquots were taken for indirect immunofluorescence staining of mitotic spindles with the YOL1/34 anti-tubulin antibody and for DAPI staining of nuclear DNA. The stained samples shown are from aliquots taken when the untreated cells underwent mitotic spindle elongation.
personal communication and Steve Elledge, personal communication). I tested the S phase checkpoint in \textit{swi4A} mutants; Figure 2-4 shows that wild-type cells grown in YPD undergo elongation of mitotic spindles (as assayed by indirect immunofluorescence of tubulin). However, in the presence of hydroxyurea, this spindle elongation was inhibited. The \textit{swi4A} strain showed a similar inhibition of mitotic spindle elongation in the presence of hydroxyurea (Figure 2-4). Therefore, I conclude that the S phase checkpoint is intact in \textit{swi4A} mutants.

Because the hydroxyurea-sensitivity of \textit{hrr25A} and SBF-deficient cells is not due to a defective checkpoint function, I used northern blot analysis to test the induction of \textit{RNR} (ribonucleotide reductase) gene expression in \textit{swi4A, swi6A, hrr25A, mbp1A} and wild-type cells upon treatment with hydroxyurea. Three genes have been identified for subunits of ribonucleotide reductase in yeast: \textit{RNR1} and \textit{RNR3} encode the large subunit of the enzyme while \textit{RNR2} encodes the small subunit (Elledge et al., 1992). \textit{RNR3} transcription was induced 4- to 6-fold following hydroxyurea-treatment of the wild-type strain (Figure 2-5). In contrast, \textit{RNR3} transcriptional induction was greatly reduced in \textit{swi4A, swi6A} and \textit{hrr25A} mutants (Figure 2-5, 2-6). \textit{SWI4} and \textit{SWI6} were also required for maximal induction of \textit{RNR3} expression after treatment with MMS (data not shown). \textit{hrr25A} mutants also failed to induce \textit{RNR2} expression; in \textit{swi4A} and \textit{swi6A} mutants, the defect in transcriptional induction of \textit{RNR2} was less dramatic than the defect for \textit{RNR3} expression. The \textit{RNR1} gene was not transcriptionally induced in response to hydroxyurea in the strain background used (data not shown). Consistent with the relative resistance of the \textit{mbp1A} mutant to MMS and HU (Table 2-2A), \textit{RNR2} and \textit{RNR3} expression was comparable to wild type following HU-treatment of
Figure 2-5: Transcriptional Induction of Genes After Treatment with 200mM Hydroxyurea in swi6Δ, swi6Δ and mbp1Δ Strains.

(A) Yeast strains (indicated at the top of each panel) were grown in minimal media to early log phase and a 0 time point was taken before hydroxyurea was added to 200mM. Aliquots of cells were taken after hydroxyurea addition at the time points specified (min.). Total RNA was extracted and northern hybridization analysis was performed with the probes indicated to the left of each panel. All time points shown for each probe are from the same exposure of the northern blot. Blots were sequentially hybridized with the different probes. (B) Phosphorimager analysis of the northern blots shown in (A). The RNA levels of RNR2, RNR3 and UBI4 relative to ACT1 were determined and plotted versus time after hydroxyurea addition. ACT1 encodes actin and served as a loading control. swi6- (SWI4) indicates a swi6 deletion strain transformed with a high copy SWI4 plasmid.
B

**RNR3**

**RNR2**

**UBI4**

- **WT**
- **swi6**
- **swi4**
- **mbp1**
- **swi6 (SWI4)**
Figure 2-6: Transcriptional Induction of \textit{RNR2}, \textit{RNR3} and \textit{UBI4} After Treatment with 200mM Hydroxyurea in an \textit{hrr25A} Strain.

(A) RNA was isolated from the strains indicated at the top of each panel after treatment with hydroxyurea as described in the legend to Figure 3. Total RNA was extracted and northern hybridization analysis was performed with the probes indicated to the left. (B) Phosphorimager analysis of the northern blots shown in (A). The RNA levels of \textit{RNR2}, \textit{RNR3} and \textit{UBI4} relative to \textit{ACT1} were determined and plotted versus time after hydroxyurea addition.
an mbp1Δ mutant. The failure to induce RNR3 transcription was not due to low viability or slow response of the mutant cells because UBI4 expression was induced normally in swi4Δ and swi6Δ mutants and also in hrr25Δ cells, although not to wild-type levels (Figure 2-5, 2-6). UBI4 encodes polyubiquitin and is transcriptionally induced in response to a variety of physiological stresses (Treger et al., 1988) through mechanisms that appear distinct from those controlling the damage induction of RNR genes (Kiser and Weinert, 1996; Zhou and Elledge, 1993).

Since overproduction of SWI4 rescued the inviability of swi6Δ mutants in the presence of HU, I assayed RNR2 and RNR3 gene expression in swi6Δ mutants transformed with a high copy plasmid containing SWI4. Overproduction of SWI4 in the swi6Δ strain partially restored the expression of both RNR2 and RNR3, although not to wild-type levels (Figure 2-5). It is possible that rescue of swi6Δ cells by SWI4 overproduction is due to a prolonged transcriptional response occurring over a much longer period of time than I used in my analysis of RNR2/3 induction (see Discussion). In an hrr25Δ strain, ectopic expression of SWI4 had a more drastic effect on RNR gene expression. RNR2 induction was almost completely rescued, while RNR3 expression was increased relative to the wild-type strain after 200 minutes in the presence of hydroxyurea (Figure 2-6). Swi4 overproduction in the hrr25Δ mutant also increased basal UBI4 transcription in untreated cells (figure 2-6B, 0 min. time point), but the induction of UBI4 over time was similar to that seen in hrr25Δ mutants not overexpressing SWI4.
Figure 2-7: Localization of Swi6 Protein After Methylmethanesulfonate (MMS) Treatment

The strain JO42 (swi6Δ) overproducing Swi6 from a 2μ high copy plasmid (YEpSWI6) was treated with or without 0.05% MMS for 1 hour, as indicated. The cells were fixed and stained with α-Swi6 antibodies for indirect immunofluorescence and DAPI to stain for nuclear DNA. The stained cells were compared with the same cells observed under Nomarski optics (DIC). JO42 cells transformed with YEp13 (vector backbone for YEpSWI6) were used as a negative control for Swi6 staining and did not show any significant signal (not shown).
<table>
<thead>
<tr>
<th>untreated</th>
<th>1hr. MMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIC</td>
<td></td>
</tr>
<tr>
<td>α-Swi6</td>
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<td>DAPI</td>
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After MMS Treatment, the Swi6 Protein Becomes Predominantly Nuclear-Localized, Even in G2 Cells.

The subcellular localization of Swi6 changes during cell cycle progression (Sidorova et al., 1995). The protein is present throughout the cell cycle but is largely cytoplasmic in G2. At around mitosis, Swi6 enters the nucleus and remains nuclear throughout G1 until the next G2 phase. Since treatment with MMS causes cells to arrest primarily in G2 (Siede, 1995), Swi6 may need to be relocated to the nucleus in G2 phase in response to DNA damage in order to induce transcription. I tested this idea using anti-Swi6 indirect immunofluorescence to assay the cellular localization of Swi6. I found that the Swi6 protein is predominately nuclear in MMS-treated cells. Figure 2-7 shows that after MMS treatment, G2 cells (cells with buds), that normally show cytoplasmic localization of Swi6, now showed strong nuclear localization. This observation suggests that Swi6, which is normally cytoplasmic in G2 cells, can relocalize to the nucleus after MMS treatment.
DISCUSSION

I have made two sets of observations that suggest a functional interaction between the Hrr25 protein kinase and the Swi6 transcription factor. First, Hrr25 interacts with and phosphorylates Swi6 in vitro, and second, hrr25Δ and swi6Δ mutants share an in vivo defect in the induction of RNR gene expression after treatment with the DNA synthesis inhibitor, hydroxyurea. In addition, I found that swi4Δ mutants, but not mbp1Δ mutants, are defective in the induction of RNR genes in response to HU. Overproduction of SWI4 rescues the HU- and MMS-sensitivity of swi6Δ mutants and partially alleviates the transcriptional induction defect of both swi6Δ and hrr25Δ mutants. Thus, although my data suggest that Swi6 is a target of Hrr25, the SBF complex, but not MBF, appears to be involved in the RNR transcriptional response.

Four observations support a role for Hrr25 and SBF that is specific to RNR transcriptional induction in response to DNA damage, and not simply a role in providing a basal transcriptional activity. First, I found that basal, uninduced levels of RNR2 and RNR3 expression are not affected by mutation of SWI4, SWI6 or HRR25 (Figures 2-5, 2-6). Second, I found that the activity of SCB::lacZ and MCB::lacZ reporter genes (which are completely dependent on SWI6 for expression) in untreated cells was not reduced by mutation of HRR25 (Y.H., unpublished data). This demonstrates that the cell cycle function of SWI6 is intact in an hrr25Δ mutant. Therefore, the interaction between Hrr25 and Swi6 is not necessary for Swi6 function in untreated cells; the interaction is likely to reflect a DNA damage-specific role. I conclude that Hrr25 and SBF are involved
specifically in mediating the transcriptional induction of RNR2/3 in response to HU and are not simply providing a basal transcriptional activity that is modulated by another damage-responsive factor. Third, overproduction of SWI4 in the hrr25∆ mutant rescued the RNR2/3 induction defect, but it did not increase basal levels of RNR2/3 expression (Figure 2-5, 2-6). This result is consistent with a direct role for SWI4 in the induction of RNR genes in response to DNA damage. Therefore, not only are SWI4 and SWI6 required for maximal induction of RNR2/3 in response to DNA damage, but SWI4 is sufficient, when overexpressed, to restore damage-inducibility of RNR2/RNR3 to the induction-defective hrr25∆ mutant (the further ramifications of the suppression by SWI4 are discussed below). Fourth, the Swi6 protein is relocalized to the nucleus in G2 after DNA damage, indicating that Swi6 is actively regulated in response to DNA damage. In summary, these observations point to a direct and active role for SBF in RNR2/3 transcriptional induction in response to DNA damage, as opposed to a passive role in providing a basal expression level. Furthermore, the rescue of the hrr25∆ induction defect by ectopic SWI4 expression and the cellular relocalization of Swi6 suggest that both Swi4 and Swi6 are actively regulated in response to DNA damage.

Previous studies have shown that RNR2 inducibility is not blocked by protein synthesis inhibitors, suggesting that a pre-existing factor is likely responsible for DNA damage-induced transcription (Elledge and Davis, 1989; Hurd and Roberts, 1989). My finding that SBF was involved in upregulating RNR gene expression in response to HU suggests that SBF may be one such factor acting directly on the RNR promoters. The RNR3 gene contains three matches to the SCB consensus within the 350 bp upstream of the ATG. Likewise, the RNR2 gene contains one near match in its upstream sequences
Both promoters also contain matches, or near matches, to the MCB consensus element (Elledge et al., 1993). Although several observations suggest that, at least in certain promoter contexts, the principle binding sites for SBF are SCB sequences, other data suggest that SBF may also act through MCB elements (reviewed in Breeden, 1995; Partridge et al., 1997).

Alternatively, SBF may be acting through another, unidentified element to mediate the DNA damage response. Dissection of the SWI4 and CLN2 promoters has provided evidence that both Swi4 and Swi6 may act through upstream sequences distinct from SCB or MCB elements (Cross et al., 1994; Foster et al., 1993; Stuart and Wittenberg, 1994). In support of an alternative element mediating the SBF-dependent DNA damage response, I and others do not see elevated expression from SCB or MCB::lacZ reporter genes after DNA damage (Y.H., unpublished and MCB reporter also cited in Elledge et al., 1993; Johnston and Johnson, 1995). Moreover, DNA damage-induced expression of CDC9 was still seen when MCB elements were deleted from its promoter (Johnston and Johnson, 1995), and DNA damage-inducibility of RNR2 is maintained in a promoter deletion mutant lacking the putative SCB sequence (Hurd and Roberts, 1989). The HRR25 and SBF-dependence of the RNR promoter mutants lacking SCB and MCB sequences have yet to be assessed.

Promoter analyses of damage-inducible genes have uncovered numerous DNA elements and DNA-binding proteins that are involved in the transcriptional response to DNA damage (see Introduction, also reviewed in Bachant and Elledge, 1996). Also, genetic screens have identified mutants defective in regulation of damage-inducible promoters (Huang et al., 1998; Zhou and Elledge, 1993; Zhou and Elledge, 1992). It will
be interesting to assess the relationship between these previously studied mechanisms and regulation by *SWT4, SWI6* or *HRR25*. It is possible that different elements in the complex promoters of damage-inducible genes may mediate the response to different types of DNA damage. For example, although *RNR2* and *RNR3* are induced in response to UV or UV-mimetic agents (Elledge and Davis, 1989; Elledge and Davis, 1990; Hurd and Roberts, 1989), neither *hrr25Δ, swi4Δ* nor *swi6Δ* mutants display any UV-sensitivity (Hoekstra et al., 1991, and M. Hoekstra, unpublished data; Johnston and Johnson, 1995).

