Start-specific transcriptional regulation of the budding yeast cell cycle

A Thesis presented by
Craig Talbot
in part fulfilment of the requirements
of University College London
for admittance to the degree of
DOCTOR OF PHILOSOPHY
Abstract

The control of gene expression at the G1/S transition point (Start) of the cell cycle has been examined using the budding yeast *Saccharomyces cerevisiae* as a model eukaryote. I have shown that the Swi6 protein is the primary activator of the SBF and MBF transcription factors which function at this stage of the cell cycle. The transcriptional activity of Swi6 fluctuates in a cell cycle dependent manner, peaking at the G1/S transition point, around the time of expression of two G1 cyclins, Cln1 and Cln2, as well as other genes under the control of SBF and MBF. Furthermore, the transcriptional activation properties of Swi6 are dependent upon the G1 cyclin Cln3 with which Swi6 interacts *in vivo*. At the cellular level, Cln3 activity at Start requires the presence of Swi6. Together this thesis demonstrates the pivotal roles the transcription factor, Swi6, and the G1 cyclin, Cln3, play in regulating gene transcription at Start.
Dedication

The continuing support of my parents
Acknowledgements

I would like to thank Steve Sedgwick and Lee Johnston for excellent supervision, with a smile. I would also like to thank past and present members of the Division of Yeast Genetics, in particular Rick Fagan, Nic Bouquin, Jeremy Toyn and Jonathan Millar for useful suggestions and discussion. I thank also Ad Spanos, Tony Johnston, Rick Fagan, P.J. Fitzpatrick and Marc Wilkinson for technical advice and directions on political correctness. A large thank you to Vicky Buck for essential comments on the construction of this thesis and to Ena Heather for help and advice. A big thanks to all my friends from the ‘real world’ in particular Roy who helped me in a way that only a true friend could. Finally, big kisses to Jenny for her loving support and putting up with me over the past years, and gently prodding me to finish this little bundle of joy.
Chapter One

Introduction

1.1 The Eukaryotic cell cycle

1.1.1 Key features of the eukaryotic cell cycle 1
1.1.2 Control of the cell cycle by the Cdc2 protein kinase family 3
1.1.3 Cdc2 and cyclins 4
1.1.4 Phosphorylation of Cdc2 4
1.1.5 Inhibitors of Cdk activity 5

1.2 The budding yeast cell cycle 5

1.2.1 Key events in the budding yeast cell cycle 6
1.2.3 Cdc28 and its associated cyclins 8
1.2.4 Control of Start by Cdc28/Cln 8
1.2.5 Start specific gene transcription 10
1.2.6 Structural similarities of the Swi6 family of transcription factors 12
1.2.7 Alternative mechanisms for SCB and MCB driven gene activation 13
1.2.8 Activation of SBF and MBF 15
1.2.9 Genes that can bypass the requirement for G1 cyclins 16

1.3 Gene Transcription 18
1.3.1 Eukaryotic gene activation 18
1.3.2 RNA polymerase II 19
1.3.3 Pol II and gene promoter recognition 19
1.3.4 Yeast RNA polymerase II 20
1.3.5 TBP-associated factors 22

1.4 Summary 23

Chapter Two
Materials and Methods

2.1 Bacterial Strains 24
2.2 Yeast Strains 24
2.3 Media and growth conditions 26
2.3.1 *E. coli* 26
2.3.2 *S. cerevisiae* 26

2.4 Standard buffers 27

2.5 Isotopes 27

2.6 DNA manipulations 28
2.6.1 Restriction endonucleases and DNA modifying enzymes 28
2.6.2 DNA ligations 28
2.6.3 Recovery of DNA fragments from agarose gels 28
2.6.4 Agarose gel electrophoresis 28
2.6.5 Southern hybridisation 29
2.6.6 Polymerase chain reaction 29
2.6.7 Transposition-mediated plasmid manipulation 29

2.7 Plasmids 30
2.7.1 Construction of a luciferase reporter plasmid 33
2.7.2 Construction of a galactose inducible LexA-Swi6 plasmid 33
2.7.3 Construction of a LexA-Bck2 plasmid 34
2.7.4 Construction of a galactose inducible *CLN1* plasmid 34
2.7.5 Construction of a galactose inducible *CLN2* plasmid 34
2.7.6 Construction of a galactose inducible *CLN3* plasmid 35
2.7.7 Construction of a *BCK2* genomic clone expressing plasmid 35

2.8 RNA manipulation 35
2.8.1 RNA extraction 35
2.8.2 RNA transfer 36
2.8.3 Hybridisation and probing of blots 36
2.8.4 Radiolabelling of probes 36
2.8.5 Visualisation and quantitation 37
2.8.6 DNA fragments used for probes 37

2.9 Western blots 37
2.9.1 Cell lysis 37
2.9.2 Polyacrylamide gel electrophoresis 38
2.9.3 Immunoblot analysis 38

2.10 Bacterial techniques 39
2.10.1 Transformation by CaCl₂ shock 39
2.10.2 Electro-transformation of \textit{E. coli} 40
2.10.3 Preparation of plasmid DNA 40

2.11 Yeast techniques 42
2.11.1 Lithium acetate transformation 42
2.11.2 Small scale yeast plasmid preparation 42
2.11.3 Isolation of yeast genomic DNA 42
2.11.4 Gene disruption 43
2.11.5 Gene replacement 43
2.11.6 Growth synchronisation methods 46
2.11.7 Determination of budding index and cell numbers 47
2.11.8 DAPI staining for fluorescent microscopy 47
2.11.9 Relative cell volume comparisons by FACS analysis 48
2.11.10 Cell volume determination by microscopy 48
2.11.11 Luciferase activity assays 48
2.11.12 β-galactosidase assays in liquid culture 49
RESULTS

Chapter Three

Activation properties of Swi6 and Swi4

| 3.1 Introduction | 51 |
| 3.2 LexA-Swi6 is a transcriptional activator | 51 |
| 3.3 ADH-LexA-Swi6 is functional | 53 |
| 3.4 LexA-Swi6 can activate transcription in the absence of Mbp1 or Swi4 | 55 |
| 3.5 LexA-Swi6 can activate independently of association with Mbp1 and Swi4 | 57 |
| 3.6 LexA-Swi6 activity is not dependent upon two potential Cdc28 sites | 60 |
| 3.7 LexA-Swi4 is a weak transcriptional activator | 60 |
| 3.8 LexA-Swi4 is functional | 62 |
| 3.9 Discussion | 63 |

Chapter Four

Developing and using a reporter system for periodic studies

| 4.1 Introduction | 65 |
| 4.2 Using the lexAop-lacZ reporter construct for periodic studies | 65 |
4.3 The development of a luciferase reporter plasmid 67
4.4 LexA-Swi6 activates luciferase expression 69
4.5 The relationship between luciferase activity and the amount of total cell protein is linear 69
4.6 Luciferase activity measurements does not decay rapidly after the addition of substrate 71
4.7 Luciferase activity is unstable in yeast 72
4.8 α-factor synchrony shows luciferase activity to fluctuate 74
4.9 dbf2-2 induced synchrony demonstrates that luciferase activity is periodic 75
4.10 Northern analysis of a dbf2-2 induced synchronous culture showed luciferase mRNA to fluctuate periodically 77
4.11 Discussion 79

Chapter Five

Transcriptional activation by Swi6 requires the G1 cyclin

Cln3

Page

5.1 Introduction 82
5.2 MBF activity is dependent on Cln3 82
5.3 SBF activity is dependent on Cln3 83
5.4 The need for a plasmid expressing low levels of LexA-Swi6 83
5.5 GLU-LexA-Swi6 is functional 85
5.6 GLU-LexA-Swi6 activity is dependent on Cln3 86
5.7 The dependency of Cln3 is specific to Swi6 88
5.8 Discussion 90
Chapter Six

Investigating the activation properties of Cln3

6.1 Introduction 92
6.2 LexA-Cln3 is a very weak transcriptional activator 92
6.3 Stable LexA-Cln3 is a potent transcriptional activator 94
6.4 LexA-Cln3ΔPEST activity is dependent on the cyclin box 94
6.5 LexA-Cln3 is dependent upon Swi6 for transcriptional activation 96
6.6 Functionally tests on LexA-Cln3 constructs 98
6.7 Discussion 98

Chapter Seven

Cln3 interaction with SBF is primarily through Swi6

7.1 Introduction 101
7.2 ADH-LexA-Swi6Δ345 interacts with Gal4<sub>ACT</sub>-Cln3 103
7.3 Gal4<sub>ACT</sub>-Clns are functionally expressed 105
7.4 Full length LexA-Swi6 interacts with Gal4<sub>ACT</sub>-Cln3 106
7.5 Deletion analysis of LexA-Swi6 interactions with Gal4<sub>ACT</sub>-Cln3 106
7.6 Two-hybrid assay between Swi4 and G1 cyclins 109
7.7 Discussion 110
Chapter Eight
Further analysis of Swi6 and Cln3 interdependency

8.1 Introduction 113
8.2 Swi6 activation domain 1 is Cln3 dependent for transcriptional activation 113
8.3 Functional dependence of Cln3 and Swi6 115
8.4 Discussion 118

Chapter Nine
Bck2 and its effect on transcription

9.1 Introduction 121
9.2 SBF and MBF activation is Bck2 dependent 123
9.3 LexA-Swi6 activity is dependent on Bck2 123
9.4 The TAR1 region of Swi6 is dependent on Bck2 for activity 125
9.5 LexA-Cln3ΔPEST activity is not dependent on Bck2 126
9.6 Reduction in transcriptional activation in a bck2 mutant is not specific to Swi6 126
9.7 ADH-LexA-Bck2 can activate transcription 128
9.8 Transcriptional activation by ADH-LexA-Bck2 is dependent on Cln3 but not Swi6 130
9.9 Discussion 132
Chapter Ten

General discussion

10.1 General discussion 136

Appendix I

Plasmid maps 143

References 153

List of figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>The cell cycle of <em>Saccharomyces cerevisiae</em> 2</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>A model illustrating the mode of action of SBF and MBF 11</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>Conserved and related features of the Swi6 family of transcription factors 14</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>A simplified model for eukaryotic gene transcription 21</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>PCR based gene knock out in yeast 45</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>The “one-hybrid” assay for transcriptional activation 52</td>
</tr>
<tr>
<td>Figure 3.2a</td>
<td><strong>ADH-LexA-Swi6</strong> can activate transcription of a <em>lexAop-lacZ</em> reporter plasmid in yeast strain W303-1a 54</td>
</tr>
<tr>
<td>Figure 3.2b</td>
<td>Activation of reporter strain CTY10-5d by <em>ADH-LexA-Swi6</em>, 54</td>
</tr>
</tbody>
</table>
ADH-LexA-Swi6ΔP and ADH-LexA-Swi6Δ691

Figure 3.3 Functionality test on ADH-LexA-Swi6 plasmids

Figure 3.4a Transcription by ADH-LexA-Swi6 is only reduced slightly in the absence of Swi4 or Mbp1

Figure 3.4b Reporter gene activation by LexA-p53 and LexA-E2F is only slightly reduced in the absence of Swi4 or Mbp1

Figure 3.5 ADH-LexA-Swi4 is a weak transcriptional activator

Figure 3.6 ADH-LexA-Swi4 fusion is functional

Figure 4.1a lacZ mRNA is transcribed to the same extent in cells expressing LexA-Swi6 or the LexA- only control

Figure 4.1b In the absence of a LexA DNA binding protein, the levels of lacZ mRNA are elevated further

Figure 4.2 ADH-LexA-Swi6 can activate a lexAop-luciferase reporter gene

Figure 4.3 The relationship between luciferase activity and cell extract is linear

Figure 4.4 Activity of luciferase is constant for up to 5min after addition of substrate

Figure 4.5a Luciferase activity is reduced after the addition of α-factor

Figure 4.5b A successful G1 arrest is characterised by a population of unbudded cells

Figure 4.6 G1 synchronised cells show ADH-LexA-Swi6 activity to fluctuate

Figure 4.7 Mitotically synchronised cells show ADH-LexA-Swi6 activity to be cell cycle regulated

Figure 4.8 Expression of luciferase mRNA is cell cycle regulated

Figure 5.1 MBF activity is Cln3 dependent

Figure 5.2 SBF activity is Cln3 dependent

Figure 5.3 GLU-LexA-Swi6 fusion is functional

Figure 5.4 GLU-LexA-Swi6 activity is Cln3 dependent

Figure 5.5 Levels of GLU-LexA-Swi6 protein are unchanged in G1 cyclin mutants
Figure 5.6a LexA-p53 activity is not Cln3 dependent
Figure 5.6b LexA-pE2F activity is not Cln3 dependent

Figure 6.1 ADH-LexA-Cln3 is a weak transcriptional activator
Figure 6.2 Stabilised ADH-LexA-Cln3 by deletion of the PEST sequence can activate transcription, and its activity is cyclin box dependent
Figure 6.3 Drawings of LexA-Cln3 constructs used
Figure 6.4a The activity of ADH-LexA-Cln3ΔPEST is Swi6 dependent
Figure 6.4b The activity of LexA-E2F and LexA-p53 is high in a Swi6 mutant
Figure 6.5 Functionality tests on LexA-Cln3 construct

Figure 7.1 A drawing of the ‘two-hybrid’ system
Figure 7.2 C-terminal deletion of Swi6 interacts with Cln3
Figure 7.3 Cyclin construct proteins are expressed
Figure 7.4 Full length Swi6 interacts with Cln3
Figure 7.5 Drawing of LexA-Swi6 deletions used in Cln3 interactions
Figure 7.6 Deletions of LexA-Swi6 reveal a Cln3 interaction domain
Figure 7.7 LexA-Swi4 does not interacts with the G1 cyclins

Figure 8.1 Transcriptional activation region 1 of Swi6 is Cln3 dependent and requires Cln2 for optimal activity
Figure 8.2 Transcriptional activation region 1 and Cln3 interaction domain of Swi6 correspond to the same region

Figure 9.1a MBF requires Bck2 for optimal activity
Figure 9.1b SBF requires Bck2 for optimal activity
Figure 9.2 GLU-LexA-Swi6 activity is Bck2 dependent
Figure 9.3 Plasmid expression Bck2 under its promoter is functional
Figure 9.4 Transcriptional activation region 1 of Swi6 is Bck2 dependent
Figure 9.5 LexA-Cln3 activity is not Bck2 dependent
Figure 9.6 LexA-p53, LexA-E2F but not LexA-SLN1 is dependent on...
Bck2 for activity

Figure 9.7 ADH-LexA-Bck2 can activate transcription 129
Figure 9.8 ADH-LexA-Bck2 is functional 131
Figure 9.9 ADH-LexA-Bck2 activity is dependent on Cln3 but not Swi6 131
Figure 9.10 Transcriptional interdependency between Swi6, Cln3 and Bck2 135

**List of tables**

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1</td>
<td>Yeast Strains used in this study</td>
<td>25</td>
</tr>
<tr>
<td>Table 2.2</td>
<td>Plasmids used in this study</td>
<td>30</td>
</tr>
<tr>
<td>Table 2.3</td>
<td>DNA fragments used for northern hybridisation</td>
<td>37</td>
</tr>
<tr>
<td>Table 7.1</td>
<td>Expression of Gal4act-Cln fusions are functional</td>
<td>105</td>
</tr>
<tr>
<td>Table 8.1</td>
<td>Cln3 can not accelerate Start in the absence</td>
<td>118</td>
</tr>
</tbody>
</table>

**Abbreviations**

- **ADH**: Alcohol dehydrogenase
- **β-gal**: β-galactosidase
- **bp**: Base Pair
- **BSA**: Bovine serum albumin
- **Cdk**: Cyclin dependent kinase
- **Cdki**: Cyclin dependent kinase inhibitor
- **CPM**: Counts per minute
- **C-terminal**: Carboxyl-terminal
- **DAPI**: Diamidino-2-phenylindole
- **DNA**: Deoxyribonucleic acid
- **DMSO**: Dimethyl sulphoxide
FACS Fluorescence activated cell sorter

g Gram

Gal4ACT Transcriptional activation domain of the Gal4 protein

h Hour

Kb Kilobase

lacZ E. coli β-galactosidase gene

M Molar

MBF MCB binding factor

MCB MluI cell cycle box

mg Milligram

mid-log Mid-logarithmic

min Minute

ml Millilitre

mM Millimolar

mRNA Messenger RNA

OD Optical density

ONPG o-nitrophenol-β-D-galactopyranoside

ORF Open reading frame

PBS Phosphate buffered saline

PCR Polymerase chain reaction

RNA Ribonucleic acid

RT Room temperature

SCB Swi4/6 cell cycle box

S. cerevisiae Saccharomyces cerevisiae

SBF SCB binding factor

SDS Sodium dodecylsulphate

S. pombe Schizosaccharomyces pombe
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCP</td>
<td>Total cell protein</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>ts</td>
<td>Temperature sensitive</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Polyoxrthlenesorbitan monolaurate</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast extract plus dextrose</td>
</tr>
</tbody>
</table>
CHAPTER ONE

INTRODUCTION

1.1 THE EUKARYOTIC CELL CYCLE

1.1.1 Key features of the eukaryotic cell cycle

Eukaryotic cells propagate by alternately repeating DNA synthesis (S phase) and cell division (M phase). These cellular events are separated by two gaps phases, named G1 and G2 (figure 1.1). Apart from the four phases of the cell cycle, the majority of cells in multicellular organisms are in a state of quiescence or rest known as G0 but can be induced to re-enter the cell cycle at G1 by growth stimuli. Subsequent passage from G1 into S phase is governed by a complex control mechanism (Murray, 1987), dictating whether cells should “Start” a new cell cycle or arrest at the G1/S boundary. Once cells have entered the cell cycle, they make an irreversible commitment to a whole round of DNA replication and segregation and cells traverse the G1/S boundary entering into S phase where they replicate their genome. S phase is followed by G2, in which cells prepare for the onset of mitosis. Nuclear and cell division are initiated in M and the two daughter cells return to G1.

Rigorous control mechanisms known as checkpoints ensure the phases of the cell cycle are ordered: for example, it would be detrimental to cell viability if the chromosomes were to segregate before the genome had completely replicated. Checkpoints govern
The cell cycle is divided into four phases, G1, S (DNA synthesis), G2, and M (Mitosis). Other events are cytokinesis, the final separation of the two daughter cells, and Start, an event that commits the cell to a full round of division.
entry into S and M phases, hence G1 and G2 are extremely important in terms of cell cycle regulation.

There has been rapid progress in understanding the molecular events that control the eukaryotic cell cycle in many organisms from yeast to humans. Research has identified highly conserved control mechanisms common to all eukaryotes.

1.1.2 Control of the cell cycle by the Cdc2 protein kinase family

The Cdc2-related protein kinase family has come to occupy a central position in our current understanding of the eukaryotic cell cycle. The view that Cdc2 is a master regulator of cell cycle progression has evolved over the last 10 years as a result of the convergence of work from distantly related organisms. Studies of the fission yeast *Schizosaccharomyces pombe* and frog *Xenopus* oocytes revealed that the *S. pombe cdc2* gene product was homologous to the p34 subunit of *Xenopus* Maturation Promoting Factor (MPF) (Dunphy *et al.*, 1988; Gautier *et al.*, 1988). Mammalian Cdc2 was first isolated from human cells by phenotypic complementation of a temperature-sensitive *cdc2* mutant of the fission yeast (Lee and Nurse, 1987) and is highly homologous in structure and function to *S. pombe* Cdc2 and *S. cerevisiae* Cdc28.

Since the discovery of the first human Cdc2 homologue, it has become clear that higher eukaryotes utilise a whole family of Cdc2-like proteins to regulate the cell cycle. Different Cdc2-like kinases regulate specific stages of the cell cycle, whilst family members are expressed differentially dependent on the cell type (Bates *et al.*, 1994; Meyerson and Harlow, 1994).
Because of the irreversible nature of both DNA replication and mitosis, activation of the Cdc2 kinases is carefully controlled, being subject to multiple regulations (Nigg, 1995). One important regulatory mechanism is the association with an unstable regulatory subunit called a cyclin, hence, the Cdc2 family of protein kinases are known as cyclin dependent protein kinases (Cdks). Cyclins were initially discovered as proteins whose abundance oscillates dramatically during the cell cycle of sea urchin embryos (Evans et al., 1983). Cyclins are now divided into a number of classes based on sequence similarity and physiological function. Each Cdk absolutely requires the association with a cyclin subunit, and binding of the cyclin to the Cdk causes a conformational change that affects the active site of the Cdk, such that the kinase activity is increased (Jeffrey et al., 1995). Association with a cyclin is also thought to contribute towards the Cdk’s substrate specificity. The majority of cyclins are only present at particular times of the cell cycle, and this is achieved by temporal gene transcription. In addition to cyclin messenger RNA being transcriptionally regulated by sequence specific transcription factors, cyclins are subjected to targeted destruction.

**1.1.4 PHOSPHORYLATION OF CDC2**

In addition to cyclin association, the Cdks are regulated by phosphorylation. Phosphorylation of a given amino acid in a protein can have a variety of effects, activating or inactivating a protein’s enzymatic activity, or increasing or decreasing its affinity for binding to other proteins. Phosphorylation on one site in Cdc2 inhibits its kinase activity while phosphorylation on another site is absolutely required for kinase activity. For
example the key phosphorylated residues of fission yeast Cdc2 are tyrosine 15 and threonine 167. Phosphorylation on tyrosine 15 by the Wee1 and Mik1 kinases negatively regulates Cdc2 kinase activity (Lundgren et al., 1991; Russell and Nurse, 1987), whereas phosphorylation of threonine 167 has a positive effect (Gould et al., 1991). The inhibitory phosphorylation is dominant and hence Cdc2 phosphorylated on both tyrosine 15 and threonine 167 lacks kinase activity. The positive phosphorylation on threonine 167 is itself performed by a cyclin/Cdk complex called CAK (Cdk Activating Kinase), which is composed of a cyclin named Mcs2 and a Cdk called Crk1 (Buck et al., 1995).

1.1.5 INHIBITORS OF CDK ACTIVITY

Besides the availability of cyclin partners and their phosphorylation status, Cdks are also regulated by a group of protein kinases inhibitors, such as Rum1 in fission yeast, Sic1 of budding yeast and the p21 family in mammalian cells (Schwob et al., 1994; Harper et al., 1993; Lee et al., 1995; Matsuoka et al., 1995; Moreno et al., 1994; Polyak et al., 1994; Toyoshima and Hunter, 1994; Xiong et al., 1993), these are known as Cdkis (Cyclin dependent kinases inhibitors). Cdkis can differ in their mode of action, either by forming tertiary complexes with cyclin/Cdk or inhibiting cyclin/Cdk complex formation (Guan et al., 1994).

1.2 THE BUDDING YEAST CELL CYCLE

Much of our understanding of the eukaryotic cell cycle has come from the study of two distantly related yeasts, *Schizosaccharomyces pombe* or fission yeast and *Saccharomyces*
*cerevisiae* or budding yeast. Study of the fission yeast has resulted in great progress in clarifying the control mechanisms of mitosis, whereas much of our insight into the G1/S phase has come from the study of budding yeast.

### 1.2.1 Key events in the budding yeast cell cycle

The primary control point in budding yeast is exerted at a point in late G1 known as Start (Pringle and Hartwell, 1981), where environmental factors such as nutrient availability and the presence of mating pheromones are assessed. In addition, Start cannot be passed until a critical cell size has been attained. Cells in late G1 have three potential fates. They can continue through the mitotic cell cycle, they can exit the cell cycle into a quiescent stage (G0), or they can conjugate and mate with cells of the opposite mating type and enter the sexual differentiation cycle. However, once a cell has passed Start, it is committed to the mitotic pathway, it becomes resistant to mating pheromones and can not become quiescent until cells have passed through a complete cell cycle and re-enters G1. After the initiation and completion of DNA synthesis in S phase, the cell enters G2, the end of which is marked by the migration of the replicated nucleus to the neck of the budded cell. Mitosis is characterised by the segregation of the replicated DNA into the two cells which is followed by cytokinesis, where the new daughter cell buds from the mother. In budding yeast, the first visible stage of cell division is taken to be bud formation which occurs at the G1/S boundary. One consequence of bud formation is an asymmetry of the progeny, one of the products of division (the mother cell) being typically much larger than the other (the daughter cell). As a result, the large mother cell
spends only a short time in G1, requiring less time to overcome the size requirement for traversing Start (Hartwell and Unger, 1977).

1.2.2 Cell division cycle mutants

In the *S. cerevisiae* cell cycle, both haploid and diploid cells undergo mitosis, permitting the isolation of recessive gene mutations in haploids and the analysis of genes by complementation in diploids. Using this method, over 70 genes have been identified that are required for progression through the cell cycle (Pringle and Hartwell, 1981). Cell Division Cycle (cdc) mutants were originally isolated as conditional mutants which are temperature-sensitive (ts) for growth. Temperature sensitive mutant strains have a wild type phenotype at the permissive temperature but arrest at the restrictive temperature. As originally defined, cdc mutants result in a defect in a particular stage-specific function of the cell cycle, this can be seen in an asynchronously growing culture. For example, in a Cdc28ts mutant, cells will arrest at the same point in the cell cycle, at the G1/S boundary, regardless of their stage at the time they were shifted from the permissive to the restrictive temperature.

One of the main conclusions that has been drawn from the study of cdc mutants is that there are only a few major rate-limiting steps in the cell cycle, and only when these are complete can other dependent events take place (Nurse and Bisset, 1981). Whether a gene product is involved in a rate-limiting step can be answered empirically by determining whether speeding up the rate at which the product acts significantly advances progress through the cycle. For example, isolation of alleles of cdc2+ in fission yeast that advance the onset of mitosis suggested that this gene controls an important rate-limiting
step in M phase (Draetta, 1990). Similarly, dominant alleles of the G1 cyclin \textit{CLN3} gene in budding yeast cause cells to divide at a smaller size (Sudbery \textit{et al.}, 1980), which can also be achieved by over-expression of the G1 cyclins (Lew \textit{et al.}, 1992; Richardson \textit{et al.}, 1989). This mutant phenotype can be viewed as an advance of Start in cycling populations, thus implying that the G1 cyclins are rate-limiting for execution of this control point.

1.2.3 \textbf{Cdc28 and its associated cyclins}

In budding yeast, a single Cdk, Cdc28, which is homologous to Cdc2 of fission yeast, controls the ordered progression through the cell cycle. Cdc28 is thought to phosphorylate different sets of target proteins at specific times in the cell cycle. This temporal target specificity is thought to be controlled by its association with particular cyclin partners of which there are at least nine (reviewed in Nasmyth, 1993). Passage through Start is dependent upon association of Cdc28 with the G1 cyclins, Cln1, Cln2 and Cln3 (Cross, 1990; Richardson \textit{et al.}, 1989). During S phase, the Cdc28 Cdk is associated with the B type cyclins, Clb5 and Clb6 (Schwob and Nasmyth, 1993). Finally, mitosis is regulated by complexes of Cdc28 and the mitotic cyclins, Clbs 1-4 (Richardson \textit{et al.}, 1992; Surana \textit{et al.}, 1991).