This implies that either (i) *RNR* induction is not required for maximum viability after UV irradiation or (ii) UV-irradiation induces transcription of the *RNR* genes through a pathway independent of *HRR25/SBF*. The cellular sensing apparatus for UV-irradiation is believed to be different from the sensors for DNA-damage and ribonucleotide depletion (Devary et al., 1993; Kiser and Weinert, 1996). It would be interesting to test the UV-inducibility of the *RNR* genes in *swi4Δ, swi6Δ* and *hrr25Δ* mutants.

I found that Swi4 overproduction in the *swi6Δ* mutant rescued its drug-sensitivity but only induced a modest increase in *RNR2/3* gene expression in response to HU. Can this modest increase in *RNR2/3* transcriptional induction be responsible for the observed rescue of *swi6Δ* inviability on HU and MMS plates? Hydroxyurea inhibits DNA synthesis through depleting the cellular deoxyribonucleotide pool; most HU-sensitive mutants are defective for either the S phase checkpoint and/or the transcriptional induction of *RNR* genes (Allen et al., 1994; Navas et al., 1996; Weinert et al., 1994; Zhou and Elledge, 1993). The S phase checkpoint in *swi4Δ, swi6Δ* and *hrr25Δ* mutants appears intact (see Results section for *swi4Δ*; Merl Hoekstra, personal communication, *swi6Δ*; Steve Elledge, personal communication, *hrr25Δ*). However, *swi4Δ, swi6Δ* and
hrr25Δ mutants do show defects in RNR2/3 transcriptional induction. Therefore, I suggest that overproduction of SWI4 rescues the inviability of the swi6Δ mutant on HU through either (i) its effect on RNR transcription which may become significant over a prolonged period or (ii) the induction of other genes that may be required to survive HU treatment—whose expression can be stimulated directly or indirectly by SWI4. Consistent with SWI4 being important for survival or recovery after prolonged exposure to DNA damaging agents, the viability of a swi4Δ mhp1 mutant is not affected by exposure to MMS that is transient (Johnston and Johnson, 1995).

Although overexpression of SWI4 only partially rescued the transcriptional defect of the swi6Δ mutant, it completely rescued the lack of RNR2/3 inducibility in the hrr25Δ mutant. The effect on RNR2/3 transcriptional inducibility appears specific to the RNR promoters since ectopic SWI4 expression increased the basal level of UBI4 expression in the hrr25Δ mutant, but it had little effect on UBI4 inducibility (Figure 2-6). Despite restoring RNR2/3 inducibility, Swi4 overproduction failed to rescue the inviability of the hrr25Δ mutant on hydroxyurea plates. However, the pleiotropic phenotypes of the hrr25Δ mutant suggest that in addition to regulating RNR gene expression, HRR25 involved may also be involved in other aspects of the stress response required for surviving sub-optimal conditions.

Together, my data support a model whereby the phosphorylation of Swi6 by Hrr25 promotes SBF-dependent induction of DNA repair genes in response to DNA damage or HU-induced depletion of deoxyribonucleotides. Modification of Swi6 by Hrr25 may be necessary to allow SBF to function at times in the cell cycle when it is not normally active. In support of this notion, I found that Swi6 protein, which is
predominately cytoplasmic in untreated G2 cells, becomes predominately nuclear after MMS treatment. It would be interesting to see if phosphorylation of Swi6 by Hrr25 promotes redistribution of Swi6 to the nucleus in response to DNA damage. In addition, my observations suggest that Swi4 may be regulated in response to HU by an HRR25-independent mechanism since overexpressed Swi4 is sufficient to confer RNR gene inducibility in the absence of HRR25 function. The mechanism by which ectopically expressed Swi4 restores RNR inducibility is likely to be through the SBF complex, and not the Swi4 monomer, since overproduced Swi4 only restores a small RNR induction in the absence of SWI6. Full restoration of RNR inducibility by SWI4 overexpression in an hrr25A mutant may reflect the ability of the overproduced Swi4 to form SBF heterodimers with Swi6. Therefore, SBF may receive DNA damage signals through both subunits: an HRR25-SWI6 pathway and an HRR25-independent pathway through SWI4 (Figure 2-8). In addition to SWI4, SWI6 and HRR25 as discussed here, RAD9 and the MEC1, RAD53 and DUN1 kinases are also required for RNR gene induction in response to DNA damage (reviewed in Allen et al., 1994; Bachant and Elledge, 1996; Kiser and Weinert, 1996; Weinert et al., 1994; Zhou and Elledge, 1993). It will be of interest to delineate the relationship between the HRR25/SWI6 and HRR25-independent pathways shown in Figure 2-8 and the RAD9/MEC1/RAD53/DUN1 pathway (Figure 2-1) for damage-inducible gene expression.

My studies suggest that the SBF transcription factor complex may not be a dedicated cell cycle regulator but rather a transcription factor with multiple roles. My findings suggest a role for SBF in mediating the transcriptional activation of repair genes in response to inhibition of DNA synthesis and to DNA damage. Others have reported
Figure 2-8: Model for the Role of Hrr25 and SBF (Swi4/Swi6) in the Transcriptional Response to DNA Damage.

The Hrr25 protein kinase is proposed to phosphorylate Swi6 in response to DNA damage. Phosphorylation by Hrr25 may cause cytoplasmic Swi6 in G2 cells to relocalize to the nucleus. An Hrr25-independent pathway may also function through Swi4. These two pathways serve to activate SBF to promote the transcriptional induction of repair genes.
Hydrogenase

H2-independent
pathway
that SBF may also play a role in the induction of meiosis-specific genes (Leem et al., 1998). SBF may belong to a family of transcription factors, like SRF in mammals and \textit{MCM1} and \textit{SWI5} in yeast, that play multiple, seemingly unrelated roles in transcriptional activation (Aerne et al., 1998; Tennyson et al., 1998; Treisman and Ammerer, 1992). E2F, the functional analogue of SBF in mammals, appears to also play multiple roles. Contrary to the proposed role of E2F as an activator for cell cycle entry, mice that are deficient for E2F-1 develop and reproduce normally; surprisingly, the E2F-deficient mice also display a defect in apoptosis and develop a broad spectrum of tumours (Field et al., 1996; Yamasaki et al., 1996). Clearly, the proposed role of E2F as a transcription factor at the restriction point does not account for the phenotypes of the E2F-deficient mice. Perhaps, like SBF in budding yeast, E2F is also a transcription factor with multiple roles. Mutants defective for SBF function are deficient in the transcriptional response to DNA damage (my data); a proposed, analogous role for E2F in the DNA damage response would certainly account for its apparent role as a tumour suppressor in the E2F-deficient mice.
CHAPTER III

Activation of Transcription at the S. cerevisiae Start Transition by Stb1, a Swi6-Binding Protein

I did the experiments in this chapter with the following exceptions:

1) Microsequencing of the p48 peptide shown in Figure 3-1B was done by Ryuj Kobayashi; 2) Lynda Moore compiled the data for Figure 3-2; 3) Lynda Moore and Brenda Andrews constructed the stb1Δcln3Δ mutant; 4) Brenda Andrews and I constructed and analyzed the stb1Δswi6Δ, stb1Δswi4Δ and stb1Δmbp1Δ mutants.
ABSTRACT

In Saccharomyces cerevisiae, gene expression late in G1 phase is activated by two transcription factor complexes, SBF and MBF. SBF consists of the Swi4 and Swi6 proteins and activates transcription of the G1 cyclin genes, cell wall biosynthesis genes and the HO gene, mainly through a repeated upstream sequence element called the SCB. MBF is composed of Mbp1 and Swi6, and activates transcription of genes required for DNA synthesis through a distinct element called the MCB. Mbp1 and Swi4 are the DNA binding subunits for the MBF and SBF complexes while the common subunit, Swi6, is presumed to play a regulatory role. I show that Stb1, a protein previously reported to be complexed with the transcriptional repressor Sin3, binds to Swi6 in vitro. The in vivo accumulation of Stb1 phosphoforms was dependent on the CLN1, CLN2 and CLN3 genes, which encode G1-specific cyclins for Cdc28, and Stb1 was phosphorylated by Cln-Cdc28 kinases in vitro. Deletion of STB1 in a cln3A mutant background caused an exacerbated delay in G1 progression and the onset of Start transcription. Hyperphosphorylated forms of Stb1 appeared coincidently with initiation of Start transcription in a cln3A mutant. I propose that in the absence of CLN3, phosphorylation of Stb1 by Cln1,2-associated kinases contributes to the activation of Start transcription.
INTRODUCTION

In the budding yeast *Saccharomyces cerevisiae*, commitment to enter the mitotic cell cycle occurs during late G1 phase; this commitment phase of the cell cycle is designated 'Start' (reviewed in Nasmyth, 1996). Start is marked by the transcriptional induction of a subset of genes that catalyze entry into the mitotic cell cycle. Events that ensue once a yeast cell has passed through Start include initiation of DNA synthesis, budding and spindle-pole body duplication (see Chapter 1).

The transcriptional activation of genes important for the Start transition is dependent upon two transcription factor complexes called SBF and MBF (reviewed in Breeden, 1995). The SBF (SCB-binding factor) complex contains the Swi4 and Swi6 proteins and activates transcription mainly through a cis-acting sequence element called the SCB (Swi4/6 cell cycle box; consensus=CACGAAA). Genes activated by SBF include the G1 cyclins (*CLN1,2* and *PCL1,2* Espinoza et al., 1994; Measday et al., 1994; Ogas et al., 1991), the *HO* endonuclease gene (Nasmyth, 1985), the gene encoding the Swel protein kinase (Sia et al., 1996) and a number of genes required for cell wall biosynthesis (Igual et al., 1996). The Swi4 protein is the component of SBF responsible for specific binding to SCB sequences, while Swi6 does not bind DNA specifically. In the absence of Swi4 or Swi6, *HO* is not expressed, while G1 cyclin and cell wall biosynthetic gene expression is reduced. Swi6 also interacts with a second DNA binding protein, Mbp1, to form the MBF transcription factor (MCB-binding factor, also known as DSC1 (Koch et al., 1993). MBF/DSC1 recognizes the so-called MCB element (MluI cell
cycle box; consensus =ACGCGTNA) and activates G1-specific transcription of the S-phase cyclin genes, \textit{CLB5} and \textit{CLB6} (Epstein and Cross, 1992; Schwob and Nasmyth, 1993), the \textit{SWT4} gene (Breeden and Mikesell, 1991), as well as many genes needed for DNA synthesis such as \textit{CDC9} (Barker et al., 1985; Peterson et al., 1985) and \textit{POL1} (Johnston et al., 1987). While \textit{SWT4}, \textit{SWT6} and \textit{MBP1} are not essential genes, cells lacking both \textit{SWT4} and \textit{SWT6} or both \textit{SWT4} and \textit{MBP1} are inviable, arresting prior to DNA synthesis (Koch et al., 1993; Nasmyth and Dirick, 1991). In both double mutants, the death results from inadequate expression of G1 cyclins since ectopic expression of \textit{CLN2} can rescue the lethality.

Both passage through Start and the activation of SBF and MBF require the Cdk \textit{CDC28} and one of the \textit{CLN} G1 cyclins, \textit{CLN1}, \textit{CLN2} or \textit{CLN3}. Although any one of the \textit{CLN} cyclins is, by itself, sufficient to drive Start, \textit{CLN3} appears to be responsible for activation of gene expression at Start, while \textit{CLN1} and \textit{CLN2} are important for the proper execution of post-Start events such as budding and DNA synthesis (Dirick et al., 1995; Stuart and Wittenberg, 1995). Although genetic studies indicate a key role for \textit{CLN3} in activating SBF and MBF at Start, there is as yet no evidence that Cln3-Cdc28 acts to directly phosphorylate or interact with components of SBF or MBF. Also, a \textit{cln3A} strain is viable, indicating that alternative mechanisms must function to activate Start transcription (Nasmyth and Dirick, 1991). The \textit{BCK2} gene (Bypass of C-Kinase mutation) appears to be involved in such an alternative pathway(s). Compared to \textit{bck2A} and \textit{cln3A} single mutants, the \textit{bck2Acln3A} double mutant has a severe growth defect and shows an exacerbated delay in the onset of Start transcription, suggesting that \textit{BCK2} acts in parallel to \textit{CLN3} to activate Start transcription (DiComo et al., 1995; Epstein and
Cross, 1994). In this Chapter, I present evidence to implicate another gene, STB1, as an important activator of Start transcription in the absence of CLN3.

I identified Stb1 as a protein that bound to Swi6 \textit{in vitro}. \textit{STB1} (Sin three-binding protein) encodes a novel protein first identified in a two-hybrid screen with the general transcriptional repressor, Sin3 (Kasten and Stillman, 1997). Like \textit{BCK2}, \textit{STB1} mutants do not show any appreciable phenotypes. However, I found that, like the \textit{bck2ΔcDN3Δ} mutant, the \textit{stb1ΔcDN3Δ} double mutant showed a more severe G1 progression defect and delay in Start transcription seen than in the \textit{cDN3Δ} single mutant. Stb1 was phosphorylated by Cln-associated kinases \textit{in vitro}, and Stb1 phosphoforms \textit{in vivo} were dependent on \textit{CLN} function. Phosphorylation by Cln-kinases likely activate \textit{STB1}-dependent activation of Start transcription since in the \textit{cDN3Δ} mutant, where \textit{STB1} is important for the timing of Start, Stb1 phosphoforms appeared coincidentally with the onset of Start transcription. I suggest that Cln-associated kinases phosphorylate Stb1 to activate transcription at Start, and that this parallel pathway for Start entry is important in the absence of \textit{CLN3}-dependent activation.
MATERIALS AND METHODS

Yeast Strains.