1.2.4 Control of Start by Cdc28/CLN

The G1 cyclins which control the G1/S transition were initially thought to perform overlapping functions because cells could still grow when any two of the three \textit{CLN} genes were inactivated (Cross, 1990; Richardson \textit{et al.}, 1992), but arrested in G1 if all three cyclins were absent (Cross, 1990; Richardson \textit{et al.}, 1992). Additionally, there is a delay
in Start in the absence of any one of the Clns (Hadwiger et al., 1989; Nash et al., 1988).

In turn, over-expression of any Cln results in a shortened G1 and accelerates passage through Start (Lew et al., 1992; Richardson et al., 1989). Despite this apparent functional redundancy, there are distinct differences between the G1 cyclins. Although all three share some sequence homology, CLN1 and CLN2 are far more closely related to each other than to CLN3 (Hadwiger et al., 1989; Nash et al., 1988). Levels of CLN1 and CLN2 mRNA, protein and associated protein kinase activity all vary through the cell cycle, rising to a maximum as cells pass Start then decreasing to a comparatively low level in the remainder of the cell cycle (Tyers et al., 1993; Wittenberg et al., 1990). In contrast, amounts of CLN3 mRNA, protein and associated kinase activity are low and show little periodicity throughout the cell cycle (Tyers et al., 1993; Tyers et al., 1992; Wittenberg et al., 1990).

Because of the functional redundancy of the Clns, it was initially proposed that any one of the G1 cyclins was sufficient for the Cdk activity required for passage through Start. The model proposed that once the Cdc28 protein was activated by the G1 cyclins, this would lead to the transcription of more Clns via a positive feedback loop and as a consequence induce the rapid and irreversible execution of Start (Cross and Tinkelenberg, 1991; Nasmyth and Dirick, 1991). Recently, the self induction of Cln1 and Cln2 has been disproved by the observation that the induction of Cln1 and Cln2 is largely Cln3 dependent (Stuart and Wittenberg, 1995). The demise of the positive feedback model has led to the proposal that levels of Cln3 accumulating as cells increase in mass during late G1 activate the Cdc28 kinase leading to the transcription of CLN1 and CLN2, and passage
through Start (Dirick et al., 1995; Stuart and Wittenberg, 1995). Thus, the current view is that Cln3, and not Cln1 and Cln2, initiates the execution of Start. Since Cln3-associated kinase activity leads to Cln1 and Cln2 expression, attention now focuses on whether Cln3 has some regulatory effect on the transcription factors controlling expression of Cln1 and Cln2.

1.2.5 Start specific gene transcription

Two related heterodimeric transcription factors control the transcription of many of the genes, including CLN1 and CLN2, which are periodically expressed as cells pass Start. The first of these, SBF (SCB Binding Factor), is composed of Swi6 and Swi4 (Andrews and Herskowitz, 1989; Taba et al., 1991) and the second, MBF (MCB Binding Factor), is composed of Swi6 and Mbp1 (Dirick et al., 1992; Koch et al., 1993). Peak expression of the CLN1 and CLN2 genes is dependent on SBF (Nasmyth and Dirick, 1991; Ogas et al., 1991). The Swi4 protein of SBF contains a DNA binding domain which specifically recognises a DNA motif called the SCB (Swi4/6 Cell cycle Box) with a consensus nucleotide sequence of CACGAAA. This cis-acting regulatory motif has been identified in the upstream promoter regions of CLN1, CLN2, the HO endonuclease gene (which is involved in mating type switching), some of the cyclin-like PCL genes (Frohlich et al., 1991; Nasmyth, 1985; Ogas et al., 1991) and in genes involved in cell wall integrity (Igual et al., 1996). In the second transcription factor, MBF, the DNA binding moiety is Mbp1 (Dirick et al., 1992; Koch et al., 1993; Lowndes et al., 1991). Mbp1 recognises the DNA motif ACGCGT otherwise known as MCB (MluI Cell cycle Box). MCB elements are found in the promoters of many, if not all the genes involved with DNA synthesis as well
SBF consists of Swi4 and Swi6, and specifically recognises a DNA sequence called the SCB element in the upstream regions of genes needed for passage through Start. The second transcription factor complex is made of Mbp1 and Swi6. Many genes essential for DNA synthesis, together with the B-type cyclins genes, $CLB5$ and $CLB6$ contain MCB elements in their upstream promoter regions, which are specifically recognised by MBF.
as in the promoter regions of *SWI4* and the B-type cyclin genes, *CLB5* and *CLB6*

(Andrews and Herskowitz, 1990; Breeden, 1988; Epstein and Cross, 1992; Johnston and Lowndes, 1992; Kuhne and Linder, 1993; Schwob and Nasmyth, 1993). Both SCB and MCB elements can confer late G1-specific gene expression to otherwise inactive promoters (Lowndes et al., 1991; McIntosh, 1993; McIntosh et al., 1991). Figure 1.2 shows the mode of action of SBF and MBF.

1.2.6 **STRUCTURAL SIMILARITIES OF THE SWI6 FAMILY OF TRANSCRIPTION FACTORS**

There are distinct similarities between Swi6, Swi4 and Mbp1, figure 1.3 shows key features. Swi4 and Mbp1 both share a conserved amino terminal DNA binding domain, which is not present in Swi6. Swi6 itself has no specific DNA binding ability, but band shift assays have demonstrated that Swi6 is needed for the efficient binding of full length Swi4 to DNA (Sidorova and Breeden, 1993). Furthermore, it would appear that the DNA binding domain of Swi4 can bind DNA independently of Swi6 (Primig et al., 1992). Hence, Swi6 may induce some conformation change in Swi4 to allow it to bind its specific DNA sequences. The region of Swi6 necessary for Swi4 and Mbp1 interaction has been attributed to the carboxyl terminal (Andrews and Moore, 1992; Primig et al., 1992; Siegmund and Nasmyth, 1996). Similarly, the C-terminals of Swi4 and Mbp1 are sufficient for binding to Swi6 (Andrews and Moore, 1992; Koch et al., 1993; Primig et al., 1992). In the central region of each protein are 4, possibly 5, copies of the ankyrin repeat motif. This 33 amino acid motif is evolutionary conserved, it is present in many proteins with diverse functions (Bork, 1993), and is thought to mediate protein-protein interactions. Point mutations in Swi6 ankyrin coding sequences results in a temperature
sensitive phenotype (Ewaskow et al., 1998). The ankyrin repeat domain of Swi4 is known to interact with the Clb2-Cdc28 kinase (Siegmund and Nasmyth, 1996), and this has been postulated to inhibit Swi4-Swi6 transcriptional activity at SCB elements during the G2/M phase of the cell cycle.

1.2.7 ALTERNATIVE MECHANISMS FOR SCB AND MCB DRIVEN GENE ACTIVATION

Although SCB containing genes are thought to be regulated by the binding of SBF and genes with MCB elements are under the control of the MBF transcription complex, there appears to be some redundancy in this simple model. For example, in the absence of normal SBF activity the SKN7 gene product can promote expression from a multicopy SCB-lacZ reporter plasmid (Morgan et al., 1995). Furthermore, several other proteins are able to promote expression from SCB and MCB containing promoters including the meiotic regulator Rme1 (Toone et al., 1995) and Bck2, a protein of unknown function (Di Como et al., 1995).

As well as factors other than SBF and MBF which can activate MCB and SCB mediated gene expression, there appears to be some degree of cross talk between the specificity of SBF and MBF. Several observations support this cross regulation theory. The DNA binding domain of Swi4 can bind MCB sites, and the DNA binding domain of Mbp1 can bind SCB and MCB sites with equal efficiency (Koch et al., 1993). Furthermore, the MCB-dependent Clb5 gene is still cell cycle regulated in a mbp1Δ (Koch et al., 1993), similarly, Cln1 expression driven by Swi4 occurs from its MCB elements (Partridge et al., 1997). In addition, gel retardation assays demonstrated that SCB probes
Figure 1.3
Conserved and related features of the components of yeast SBF and MBF transcription factors. Each member has a conserved centrally located domain containing four ankyrin repeats, shown in red. Swi4 and Mbp1 have a conserved amino terminal DNA binding domain which is absent in Swi6, shown in yellow. SBF is complexed by association of Swi4 and Swi6 through their carboxyl terminals, shown in green. Similarly, Mbp1 and Swi6 associate through their carboxyl terminals to form MBF.
can compete for MBF complexes, and vice versa. These observations help to explain the viability of *swi4, mbp1* or *swi6* strains.

### 1.2.8 Activation of SBF and MBF

In *S. cerevisiae*, several mechanisms for the activation of SBF and MBF have been proposed. The periodic expression of *SWI4* (Breeden and Mikesell, 1991) may have some regulatory role in the activation of SBF, but *CLN1* and *CLN2* periodic expression can occur under conditions when Swi4 protein synthesis is blocked (Marini and Reed, 1992). The periodic binding of Swi4 to SCB elements has been demonstrated, but not at the time of *CLN1* and *CLN2* expression and this binding is not Cdc28 dependent (Koch *et al.*, 1996). Furthermore, SBF and MBF have been detected at all stages of the cell cycle (Dirick *et al.*, 1992; Taba *et al.*, 1991). The activity of these transcription factors could therefore be post-translationally regulated, so that they are active in late G1 and inactive in G2.

Ultimately, Swi6 has emerged as the most attractive candidate for a regulatory role in the complexes. One reason for this view is the presence of Swi6 in both transcription factors. Secondly, in the absence of Swi6, some genes having SCB and MCB promoter elements are no longer periodically expressed although they maintain a constitutive and intermediate level of expression (Dirick *et al.*, 1992; Foster *et al.*, 1993; Lowndes and Johnston, 1992; Lowndes *et al.*, 1992). This intermediate level suggests that Swi6 may have both positive and negative regulatory effects on the expression of these genes.

Transcriptional activation via SCB elements in the *HO* promoter requires both Swi6 and Cdc28 activity (Breeden and Nasmyth, 1985; Nasmyth, 1985). *CLN1* and *CLN2* as well
as MCB-regulated genes are also maximally expressed after Cdc28 activation in G1 (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Johnston and Thomas, 1982). These observations suggest that the periodic activity of SCB and MCB elements is Cdc28 dependent. Indeed, the Swi6 protein is highly phosphorylated in vivo and has several potential Cdc28 phosphorylation sites, one of which, Serine 160, appears to be periodically phosphorylated (Sidorova et al., 1995). Serine 160 resides within a putative Cdc28-consensus phosphorylation site and its phosphorylation is required for Swi6 exit from the nucleus after S phase. In reality, however, phosphorylation at this site is not Cdc28-dependent nor does it influence G1/S specific transcription. Thus, phosphorylation of Swi6 may not be responsible for its transcriptional activity and it remains unclear what activates the SBF and MBF transcription factors.

1.2.9 Genes that can bypass the requirement for G1 cyclins

The requirement for at least one of the three CLN genes for cell viability appears to identify a requirement for G1 cyclin in Cdc28 activation. A screen was initiated in an attempt to identify either genes acting downstream of the CLN requirement or genes that activate a parallel pathway making CLN genes unnecessary (Epstein and Cross, 1994). A mutation in one gene named BYCl (for bypass for cln requirement) was found to be a dominant mutation in the previously identified gene SIC1. Sic1 is a Cdk inhibitor, specific to Cdc28/B-type cyclin kinase complexes (Donovan et al., 1994; Mendenhall, 1993; Schwob et al., 1994). After cells pass Start, B-type cyclin/Cdc28 kinases such as Clb5/Cdc28 and Clb6/Cdc28 must be activated to allow replication of DNA (Schwob et al., 1994). Although Clb5- and Clb6/Cdc28 complexes are present in G1 phase they are
initially inactive because of inhibition by the Sic1 protein (Mendenhall, 1993; Schwob et al., 1994). Activation of Clb5- and Clb6/Cdc28 occurs after Sic1 is targeted for proteolysis by the ubiquitin-conjugating enzyme Cdc34 (Schwob et al., 1994). As cells pass Start the kinase activity associated with Cln/Cdc28 phosphorylates Sic1, thus making it a target for Cdc34. Biochemical reconstitution experiments have revealed that Cdc4, Cdc53 and Skp1 constitute a ubiquitin ligase complex that collaborates with the ubiquitin-conjugating enzyme, Cdc34, and the G1-specific Cdk, Cln2/Cdc28, to promote the ubiquitination of Sic1 (Verma et al., 1997). If a major function of Clns is to promote proteolysis of Sic1, then Clns should be less important in a sic1 mutant. Indeed, a sic1 mutation can suppress the lethality of a cln1 cln2 cln3 triple null mutation (Epstein and Cross, 1994; Schneider et al., 1996; Tyers, 1996). Thus, the only non-redundant essential function of the Clns is to inactivate Sic1.