Except where indicated, the genotype of the parental strain for experiments presented in this chapter is MATα TRP+ ura3-52 lys2-801a ade2-1070 his3Δ200 leu2-Δ1 (By263, an S288C derivative). The cln3ΔURA3 deletion strain (BY655) was constructed by transformation of strain BY263 (Measday et al., 1997) with a cln3ΔURA3 disruption cassette (Lew et al., 1991). For construction of an isogenic stblΔ strain, the polymerase chain reaction (PCR) was used to generate a DNA fragment from primers with 45 bases corresponding to sequences at the 5' and 3' ends of the STBL gene followed by homology to pBluescript sequences that straddle the URA3 marker gene in template plasmid pJJ242 (Jones and Prakash, 1990). The primers used were:

CAT GAC GCT CTT TCA CCT TTC CTC TCA TAA TTT TGA TAT CAC GCG AAC AGC TAT GAC CAT G (sense)

and

GTC CAT TTC AGG AAG TAT GGA ATC ATC AAC TGA TAT GGC CGT CAA GTT TTC CCA GTC ACG AC (antisense).

The italicized sequences at the 3' ends of the primers represent bases that hybridize to the URA3-containing template. The resulting fragment was used to transform strain BY448 (MATα derivative of BY263) to create strain BY805 (MATα stblΔURA3 trp1Δ63 ura3-52 lys2-801a ade2-1070 leu2-Δ1 his3Δ200). A stblΔTRP1 strain was constructed by transformation of BY805 with a URA3-TRP1 switcher plasmid (pUT11) as described (Cross, 1997) to create strain BY806 (MATα stblΔTRP1 trp1Δ63 ura3-52 lys2-801a ade2-1070 leu2-Δ1 his3Δ200). To
construct a \textit{stb1\Delta cln3\Delta} double mutant, strains BY806 (\textit{\alpha stb1\Delta TRPI}) and BY655 (a \textit{cln3\Delta URA3}) were mated and BY822 (\textit{MATa stb1\Delta TRPI cln3\Delta URA3}) was recovered by dissecting tetrads. All gene disruptions were confirmed by Southern blot analysis.

For Western blot analysis of Stb1 phosphoforms, BY263 was transformed with either vector pRS425 (Christianson et al., 1992) or pM2517 (gift from D. Stillman). Plasmid pM2517 contains a 3.1kb genomic fragment of \textit{STB1} in pRS425 (Kasten and Stillman, 1997). For western blot analysis of Stb1 phosphoforms in \textit{cln\Delta} strains, the wild-type strain used was BF305-15d (\textit{MATa ade1 arg5,6 his3 leu2 met14 trp1 ura3}, Tyers, 1996). The \textit{cln-sicl-} strain was isogenic to BF305-15d except \textit{cln1\Delta HIS3 cln2\Delta TRPL cln3\Delta ura3-pGAL1-CLN3 sicl\Delta URA3} (Tyers, 1996). These strains were transformed with pM2517 for \textit{STB1} overexpression. For northern blot experiments, the wild-type strain used was W303 (\textit{MATa ura3 trp1-l ade2-l his3-11,15 leu2-3,112 can1-100}) and the \textit{stb1\Delta TRPI} mutant strain was otherwise isogenic (gift from D. Stillman Kasten and Stillman, 1997).

Strains used for \textit{in vitro} kinase assays with immunoprecipitated cyclin-Cdc28 complexes were in the W303 strain background. The \textit{HA-CLNI} (MT235), \textit{HA-CLN2} (MT244) and the \textit{HA-CLB2} (MT537) strains were gifts from Mike Tyers. The cyclin genes were tagged and integrated into the genome basically as described (Tyers et al., 1993).

\textbf{Protein Affinity Chromatography and Microsequencing of p48.}

Swi6 protein affinity chromatography was done as previously described (Ho et al., 1997; Chapter II). The Swi6\Delta M protein is deleted for 285 internal amino acids which include the ankyrin repeats. The deletion was created by digestion of the full-length Swi6-
containing plasmid with SacI followed by fill-in synthesis with Klenow polymerase, resulting in an in-frame 860bp deletion. The full-length Swi6 and Swi6ΔM proteins were coupled to AffiGel-10 resin (Bio-Rad) as recommended by the manufacturer to a column protein concentration of 40µM. Yeast extracts for the column chromatography were made from a protease-deficient strain, BJ2168 (MATa ura3-52 leu2 trp1 prb1-122 pep4-3 prcl-407) grown to mid-log phase in YPD medium (Kaiser et al., 1994). The cells were lysed in Lysis Buffer (100mM Tris pH 8.0, 100mM NaCl, 10mM MgCl₂, 1mM EDTA, 10% glycerol, 1mM DTT, 20mM NaF, 50mM β-glycerophosphate, 2mM benzamidine, 2µg/mL aprotinin, 2µg/mL leupeptin, 1µg/mL pepstatin, 1mM PMSF) using agitation in the presence of glass beads.

For preparative scale chromatography, a 1.25mL Swi6 affinity column and a 1.25mL control column (resin with no coupled protein) were used. 180 g of wet cell pellet were lysed in a Biospec Beadbeater (Biospec Products, Bartlesville, OK) using 16X30 sec bursts in the 400mL lysis chamber. After centrifugation for 1 hr at 100 000X g, 80mL of 28.5mg/mL clarified extract were loaded onto each column that had been previously washed in SB buffer (20mM Hepes pH 7.2, 10% glycerol, 0.1mM DTT, 0.1mM PMSF) with 1M NaCl (SB-1000) and equilibrated with SB-100 (SB with 100mM NaCl). The columns were loaded at 4°C over 14 hours. Each column was then washed with 10 column volumes of SB-100, followed by 4 volumes of SB-1000 before eluting with 4 volumes of SB buffer containing 1% SDS. Eluates determined by Farwestern blotting (see below) to contain p48 were pooled, dialyzed to remove SDS and concentrated by precipitation with 1:1 MeOH:acetone before loading onto SDS-PAGE gels. The p48 band could not be visualized by Coomassie Blue or silver staining because
it co-migrated with a protein that bound to the AffiGel-10 resin. Gel slices at around 48kDa were taken from both the Swi6 column and control column eluates for microsequencing.

Microsequencing of p48 was performed essentially as described (Collins et al., 1995) with the following exceptions. The 48kDa gel slices from both the Swi6 and control column eluates were digested with *Achromobacter* protease I, and the resulting peptides from each digest were resolved on two different HPLC columns. Peptide peaks specific to the digest for the Swi6 column eluates were then sequenced using an automated sequencer (Applied Biosystems models 470, 473 and 477).

**Farwestern Blot Analysis.**

To develop a Swi6 derivative useful for Far-Western experiments, a 10xhistidine tag and an HMK (heart muscle kinase) phosphorylation site were engineered at the N-terminus of Swi6. To insert the HMK and His tags into Swi6, a 190bp His-HMK cassette was amplified using PCR from plasmid pET-HMK (gift from B. Dietrich c/o H. Krause lab). The primers used for amplifying the His-HMK cassette contained NcoI sites that allowed for cloning of the cassette into the unique NcoI site at the N-terminus of Swi6 in the Swi6 expression vector, pBA789 (see above). The resulting plasmid, pET-HMKFL6, was transformed into *E. coli* strain BL21(DE3) pLysS (Novagen) and transformants were grown in LB with 70μg/mL chloramphenicol and 200μg/mL ampicillin to an OD600 of 0.8. Protein expression was induced by addition of IPTG to a final concentration of 0.5mM final concentration and incubation at 25°C for 3 hours. The His-HMK-Swi6 protein was then purified under native conditions by Ni²⁺-NTA chromatography according to the manufacturer’s recommendations (QIAGEN).
The labelling of the probe and the farwestern experiments were done essentially as described (Blanar and Rutter, 1992). One microgram of purified His-HMK-Swi6 was labelled by incubation at 37°C for 1 hour with 200 units of the catalytic subunit of bovine HMK (heart muscle kinase, Promega) and 100μCi γ-32P-ATP in a buffer containing 20 mM Tris pH 7.5, 100mM NaCl, 12mM MgCl₂, 1mM dithiothreitol. The probe was then purified by size exclusion chromatography on a Sephadex G-50 column.

Affinity column eluates were resolved on SDS-polyacrylamide gels and blotted onto nitrocellulose membrane (Ausubel et al., 1994). The resulting blot was renatured according to Vinson et al. (1988). Blots were incubated in HBB buffer (25mM Hepes pH 7.7, 25mM NaCl, 5mM MgCl₂, 1mM dithiothreitol) with 6M guanidine HCl, followed by HBB +3M guanidine HCl. Blots were then sequentially washed in HBB with the concentration of guanidine HCl reduced 2-fold relative to the previous wash until the concentration of guanidine HCl reached 0.19M. The blot was then rinsed in HBB and blocked with HBB +5% milk, before probing with radio-labelled Swi6 at 450 000 cpm/mL in Hyb75 (20mM Hepes 7.7, 75mM KCl, 0.1mM EDTA 2.5mM MgCl₂) with 0.05% NP-40 +1% milk. After an overnight incubation at 4°C, the blot was washed once at room temperature for 15 min. with Hyb75 +0.05% NP-40 +1% milk, followed by three 5 min. washes. The blot was then exposed to autoradiographic film (XAR-5, Kodak).

**Synchronization of Cells and Northern Blot Analysis.**

For synchronization by α-factor block-and-release, cells were arrested in late G1 by treatment with α-factor mating pheromone until greater than 95% of the cells showed a ‘shmoo’ morphology. The cells were then washed and released into media for collection of synchronous cell samples as previously described (Measday et al., 1994).
For synchronization by centrifugal elutriation, cells were grown in YP medium containing sucrose to an O.D. of 0.6-0.8. The cultures were concentrated to approximately 100 mL final volume and loaded at 12-14 mL/min. into a Beckman JC-MI centrifugal elutriator (rotor type JE-5.0) running at 2400 r.p.m. cooled to 4°C. The cells were elutriated by pumping water into the rotor at the loading speed with increases of 2 mL/min. after each 100-150 mL of elutriated sample was recovered. The elutriated samples were checked by microscopy and by Coulter channelizer analysis. The elutriated cells were quickly centrifuged and resuspended in fresh, pre-warmed YPD for collection of synchronous samples at the time points specified.

For both α-factor and elutriation synchronization experiments, 15 mL of cells were collected at the specified time points, and RNA was prepared and analysed by Northern blotting as described (Kaiser et al., 1994). Cells were analysed for DNA content by fluorescence-activated cell sorting (FACS) as described previously (Tyers et al., 1993), and the results were analyzed with LYSYS II software (Becton Dickson). In elutriation synchrony experiments, cells at each time point were analyzed by Coulter channelizer, and the mean cell size was determined.

The probes used for Northern blot analysis were a 600 bp EcoR1-HinDII fragment of the ACT1 gene (Harrington and Andrews, 1996); a 1.07 kB EcoRI-HinDIII fragment containing the STB1 gene from pM2517; a 440 bp EcoRI fragment of CLB5 from vector pMT895 (gift from M. Tyers); a 864 bp PCR product containing the PCL1 coding sequence (Measday et al., 1997); a 1.7 kb BgII-EcoRI fragment of RNRI (Elledge and Davis, 1990) a 2.5kb EcoRI fragment of CLN1 (Hadwiger et al., 1989) and a 1.3 kb XhoI-NcoI fragment of CLN2 (Harrington and Andrews, 1996). Probes were labelled
using random primed synthesis with Klenow DNA polymerase in the presence of $\alpha^32P$-dATP.

For RNA quantitation, Northern blots were exposed on a Molecular Dynamics screen and scanned with a Molecular Dynamics PhosphorImager and IMAGEQUANT software (version 3.3).

**Preparation of Antibodies to Stb1 and Western blotting.**

To construct a vector for preparation of recombinant Stb1 in bacteria, a 1.3kb HindIII fragment containing *STB1* was isolated from vector pM2517 (Kasten and Stillman, 1997) and inserted into the HindIII site of pRSET-B (Invitrogen). The resulting plasmid, pRSET-StbH/H, expresses a derivative of Stb1 with an N-terminal His-tag but lacking the first 85 N-terminal amino acids of full-length Stb1. pRSET-StbH/H was transformed into *E. coli* strain *BL21(DE3)* and the His-Stb1 fusion protein was purified under denaturing conditions using Ni$^{2+}$-NTA affinity chromatography as recommended (QIAGEN). The purified protein was dialyzed to 1M urea and used to immunize rabbits (Faculty of Medicine, University of Toronto). Antisera were then affinity purified as described (Andrews and Herskowitz, 1989). For Western blotting, proteins were separated by 9% SDS-PAGE and transferred to nitrocellulose using a semi-dry transfer apparatus (BioRad). Immunoblots were probed with a 1:1000 dilution of affinity-purified Stb1 antibody and subsequently with a 1:5000 dilution of goat-anti-rabbit Ig-peroxidase (BioRad). Proteins were visualized using enhanced chemiluminescence (Renaissance detection system, NEN) followed by exposure to X-ray film (Xar-5, Kodak).