In addition to the mutation in Sic1 which can bypass the requirement for G1 cyclins at Start, the expression of another gene, BCK2, at low copy can suppress the growth defect of a triple cln mutant. The BCK2 gene was originally isolated as a high copy suppressor of the temperature sensitive cell lysis defect of mutations in the protein kinase C pathway (Lee et al., 1993). Furthermore, in cells lacking BCK2 the additional loss of CLN3 or SWI6 resulted in a reduction or loss of viability respectively (Di Como et al., 1995). This would suggest that Bck2, functions in a parallel pathway to Cln3 or Swi6 in activating Start. Indeed, the over-expression of BCK2 results in an increase in CLN1 and CLN2 RNA accumulation, but this increase appears to be Swi6 and Swi4 dependent (Di Como et al., 1995). Conversely, bck2 mutants have a reduction in CLN1 and CLN2...
RNA. Hence, it would appear that \textit{bck2} mutants have a \textit{CLN1} and \textit{CLN2} expression defect. Furthermore, Bck2 appears to be acting through SCB and MCB elements found in the promoters of these genes because the synthetic lethality of \textit{bck2 cln3} mutants can be suppressed by overexpression of plasmid borne \textit{CLN2} expressed under the \textit{ADH1} promoter but not its own natural SCB containing promoter. The role that Bck2 plays at Start is unclear, but it appears to influence \textit{CLN1} and \textit{CLN2} transcription in mainly a SCB/MCB dependent manner, although there is also evidence for an alternative independent mechanism.

1.4 GENE TRANSCRIPTION

The Swi6 component of the two transcription factors SBF and MBF together with the G1 cyclin Cln3 have been implicated in the regulation of G1/S specific gene expression (Dirick \textit{et al.}, 1995; Stuart and Wittenberg, 1995). However, the way in which regulated gene expression is affected by these components is unknown. To better understand the potential mechanisms of gene regulation, it is necessary to appreciate the basic mechanics of transcriptional activation.

1.3.1 EUKARYOTIC GENE ACTIVATION

Eukaryotes contain three distinct RNA polymerase enzymes, each responsible for the transcription of nuclear genes. All three polymerases maintain considerable sequence similarity in their largest subunits (Allison \textit{et al.}, 1985) and actually have five subunits in common (McKune and Woychik, 1994; Woychik \textit{et al.}, 1990; Young, 1991). Despite these similarities, each interacts with distinct sets of transcription factors to mediate
accurate transcription of a subclass of nuclear genes. RNA polymerase I only transcribes genes encoding large ribosomal RNAs. RNA polymerase II transcribes all of the cell’s protein-coding messenger RNAs (mRNA) and other small RNAs (snRNAs). RNA polymerase III synthesises tRNA and the 5S RNA component of ribosomes.

1.3.2 RNA POLYMERASE II

In the RNA polymerase I (Pol I) and Pol III systems, the association of the polymerase with the promoter signals the completion of the assembly process (Buratowski and Zhou, 1992; Lofquist et al., 1993). The Pol II system differs in that even after the polymerase has been loaded onto the promoter, the complex is not competent to initiate transcription. This requires the association of two more general transcription factors (GTFs), TFIIE and TFIIH. TFIIE is a heterodimer and TFIIH is a multi-subunit factor. Pol II contains a unique motif known as the CTD. CTD refers to a series of tandem repeats (52 in humans, 43 in *Drosophila*, and 26 in yeast), in the C terminal of the largest subunit of Pol II. Phosphorylation of the serine, threonine, and/or tyrosine residues within the CTD appears to promote a transition from transcription initiation to elongation. TFIIH contains a kinase activity capable of phosphorylating CTD, and purification of TFIIH from different organism revealed that a Cdk and a cyclin partner were components of the complex.

1.3.3 POL II AND GENE PROMOTER RECOGNITION

The transcriptional control regions of eukaryotic protein coding genes can be separated into at least two categories; a core promoter and upstream (or downstream) regulatory elements. Each gene carries a unique array of proximal and distal enhancer elements that
are recognised by sequence specific DNA-binding factors critical for activating or repressing transcription initiation (Tjian and Maniatis, 1994). The core promoter nucleates the assembly of an initiation complex containing RNA polymerase II and a number of accessory factors TFIIA, TFII B, TFIID, TFII E, TFII F, and TFII H that can direct a low level of basal transcription \textit{in vitro} (Buratowski, 1994; Conaway and Conaway, 1993; Zawel and Reinberg, 1992) (figure 1.4 shows Pol II holoenzyme). Most of the genes transcribed by Pol II have a DNA sequence called the TATA box as the core promoter element, whose sequence is recognised by the TATA box-binding protein (TBP) subunit of TFIID. Detailed biochemical studies of TFIID have revealed that this transcription factor consists of TBP and a number of TBP-associated factors (TAFs) (Tjian and Maniatis, 1994).

1.3.4 \textbf{YEAST RNA POLYMERASE II}

The mechanism of eukaryotic transcription activation and repression is complicated by the large number of proteins involved in the process. Prior to promoter assembly, Yeast RNA polymerase II is associated with a number of proteins, including the general transcription factors TFII B, TFI IH and TFII F. Polymerase II is not, however, associated with the TATA-box binding protein (TBP) and its associated factors (TAFs) or TFII E. Transcription by polymerase II involves an activator protein binding to specific sites, often present in multiple copies, upstream of the TATA-box. The binding of these activators may promote transcription by recruiting the polymerase complex to a particular gene (Ptashne and Gann, 1997).
Figure 1.4
A simplified drawing of the proteins involved in eukaryotic gene transcription
1.3.5 TBP-ASSOCIATED FACTORS

Although the essential role of TBP of different eukaryotes has been extensively analysed in vivo and in vitro (Hernandez, 1993; Struhl, 1995), the function of TAFs is less clear. In vitro, TAFs are dispensable for basal transcription but are required for the response to activators (Tjian and Maniatis, 1994). This tentative link to activator dependent transcription suggests that TAFs may act as molecular bridges between particular activators and the general transcription machinery (Chen et al., 1994; Sauer et al., 1995). Indeed, the budding yeast Taf145 and its higher eukaryotic homologue, TAF1250, are known to contact TBP directly.

Recently it has been suggested that TAFs may be required to perform specialised functions within the transcription complexes on specific promoters. Indeed, it has been demonstrated that yeast TAFs are not needed for general transcription (Moqtaderi et al., 1996; Walker et al., 1997).

The most extensively studied yeast TAF is Taf145 which encodes an essential gene. Cells containing a temperature sensitive mutant of Taf145 arrest as large unbudded cells at the restrictive temperature, typical of a mutation in a gene required at Start (Walker et al., 1997). This cdc-like phenotype is likely to result from the reduced CLN1 and CLN2 transcription observed in these cells. This apparent link between Taf145 and the cell cycle is extremely interesting and suggest a possible crude mechanism for Cdk/cyclin and SBF/MBF gene activation at Start. It is possible that Taf145 could activate transcription by recruiting the specific factors necessary to the promoter region. Furthermore, Taf145 could promote this transcription by somehow remodelling
histones, for Taf145 has histone acetyltransferase activity (Mizzen et al., 1996). The past year has seen much progress in the understanding of chromatin and transcription, showing that different histone acetylation states play a role in gene activation (Gregory and Horz, 1998).

1.4 SUMMARY

Recent work has suggested pivotal roles for Cln3 and Swi6 in the control of the G1/S transition point in budding yeast. This report attempts to clarify our present understanding of Start in this yeast, and the mechanisms which control gene expression at this fundamental control point.
CHAPTER TWO

MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

The bacterial strain used throughout this study was *E. coli* DH5α F' φ80lacZΔM15 (lacZYA-argF) U169 endA1 recA1 hsdR17 (rK’mK’') deoR thi-1 supE44 gyrA96 relA1. In addition, bacterial strains used for transpositional disruption of plasmid genes were according to Morgan *et al.*, (1996).

2.2 YEAST STRAINS

Details of yeast strains used in this study are given below, and list of strains used is in Table 2.1

Most strains are derivatives of W303-1a: *MATa ade2-1 trp1-1 can1-100 leu2-3,112, his3-11,15 ura3 GAL psi+ ssd1-d*. Those marked with an asterisk are derived from L181-6B: *MATa dbf2-2 ura3 leu2, trp1*. Strains marked with a double asterisk are derived from CTY10-5d: *MATa trp1 leu2, his3 gal4 gal80URA3::LexAop-LacZ*. The strain MDS4: *ade2-1 trp1-1 leu2, his3 mbp1::URA3 swi4ts*, was a kind gift from Mark Toone. Strain K2003 is *MATa ade2 his3 mat trp1 ura3 swi4ts swi6::TRP1*. Strain BF305-15d is *leu2-2,112, his3-11,15 ura3-52 trp1 ade1 met14 arg5,6*. CLN1 and CLN2 are disrupted by
HIS3 and TRP1 respectively. CLN3 is under the control of GAL1 promoter, integrated at the URA3 locus. Strain BF411-2C is ade1 URA3::GAL-CLN3 ura3 cln1::HIS3 cln2::LEU2 trp1 arg5,6. This strain is ura' due to selection on 5FOA.

Table 2.1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303-1a</td>
<td>Lab stock</td>
</tr>
<tr>
<td>YAT2, cln1::HIS3</td>
<td>Mark Toone</td>
</tr>
<tr>
<td>YAT3, cln1::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>YAT4, cln2::LEU2</td>
<td>Mark Toone</td>
</tr>
<tr>
<td>YAT5, cln3::URA3</td>
<td>Mark Toone</td>
</tr>
<tr>
<td>YAT7, cln3::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>YAT8, cln3::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>YAT9, cln1::HIS3 cln2::LEU2</td>
<td>Kate Kramer</td>
</tr>
<tr>
<td>YAT13, mbp1 point mutation</td>
<td>Nicolas Bouquin</td>
</tr>
<tr>
<td>YAT12, swi4::ADE2</td>
<td>Kate Kramer</td>
</tr>
<tr>
<td>YAT11, swi6::TRP1</td>
<td>Gary Merrill</td>
</tr>
<tr>
<td>YAT18, bck2::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>YAT68, bck2::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>L181-6B*</td>
<td>(Toyn and Johnston, 1993)</td>
</tr>
<tr>
<td>CTY10-5d**</td>
<td>(Bartel et al., 1993)</td>
</tr>
<tr>
<td>YAT21**, cln1::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>YAT22**, cln2::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>YAT24**, cln3::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>YAT25**, swi6::HIS3</td>
<td>This study</td>
</tr>
</tbody>
</table>
This study

Mark Toone

(Nasmyth and Dirick, 1991)

(Xiong et al., 1991)

Bruce Futcher

2.3 MEDIA AND GROWTH CONDITIONS

2.3.1 E. coli

E. coli strain DH5α was grown in Luria-Bertani broth (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl pH7.5) with the addition of 50μg/ml ampicillin and 50μg/ml methicillin from filter sterilised stock solution for the selection of plasmids. Liquid cultures were grown at 37°C with continuous agitation. For solid media, 1.5% Difco agar was added and the agar plates incubated in a constant temperature incubator at 37°C. Strains were stored short-term at 4°C on LB agar (with ampicillin as required) and for long term storage, were grown to stationary phase in LB and kept at -70°C in 20% sterile glycerol.

2.3.2 S. cerevisiae

Cells were grown in YPD rich medium (1% yeast extract, 2% bacto-peptone, 2% glucose) or, for plasmid selection, in synthetic medium (0.67% yeast nitrogen base, 2% glucose) supplemented with the appropriate amino acids at 20μg/ml. In the case of gene induction from the GAL1-10 promoter, synthetic medium was supplemented with 2% galactose instead of glucose. Liquid cultures were grown with continuous agitation; for solid media, 2% Difco agar was added and the plates placed in a constant temperature...
incubator. The standard growth temperature for wild type yeast strains was 30°C.

Temperature sensitive strains were grown either at the permissive temperature of 25°C or the restrictive temperature of 37°C.

Yeast strains were stored short-term on YPD or minimal agar plates at 4°C. For long term storage, freshly grown cells were removed from agar plates, inoculated into 1ml of storage medium (1% yeast extract, 1% bacto-peptone, 2% glucose and 25% glycerol) and kept at -70°C.

2.4 STANDARD BUFFERS

TE 10mM Tris HCl, 1mM EDTA, pH8.0
TAE 40mM Tris base, 20mM glacial acetic acid, 1mM EDTA pH8.3
DNA loading buffer 0.1% bromophenol blue, 50% glycerol, 50mM EDTA
RNA loading buffer 0.4% bromophenol blue, 50% glycerol, 0.01M PO₄ (pH6.5)

2.5 ISOTOPES

Radiolabelled isotope [α-³²P] dCTP 111Tbq/mol (3000Ci/mol) was obtained from Dupont.
2.6 DNA MANIPULATIONS

2.6.1 RESTRICTION ENDONUCLEASES AND DNA MODIFYING ENZYMES

DNA was incubated with restriction endonucleases (BRL, NEB) in the appropriate restriction endonuclease buffer at the recommended temperature, with the addition of 1xBSA according to manufacturer’s recommendation. Klenow Taq polymerase (Amersham), T4 DNA ligase (BRL) and calf intestinal phosphatase (Boehringer Mannheim) were used according to the manufacturers’ recommendations.

2.6.2 DNA LIGATIONS

Ligation of DNA fragments was carried out in a 20μl volume in 1x ligation buffer (50mM Tris-HCl pH7.6, 10mM MgCl₂, 1mM ATP, 1mM DTT, 5%(w/v) polyethylene glycol-8000) and 0.4 units of T4 DNA ligase (GIBCO BRL). Ligations were incubated at room temperature for 2h, or alternatively overnight at 16°C before transformation into *E. coli*.

2.6.3 RECOVERY OF DNA FRAGMENTS FROM AGAROSE GELS

Glass milk extraction was used to remove fragments of DNA greater than 200bp from TAE agarose gels following the recommendations of the manufacturer (BIO 101, Geneclean II).