**In vitro Kinase Assays with Cln-Associated Kinase.**
Cln1, Cln2 and Clb5-associated kinases were immunoprecipitated from yeast strains bearing hemagglutinin-tagged (HA) forms of each cyclin, using the 12CA5 antibody. The immunoprecipitation-kinase assay was performed as described in Tyers et al. (1993). In each reaction, 0.5µg of purified StbH/H protein (see section on preparation of anti-Stb1 antibodies) and 1.0 µg Histone H1 were added as exogenous substrates.

**Phosphatase Analysis of Stb1 Phosphoforms.**

For phosphatase treatment of cell extracts, 15 mL of log-phase yeast culture were lysed in a modified Lysis Buffer (100mM Tris pH 8.0, 100mM NaCl, 2mM MnCl₂, 10% glycerol, 1mM DTT and protease inhibitors as described above for Lysis Buffer) using agitation in the presence of glass beads. Approximately 150µg of each extract was treated with 1600 units of Lambda Protein Phosphatase (NEB) for 0.5 hour at 30°C. Where indicated, phosphatase inhibitors were present at 5mM EDTA, 50mM NaF, 50mM β-glycerophosphate and 1mM sodium vanadate. The phosphatase reactions were stopped by addition of an equal volume of SDS sample buffer and electrophoresed on 9% SDS-PAGE gels for western blot analysis (Ausubel et al., 1994).

**Microscopy.**

Cells were grown in YPD medium to log phase and observed at a magnification of 600X, using Nomarski optics and a charge-coupled device camera mounted on a Leica DM-LB microscope. Images from the camera were captured and analysed using Northern Exposure Imaging system (Empix Imaging, Inc., Mississaugua, Ontario).
RESULTS

Interaction of Stb1 with Swi6

As described in Chapter 2, to investigate the function and regulation of Swi6, I used protein affinity chromatography to look for proteins in crude yeast extracts that physically associate with Swi6. Since some specific Swi6-binding proteins may be obscured by yeast proteins that bind non-specifically to the resin (Chapter 2, Figure 2-1A), I blotted the Swi6 affinity column eluates to nitrocellulose and probed the blot with radio-labelled Swi6 protein. Using this ‘far-western’ protocol, I detected a 48kDa protein (p48) in eluates from the Swi6 column (Figure 3-1A, lane 2, indicated by arrow) but not in eluates from the control column (column resin with no coupled protein, Figure 3-1A, lane 1). The p48 band was absent in eluates from a column conjugated with Swi6 protein deleted for the central ankyrin repeat region (Figure 3-1A, lane 3). Swi4 and Hrr25, two other Swi6-binding proteins, still bound to the Swi6Δmotif ligand (Ho et al., 1997, unpublished, and Figure 3-1A, lane 3), indicating that the Swi6Δmotif protein is sufficiently folded to maintain these protein interactions.

Microsequencing of p48 yielded an 11 amino acid peptide (Figure 3-1B) that matched perfectly with amino acids 288-298 of Stb1, a novel protein that is observed in Sin3 complexes (Kasten and Stillman, 1997). Other peptides obtained in the microsequencing analysis corresponded to a major protein contaminant that bound non-
Figure 3-1: Binding of Yeast Extract Proteins to Swi6 Protein Affinity Chromatography Columns.

(A) Yeast extracts were passed over a control column with resin alone (lane 1), a column coupled with Swi6 protein (lane 2) and a column coupled with a Swi6 ligand deleted for the central ankyrin region (Swi6ΔM, lane 3). The washed columns were then eluted with 1% SDS. The eluates were run on SDS-PAGE, blotted onto nitrocellulose and probed with a radioactively-labelled Swi6 protein probe (see Materials and Methods). The 48 kDa protein band is denoted by the arrow. (B) Results from microsequencing of an 11 amino acid peptide from the 48 kDa protein band. (C) Swi6 protein affinity chromatography with extracts from stb1Δ cells.
Sequenced p48 peptide..............PSASSPQSTFK Stb1 (aa. 288-298).............. PSASSPQSTFK
specifically to the column control resin at around 48kDa in both control and Swi6-column lanes (See Figure 2-2A). Due to the presence of this contaminant, the farwestern blot was necessary to visualize p48 in the column eluates. p48 was absent in eluates from Swi6 affinity columns loaded with an extract from a stb1Δ strains (Figure 3-1C), confirming that p48 was indeed Stb1. The binding of Stb1 to Swi6 in the farwestern blot assay indicates that the interaction is direct. I conclude that Stb1 interacts directly with Swi6 in vitro, and that this interaction requires the ankyrin repeats in Swi6.

**Genetic Interactions with stb1Δ**

The finding that Stb1 interacts in vitro with Swi6, a regulator of Start transcription, prompted me to look for cell cycle defects in the stb1Δ mutant. I found no defects in the stb1Δ mutant in terms of growth rate, cell cycle progression or in the transcription levels or cell cycle periodicity of a panel of Start-expressed genes (data not shown). Similarly, another study found no requirement for STB1 in expression from the HO gene (Kasten and Stillman, 1997).

As mentioned earlier (Chapter 1), certain combinations of mutations affecting the components of SBF and MBF exhibit lethal genetic interactions with one another (ie. swi4Δswi6Δ and swi4Δmbp1Δ strains are inviable). Because I found that Stb1 interacts physically with Swi6, I tested for genetic interactions between STB1 and SWI4, SWI6 and MBP1. I found no obvious additional defects in stb1Δswi4Δ, stb1Δswi6Δ or stb1Δmbp1Δ strains (data not shown).

As discussed in Chapter 1, a number of genetic observations suggest that the Cln3-Cdc28 kinase is required for efficient activation of transcription at Start. However,
Figure 3-2: Growth Characteristics of the \textit{stb1}\(\Delta\) \textit{cln3}\(\Delta\) Double Mutant.

\textit{(A)} Wild-type (BY263), \textit{stb1}\(\Delta\) (BY805), \textit{cln3}\(\Delta\) (BY655) and two isolates of the \textit{stb1cln3}\ double mutants (BY822 and BY824) were streaked onto a YPD plate and incubated at 30\(^\circ\)C. \textit{(B)} Nomarski microscopy of the wild-type and the various mutant cells (\textit{stb1}\(\Delta\)\textit{cln3}\(\Delta\) strain shown is BY822). The photographs shown are all taken at the same magnification. \textit{(C)} FACS analysis and budding index (calculated from at least 300 cells) of the wild-type and mutant strains (\textit{stb1}\(\Delta\)\textit{cln3}\(\Delta\) strain shown is BY822).
Figure 3-3: Cell Cycle Northern Blot Analysis of the STB1 Transcript.

(A) The wild-type strain (W303) was synchronized by α-factor G1 block and release into YPD medium. RNA was prepared from samples taken every 15 min. for Northern analysis with STB1, CLB5 and ACT1 (actin) probes. (B) The Northern signals were quantitated by a phosphorimager, and the values were normalized to the ACT1 loading control before plotting versus time after α-factor release (STB1, solid line; CLB5, broken line).
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C'3 is not absolutely required for activation of SBF and MBF-dependent gene expression. Rather, in the absence of cln3, activation of SCB- and MCB-dependent transcription is delayed. These observations suggest that other regulators, such as BCK2, can activate SBF and MBF in the absence of CLN3 (see Chapter 1, section 2c). To test whether STB1 and CLN3 might function in parallel to activate Start transcription, I constructed a stb1Δcln3Δ double mutant. When cultured in rich or minimal media, both the cln3Δ strain and the stb1Δ mutant had growth rates comparable to the wild-type strain (Figure 3-2A and not shown). The stb1Δcln3Δ double mutant grew much more slowly than the wild-type strain, with an average doubling time in rich media of 4.5 hours--compared with 1.5 hours for the wild-type, cln3Δ and stb1Δ strains (Figure 3-2A and data not shown). Analysis of cell morphology and DNA content in log phase cultures revealed that the stb1Δcln3Δ double mutant cells accumulated in G1 phase as predominantly large, un budded cells (Figure 3-2B, C). Consistent with a role for STB1 during late G1 phase, I found that STB1 transcript levels fluctuated during the cell cycle with peak levels in late G1, coincident with maximal expression of other Start-regulated genes (Figure 3-3, shown is CLB5). Taken together, these results suggest that STB1 plays a role in G1 progression.

**STB1 is Required for Timely Expression of Start Genes in a cln3Δ Mutant**

The phenotype of the stb1Δcln3Δ double mutant suggested that, like BCK2, STB1 may act in a parallel pathway with CLN3 for activating transcription at Start. To test this hypothesis, I isolated small G1 cells from wild-type, cln3Δ, stb1Δ and stb1Δcln3Δ strains by centrifugal elutriation. Cells were inoculated into fresh medium and Start transcription, cell size and budding index were monitored as the cells progressed synchronously through the cell cycle. I analyzed the expression of the CLN1, CLN2 and
RNRI genes. CLN1 transcription is regulated through MCB elements that are dependent upon SBF (Swi4/Swi6, Partridge et al., 1997), CLN2 is regulated through both SCB and MCB elements, and also through a novel promoter element(s) that is dependent on SWI4 (Cross et al., 1994; Stuart and Wittenberg, 1994), and RNRI is controlled through MCB elements that are dependent on MBF (Mbp1/Swi6, Elledge and Davis, 1990). I found that the stb1Δ mutant initiated transcription of CLN1, CLN2 and RNRI at the same cell volume as wild type—at approximately 25fL (Figure 3-4A, B). As previously reported (Tycrs et al., 1993), activation of these genes was significantly delayed in the cln3Δ mutant, with transcripts accumulating at a cell volume of roughly 40fL. The delay in transcriptional activation was even more pronounced in the stb1Δcln3Δ double mutant; the CLN1 and CLN2 transcripts began to accumulate at a cell size of about 60fL. There was no clear induction of RNRI in the stb1Δcln3Δ mutant, and the cln3Δ single mutant only showed a minor induction of RNRI transcription. I conclude that although Start transcripts are induced with similar kinetics in wild type and stb1Δ strains, STB1 is important for the timing of Start transcription in a cln3Δ strain. The defect in the timely activation of Start transcription in the stb1Δcln3Δ double mutant could account for the phenotypic defects seen in the stb1Δcln3Δ double mutant, namely the large cell size, the compromised G1 progression and the growth defect. Indeed, overexpressed CLN1 and ectopically expressed CLN2 can suppress the slow growth phenotype of the stb1Δcln3Δ mutant (M. Costanzo, unpublished data), suggesting that the slow growth phenotype is due to improper expression of CLNs. The observed physical interaction between Stb1 and Swi6 suggests that Stb1 may affect Start transcription directly through SBF and/or MBF.
Figure 3-4: Activation of Start Transcription in \textit{stb1Δcln3Δ} mutants

(A) Wild type (BY263), \textit{stb1Δ} (BY805), \textit{cln3Δ} (BY655) and \textit{stb1Δcln3Δ} (BY822) cultures were synchronized by isolating small G1 daughter cells by centrifugal elutriation. The isolated small daughter cells were inoculated into YPD medium, and aliquots were taken at 15 or 20 min. intervals for total RNA isolation, budding analysis and FACS analysis of DNA content (not shown). \textit{CLNI}, \textit{CLN2}, \textit{RNRI} and \textit{ACT1} expression was determined by Northern blot analysis of the extracted total RNA. Budding percentage was counted from at least 300 cells, and the mean cell size was estimated from the cell size peak determined by a Coulter channelizer. (B) The Northern blot data from A were quantitated by phosphorimager scanning. The quantitated levels of \textit{CLNI}, \textit{CLN2} and \textit{RNRI} expression were normalized against \textit{ACT1} expression as an internal control, and the normalized expression levels were plotted versus mean cell size.
A budding (%):  
clen size (fl):  

**WT**

* time after release (min.):  

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**stb1Δ**

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**cln3Δ**

* time after release (min.):  

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<td>54</td>
<td>58</td>
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There is a slight difference in the peak height of the transcript levels between the $stb1\Delta$ mutant and wild type, although there is little difference in the integrated area for each peak. This difference between $stb1\Delta$ and the wild type cells may reflect poorer synchronization of the $stb1\Delta$ strain by centrifugal elutriation, since $stb1\Delta$ cells synchronized by $\alpha$-factor block-and-release exhibited identical Start transcription profiles with wild type (not shown).

**Phosphorylation of Stb1 by Cln-Associated Kinases in vivo and in vitro**

To further explore the function of Stb1, I investigated the relationship between Stb1 and Cln-Cdc28 kinase complexes. The Stb1 amino acid sequence contains 5 putative Cdc28 phosphorylation sites (S46, S111, S128, T191 and T351; using the consensus S/T,P,x,basic; ref. Morgan, 1998). I used epitope-tagged cyclins to immunoprecipitate Cln1-Cdc28 and Cln2-Cdc28 kinases from yeast extracts (Tyers et al., 1993; Tyers et al., 1992). The immunoprecipitates were incubated with $\gamma^{32}$P-ATP and tested for kinase activity towards purified, recombinant Stb1. Histone H1, a substrate commonly used to assay Cdc28 kinase activity, was included as a control in the kinase reactions. Stb1 was an excellent substrate for both the Cln1-Cdc28 and Cln2-Cdc28 kinases in vitro and was a better in vitro substrate than Histone H1 (Figure 3-5, lanes 3-6). Stb1 was also phosphorylated by Cln3-Cdc28 kinase complexes purified from insect cells (J. Wang and M. Tyers, personal communication). In contrast, Stb1 was less effectively phosphorylated by Cdc28 associated with the Clb2 mitotic
Figure 3-5: Stb1 as a Substrate for Cyclin-Associated Kinases.