2.6.4 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis was carried out in 0.8% w/v agarose gels with TAE electrophoresis buffer. DNA was loaded onto gels with 1/6 volume DNA loading buffer and electrophoresed with a constant current of 50-100mA. DNA was stained by including 1μl of ethidium bromide (10mg/ml) per 100ml of molten agarose and visualised
under short wave (nm) UV using a Uniscience TF-20M transilluminator. The molecular size of DNA fragments was determined by comparison to DNA size markers (Life Technologies).

2.6.5 SOUTHERN HYBRIDISATION

Samples (2-5μg) of genomic DNA were digested with appropriate restriction enzymes and separated by electrophoresis through a 0.8% agarose gel. DNA transfer and hybridisation to specific probes was carried out according to Sambrook et al., (1989). GeneScreen transfer membranes (Dupont, NEN Research Products) were used as described in the manufacturer’s instructions.

2.6.6 POLYMERASE CHAIN REACTION

Reactions were carried out in 100μl total volume, 0.5-2μg of genomic or 5ng plasmid DNA, 1μl of 10mM dNTP stock (Pharmacia Biotech), 1μl of 0.1-0.5nMol/μl of each primer, 1x PCR buffer containing 15mM MgCl₂ (Perkin Elmer) and 2 Units of Taq Polymerase (Perkin Elmer). All PCR reactions were performed on a Biometra TRIO-Therobloc. Reactions were incubated using the same general conditions namely; 94°C 5min, 29(94°C 1min, 50°C 2min, 72°C 3min), 72°C 7min, then held at 20°C for up to 12h.

2.6.7 TRANSPOSITION-MEDIATED PLASMID MANIPULATION

Plasmid genes were disrupted by transposition according to Morgan et al., (1996). Additionally, the same method was used to produce truncated protein products from plasmid genes.
2.7 PLASMIDS

The plasmids used in this study are summarised in table 2.2.

Details of plasmids constructed in this study are given in section 2.7.1 to 2.7.7. Maps of some plasmid are in the plasmid appendix on page 145.

Table 2.2

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSH18-34</td>
<td>lacZ reporter plasmid: 8lexA motifs, GAL1-10 minimal promoter: URA3, 2µ; (Yocum et al., 1984).</td>
</tr>
<tr>
<td>pRS316</td>
<td>URA3, CEN cloning vector; (Sikorski and Hieter, 1989).</td>
</tr>
<tr>
<td>pLUC1</td>
<td>Contains the firefly luciferase gene a gift from Andrew Furley; (de Wet et al., 1987).</td>
</tr>
<tr>
<td>pBH1</td>
<td>As pRS316 with, GAL1-10:: 8lexA promoter cloned upstream of the luciferase coding sequence.</td>
</tr>
<tr>
<td>pTR27</td>
<td>LexA DNA binding domain: TRP1, 2µ.</td>
</tr>
<tr>
<td>pTR28</td>
<td>As pTR27, expressing LexA-Swi6 fusion.</td>
</tr>
<tr>
<td>pTR432</td>
<td>As pTR27, expressing LexA-Bck2 fusion.</td>
</tr>
<tr>
<td>pTR40</td>
<td>As pTR27, expressing LexA-Swi4 fusion.</td>
</tr>
<tr>
<td>pTR434</td>
<td>As pTR27, expressing LexA-Cln3 fusion.</td>
</tr>
<tr>
<td>pTR437</td>
<td>As pTR434 expressing C-terminal PEST deletion mutant Cln3.</td>
</tr>
<tr>
<td>pTR439</td>
<td>As pTR437 expressing N-terminal deletion up to cyclin box III.</td>
</tr>
</tbody>
</table>
pACTI     GAL4 activation domain: LEU2, 2μ; (Durfee et al., 1993).

pACTIII  As pACTI with altered polylinker sequence
          (CATATGGCCATGGAGGCCCCGGGGATCT
          ATGCATTGGGATCCGGATATCAAGATCC
          GAATTCGAAGCTCAGAGATCT).

pTR290   As pACTIII, expressing Gal4-Cln1 fusion.

pTR291   As pACTIII, expressing Gal4-Cln2 fusion.

pTR212   As pACTI, expressing Gal4-Cln3 fusion.

pTR83    MCB-lacZ: URA3 2μ. MCB denotes 3 MluI restriction sites.

pTR241   As pTR83 with mutated MCB.

pTR228   SCB-lacZ: HIS3, 2μ. SCB denotes bp -501 to -367 of the HO
          regulatory region encompassing four SBF binding sites.

pV44ER   GAL1-10 promoter upstream of sequence encoding the LexA
          DNA binding domain: TRP1 CEN (Jayaraman et al., 1994).

pTR240   As pV44ER expressing LexA-Swi6 fusion.

pΔP      pTR28-based, Cdc28 sites of Swi6 mutated, S160A S228A.

pΔ691    pTR28-based transposition with TnHIS resulting in a truncation at
          residue 691 of Swi6.

pΔ345    pTR28-based with transpositional insertion of TNHIS3 resulting in
          a truncation at residue 345 of Swi6.

pΔ145    pTR28-based with transpositional insertion of TnHIS3 resulting in
a truncation at residue 145 of Swi6.

pTR29  pTR28-based with internal Nsil region of SWI6 deleted resulting in the expression of a protein with the central region of Swi6 deleted between residues 103-729.

pTR250  As pTR27 expressing LexA-E2F fusion.

pTR249  As pTR27 expressing LexA-SLN1 fusion.

pTR345  As pTR27 expressing LexA-p53 fusion.

pTR433  TRP1::TnHIS3 derivative of YEplac112-CLN3. (YEplac112-CLN3 was a kind gift from C Wittenberg and has a single copy of CLN3 under the control of its own promoter).

YCplac181  Shuttle vector; (Gietz and Sugino, 1988).

pTR476  As YCplac181 with SphI-XbaI 4.5kb fragment containing BCK2 gene and flanking sequences cloned from YEp352-BCK2; (Lee et al., 1993).

pGEMlacZ  Contains BamHI-BamHI lacZ fragment, a kind gift from Miguel Manzanares.

pSE738  Plasmid containing RNR1 gene; (Elledge and Davis, 1990)

pYA301  ACT1 containing plasmid; (Gallwitz and Sures, 1980).

TRT1  Plasmid containing H2B gene; (Hereford et al., 1979)

pSD06a  CYC-GAL-VP16 (Dalton and Treisman, 1992).

pTR303  BamHI-Xbal CLN1 PCR fragment cloned into
$\textit{BamHI}-X\textit{ba}l$ sites of pSD06a.

pTR304  $Bgl\text{II}-Bag\text{II}$ $\textit{CLN2}$ PCR fragment cloned into $Bam\text{HI}-Bam\text{HI}$ sites of pSD06a.

pTR304  $Bam\text{HI}-Hind\text{III}$ $\textit{CLN3}$ PCR fragment cloned into $Bam\text{HI}-Hind\text{III}$ sites of pSD06a.

pTR477  pTR28 based $\textit{HIS3::swi6}$ disruption plasmid.

pTR251  $\textit{CLN2}$ under control of $S.\textit{pombe}$ $\textit{ADH}$ promoter ($\textit{LEU2}$);

(Fernandez Sarabiae et al., 1992).

2.7.1 CONSTRUCTION OF A LUCIFERASE REPORTER PLASMID

pBH1 was derived from the CEN $\textit{URA3}$ plasmid pRS316 (Sikorski and Hieter, 1989) and carries the firefly luciferase gene positioned downstream from the $\textit{GAL1-10}$ minimal promoter and $\textit{LexA}$ binding site sequences. The firefly luciferase gene was excised from pLUC1 (a gift from Andrew Furley, from de Wet et al., 1987) on a $\textit{HindIII}$-$\textit{KpnI}$ restriction fragment whilst a $\textit{BamHI}$-$\textit{HindIII}$ fragment containing the $\textit{GAL1-10}$ minimal promoter with the $\textit{lexAop}$ was purified from pSH18-34 (Yocum et al., 1984). A three-way ligation of these fragments with pRS316 cut with $\textit{BamHI}$-$\textit{KpnI}$ produced the $\textit{lexAop}$-$\textit{GAL1-10}$-luciferase reporter plasmid, pBH1.

2.7.2 CONSTRUCTION OF THE GALACTOSE INDUCIBLE $\textit{LEXA-SWI6}$ PLASMID

pTR240 was made by cloning the $\textit{SWI6}$ gene downstream of the DNA sequence coding for the bacterial LexA DNA binding protein. The CEN vector pV44ER (Jayaraman et al., 1994) encodes the LexA protein expressed from a galactose inducible promoter. A
2409bp BamHI restriction fragment containing the SWI6 coding sequence was purified and cloned into the BglII site in pV44ER, in frame with the LexA coding sequence.

2.7.3 CONSTRUCTION OF THE LEXA-BCK2 PLASMID

pTR432 was made by cloning the BCK2 gene downstream of the DNA sequence coding for the bacterial LexA DNA binding protein. A 2.56kb PCR product containing the BCK2 gene flanked by 5’BclI and 3’XhoI sites was digested with these enzymes, purified through agarose and cloned into the BamHI and SalI sites of the vector pTR27 to produce an in-frame LexA-Bck2 fusion.

2.7.4 CONSTRUCTION OF A GALACTOSE INDUCIBLE CLN1 PLASMID

pTR303 was made by cloning a PCR fragment encoding the CLN1 gene into the pSD06 (Dalton and Treisman, 1992). pSD06a was cut with BamHI and XbaI resulting in the excision of the VP16 coding region. A PCR fragment coding for CLN1 with 5’ BamHI and 3’ XbaI ends was digested and cloned into prepared pSD06a vector, resulting in CLN1 under the control of the GAL-CYC promoter.

2.7.5 CONSTRUCTION OF A GALACTOSE INDUCIBLE CLN2 PLASMID

pTR304 was made by cloning a restriction fragment encoding the CLN2 gene into the pSD06a. pTR291 was digested with BglII yielding a 1.6kb restriction fragment encoding the CLN2 gene. This fragment was cloned into pSD06a cut with BamHI. A stop codon before the sequence encoding VP16 resulting in CLN2 under the control of the GAL-CYC promoter.
2.7.6 Construction of a Galactose Inducible CLN3 Plasmid

pTR303 was made by cloning a PCR fragment encoding the CLN3 gene into the pSD06a. pSD06a was cut with BamHI and HindIII resulting in the excision of the VP16 coding region. A PCR fragment coding for CLN3 with 5′ BamHI and 3′ HindIII ends was digested and cloned into prepared pSD06a vector, resulting in CLN3 under the control of the GAL-CYC promoter.

2.7.7 Construction of a BCK2 Genomic Clone Expressing Plasmid

The plasmid pTR476, expressing BCK2 under its own promoter was constructed. A 4.5kb BCK2 containing SphI-XbaI restriction fragment was cloned from YEp352-BCK2 (Lee et al., 1993) into the yeast shuttle vector, YEplac181 (Gietz and Sugino, 1988), digested with SphI-XbaI. The resulting plasmids marker gene was compatible with the CTY10-5d reporter strain.

2.8 RNA Manipulation

2.8.1 RNA Extraction

$10^8$ synchronously or asynchronously mid-logarithmically growing cells were harvested, washed in 0.9% cold saline solution and rapidly frozen in dry ice. Total RNA was prepared by the hot phenol extraction method (Aves et al., 1985). The quality and quantity of total RNA was assessed by UV spectrometry at $A_{260}$ using a Shimadzu UV-160A UV-visible recording spectrophotometer.
2.8.2 RNA TRANSFER

5μg samples of total RNA were denatured in the presence of 0.5M deionised glyoxal before the addition of 4μl of loading buffer (Sambrook et al., 1989). Size separation was by electrophoresis through 1.2% agarose gels (15mM Na(PO₄) buffer pH6.5). RNA was transferred by capillary action (25mM Na(PO₄) buffer pH6.5) to a GeneScreen hybridisation membrane (Dupont, NEN Research Products) as described in the manufacturer’s instructions. RNA was fixed to the membrane by UV crosslinking using a Stratagene UV Stratalinker at 1800/2400.

2.8.3 HYBRIDISATION AND PROBING OF BLOTS

Further treatment of the GeneScreen membrane, hybridisation in the presence of Dextran Sulphate, washing and rehybridisation were performed exactly as described by the manufacturer (Dupont, NEN Research Products). To remove redundant bound probes from the membranes for further hybridisation, the membranes were incubated for 30min at 60°C in 96% deionised formamide, 10mM Tris-HCl pH8.0, 10mM EDTA pH8.0.

2.8.4 RADIONLABELLING OF PROBES

RNA blots were subjected to hybridisation with radiolabelled single stranded DNA restriction fragments or PCR products internal to the gene concerned. DNA was radiolabelled with ³²P-dCTP using the oligolabelling protocol and kit from Amersham International.
2.8.5 Visualisation and Quantitation

For visualisation, hybridised probes were subjected to autoradiography. X-ograph Imaging System High Definition X-ray films were exposed to membranes at -70°C in conjunction with X-ograph Hi-speed-X intensifying screens. Quantitation of transcripts was by densitometry using Molecular Dynamics Imagequant version 3.3.