Strains bearing HA (hemagglutinin)-tagged alleles of CLNI, CLN2 and CLB2 were lysed, and the tagged cyclin-Cdk complexes were immunoprecipitated with anti-HA antibodies. γ-32P-ATP and exogenous substrates (1µg Histone H1 to all reactions and 0.5µg Stb1 protein to reactions indicated) were added to the immunoprecipitates. The reactions were allowed to proceed for 20 min. at 30°C.
cyclin (Figure 3-5, lane 8). The kinase activities of all three forms of Cdc28 tested in Figure 3-5 were normalized with respect to phosphorylation of Histone H1.

The ability of G1-specific forms of Cdc28 to phosphorylate Stb1 \textit{in vitro} raised the possibility that Stb1 may be a downstream target of Cln-Cdc28 kinases \textit{in vivo}. To test this possibility, I first asked whether Stb1 was a phosphoprotein \textit{in vivo}. Immunoblot analysis with affinity-purified antibodies to Stb1 (\(\alpha\)-Stb1 antibodies) recognized several isoforms of Stb1 in lysates from log phase yeast cells (Figure 3-6A, lane 2). The antibodies recognized endogenous Stb1 (data not shown) but the Stb1 isoform signals were more easily detected in a strain bearing a high-copy plasmid expressing STBI from its own promoter (see Materials and Methods, Figure 3-6A). Phosphatase treatment of the extract resulted in loss of the slower migrating species (upper bands \(a\) and \(b\)) and an increase in the fastest migrating isoform (lower band \(c\), Figure 3-6A, lane 3). The presence of phosphatase inhibitors in the phosphatase reaction prevented the conversion to the fastest migrating isoform (Figure 3-6A, lane 4). I conclude that Stb1 is a phosphoprotein \textit{in vivo}, and that the slower migrating species detected on SDS-PAGE correspond to hyper-phosphorylated forms of Stb1.
Figure 3-6: Analysis of Stb1 Phosphoforms by Western Blot Analysis.

(A) Anti-Stb1 Western blots were done on extracts from cells with Stb1 overexpressed from a 2µ vector (lane2) or with vector only (lane1). Samples of cell extracts were treated with protein phosphatase with (lane3) or without (lane4) phosphatase inhibitors prior to Western blot analysis. (B) Anti-Stb1 western blot on wild-type and cln1Δcln2Δcln3Δ extracts (The sic1Δ mutation allows the cln1Δ,2Δ,3Δ strain, which would otherwise die, to be viable. The strain also had a genomic GAL-CLN3 gene which allowed CLN3 expression to be controlled so that the cells were not unnecessarily stressed during growth before assaying.). Extracts were made from wild type and clnAsicΔGAL-CLN3 cells during growth in medium with galactose, when GAL-CLN3 is induced, and after 2 and 3 hours after transfer to medium with glucose to shut off CLN3 expression. The various forms of Stb1 are denoted a, b and c.
Because I saw *in vitro* phosphorylation of Stb1 by Cln-associated kinases, I tested whether the Stb1 phosphoforms I detected *in vivo* were genetically dependent on the *CLN* genes. Although each *CLN* normally plays distinct roles in wild type yeast cells, mutational analyses have shown that the *CLN* genes are genetically redundant. That is, any single *CLN* gene is sufficient to support cell viability and cell cycle progression. Given this genetic redundancy, I assayed Stb1 phosphoforms in yeast extracts from strains deleted for all three *CLN* genes. Since a strain lacking *CLN1,2* and 3 would normally arrest at Start, the cells were kept alive by deletion of the B-type Cdk inhibitor *SIC1* (Schneider et al., 1996; Tyers, 1996) and by expression of *CLN3* from the inducible GAL promoter (GAL-CLN3, Tyers et al., 1992). The *cln1,2,3Δ sic1Δ GAL-CLN3* strain was first grown on galactose-containing medium, to turn on *CLN3* expression (Figure 3-6B, lanes 1-3) and later transferred to glucose-containing medium to repress *CLN3* expression, thereby depleting all Clns from the yeast cell (lanes 4-7). I used α-Stb1 immunoblots on extracts from the Cln-depleted cells to show that the phosphorylated isoforms of Stb1 were dependent on *CLN* function (Figure 3-6B). When Cln 3 is the only source of Clns, I saw only the two lower phosphoforms of Stb1 (bands b and c; Figure 3-6B, lane 3). Relative to the lower band c, band b in this strain was not as intense as in the wild type strain. When *GAL-CLN3* expression was shut off in the presence of glucose, the two upper bands (a and b) disappeared, and the lower band c increased in intensity.
Figure 3-7: Cell cycle analysis of the Stbl phosoforms.

(A) Anti-Stbl Western blots were done on extracts from wild type cells (BY263) that were synchronized by α-factor G1 block and release. Extracts were made from samples taken at 15 min. intervals. Various forms of Stbl are denoted a, b and c. The Western blots were compared to PCLI Northern analysis on the same cultures. The timing of PCLI transcript appearance acted as a marker for Start (ie. when Start genes are transcribed). (B) FACS analysis of DNA content of the culture at each time point after release from α-factor.
A

Time after release (min.)

<table>
<thead>
<tr>
<th>Vector</th>
<th>Log arrest</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>75</th>
<th>90</th>
<th>105</th>
<th>120</th>
<th>135</th>
<th>150</th>
<th>165</th>
</tr>
</thead>
</table>

α-Stb1

PCL1 RNA

B

α-factor

0 min.

15 min.

30 min.

45 min.

60 min.

75 min.

90 min.

105 min.
Therefore, Stb1 phosphoforms are dependent on CLN function in vivo. In conjunction with the specific phosphorylation of Stb1 by Cln-kinases in vitro, this result strongly suggests that Stb1 is a direct target of Cln-associated kinases in vivo.

**Cell Cycle-Dependent Phosphorylation of Stb1**

Because my analysis with asynchronous cultures showed that Stb1 was phosphorylated in a Cln-dependent manner, I next asked if phosphorylation of Stb1 was cell cycle-periodic to reflect the periodic abundance of Clns. To examine phosphorylation of Stb1 throughout the cell cycle, I used α-factor mating pheromone to arrest cells in G1 phase before Start, released the cells into fresh medium and prepared extracts from samples as the culture progressed synchronously through the cell cycle (Breeden, 1997). Figure 3-7 shows an immunoblot with anti-Stb1 antibodies on samples isolated from a synchronous culture. The slower-migrating, hyper-phosphorylated forms of Stb1 (bands a and b) were absent in cells arrested by α-factor treatment (Figure 3-7A, lane 2). The upper phosphoforms then gradually reappeared as the cells progressed synchronously through the cell cycle (Figure 3-7A, lanes 2-6). To monitor the timing of Stb1 phosphorylation in the cell cycle, I used the accumulation of the SBF-regulated \textit{PCLI} transcript as a marker for Start transcription (Figure 3-7A), and I used FACS analysis of cellular DNA content as an indicator of DNA synthesis and cell cycle position (Figure 3-7B). I found that Stb1 phosphorylation reappeared after the appearance of \textit{PCLI} transcripts (Figure 3-7A, lanes 4 and 5), but before DNA replication (Figure 3-7B, 30 min.). During the peak of Stb1 phosphorylation, only the two upper phosphoforms (a
Figure 3-8: Timing of Stb1 Phosphorylation in Wild Type Versus \textit{cln3}Δ Cells

Wild type (BY263) and \textit{cln3}Δ (BY655) cells were synchronized by arrest in late G1 with α-factor mating pheromone, followed by release into synthetic glucose medium (see Materials and Methods). Sample aliquots were collected at 15 min. intervals for Western blot analysis of Stb1 phosphoforms and Northern blot analysis of initiation of Start transcription. The protein extracts were probed with α-Stb1 antibodies (various forms of Stb1 are denoted \textit{a}, \textit{b} and \textit{c}). Western blot analysis of Stb1 phosphoform accumulation was compared to the onset of Start transcription as assayed by \textit{CLB5} expression.
WT

$cln3\Delta$

CLB5 northern
α–Stb1 western
CLB5 northern
α–Stb1 western
and b) were seen (lanes 5-7). The Stb1 phosphoforms then began to disappear in G2 phase (Figure 3-7AB; lane 8, 75min.). Thus, Stb1 is phosphorylated in a cell cycle-dependent manner in vivo, with Stb1 phosphoforms beginning to accumulate early in the cell immediately after the appearance of Start-dependent transcripts. The timing of Stb1 phosphorylation is consistent with my data suggesting that Stb1 is a substrate for Cln-associated kinases.

The appearance of Stb1 phosphoforms after Start gene expression suggested that Stb1 phosphorylation may not be involved in the STB1-dependent transcriptional activation that I observed in my gene expression analysis. However, my data suggest a requirement for STB1 in timing of Start transcription only in cells deleted for CLN3 (Figure 3-2, -4). I therefore checked the timing of the appearance of Stb1 phosphoforms in the cln3Δ mutant, where STB1 is important for the proper timing of Start. In contrast to the wild type strain, Stb1 phosphoforms in the cln3Δ mutant appeared coincidentally with CLB5 transcription (marker for transcription at Start; Figure 3-8, lane 8). The intermediate Stb1 phosphoband b appeared as CLB5 transcripts began to accumulate (lane 8). The slowest migrating phosphoband a appeared as CLB5 transcription peaked (lane 10). Therefore, under conditions where STB1 is important for G1 progression and Start (ie. in the cln3Δ mutant), Stb1 phosphorylation is coincident with the activation of Start transcription. Together, my analysis of Stb1 phosphorylation suggests that phosphorylation of Stb1 by Cln-Cdc28 contributes to the activation of transcription at Start.
DISCUSSION

My data imply a role for Stb1 in the activation of transcription at Start; specifically, STB1 is important for Start transcription when CLN3 is deleted. Compared with the cln3Δ mutant, the stb1Δcln3Δ mutant is slower growing, has a larger cell size, shows a pronounced G1 delay by FACS analysis and undergoes Start transcription at a larger cell volume. Stb1 may affect Start transcription through its interaction with Swi6. The Stb1 protein is phosphorylated by Cln-associated kinases in vitro and is phosphorylated in vivo in a CLN-dependent manner. In the cln3Δ mutant, where STB1 becomes important for G1 progression, the in vivo phosphorylation of Stb1 coincides with the activation of Start transcription, suggesting that Cln-dependent phosphorylation of Stb1 mediates STB1-dependent activation of transcription at Start.

The Role of the Swi6 Ankyrin Repeats in Binding Stb1

In this study, I describe a physical interaction between Swi6 and Stb1 in vitro. I show that the Swi6 ankyrin repeats are required for binding of Stb1 to a Swi6 column, and that Swi6 binds directly, in the absence of DNA or other proteins, to Stb1 in a farwestern blot assay. A small amount of Swi6 can be immunoprecipitated from yeast extracts with α-Stb1 antibodies (M. Costanzo, personal communication). In choosing to study the biological role of STB1, I did not further analyze the regions of Stb1 or Swi6 required for their interaction.

However, it would be interesting to see if Stb1 also binds to Swi4 or Mbp1 which also contain ankyrin repeats. The close conservation of this region in the cell cycle
regulatory transcription factors of three yeast species suggests that the ankyrin motif region in these proteins may bind a similar or conserved protein (see Chapter 1, section 3). In Swi4, the ankyrin repeats are both necessary and sufficient for binding to the mitotic cyclin, Clb2; Clb2 is required for down-regulation of SBF-driven gene expression in G2 (Amon et al., 1993). Clb2-associated kinase phosphorylates Swi4 in vitro, but whether this phosphorylation is required for down-regulation of SBF is unknown. Clb2 does not bind to Swi6 or Mbp1, suggesting that in the case of Swi4-Clb2 binding, the Swi4 ankyrin repeats are mediating an interaction that is specific to the Swi4 motifs (Siegmund and Nasmyth, 1996).

Mutational analyses of the ankyrin repeat region of Swi6 suggest roles for this region in both activation and repression of transcription. First, functional domain mapping of the Swi6 protein implicates the Swi6 ankyrin repeat domain in transcriptional repression (Sedgwick et al., 1998). Point mutants have been isolated in the ankyrin repeat region of Swi6 that reduce SBF-dependent transcription (Ewaskow et al., 1998). Many of these mutants appeared to be defective in protein folding, consistent with the ankyrin repeats forming a protein structural motif (see Chapter 1, section 3b). One substitution, G347D, was predicted to have little effect on the protein structure and may instead affect a protein-protein interaction required for transcriptional activation. Whether the binding of Swi6 to Stb1 is affected by the G347D mutation remains to be tested. SWI6 mutants that are defective for Stb1 binding may not have been isolated in this study since swi6 mutants that were defective for HO::LacZ expression were selected, and stb1Δ mutants exhibit no defects in HO or SCB::LacZ expression (Kasten and Stillman, 1997, and Yuen Ho, data not shown).
Regulation of Start Transcription by Stb1

I found that Stb1 and Swi6 interact directly in vitro, suggesting that Stb1 may activate gene expression by interacting with Swi6, a common subunit in SBF and MBF. Stb1 (Sin three-binding protein) was originally identified as a protein that bound the Sin3 general transcription repressor (Kasten and Stillman, 1997). A possible model is that Stb1, bound to promoters via Swi6, may effect Start transcription through Sin3. However, no direct role has been reported for Stb1 in Sin3-dependent transcriptional repression. Moreover, HO is the only Start-expressed gene whose expression has been reported to be dependent on SIN3. Although SIN3 does affect HO transcription, a role for SIN3 in SBF-mediated transcription of HO has not been established. Mutation of SIN3 does not de-repress expression of the HO gene in swi6Δ or swi4Δ cells (Sternberg et al., 1987), but the cell cycle regulated expression of HO or any Start gene has not been examined in a sin3Δ mutant. Sin3 was not detected in the Swi6 affinity column eluates (Y. Ho., unpublished data), suggesting that Sin3 is not present in Swi6-Stb1 complexes.

Although a role for Stb1 in transcriptional activation or repression has not been ruled out, I suggest that Stb1 is a regulator for the timing of Start transcription. The levels of Start transcripts in log phase stb1Δ and stb1Δsin3Δ cells are virtually identical to wild type cells (data not shown). Although stb1Δ cells synchronized by centrifugal elutriation show a slightly different peak in accumulation of Start genes (Figure 4), stb1Δ cells synchronized by α-factor block-and-release show an identical cell cycle transcription profile to wild type (not shown). Therefore, Stb1 does not affect the transcriptional activation of Start genes per se, but only the timing of Start transcription,
as manifest in the \textit{stb1\Delta cln3\Delta} mutant. I suggest that Stb1, like Cln3, is a regulator for the timing of Start.

**The Role of Cln-Dependent Phosphorylation of Stb1**

My analysis of \textit{STB1} suggests that Stb1 is an \textit{in vivo} substrate for Cln-kinases, and that it plays a role in activating transcription at Start. To integrate these two observations, I suggest that \textit{CLN}-dependent phosphorylation of Stb1 activates \textit{STB1}-dependent transcription. However, the induction of \textit{STB1} expression at Start and the apparent peak in Stb1 phosphorylation after Start in the wild type strain seem to suggest a role for Stb1 phosphorylation after transcriptional induction at Start. A solution to this apparent contradiction lies in a re-examination of the role of the \textit{CLNs} in the activation of transcription at Start (discussed earlier in Chapter 1, section 2). The current linear model for Start holds that \textit{CLN3} is primarily responsible for activating Start transcription, while \textit{CLN1} and \textit{CLN2} are involved in post-Start events such as bud formation and DNA synthesis (Dirick et al., 1995; Stuart and Wittenberg, 1995). However, activation of Start transcription still occurs in a \textit{cln3\Delta} mutant, albeit at a larger cell size (Tyers et al., 1993), suggesting that other pathways can activate Start transcription in the absence of \textit{CLN3} (discussed further below). My data, and observations by others, support the existence of other, dedicated pathways that can activate Start transcription in the absence of \textit{CLN3}. Studies of \textit{BCK2} (Bypass of \textit{C-Kinase} mutation), and my analysis of \textit{STB1}, implicate these two genes in the \textit{CLN3}-independent pathway(s) for activating Start (DiComo et al., 1995; Epstein and Cross, 1994, and this study). Neither \textit{bck2\Delta} nor \textit{stb1\Delta} mutants show any appreciable phenotypes in the presence of \textit{CLN3}. However, mutation of these genes

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in a cln3Δ mutant causes a pronounced delay in G1 progression and Start transcription, suggesting that BCK2 and STB1 are specific activators of Start transcription that can act in the absence of CLN3.

Previous observations by others, and my analysis of STB1, lead me to propose a role for CLN1,2 in the CLN3-independent pathways for transcriptional activation at Start. Transcription at Start is genetically dependent on CLN1 and CLN2 since a cln1Δcln2Δcln3Δ triple mutant arrests in late G1 before the activation of Start transcription (Wittenberg et al., 1990). Promoter swap experiments suggest that CLN2 and CLN3 differ in their biological roles but cannot formally exclude the possibility that, in the absence of CLN3, the genetic requirement for CLN1,2 in activating Start transcription is just due to the gradual accumulation of Cln1,2 in G1 which, due to overlapping biochemical specificities, act to substitute for the function of CLN3 (Levine et al., 1996). However, CLN3-dependent activation of Start transcription and CLN1,2-dependent activation (in the cln3Δ mutant) are clearly distinct in that they have different requirements. First, CLN3-dependent activation of Start is not affected by mutation of BCK2 and STB1 (bck2Δ, stb1Δ, bck2Δcln1Δcln2Δ and stb1Δcln1Δcln2Δ mutants show no obvious defects, ref. DiComo et. al., 1995 and this study), whereas the timing of Start transcription in a cln3Δ mutant (that requires CLN1,2 for Start transcription) is greatly affected by further mutation of BCK2 and STB1. This requirement is not because mutation of BCK2 or STB1 causes a decrease in CLN1,2 levels (DiComo et. al. 1995 and Y.Ho, unpublished). As such, CLN3- and CLN1,2-dependent activation of Start transcription have different genetic requirements. Second, Start transcription driven by CLN2 as the only CLN requires different promoter elements than CLN3-driven
transcription (Stuart and Wittenberg, 1995). Therefore, the activation of Start transcription in a cln3A mutant is unlikely to be due to CLNI,2 substituting for CLN3 function. Rather, CLNI,2 likely play a specific role in activating transcription at Start. Since, BCK2, STB1 and CLNI,2 are either important or required for Start gene expression in the cln3A mutant, I propose that BCK2, STB1 and CLNI,2 are components of CLN3-independent pathway(s) that activate Start in the absence of CLN3 function.

Although CLN3cln1Δcln2Δ cells still accumulate Stb1 phosphoforms (data not shown and Figure 3-6, lane 3), Cln1- and Cln2-Cdc28 are likely the physiological kinases for Stb1 phosphorylation: (i) a cln1Δcln2ΔGAL-CLN3 strain only shows low level phosphorylation of Stb1, despite overproduction of Cln3 (Figure 3-6B, lane 3), (ii) the phosphoforms in a CLN1CLN2cln3Δ strain are comparable to those in a wild type strain, (iii) in the wild type cell, Stb1 is phosphorylated after Start, at the time when Cln1 and Cln2 levels are induced, and (iv) in the cln3Δ mutant, Cln1 and Cln2 are sufficient to phosphorylate Stb1 at the time of Start transcription. Since both CLN1/2 and STB1 are important for G1 progression in the absence of CLN3, the simplest model to explain Cln-dependent phosphorylation of Stb1 is that Cln1,2-associated kinases phosphorylate Stb1 to activate Start transcription. Consistent with this notion, when STB1 is important for Start (ie. in the cln3Δ mutant), phosphorylation of Stb1 is coincident with activation of Start transcription. The phosphorylation of Stb1 by Cln1,2-kinases may partially explain the requirement for CLNI,2 for Start in the cln3Δ mutant. However, phosphorylation of Stb1 does not account for the absolute requirement of CLNI,2 in the cln3Δ mutant since the stb1Δcln3Δ mutant is still alive. Other regulators of Start, such as BCK2, may also be targets of CLNI,2.
A Proposed Positive-Feedback Loop Required for Activation of Start Transcription in the Absence of CLN3

As discussed above, in the absence of CLN3, Cln1,2 are required for their own transcription. This requirement suggests that a positive feedback loop functions to activate transcription at Start. Moreover, STB1 is transcriptionally induced at Start. Since Stb1 is also an activator of Start transcription, it may also function as a component of a positive feedback loop. In Figure 3-9, I show a model in which Cln1,2-Cdc28 complexes phosphorylate Stb1 to activate STB1-dependent transcription at Start. The newly synthesized Cln1,2 and Stb1 at Start further activate their own expression. I suggest that this positive feedback loop for activation of Start transcription was difficult to appreciate experimentally because it is only required in the cln3Δ mutant, and not in wild type cells (Dirick et al., 1995; Stuart and Wittenberg, 1995). This model explains why Stb1 is phosphorylated after the appearance of Start transcripts in the wild type cell.

I suggest that CLN3-dependent activation of Start occurs at a smaller cell size than that required to activate the CLN1,2/STB1-dependent pathway, so the CLN3-dependent pathway may override or 'mask' the CLN1,2/STB1 pathway for activation of Start transcription. I suggest the CLN3-dependent burst of CLN1,2 expression at Start causes a rapid accumulation in Stb1 phosphoforms after the appearance of Start transcripts in the wild type cell. In the cln3Δ mutant, CLN1/CLN2 are required for their own synthesis, so the accumulation of Start transcripts reflects the accumulation of Cln-dependent phosphorylation of Stb1.
Figure 3-9: Model for the Role of \textit{STBI} in Activation of Transcription at Start

The above model incorporates my findings with \textit{STBI} and previous observations on \textit{CLNI} and \textit{CLN2} (see text). \textit{BCK2}, \textit{STBI} and \textit{CLNI,2} play important roles for the activation of Start transcription in the absence of \textit{CLN3} function. \textit{Cln1,2}-dependent phosphorylation of Stb1 is proposed to activate \textit{STBI}-dependent activation of Start transcription. Stb1 likely effects transcriptional activation through interaction with the Swi6 subunit of SBF and MBF. Since both \textit{STBI} and \textit{CLNI,2} are activators of Start transcription and are themselves transcribed at Start, I propose a positive feedback loop mechanism for activation of Start in the absence of \textit{CLN3}. 

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**CLN3-Independent Pathway(s) for Activating Start**

In Figure 3-9, I diagram \( BCK2 \), \( STB1 \) and \( CLN1,2 \) as components of the \( CLN3 \)-independent pathway(s) for Start transcription. There are likely at least three pathways for activating Start transcription because as mentioned earlier, \( stb1Δcln3Δ \) and \( bck2Δcln3Δ \) double mutants still undergo Start transcriptional activation, albeit at a much larger cell size. How \( BCK2 \), \( STB1 \) and \( CLN1,2 \) fit into these various pathways remains to be elucidated. However, \( STB1 \) is likely a downstream component of one these pathways because it interacts directly with the Swi6 transcription factor. \( CLN1,2 \) likely act directly upstream of \( STB1 \) because Cln1,2-kinases phosphorylate Stb1; however, as discussed above, Cln1,2-kinases must phosphorylate other substrates for Start activation. Clearly, the activation of transcription at Start is a complex phenomenon, and more work needs to be done to construct a more detailed model for activation of Start transcription (see Chapter 4).

In a wild type cell, deletion of \( BCK2 \) or \( STB1 \) has little effect on the timing of Start (DiComo et al., 1995, and this study), whereas \( cln3Δ \) mutants undergo Start at a larger cell size (Tyers et al., 1993). Does this mean that \( CLN3 \) is the ‘biologically relevant’ activator of Start, and that the \( CLN3 \)-independent pathways are only important in the \( cln3Δ \) mutant as a ‘back-up’ pathway? Most studies done with \( CLN3 \), \( STB1 \) and \( BCK2 \) mutants used optimal growth conditions that may not reflect the natural habitats of \( S. cerevisiae \). Perhaps, under these optimal growth conditions, \( CLN3 \) is the most important activator of Start. However, there is no evidence that deletion of \( CLN3 \) affects the timing of Start in cells grown under sub-optimal conditions. Studies on the
regulation of \textit{CLN3} suggest that it may play a more limited role in the cell cycle than previously thought. As mentioned in Chapter 1 (Introduction), Cln3 is regulated in response to translation rates and to nitrogen and carbon sources. Cln3 is downregulated—transcriptionally, translationally and by protein degradation—in response to poor nitrogen and carbon sources (Gallego et al., 1997; Parviz and Heideman, 1998; Parviz et al., 1998; Polymenis and Schmidt, 1997). Paradoxically, cells grown in poor carbon sources, where Cln3 is activity is low, undergo Start at a smaller cell size (Jagadish and Carter, 1977; Nash et al., 1988). This result is unexpected since \textit{cln3}Δ cells are large, and cells overexpressing \textit{CLN3} are small (Nash et al., 1988; Tyers et al., 1992). This observation suggests that, in poor carbon and/or nitrogen sources, \textit{CLN3} is not important for activating Start. Rather, another \textit{CLN3}-independent pathway likely becomes active in poor carbon/nitrogen sources to drive Start entry at a smaller cell size. Indeed, Hall \textit{et. al.}(1998) suggest that \textit{CLN3} acts specifically in rich media to shorten the length of G1. Thus, in cells grown in rich media, we may be looking at activation of Start under conditions where \textit{CLN3} is specifically up-regulated to drive the cell cycle while inputs from other pathways are overridden. In this situation, \textit{BCK2}, \textit{STB1} and \textit{CLN1/2} would appear to play secondary, ‘back-up’ roles for activation of Start transcription. Although \textit{bck2}Δ, \textit{stb1}Δ and \textit{cln1Δcln2Δ} mutants do not show a delay in Start transcription when grown in rich medium, my model predicts that these mutants will show a delay when grown in poor medium.