2.8.6 DNA Fragments Used for Probes

The DNA fragments used for probes are shown in table 2.3 below.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Fragment</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTIN</td>
<td>563bp Clal-Clal</td>
<td>(Gallwitz and Sures, 1980)</td>
</tr>
<tr>
<td>RNR1</td>
<td>2.6kb EcoRI-EcoRI</td>
<td>(Elledge and Davis, 1990)</td>
</tr>
<tr>
<td>H2B</td>
<td>1.6kb SstI-SstI</td>
<td>(Hereford et al., 1979)</td>
</tr>
<tr>
<td>LUC</td>
<td>1.9kb HindIII-KpnI</td>
<td>This study, from pLUC1</td>
</tr>
<tr>
<td>lacZ</td>
<td>2.9kb BamHI-BamHI</td>
<td>Cut from pGEMlacZ</td>
</tr>
</tbody>
</table>

2.9 Western Blots

2.9.1 Cell Lysis

15ml of 5x10^6 synchronously or asynchronously mid-logarithmically growing cells were harvested and frozen on dry ice until needed. Yeast total protein extracts were prepared by resuspending cells in 100µl ice-cold lysis buffer (20mM Tris-Cl pH7.5, 100mM NaCl, 10mM EDTA, 1% Trition X-100), 1mM each of NaF, sodium pyrophosphate, sodium
orthovanadate, β-glycerophosphate and EGTA, with the addition of protease inhibitors 2mM PMSF, 10µg/ml each of Aprotinin, Leupeptin, Antipain and Pepstatin A. Cells were broken by vortexing with 0.5mm diam. chromic acid-washed Ballotini glass beads (4x30sec), after which the lysate was cleared by centrifuging for 5min at 12,000rpm and the supernatant removed and held on ice. A second 100µl of lysis buffer was added to the pellet, mixed and the sample re-centrifuged. This supernatant was added to the first extract. Total protein was determined by Lowry assay (Peterson, 1983). Samples were kept at -70°C until needed.

2.9.2 Polyacrylamide gel electrophoresis

Gels for electrophoresis of proteins through polyacrylamide were prepared as described in Sambrook et al., (1985), the precise proportions and percentages were varied depending upon the proteins to be analysed.

2.9.3 Immunoblot analysis

Transfer of proteins onto nitrocellulose membranes (Schleider & Schuell) was by semi-dry electroblot as described in Sambrook et al., (1985). Following transfer, the membrane was rinsed with PBS and blocked for 1hr in 5% w/v dried milk powder/0.1% tween in PBS, followed by three 5min rinses with 0.1% tween in PBS. The membranes were incubated with the primary antibody for 1h at room temperature or overnight at 4°C in 20ml 5% milk/0.1% tween in PBS. All primary antibodies used were at a final concentration of 1µg/ml. Membranes were washed three times for 5min with PBS/0.1% tween before the addition of secondary anti-mouse Ab (1 in 20,000 dilution) or anti-rabbit
Ab (1 in 20,000 dilution) in 20ml PBS/0.1% tween and incubated for 1hr at room temperature. The membranes were washed three times for 5min in PBS/0.1% tween and once for 5min in PBS. Luminescence of the secondary antibody was visualised using ECL Western Blotting method according to manufacturer's specifications (Amersham LIFE SCIENCE).

2.10 BACTERIAL TECHNIQUES

2.10.1 Transformation by CaCl₂ shock

1ml of an overnight culture of *E. coli* was inoculated into 99ml of fresh LB medium and grown at 37°C with shaking for 2-3h to an O.D.₆₀₀ of 0.2. Cells were harvested (4000rpm for 5min in a pre-chilled rotor) and resuspended in 20ml of ice-cold 100mM CaCl₂. The cells were chilled of ice for 30min, harvested and resuspended in 2ml of ice-cold 100mM CaCl₂, 20% glycerol solution. Competent cells were used immediately or frozen at -70°C in 50μl aliquots for future use.

For transformation, 10-20ng of plasmid DNA was added to 50μl of competent cells prepared by the CaCl₂ method. The cell/DNA mixtures were held on ice for 30min and then heat shocked at 42°C for 10min before the addition of an equal volume of LB medium. The cells were then incubated at 37°C for 30-60min before plating out on to LB agar containing the appropriate antibiotic.
2.10.2 **Electro-transformation of *E. coli***

DH5-α cells were grown overnight in LB medium, inoculated into 500ml fresh medium at 1/100th volume and grown at 37°C with shaking to an O.D.₆₀₀ of 0.5-1.0. After 30min on ice, the cultures were harvested at 4000rpm for 15min in pre-chilled rotors and resuspended in 250ml iced water. The cells were reharvested and resuspended in 10ml ice-cold 10% filter sterilised glycerol. The cells were harvested again and resuspended in 1ml 10% glycerol. Electro-competent cells were used immediately or aliquots of 40μl were stored in pre-cooled eppendorfs tubes at -70°C, for future use.

Electro-transformation was used to increase transformation efficiency in cases where amounts of DNA were limiting. DNA to be transformed was first de-salted by ethanol precipitation and washing in 70% ethanol before resuspending in distilled water. Transforming DNA in a maximum of 5μl water was added to 40μl of gently thawed electrocompetent cells. The mixture was transferred to electro-cuvettes (Bio-Rad) and pulsed at 25μF and 2.3kV set to 200Ω (BIO-RAD Gene Pulser™). Immediately following the pulse 1ml of SOC was added (2% bacto-tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄, 20mM glucose) and the cells incubated for 1h at 37°C before plating onto LB agar containing the appropriate antibiotics.

**2.10.3 Preparation of plasmid DNA**

Small scale plasmid preparations from transformed *E. coli* cells were done by the alkaline lysis protocol of Ish-Horowitz and Burke (1981). 1ml of cells were grown overnight in
LB plus the appropriate antibiotic, harvested and resuspended in 100μl of solution I (50mM glucose, 25mM Tris-HCl pH8.0, 10mM EDTA pH8.0). 200μl of fresh solution II was added (0.2M NaOH, 1% SDS) and the solution gently mixed by inversion and left at room temperature for 5min. Finally 150μl of solution III (5M NaOAc pH4.8) was added before the mixture was shaken vigorously and incubated on ice for 5min. The precipitated SDS-protein and cell debris was pelleted by microfuging at 12,000rpm for 3min and the supernatant transferred to 0.6x volume of propan-2-ol (isopropanol). Plasmid DNA was precipitated on ice for 5min and pelleted at 12,000rpm for 10min.

The DNA was resuspended in 50μl of sterile H2O or 1xTE.

In the case of larger scale preparations, the method described above was essentially scaled up, with an additional purification step as follows:

The supernatant from solution III was vortexed with an equal volume of phenol/chloroform/isoamylalcohol (25/24/1). After brief centrifugation, the aqueous layer was removed and placed on ice for 15min following the addition of 1/10th volume of 3M NaOAc and 3X volume of cold 96% ethanol. After centrifuging the resulting pellet was washed in 70% ethanol, air dried and resuspended in 1/5 the solution III supernatant volume.
2.11 YEAST TECHNIQUES

2.11.1 Lithium acetate transformation

*S. cerevisiae* cells were transformed by lithium acetate transformation protocol (Ito et al., 1983). A 50ml culture was grown to mid-logarithmic phase (2x10^6-1x10^7 cell/ml), harvested and washed twice in 10ml of 1xTE buffer. The cells were harvested and resuspended in 300μl of LA (1xTE, 1mMLiAc, 50μg single stranded carrier DNA) and 25-50μl of the cell suspension was used for transformation. 1-10μg of DNA was added to aliquots of competent yeast followed by the addition of 6X volume of LAP (1xTE, 1mMLiAc, 40% PEG₄₀₀₀). The cells were then incubated for 30min at 30°C. Following heat shock at 42°C for 15min the transformation mixtures were plated directly onto selective agar minimal plates.

2.11.2 Small scale yeast plasmid preparation

Small amounts of yeast plasmid were prepared from 1.5ml of overnight culture as described in Robzyk and Kassir (1992). The plasmids were transformed into electro-competent *E. coli* for recovery.

2.11.3 Isolation of yeast genomic DNA

Yeast genomic DNA for Southern and PCR analysis was prepared from 10ml stationary phase yeast culture as described by Hoffman and Winston (1987). Approximately 10-20μg of DNA were obtained from each preparation.
2.11.4 Gene disruption

Yeast strain, YAT25 was created by SWI6 disruption in the strain CTY10-5d. YAT25 was constructed by the one step gene disruption method of Rothstein (1983). The 1.9kb internal NsiI fragment of SWI6 in pTR28 was replaced by a 1kb NsiI fragment containing the HIS3 gene (pTR477). A DNA fragment containing swi6::HIS3 was generated by BamHI digestion and used to transform the his3' CTY10-5d reporter strain to histidine prototrophy. The integrated swi6 disrupts were confirmed by Southern hybridisation.

The disruption alleles of cln1 and cln2 in the strains YAT3, YAT21 and YAT22 were made using plasmids provided by Dr F. Cross. Disruption was verified by Southern hybridisation.

2.11.5 Gene replacement

Figure 2.1 depicts the use of a Polymerase Chain Reaction (PCR) based method to make cln3 deletions in the strains YAT7 (W303-1a, cln3::LEU2); YAT8 (W303-1a, cln3::HIS3) and YAT24 (CTY10-5d, cln3::LEU2). Linear fragments carrying the S. cerevisiae HIS3 or LEU2 genes flanked by 40bp of 5' and 3' terminal sequence from CLN3 were generated by PCR using the following oligonucleotides.

(primer corresponding to CLN3 5' coding sequence)

5'-ATG GCC ATA TTG AAG GAT ACC ATA ATT AGA TAC GCT AAT GGA ATT
CCC GGG GAT CCG GTG-3'

43
Homology to the *CLN3* gene is shown in bold type while the remaining sequence is homologous to the polylinker sequence of the *YDp* plasmid templates carrying the marker genes (Berben *et al.*, 1991). The PCR fragments were introduced into yeast and transformants selected on medium lacking the appropriate amino acid. Genomic DNA was isolated from transformants (Hoffman and Winston, 1987) and homologous recombination resulting in the replacement of the *CLN3* gene by the appropriate marker was verified by PCR, using two pairs of primers as follows:

Where *CLN3* was replaced by *HIS3*, the primers used for generating the knock out fragment generated a PCR product of 1.4kb, but not in wild type, since the yeast genome does not contain sequences homologous to the polylinker of the *YDp* plasmid templates.

Primers specific to 30 nucleotides 5’ and 3’ to *CLN3* produced a 1.6kb fragment in wild type, corresponding to the *CLN3* gene and a 1.4kb fragment in cells where *CLN3* had been deleted and replaced by *HIS3*. The method of *CLN3* deletion replaced by *LEU2* is as described above only using the YDp-*LEU2* plasmid as a template to generate a *LEU2* gene product flanked by *CLN3* sequences.
Figure 2.1

PCR assisted gene replacement. Primers with homology to the poly-linker of YDp-H and CLN3 5' and 3' ends (I) generate a PCR fragment with the HIS3 marker gene flanked by CLN3 5' and 3' ends (II). The PCR fragment recombines with genomic CLN3 (III) resulting in cln3- and HIS+ strain (IV). Gene knockout can be confirmed using the same primers and primers to CLN3.
The same method was used in the deletion of the \textit{BCK2} gene in strains YAT18 (W303-1a, \textit{bck2::LEU2}); YAT68, (W303-1a, \textit{bck2::HIS3}) and YAT71, (CTY10-5d, \textit{bck2::HIS3}).

The following oligonucleotides used are shown below

(primer corresponding to \textit{BCK2} 5' coding sequence)

\begin{verbatim}
5'-ATG CCG AAG CAT TGT CAC CAC CAT CGT TCC AGT TCG GTT AGA ATT CCC GGG GAT CCG GTG-3'
\end{verbatim}

(primer corresponding to \textit{BCK2} 3' coding sequence)

\begin{verbatim}
5'-TTA GTT GCT ATT ATC AAA ATA AAA AGA CTG TAA ATT ATT AAA GCT AGC TTG GCT GCA GGT-3'
\end{verbatim}

Homology to the \textit{BCK2} gene is shown in bold type while the remaining sequence is homologous to the polylinker sequence of the YDp plasmid templates carrying the marker genes (Berben \textit{et al.}, 1991).

\textbf{2.11.6 GROWTH SYNCHRONISATION METHODS}

Synchronisation of growth of W303-1a cells was achieved by release from \(\alpha\)-factor-induced cell cycle arrest. Cells were grown at 30\(^{\circ}\)C in synthetic medium with appropriate supplements to a cell density of 3.5 x 10\(^6\) cells/ml. Synthetic \(\alpha\)-factor (Polypeptide synthesis lab-NIMR) was added to a final concentration of 3\(\mu\)g/ml and the cells were
incubated for a further 4h. The cells were released from the G1/S block by rapid filtration and washing followed by resuspension in fresh medium at 30°C.

A second synchronisation method relied on releasing cells from a *dbf2-2* mitotic block. *dbf2-2* cells were grown at 25°C to a cell density of 5 x 10^6 cell/ml, followed by a temperature shift to 37°C for a further 2h. Cells were released from the block by rapid temperature shift back to 25°C.

2.11.7 **Determination of budding index and cell numbers**

The budding index (the percentage of budding cells in a population) was determined microscopically using a 40x objective lens (Nikon Labophot-2). Cell samples were gently sonicated and concentrated by centrifugation. Approximately 150 cells were examined for each count. The cell density of liquid cultures was determined with a Coulter (model ZM) particle counter. 1ml samples were gently sonicated to separate clumps of cells. The samples were diluted 1:100 in saline and counted.