If \textit{CLN3} acts to sense the presence of nutrients such as carbon and nitrogen, other pathways may respond to cell size, pH, temperature, or perhaps, even the proximity of other cells. Interestingly, \textit{BCK2} has been associated with the yeast PKC (Protein Kinase
C) pathway because overexpression of BCK2 can suppress the lethality of a \( pkc1 \Delta \) mutation and the high-temperature lethality of a deletion in the gene for \( SLT2 (MPKI) \), the terminal MAP kinase of the PKC pathway (Lee et al., 1993). The PKC pathway in yeast transduces signals for membrane stress; membrane stress may be an indicator of cell size. Moreover, \( SLT2 (MPKI) \), has been observed to phosphorylate Swi6 \textit{in vitro}, and mutations in \( SLT2 \) can be suppressed by overexpression of two Start-expressed genes, \( PCL1 \) and \( PCL2 \) (Madden et al., 1997). A role for the yeast PKC pathway in driving cell cycle entry is consistent with the mitogenic role of the PKC pathway in multicellular eukaryotes.

Although previous models regarded entry into Start as a simple, linear pathway centering on \( CLN3 \) regulation, recent studies suggest that entry into the budding yeast cell cycle at Start is influenced by multiple signals that are transduced through many pathways. This makes cell cycle entry in yeast ever more similar to metazoan cell cycles which must incorporate both extra- and intracellular signals such as presence of growth factors or anti-mitogens in serum, nutrients, cell size, contact inhibition and senescence.
CHAPTER IV

THESIS SUMMARY

AND

FUTURE DIRECTIONS
Thesis Summary

In budding yeast, the Swi6 protein functions in two transcription factor complexes, SBF and MBF, to promote cell cycle-dependent gene expression early in the cell cycle. In an effort to understand how Swi6 is regulated, I used Swi6 protein affinity chromatography to identify Swi6-interacting proteins from yeast extracts.

In Chapter 2, I described the identification of Hrr25, a casein kinase I isoform, as a Swi6-interacting protein. I showed that Swi6 is an in vitro substrate for the Hrr25 kinase. Hrr25 may regulate Swi6 to activate transcription of repair genes in response to DNA damage since both Hrr25 and SBF are required for viability in the presence of DNA-damaging agents and for the induction of repair genes in response to the DNA synthesis inhibitor hydroxyurea.

In Chapter 3, I described the characterization of a second Swi6-binding protein, Stb1. Stb1 interacts with Swi6 in vitro and is required for the timely onset of Start transcription in the absence of CLN3 function. I showed evidence suggesting that phosphorylation of Stb1 by Cdc28, associated with the G1 cyclins Cln1,2, may activate STB1-dependent activation of Start transcription.

In this Chapter, I discuss future directions stemming from my work described in Chapter 3, since my studies of Stb1 suggest interesting experiments to explore mechanisms activating transcription at Start. First, I describe the analysis of the requirement for CLN3-dependent and CLN3-independent pathways for activation of Start transcription under different nutrient conditions. This analysis sets the foundation for assay conditions to be used in future experiments. Next, I describe experiments to
identify other genes involved in activating Start transcription. After defining nutrient conditions to assay activation of Start transcription and after identifying other components in both the CLN3 and CLN3-independent pathways, I propose to use genetic analysis to construct a model to integrate various regulators of Start into pathways for activating transcription at Start (as predicted in the Chapter 3 Discussion). The final section deals with how the CLN3 and CLN3-independent pathways may converge to effect Start transcription.

**Activation of Start Under Various Nutrient Conditions**

Most of the published data on the activation of Start transcription implicate CLN3 as the important physiological activator of Start (see Chapter 3 Discussion). Deletion of CLN3 causes an obvious Start delay, whereas bck2Δ, stb1Δ and cln1Δcln2Δ mutants exhibit almost no delay in the onset of Start transcription (DiComo et al., 1995; Tyers et al., 1993), and Chapter 3]. On the surface, these genetic data implicate CLN3 as the key activator of Start transcription and suggest that BCK2, STB1 and CLN1/2 act in alternative or 'back-up' pathways that are only required when CLN3 is deleted. However, entry into the mitotic cycle is a highly regulated event; the cell must integrate numerous conditions and signals, both intracellular and extracellular. I suggest that the study of transcriptional activation at Start is incomplete until we study cell cycle entry under various growth conditions.

As discussed in Chapter 3, recent studies on the regulation of CLN3 activity have helped to illuminate the role of CLN3 in the activation of Start. CLN3 transcription and translation are specifically down-regulated, and the Cln3 protein is degraded when cells
are grown in poor carbon and nitrogen sources (Gallego et al., 1997; Parviz and Heideman, 1998; Parviz et al., 1998; Polymenis and Schmidt, 1997). These data apparently contradict the observation that yeasts grown on poor carbon sources are smaller than cells grown in rich carbon sources (Jagadish and Carter, 1977; Nash et al., 1988). In rich media, cells mutated for CLN3 are larger than wild type cells. This last observation suggests that cells with low CLN3 levels due to poor growth conditions would be larger than cells grown in rich carbon sources. However, yeasts grown on poor carbon sources are, in fact, smaller than cells grown on rich carbon sources. This apparent contradiction suggests that CLN3 is not the activator of Start in yeasts grown in poor carbon medium. Another, CLN3-independent pathway must be activating Start when CLN3 is down-regulated in these cells. To explain their observations, Hall et al. (1998) proposed that CLN3 is a regulator that acts specifically in rich medium to shorten the length of G1 phase.

Strains lacking BCK2, STB1 or CLN1/2 do not show any growth defect or obvious changes in the timing of Start transcription when grown in rich media. These genes are important for activation of Start when CLN3 is deleted, suggesting that they do play a role in turning on Start transcription. I propose to test whether the physiological role of these genes is in activating Start transcription in poor media when CLN3 levels are down-regulated. I predict that mutants defective in one or more of these genes would be large and slow growing in poor media. A rigorous test of my prediction would involve assaying the timing of Start transcription in synchronized cultures of these mutants grown in poor media. Furthermore, my model predicts that cln3Δ mutants, when grown in poor media, would not show a delay in Start transcription, as compared with wild type. This
series of experiments would formally test the proposal that in poor media, \textit{CLN3} is not driving Start and that another pathway(s) involving \textit{BCK2}, \textit{STB1}, \textit{CLN1/2} becomes important for Start transcription.

**Further Experiments on \textit{STB1}**

My data show that in the absence of \textit{CLN3}, \textit{STB1} is necessary for timely activation of Start transcription (Chapter 3). Since the other activators of Start, \textit{CLN3} and \textit{BCK2}, can accelerate the onset of Start transcription when overexpressed, an obvious experiment is to test if overexpressed \textit{STB1} can also accelerate the timing of Start. An acceleration of Start by ectopic Stb1 would strengthen my argument that \textit{STB1} is an activator of Start transcription.

I found that Cln-kinases can phosphorylate Stb1 \textit{in vitro} and Stb1 phosphoforms \textit{in vivo} are dependent on the \textit{CLNs}. In Chapter 3, I proposed that Cln-kinases phosphorylate Stb1 to activate Stb1-dependent activation of Start transcription. This proposal was based on the fact that both the \textit{CLNs} and \textit{STB1} are involved in activation of Start transcription and that \textit{CLN}-dependent phosphorylation of Stb1 appears at the time of Start transcription in the \textit{cln3Δ} mutant. To show that Clns activate Start transcription through Stb1, I propose to see if \textit{CLN}-dependent acceleration of Start is dependent on \textit{STB1}. When overexpressed, \textit{CLN1}, \textit{CLN2} and \textit{CLN3} can accelerate the onset of Start transcription (Tyers et al., 1993). My model for the role of Stb1 at Start predicts that acceleration of Start by overproduced Cln1 or Cln2 would be dependent on \textit{STB1}, at least to a certain degree (see Discussion in Chapter 3). If \textit{CLN3} acts through an activation pathway that is parallel to \textit{STB1/CLN1/CLN2}, then deletion of \textit{STB1} would not affect
acceleration of Start by Cln3 overproduction. The predicted results from these experiments would not only confirm that phosphorylation of Stb1 by Cln1/2 serves to activate Start transcription, but they would also confirm the genetic placement of \textit{STB1/CLN1/CLN2} in one pathway and \textit{CLN3} in another pathway for activation of Start.

**Novel Activators of Start Transcription**

As discussed in Chapter 3, there are likely three pathways that contribute to the activation of Start transcription. In this section, I propose to find novel activators of transcription at Start.

i) **The MAP kinase \textit{SLT2} as an activator of Start transcription**

Previous experiments had implicated the MAP kinase Slt2 as a regulator of SBF: (i) Slt2 phosphorylates Swi6 \textit{in vitro}; (ii) Swi6 phosphoforms \textit{in vivo} are dependent on \textit{SLT2}; (iii) \textit{slt2Δ} mutants are rescued by overexpression of two SBF-driven genes, \textit{PCL1} and \textit{PCL2} (Madden et al., 1997), and (iv) Slt2 is activated by tyrosine phosphorylation at around the time of Start (Marini et al., 1996; Mazzoni et al., 1996). Other data further support a role for \textit{SLT2} in activation of gene expression at Start; \textit{slt2Δcln3Δ} double mutants are very slow growing, and \textit{slt2Δ} mutants are defective for expression from an \textit{MCB::LacZ} reporter (Kevin Madden, personal communication). To formally establish \textit{SLT2} as an activator of Start transcription, I propose to assay the transcriptional induction of Start genes in \textit{slt2Δ} and \textit{slt2Δcln3Δ} mutants. As discussed above, the requirement for \textit{SLT2} may only be apparent in cells grown under certain conditions. If my experiments show a requirement for \textit{SLT2} in Start activation, I will test whether overexpression of \textit{SLT2} can accelerate the timing of Start transcription. As Slt2-dependent Swi6
phosphoforms are detectable by Western blot analysis, this allows an easy assay to check if Slt2-dependent phosphorylation of Swi6 is coincident with the onset of Start transcription. This experiment will be done both in a wild type and cln3Δ strain, since CLN3-activation of Start may obscure any requirement for SLT2.

Slt2 is the terminal MAP kinase for the PKC (protein kinase C) pathway that responds to membrane stress, and membrane stress may act as a sensor for cell size (reviewed in Levin and Errede, 1995; Zarzov et al., 1996). Therefore, the PKC pathway may transduce a cell size signal to the Start transcription apparatus through Slt2. This model predicts that SLT2-dependent phosphorylation of Swi6 will appear at a set cell size in both a wild type and cln3Δ cells. I anticipate that it will be technically difficult to study the role of PKC1 in activation of Start since PKC1 is an essential gene and the PKC pathway is involved in numerous cellular processes (reviewed in Levin and Errede, 1995). However, protein kinase C isoforms can be inhibited by the staurosporine peptide inhibitor (Watanabe et al., 1994). I propose to synchronize wild type and cln3Δ cells, followed by release into medium with various concentrations of staurosporine. By monitoring the timing of Start transcription in staurosporine-containing medium, I hope to determine whether the PKC pathway has a role in activating Start in wild type or cln3Δ cells.

(ii) Genetic screen for unknown activators of Start

There are likely to be other genes with roles in the activation of Start transcription. The observation that bck2Δcln3Δ and stb1Δcln3Δ mutants still activate Start transcription, albeit at a much larger cell size, suggests that there are at least three different pathways for activation of Start transcription (DiComo et al., 1995), Chapter 3]. This is further complicated by the fact that different pathways seem to be activating Start
in cells grown under different conditions; that is, if experiments in rich media hypothetically suggest three pathways, experiments in poor media may suggest only a single pathway. Our current understanding on the activation of Start transcription under different growth conditions and on how cell status is related to the different activation pathways is meagre. To improve our understanding of the mechanisms regulating gene expression at Start, I propose a genetic screen to identify other activators of Start transcription.

Many screens for activators of Start have already been performed. I propose a simple genetic screen to identify other activators of Start that may have been missed in other screens, perhaps due to the apparent genetic redundancy of Start regulation. I propose to use a colony reporter screen, motivated by the observation that two known activators of Start, BCK2 and STB1, are defective for expression from an MCB::LacZ reporter gene (Kim Arndt, personal communication and Yuen Ho, data not shown). As mentioned above, the MAP kinase Slt2 may be a regulator of Start transcription and is also defective for MCB::LacZ reporter gene expression (Kevin Madden, personal communication). The defect is specific to MCB-driven reporters since expression from an SCB::LacZ reporter is not affected in bck2Δ, stb1Δ and slt2Δ mutants. Mutants lacking CLN3 may have a similar MCB::LacZ expression defect; in Chapter3 (Figure 3-4), peak RNRI expression in a cln3Δ mutant was only 30% of that seen in the wild type strain. RNRI expression is controlled by MCB elements in its promoter that act like isolated MCB elements in that they are dependent on SWI6 and MBPI. The other genes I tested for expression do not show any appreciable defect in the peak levels of transcription in the cln3Δ mutant: CLN2 is controlled by both MCB and SCB elements,
and CLN1 is controlled through MCB elements that are dependent on SBF, not MBF. Therefore, clin3Δ mutants may also share a similar defect in MCB::LacZ expression but not SCB::LacZ expression. In summary, four known or suspected activators of Start transcription share a similar defect in MCB reporter gene expression, but not SCB reporter gene expression. The defect for MCB reporter expression in these mutants is an empirical observation. Perhaps, transcription driven by isolated MCB elements is more sensitive than from SCB elements, requiring activation from all the pathways that activate Start transcription. I plan to directly test MCB::LacZ and SCB::LacZ reporter gene expression in activators of Start transcription that have not been tested (ie. in the clin3Δ mutant and also in the clin1Δclin2Δ mutant).