2.11.8 **DAPI staining for fluorescent microscopy**

Mid-logarithmically growing cell cultures were harvested and sonicated to separate individual cells. Cells were resuspended in PBS containing 0.1µg/ml of 4', 6-diamidino-2-phenylindole (DAPI), (Sigma). 10µl of a 1.0x10^7 cell suspension was pipetted on to a Poly-Prep™ slide (Sigma Diagnostics) and allowed to air dry for 5min. The residual liquid was removed by vacuum evaporation and of 5µl of fixing fluid (90% glycerol, 0.1%PBS, 2.5% DABCO; Sigma) was dotted onto the dried sample. A glass coverslip was fixed around the edges with nail fashion finish (Rimmel). DAPI strained nuclei were
observed using a 63X oil immersion objective on an Axiophot fluorescence microscope (ZEISS) with ultra violet illumination and the appropriate filter.

2.11.9 **RELATIVE CELL SIZE COMPARISONS BY FACS ANALYSIS**

Relative yeast cell volumes were determined by harvesting 10^7 mid-logarithmically growing cells and resuspended in 2ml PBS. The yeast suspension was then analysed using a Dickinson FACscan. Relative cell size was determined by calculating the geometric mean of cell shadows cast from each culture.

2.11.10 **CELL VOLUME DETERMINATION BY MICROSCOPY**

Cell volume (fl) was determined for yeast growing in mid-logarithmic phase. For each sample, the lateral, A, and longitudinal, B, radii of 20 cells with new buds were measured optometrically using a Nikon Filar micrometer at X200 magnification. Assuming yeast to be prolate ellipsoids, cell volume (fl) was calculated by \( V = \frac{4}{3} \pi A^2 B \).

2.11.11 **LUCIFERASE ACTIVITY ASSAYS.**

Cell pellets containing 5x10^7 to 1x10^8 mid-logarithmically growing cells were harvested, snap frozen on dry ice and stored at -70°C until needed. The cells were resuspended in 200µl of Promega 1X Cell Culture Lysis Reagent before the addition of 0.5mm diam. chromic acid-washed Ballotini glass beads to just below the meniscus. The preparation was vortexed on ice 4x20sec. A further 100µl of lysis buffer was added and the mixture microfuged for 20sec. Cell extract was removed from the surface of the beads and 20µl was used for measurement of total protein content by Lowry assay (Peterson, 1983).
For each luciferase determination, three aliquots of cell extract, ranging from 5-15µl, were made up to 50µl with lysis buffer and luciferase activity was quantitated by adding 50µl of Promega Luciferase Assay Reagent. Photon emission from the degradation of the substrate by luciferase was measured on a Beckman LS6000IC scintillation counter. A graph of µg of Total Cellular Protein (TCP) versus Counts Per Minute (CPM) of luciferase activity was plotted to ensure that assays were done in the linear range of enzyme/substrate concentrations.

2.11.12 β-GALACTOSIDASE ASSAYS IN LIQUID CULTURES.

Mid-logarithmically growing cells were harvested from liquid culture and 1x10^7 cells used in the assays. The cells were resuspended in 200µl of Z-buffer (60mM Na2HPO4, 40mM NaH2PO4, 10mM KCl, 0.1mM MgSO4, pH7.0) containing 1µg/µl of zymolyase (Arthrobacter luteus zymolyase-20T, Seikagaku Corporation) and incubated at 30°C for 10min to digest the cell wall. 20µl of lysate was removed for a Lowry protein assay (Peterson 1983). 40µl of lysate was added to 1ml of Z-buffer containing 0.8µg of the β-galactosidase artificial substrate, o-Nitrophenyl β-D-galactopyranoside (ONPG; Sigma.). The mixture was incubated at 30°C until a non-saturating development of yellow colour was observed. The reaction was stopped by adding 500µl of 1M Na2CO3. Each experimental result was repeated at least 3 times and was reproducible. Arbitrary units of ONPG were calculated according to the following formula.
2.11.13 β-Galactosidase Assays in Yeast Colonies

Freshly grown yeast cells were blotted from agar plates onto Whatman filter paper and freeze fractured by immersion in liquid nitrogen for 30 sec. The paper was then laid face upwards in an inverted petri dish containing a disc of Whatman paper pre-soaked in 2 ml of Z-buffer reaction mix (2.7 μl β-mercaptoethanol/ml Z buffer and 50 μl of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) solution at 40 mg/ml of X-gal in Z-buffer). The bottom of the petri dish was inverted over the paper and petri dish lid to form a cover. The samples were then incubated at 28°C for 20 min to 3 h and observed for the development of blue colour. The reaction was stopped by the addition of 500 μl of 1 M Na₂CO₃.
CHAPTER THREE

TRANSCRIPTIONAL ACTIVATION PROPERTIES OF SWI6 AND SWI4

3.1 INTRODUCTION

Whilst it is known that SBF and MBF can activate transcription, it was unclear whether this activity is due exclusively to the Swi6 protein, or its known DNA binding partners, Swi4 and Mbp1, or combined activities of Swi6 with either Mbp1 or Swi4. To examine this question, it was clearly important to assay the properties of Swi6 independently of its association with Swi4 or Mbp1.

The potential transcriptional activity of any given protein can be examined by using the yeast ‘one-hybrid’ test (Brent and Ptashne, 1985). This involves fusing a known DNA binding domain to the protein of interest. Transcriptional activation by this chimeric protein can then be quantified via the expression of a reporter gene situated downstream of the cognate DNA binding site (figure 3.1).

3.2 LEXA-SWI6 IS A TRANSCRIPTIONAL ACTIVATOR

The ‘one-hybrid’ test was used to examine the transcriptional activation properties of the Swi6 protein. An in-frame fusion was made by cloning full length SWI6 immediately downstream of sequences encoding the bacterial LexA DNA binding protein in the multicopy plasmid, pTR27. The resulting plasmid, pTR28, expresses the LexA-Swi6 fusion from the yeast ADH1 promoter. For purpose of clarity, expression of LexA-Swi6
Figure 3.1
The one-hybrid assay for transcriptional activation.
a) The bacterial LexA DNA binding protein, LexA<sub>BD</sub>, can bind the lexA<sub>op</sub>, but is unable to activate transcription due to the absence of an activation domain.
b) Protein X has a transcriptional activation domain but normally lacks a DNA binding domain. The LexA<sub>BD</sub> can bring the hybrid protein into the proximity of the lacZ reporter gene and the activation domain on protein X can activate transcription.
from this plasmid shall be referred to as ADH-LexA-Swi6, from here on. This construct was introduced into yeast strains carrying the bacterial lacZ gene downstream of multiple LexA binding sites, such that the transactivation potential of the hybrid could then be measured by assaying β-galactosidase activity. Two reporter systems were used. In the first, a lexAop-lacZ fusion was carried on a multicopy plasmid, pSH18-34 (Yocum et al., 1984). The advantage of this system is that a number of different recipient strains can be readily used. In the second system, the reporter was integrated as a single copy into the yeast genome and so is therefore more stable and physiologically realistic. All β-galactosidase assays in the results sections were performed on yeast cells growing in liquid culture in mid-logarithmic growth phase. Figure 3.2a demonstrates that using the plasmid-bound reporter system in wild type W303-la cells, ADH-LexA-Swi6 can activate gene expression to 11 β-galactosidase units, a 55 fold increase over the ADH-LexA control.

Similar results were obtained when the reporter strain CTY10-5d (Bartel et al., 1993) was used (figure 3.2b). Expression of ADH-LexA-Swi6 in CTY10-5d cells resulted in β-galactosidase units of 1.2, a 24 fold increase over ADH-LexA control levels.

3.3 ADH-LexA-Swi6 IS FUNCTIONAL

Before continuing investigations into the transcriptional activation properties of the ADH-LexA-Swi6 fusion protein, it was important to confirm that the ADH-LexA-Swi6 construct was functional at the cellular level. The yeast strain, K2003 (Nasmyth and Dirick, 1991), which is deleted for swi6 and carries a swi4Δ temperature sensitive mutation was used in this functionality test. Cells are viable when grown at the permissive temperature of
ADH-LexA-Swi6 can activate transcription of a lexAop-lacZ reporter gene.

Figure 3.2a shows that LexA-Swi6 can activate plasmid bound reporter gene expression in the strain W303-la.

Figure 3.2b shows that ADH-LexA-Swi6 can activate the reporter strain CTY10-5d. Additionally, ADH-LexA-Swi6 which can no longer physically associate with Swi4 or Mbp1 (LexA-Swi6Δ691), can still activate transcription. Loss of two consensus Cdc28 sites on Swi6 (LexA-Swi6ΔP), has no effect on the overall transcriptional activation properties of Swi6.
25°C, whereas incubation at the restrictive temperature of 37°C results in lethality.

K2003 was transformed with either the plasmid expressing \textit{ADH-LexA-Swi6} or the control plasmid expressing \textit{ADH-LexA}. Transformants were streaked out on selective agar and grown at 25°C, demonstrating that both strains could grow at the permissive temperature. Upon re-streaking to 37°C, cells carrying the vector plasmid failed to proliferate. Cells expressing the \textit{ADH-LexA-Swi6} fusion protein were able to grow at the restrictive temperature demonstrating that the Swi6 moiety of the LexA fusion protein could complement the mutational defects of K2003 (figure 3.3).

3.4 \textbf{LEXA-SWI6 CAN ACTIVATE TRANSCRIPTION IN THE ABSENCE OF MBP1 OR SWI4}

The results presented in section 3.2 demonstrate that Swi6, given an artificial DNA binding domain can activate reporter gene transcription. It is also known that the DNA binding specificities of SBF and MBF are provided by Swi4 and Mbp1 respectively. It could therefore be argued that the transcription activation potential of \textit{ADH-LexA-Swi6} may not require either Swi4 or Mbp1. Alternatively, it might be envisaged that the Swi6 activity is as a result from the recruitment of Swi4 or Mbp1. To investigate this possibility the transcriptional activation properties of \textit{ADH-LexA-Swi6} were assayed in \textit{swi4} or \textit{mbp1} mutants. The plasmid-based reporter system described in section 3.2 was used to examine the effect of a W303-1a isogenic \textit{swi4} deletion (\textit{Δswi4}) or \textit{mbp1} point mutation (\textit{mbp1-2}) on the transcriptional activation by \textit{ADH-LexA-Swi6}. \textit{β}-galactosidase assays in both the \textit{Δswi4} or \textit{mbp1-2} mutant strains revealed \textit{ADH-LexA-Swi6} driven reporter gene activity to be high with an activity of 8.5 units, a 85 fold increase over the
Plasmids expressing ADH-LexA-Swi6 fusion proteins were tested for functionality. ADH-LexA-Swi6 can rescue the temperature sensitive strain K2003 (△swi6 swi4ts) at the restrictive temperature of 37°C. Similarly, ADH-LexA-Swi6ΔP which is mutated at the two best consensuses Cdc28 sites can rescue. Conversely, ADH-LexA-Swi6Δ691, which is unable to interact with Swi4 and Mbp1, cannot rescue K2003.
ADH-LexA control (figure 3.4a). The activity of 8.5 units demonstrates a 1.3 fold decrease when compared to ADH-LexA-Swi6 dependent reporter gene transcription in wild type cells. Although there was an overall decrease in reporter gene expression in the Δswi4 or mbp1-2 mutant strains, ADH-LexA-Swi6 is nevertheless a potent transcriptional activator in these mutant strains.

Additionally, the transcriptional activation potential of two non-yeast transcription factors were tested using the one-hybrid approach in the swi4 and mbp1 mutants strains and compared against wild-type levels (figure 3.4b). ADH-LexA-p53 and ADH-LexA-E2F activated reporter gene expression to 46 and 85 β-galactosidase units respectively in the wild-type strain. Activity of ADH-LexA-p53 in the showed a 1.15 fold decrease in both swi4 and mbp1 strains compared to wild type levels, with β-galactosidase activities of 40 units. Similarly, there was a 1.1 fold decrease in ADH-LexA-E2F activity in the swi4 and mbp1 strains compared to wild-type, with β-galactosidase units being 77. This experiment shows that the small decrease in ADH-LexA-Swi6 activity seen in the swi4 and mbp1 mutants is not specific to Swi6.

3.5 LexA-Swi6 can activate independently of association with Mbp1 and Swi4

The previous section demonstrated that ADH-LexA-Swi6 is a potent transcriptional activator in the absence of either Swi4 or Mbp1. However, an investigation of the transcriptional activation properties of ADH-LexA-Swi6 in the simultaneous absence of Swi4 and Mbp1 was not possible due to the inviability of swi4 mbp1 double mutant. Therefore an alternative approach was devised to demonstrate that ADH-LexA-Swi6 could
Figure 3.4a
Transcription activation by ADH-LexA-Swi6 is only reduced slightly in the absence of either Swi4 or Mbp1.

Figure 3.4b
Reporter gene activation by two non-yeast transcription factors are slightly reduced in the absence of either Swi4 or Mbp1.

Figure 3.5
Transcription activation by ADH-LexA-Swi4 is weak in comparison to that seen by ADH-LexA-Swi6.
activate the transcription of a reporter gene in the absence of physical association with Swi4 and Mbp1. Previous work by ourselves and others (Andrews and Moore, 1992; Primig et al., 1992; Siegmund and Nasmyth, 1996) had demonstrated that Swi6 and its DNA binding partners Swi4 and Mbp1 interact with one another via their C-terminal residues. The plasmid which expresses ADH-LexA-Swi6 was transposed with TnHIS3 at bp 2177 (Morgan et al., 1996). The resulting plasmid expresses ADH-LexA-Swi6Δ691 with only the 691 N-terminal residues of Swi6. Assays by colleagues confirmed no interaction between Swi6Δ691 and Mbp1 or Swi4. The transcriptional activation by ADH-LexA-Swi6Δ691 was assayed in the reporter strain CTY10-5d. Figure 3.2b shows ADH-LexA-Swi6Δ691 activates reporter gene expression 30 fold compared to that of the ADH-LexA control, with β-galactosidase units of 1.5.