If the clin3Δ mutant and clin1Δclin2Δ mutants are defective for MCB::LacZ expression, then the MCB::LacZ reporter would be a useful assay for isolating new mutants in activators of Start. Cells will be mutated by transposon-mediated mutagenesis, and the white/light blue colonies will be scored as mutants. In my experience with the stb1Δ mutant, the 5-fold reduction in MCB::LacZ expression in this strain can easily be detected in a colorimetric screen for a mutant white colony against a background of wild type blue colonies (Yuen Ho, data not shown). LacZ reporter screens have been used successfully to identify mutants that fail to express HO::LacZ and SCB::LacZ reporters (Andrews and Herskowitz, 1989; Breeden and Nasmyth, 1987). Because of the genetic redundancy for activation of Start transcription, I anticipate that most unidentified activators of Start are non-essential genes. Therefore, I suggest transposon-mediated mutagenesis in this screen for quick identification of the mutated gene (Snyder et al., 1986), although essential genes will not necessarily be identified.
using this method of mutagenesis. The MCB::LacZ-defective mutants will be assayed for the timing of Start transcription. These mutants will also be examined in a cln3Δ background. The MCB::LacZ reporter screen can also be done on colonies grown in poor medium in which CLN3 is down-regulated. A screen in suboptimal growth medium may make the screen more sensitive to mutations in other Start activators by selecting conditions in which expression of Start genes is more dependent on these other activators.

I hope to obtain a collection of mutants from this screen that are involved in the activation of gene expression at Start. Identification of all activators of Start is crucial in constructing a genetic pathway for activation of Start. Moreover, the identification of any novel genes with roles in Start activation will likely shed light on how Start is regulated.

**Genetic Analysis of Start Transcriptional Activation**

In this section, I focus on genetic analysis of the known activators of Start transcription, CLN3, BCK2, STB1 and CLN1/2.

As discussed before, the current data suggest that CLN3 functions in a parallel fashion with BCK2, STB1 and CLN1,2 to activate Start transcription, but we do not know the genetic relationships between BCK2, STB1 and CLN1,2. The viability of stb1Δcln3Δ and bck2Δcln3Δ mutants suggests that there are at least three pathways for activating Start, so STB1, BCK2 and CLN1,2 are not necessarily in one pathway. The phosphorylation of Stb1 by Cln1,2 and the lack of apparent phenotype in the stb1Δcln1Δcln2Δ mutant suggest that STB1 and CLN1,2 are on the same pathway, with STB1 being downstream of CLN1,2 (Chapter 3, and Brenda Andrews and Yuen Ho, data
not shown). Since $\text{cln1} \Delta \text{cln2} \Delta \text{cln3} \Delta$ mutants are dead but $\text{stb1} \Delta \text{cln3} \Delta$ mutants are alive, $\text{CLN1,2}$ must affect other pathway(s) in addition to regulating $\text{STB1}$. Although $\text{bck2} \Delta \text{cln3} \Delta$ mutants are slow growing, there is no apparent defect in the $\text{bck2} \Delta \text{cln1} \Delta \text{cln2} \Delta$ mutant (DiComo et al., 1995). This observation is consistent with $\text{BCK2}$ and $\text{CLN1/2}$ being on the same pathway. However, this conclusion should only be considered tentative since in the $\text{bck2} \Delta \text{cln1} \Delta \text{cln2} \Delta$ mutant, $\text{CLN3}$ is fully functional and may obscure any effects of mutating the other pathways ($\text{CLN3}$ is phenotypically the most important activator of Start transcription in rich media).

I propose to refine the genetic placements of the known activators of Start. This may be a difficult task since a genetic analysis done solely on rich media will likely obscure the contributions of the $\text{CLN3}$-independent pathways to Start activation. Despite these complications, I suggest experiments that I hope will advance our understanding of Start activation.

i) Genetic analysis of mutants in Start regulation

So far, all the double mutant combinations for $\text{CLN3}$, $\text{BCK2}$, $\text{STB1}$ and $\text{CLN1,2}$ have been analyzed except for the $\text{stb1} \Delta \text{bck2} \Delta$ mutant. I propose to analyze the growth characteristics of this strain in rich and poor media. Although the $\text{bck2} \Delta \text{cln1} \Delta \text{cln2} \Delta$ and the $\text{stb1} \Delta \text{cln1} \Delta \text{cln2} \Delta$ mutants displayed no apparent growth defects when grown in rich medium, these mutants may exhibit defects in Start activation when grown in poor medium. I will analyze the growth rate and timing of Start in these mutant combination strains on poor medium. In short, I propose to do a thorough double mutant analysis ($\text{CLN1,2}$ are considered as a single gene in these experiments) on the known activators of Start transcription and to analyze all the double mutant combinations on both rich and poor medium.
ii) Analysis of multiple mutant combinations

The phenotypes of the bck2Δcln3Δ and stb1Δcln3Δ suggest that there are three independent pathways for activating Start. The cln1Δcln2Δcln3Δ mutant is dead and arrests before activation of Start transcription. I propose to ask if CLN3, STB1 and BCK2 function on separate pathways by constructing a bck2Δstb1Δcln3Δ triple mutant strain. A thorough analysis of the timing of arrest in this triple mutant will distinguish between lethality caused by general ‘sickness’ versus a specific defect in activating Start transcription. Moreover, any conclusions drawn from experiments with this triple mutant can be compared to and corroborated by data from the double mutant analyses described above.

If CLN3 is the important activator of Start in rich media, a stb1Δbck2Δcln1Δcln2Δ quadruple mutant should activate Start transcription with proper timing when grown in rich media.

iii) Epistasis analysis of Start activation by overexpression studies

The ability of overexpressed CLN3, CLN1,2 and BCK2 to accelerate the onset of Start transcription allows the analysis of Start by epistasis (DiComo et al., 1995; Tyers et al., 1993). I propose to find the genes acting downstream of CLN3, CLN1,2 and BCK2 by asking if mutating a putative downstream gene will eliminate the ability of CLN3, CLN1,2 and BCK2 to accelerate Start.

Further experiments for the genetic analysis of the pathways that activate Start transcription will likely be suggested by new developments in this field.
Analysis of the Regulation on the CLN2 Promoter

In Figure 4-1, I diagram two mechanisms by which various pathways may converge to activate gene expression at Start (only two pathways are shown for simplicity; there are likely more). In scenario A, the two pathways converge to activate SBF and MBF. The activated transcription factor complexes then promote gene expression from various Start-specific promoters. In scenario B, one pathway drives transcription through one region of the promoter, while another pathway affects a different region of the same promoter. Promoter dissection experiments suggest that scenario B may be the case for the CLN2 promoter (Cross et al., 1994; Stuart and Wittenberg, 1994). A region of the CLN2 promoter, designated UAS1, contains consensus SCB and MCB sequences. UAS1 is sufficient to confer Start-specific transcription to a heterologous promoter. CLN3-driven transcription of CLN2 is mediated primarily through the UAS1 element (Stuart and Wittenberg, 1995). However, a second region, designated UAS2, does not contain any SCBs or MCBs, but is nonetheless able to confer Start-specific regulation on a heterologous promoter. The regulation of UAS1- and UAS2-dependent expression is markedly different: (i) whereas expression driven by the native CLN2 promoter and UAS1 is still cell cycle regulated in swi4A and mbp1A mutants, cell cycle regulation of UAS2-driven transcription is entirely dependent on SWI4 but not MBP1 (although UAS2 contains no SCB elements), (ii) UAS1-driven transcription is primarily dependent on CLN3, while UAS2-driven transcription is dependent on BCK2 (Stuart and Wittenberg, 1995; DiComo et al., 1995), (iii) expression from a UAS1-driven reporter gene is dependent on Cdc28, but UAS2-driven transcription is not, and (iv) after the G1 function of Cdc28, activation of transcription through UAS1
Figure 4-1: Two Possible Mechanisms by Which the CLN3-Dependent and CLN3-Independent Pathways May Converge to Effect Transcription at Start.

(A) The CLN3-dependent and CLN3-independent pathways converge at the point of SBF/MBF activation. The activated SBF/MBF then activate the transcription of Start genes. (B) The CLN3-dependent and CLN3-independent pathways each drive transcription from different promoter elements in Start genes. Studies of the CLN2 promoter suggest that a mechanism such as that shown in (B) may be responsible for the expression of CLN2 at Start (see text for details).
A

**CLN3**

**CLN3-independent pathway(s)**

\[ \downarrow \]

SBF  MBF

\[ \downarrow \]

Start transcription

B

**CLN3**

**CLN3-independent pathway(s)**

\[ \downarrow \]

\[ \downarrow \]

\[ \downarrow \]

UAS1  UAS2
does not require de novo protein synthesis, whereas UAS2-driven transcription is blocked when protein synthesis is inhibited after release from G1 arrest caused by the cdc28-13 mutant. Although UAS2-dependent expression is not dependent on CDC28, the expression is nonetheless dependent on the CLNs. This apparent contradiction is addressed below.

I suggest that the properties of UAS2, as described above, are consistent with a role for UAS2 as a target for STB/CLNI,2-dependent activation of transcription. First, the dependence of UAS2-driven expression on the CLN genes and on de novo protein synthesis after Cdc28 function is consistent with a positive feedback loop mechanism, involving CLNI,2, acting on UAS2. In Chapter 3, I provided evidence suggesting that STB1/CLNI,2 activate Start transcription through a positive feedback loop mechanism. Second, as suggested by Stuart and Wittenberg (1994), a CLNI,2 positive feedback loop model is consistent with their observation that UAS2, but not UAS1, is insensitive to thermo-inactivation of a temperature sensitive Cdc28. To explain their observation, Stuart and Wittenberg suggested that temperature sensitive alleles may not necessarily inactivate a protein completely. Integrating the observations that UAS1 is the target of the CLN3-dependent pathway (Stuart and Wittenberg, 1995) and that Cln3 biochemical activity is weak in yeast extracts (Levine et al., 1996), I suggest that UAS1-dependent transcription would be highly sensitive to partial inactivation of Cdc28 since Cln3-Cdc28 activity would be severely compromised even by partial inactivation of Cdc28. I suggest that UAS2 may be the target of a CLNI,2-dependent pathway since CLNI,2-dependent activation likely utilizes a positive feedback mechanism, so Cln1 and Cln2 levels will be
abundant at Start, and may therefore compensate for a partially inactivated Cdc28. The abundant levels of Cln1 and Cln2 accumulated through a positive feedback loop may account for the contradictory requirement of UAS2 for CLN cyclins but not for fully functional CDC28.

In support of the CLN1,2-dependent pathway operating through UAS2, CLN2 transcription driven by CLN2 as the sole CLN was found to require both UAS1 and UAS2 (Stuart and Wittenberg, 1995). To summarize the known genetic requirements of the CLN2 UASs, CLN3 operates mainly through UAS1, BCK2 appears to operate through UAS2, and CLN2-driven transcription is dependent on both UAS1 and UAS2. I propose to find the requirement for STB1 in UAS1- and UAS2-dependent transcription. Since analysis of the CLN2 promoter elements also seems to suggest multiple activation pathways for Start transcription, I propose to further study the genetic requirements for UAS1- and UAS2-driven transcription. I also propose to study the characteristics of these promoter elements in cells grown under different nutrient conditions since my model predicts that the different pathways for Start transcription act under different nutrient conditions.

Finally, although Cross et. al. only isolated swi4 mutants in a screen for mutants defective in UAS2 reporter activity, I propose to assay the requirement of SWI6 for expression from UAS1- and UAS2-reporters since I found that Stb1 interacts with Swi6 in vitro. Mutation of SWI6 may not affect the expression of UAS2 expression in log phase cells but may have an effect on the cell cycle regulation of UAS2-dependent expression.
Concluding Remarks

My data outlined in Chapter 3 implicate \textit{CLN1,2} and \textit{STB1} as activators of Start that become important in the absence of \textit{CLN3}. Historically, the model for entry into the budding yeast mitotic cell cycle was less complex than in metazoan cells; in budding yeast, the activation of Start was believed to be a linear pathway with \textit{CLN3} sensing the critical cell size for Start, whereas metazoan cells integrated multiple intra- and extracellular signals for cell cycle entry. However, my data and observations by others suggest that entry into Start in \textit{S. cerevisiae} is a complex event that is regulated by multiple pathways (described in Chapter 3). Studying the multiple pathways that activate entry into the \textit{S. cerevisiae} cell cycle will surely shed light on the complexities of cell cycle entry in metazoan cells.


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Cvrckova, F., and Nasmyth, K. (1993). Yeast G1 cyclins CLN1 and CLN2 and a GAP-like protein have a role in bud formation. EMBO Journal 12, 5277-5286.


Epstein, C., and Cross, F., R. (1994). Genes that can bypass the CLN requirement for Saccharomyces cerevisiae cell cycle START. Molecular and Cellular Biology 14, 2041-2047.


which Cdc6 synthesis cannot promote DNA replication in yeast. Genes and Development 10, 1516-1531.


Watanabe, M., Chen, C., and Levin, D. (1994). *Saccharomyces cerevisiae* *PKCl* encodes a protein kinase C (PKC) homologue with a substrate specificity similar to that of mammalian PKC. *Journal of Biological Chemistry* 269, 16829-16836.