These results suggest that, even without the potential for binding to either Swi4 or Mbp1, the Swi6 protein remains a potent transcriptional activator. Indeed, ADH-LexA-Swi6Δ691 is 2.5 fold more active than wild type ADH-LexA-Swi6. Additionally, in a test comparable to that described in section 3.3, ADH-LexA-Swi6Δ691 was demonstrated to be non-functional at a cellular level by its inability to rescue the yeast strain K2003 (figure 3.3). This test demonstrates that while ADH-LexA-Swi6Δ691 can activate transcription, it is does not have the ability to recruit Swi4 and Mbp1, which is an essential function for cellular viability.
3.6 LexA-Swi6 activity is not dependent upon the two potential Cdc28 sites

Cell cycle progression through the G1/S transition point requires Cdc28 cyclin dependent kinase, as does SBF dependent expression of the \( HO \) gene. Additionally, Swi6 is a phosphoprotein with potential Cdc28 phosphorylation sites (Sidorova et al., 1995; Taba et al., 1991). Hence it could be possible that changes in SBF/MBF activity are attributed to Cdc28 dependent phosphorylation. To examine this possibility, the best two consensus Cdc28 phosphorylation sites on ADH-LexA-Swi6 were mutated to non-phosphorylatable residues. Serine to alanine changes at residues S160 and S225 were made by site directed mutagenesis resulting in the ADH-LexA-Swi6AP construct. The reporter strain CTY10-5d was used to determine what effect these changes had on the overall activation potential of ADH-LexA-Swi6. Reporter gene activity driven by ADH-LexA-Swi6AP was identical to that of wild type ADH-LexA-Swi6, giving 1.2 \( \beta \)-galactosidase units, a 24 fold increase over the ADH-LexA control (figure 3.2b). Thus, changes in phosphorylation of Swi6 at the two most likely targets for Cdc28 activity does not impair the overall ability of ADH-LexA-Swi6 to activate transcription.

Furthermore, in a test similar to that described in section 3.3, ADH-LexA-Swi6AP was functional at a cellular level, as determined by its ability to rescue the yeast strain K2003 (figure 3.3).

3.7 LexA-Swi4 is a weak transcriptional activator

The ‘one-hybrid’ strategy was next used to determine if Swi4 can activate transcription. The Swi4 coding sequence was cloned into pTR27 resulting in a plasmid which expresses
Figure 3.6
The $\Delta mbp1$ swi4ts fusion protein is functional. $\Delta mbp1$ swi4ts can rescue viability of the temperature sensitive strain MDS4 at the restrictive temperature of 37°C.
a LexA-Swi4 fusion protein from the ADH promoter (ADH-LexA-Swi4). The reporter strain CTY10-5d was used for detecting ADH-LexA-Swi4 activity. Figure 3.5 demonstrates that expression of ADH-LexA-Swi4 results in a β-galactosidase activity of 0.2 units, a 4 fold increase over the ADH-LexA control. Thus ADH-LexA-Swi4 can activate transcription of a reporter gene, but when compared to ADH-LexA-Swi6 in the same system, the potency of ADH-LexA-Swi4 is only one third that of ADH-LexA-Swi6.

3.8 LexA-Swi4 is functional

It could be argued that ADH-LexA-Swi4 could not activate gene transcription efficiently due to the nonfunctionality of the LexA-Swi4 hybrid construct. Therefore the ADH-LexA-Swi4 was tested for functionality by its ability to rescue the temperature sensitive strain, MDS4 (figure 3.6). MDS4 is deleted for mbp1 and carries a swi4Δ temperature sensitive mutation. Cells are viable when grown at the permissive temperature of 25°C, whereas incubation at the restrictive temperature of 37°C results in lethality.

MDS4 was transformed with either a plasmid expressing ADH-LexA-Swi4 or the control expressing ADH-LexA. Transformants were selected on minimal agar at the permissive temperature and streaked onto selective plates at either the permissive or restricted temperature. Yeast cells expressing ADH-LexA or ADH-LexA-Swi4 could grow at the permissive temperature. Additionally cells expressing ADH-LexA-Swi4 could grow at the restrictive temperature, whereas no growth was observed in cells expressing the control plasmid.
3.9 Discussion

The experiments described in this section begin an investigation into the overall transcriptional activation properties of Swi6 and to a lesser extent Swi4. It would appear that ADH-LexA-Swi6 can activate reporter gene expression and that the overall transcriptional activation properties are separate from association with its DNA binding partners Swi4 or Mbp1. An obvious interpretation of this result is that Swi6 harbours the transcriptional activation properties of SBF and MBF. This is consistent with the hypothesis that it is Swi4 and Mbp1 which bind the transcription factor complexes to the promoter region, with Swi6 having some regulatory function. Indeed, I have shown that compared to Swi4 it is Swi6 which is the major activator of the two known components of SBF. The way in which Swi6 could be activated is an important unanswered question. Phosphorylation of Swi6 by the Cln3-associated kinase, Cdc28, is an obvious possibility (Koch et al., 1996; Stuart and Wittenberg, 1995; Tyers et al., 1993), especially as Swi6 is a phosphoprotein (Sidorova et al., 1995). However, mutational inactivation of the two best consensus Cdc28 phosphorylation sites in Swi6 did not reduce the overall level of transcriptional activation observed even though the only change identified in Swi6 phosphorylation status involves the S160 residue mutated here (Sidorova et al., 1995). Swi6 also shows cell cycle dependent changes in the nuclear and cytoplasmic localisation (Taba et al., 1991) and it is not known whether LexA-Swi6 behaves in a similar manner. The lack of effect of the S160 mutation is consistent with the observation, made after this experiment was completed, that this phosphorylation event does not coincide with the peak in G1/S transcription and that the phosphorylation of S160 is not Cdc28 dependent.
(Sidorova et al., 1995). Likewise, members of our team have observed no reduction in the overall level of Swi6 phosphorylation in cln1, cln2 or cln3 mutants compared to wild type.

Changes in the phosphorylation status of Swi6 may effect the activity of SBF and MBF indirectly, by regulating a protein protein interaction between Swi6 and its DNA binding partners, perhaps in a similar way to the S. pombe Swi6 homologue, Cdc10, which requires phosphorylation to interact with the Mbp1 homologue, Res1 (Connolly et al., 1997). However, with LexA-Swi6 activity the Swi6 protein has its own artificial DNA binding domain and is no longer dependent upon association with Swi4 or Mbp1 for activity. Alternatively, Swi6 may of course be a substrate for some other uncharacterised phosphorylation event, several other possible mechanisms for transcriptional activation can be envisaged, and will be discussed in chapter 10.
CHAPTER FOUR

DEVELOPING AND USING A REPORTER SYSTEM FOR PERIODIC STUDIES

4.1 INTRODUCTION

The previous chapter investigated the overall transcriptional activation potentials of Swi4 and Swi6, revealing that of the two known SBF components, Swi6 appears to be the stronger contributor to gene activation. This revelation encouraged further studies into the activation properties of the \textit{adh-LcxA-Swi6} fusion protein. Like naturally occurring SBF and MBF, it could be possible that \textit{adh-LcxA-Swi6} is periodically active. Assaying for reporter gene activity in synchronously dividing yeast could determine if the transcriptional activity displayed by \textit{adh-LcxA-Swi6} is indeed cell cycle regulated.

4.2 USING THE LEXAOP-lacZ REPORTER CONSTRUCT FOR PERIODIC STUDIES

The previous section used liquid \(\beta\)-galactosidase assays to quantitate the expression of a \textit{lacZ} reporter gene. Since \(\beta\)-galactosidase has a 20h half life in yeast (Bachmair \textit{et al.}, 1986), it would be unsuitable as an indicator of cell cycle fluctuations in activation, and so I decided to measure levels of \textit{lacZ} mRNA. Wild type W303-1a cells were co-transformed with the \textit{lexAop-lacZ} reporter plasmid, pSH18-34, and either the plasmid expressing \textit{adh-LcxA-Swi6} or the \textit{adh-LexA} control. Northern hybridisation was performed on mid-logarithmically growing cultures to determine the suitability of assaying for \textit{lacZ} mRNA.
Figure 4.1a

Figure 4.1b

Figure 4.1
Northern hybridisation demonstrates that there is gene expression from the *lexAop-lacZ* reporter plasmid in the absence of a 'one-hybrid' activator. Figure 4.1a shows RNA analysed from W303-1a cells expressing a *lacZ* reporter plasmid and either the LexA control or the LexA-Swi6 activator. In both strains the level of *lacZ* mRNA was comparable. Additionally, figure 4.1b, shows that in the absence of the LexA DNA binding protein, the levels of *lacZ* mRNA are elevated further.
Hybridisation of an actin probe was used to normalise mRNA levels in different samples. The membrane was stripped and hybridisation with a lacZ probe revealed that as expected, there was a strong band corresponding to the lacZ message in samples expressing ADH-LexA-Swi6 (figure 4.1a). Surprisingly, there was also a comparable band of similar strength in the control strain. From these results it would appear that although β-galactosidase activity was low in the control strain, as seen in figure 3.2a from section 3.2, there was some spurious transcript expressed corresponding in size to the lacZ message and sharing lacZ sequence. This phenomenon was detected again by looking at the levels of lacZ message in wild type strains carrying the reporter plasmid alone (figure 4.1b). W303-1a cells which expressed the reporter plasmid alone had higher levels of lacZ mRNA than a strain which also expressed ADH-LexA-Swi6.

4.3 THE DEVELOPMENT OF A LUCIFERASE REPORTER PLASMID

Because of the technical and experimental difficulties encountered in assaying for lacZ mRNA this approach was abandoned, leading to the search for an alternative assay system. I settled with a reporter system based on expression of the firefly luciferase gene. This reporter appeared to be the most promising since there was a recently developed assay system for quantitating luciferase protein activity. Additionally, personal communications from a colleague suggested that the luciferase protein may have a short half-life in yeast, thus making assays for luciferase activity ideal for a cell cycle experiment. For the above mentioned reasons I constructed a lexAop-luciferase reporter
Figure 4.2

In a 'one-hybrid' assay ADH-LexA-Swi6 can activate expression of the lexA_{op}-luc reporter gene.
plasmid (section 2.7.1) in the hope of detecting changes in \( \text{ADH-LexA-Swi6} \) activity through the cell cycle.

**4.4 LEXA-SWI6 ACTIVATES LUCIFERASE EXPRESSION**

A preliminary experiment was carried out to determine if expression of the luciferase reporter plasmid could be activated by \( \text{ADH-LexA-Swi6} \). Wild type W303-1a cells were co-transformed with the luciferase reporter plasmid and either the plasmid expressing \( \text{ADH-LexA-Swi6} \) or the \( \text{ADH-LexA} \) control. Mid-logarithmic growing cells were harvested and broken as described in section 2.11.11. 20\( \mu \)l of cell extract from each strain was then assayed for luciferase activity by adding a luciferase substrate. Luciferase activity in cells expressing \( \text{ADH-LexA-Swi6} \) was \( 20 \times 10^5 \) counts per min (CPM), over 8 fold above cell expressing the \( \text{ADH-LexA} \) control (figure 4.2). This simple experiment demonstrated that the expression of the new luciferase reporter plasmid could be activated by \( \text{ADH-LexA-Swi6} \).

**4.5 THE RELATIONSHIP BETWEEN LUCIFERASE ACTIVITY AND THE AMOUNT OF TOTAL CELL PROTEIN IS LINEAR**

Once it was clear that \( \text{ADH-LexA-Swi6} \) could activate luciferase expression, it was important to examine the assay conditions to ensure that the relationship between luciferase activity and total cell protein (TCP) was linear.

Increasing volumes of extracts from W303-1a cells expressing either \( \text{ADH-LexA-Swi6} \) or \( \text{ADH-LexA} \) and the reporter plasmid were assayed for luciferase activity. Lowery assays on the cell extracts determined the amount total cell protein in each sample. Figure 4.3 demonstrates that in cells expressing the reporter plasmid and \( \text{ADH-LexA-Swi6} \), the
Figure 4.3
The relationship between luciferase activity and cell extract is linear. In cells expressing ADH-LexA-Swi6, there is an increase in luciferase activity as total cell protein levels increase.

Figure 4.4
Activity of luciferase is constant for up to 5 min after addition of substrate. The activity decay curve of W303-1a cells expressing lexArp-luc reporter plasmid, and the ADH-LexA-Swi6 activator is compared with cells expressing a ADH-LexA control and the reporter plasmid. TCP=Total Cell Protein.
relationship between total cell protein and luciferase activity is linear. The linear range was up to 20μg of total cell protein, so that luciferase activity of a given strain could be given as the slope of multiple samples in future assays. Using this method there was over a 10 fold increase in luciferase activity in cells expressing ADH-LexA-Swi6 in comparison to cells expressing the ADH-LexA only control. Luciferase activities from section 4.7 onwards are calculated as the slope of multiple samples.

4.6 Luciferase Activity Measurements Does Not Decay Rapidly After the Addition of Substrate

A second obstacle to properly quantifying luciferase activity is the decrease in light emission which occurs by substrate depletion after the start of the assay. The new Promega system promised a constant assayable level of luciferase activity after the addition of substrate, hence allowing multiple samples to be assayed at one time. Unfortunately the assays described by Promega had been performed on mammalian cells expressing luciferase reporter plasmids, with no data available for yeast. Therefore it was important to determine the rate of decay of luciferase activity after the addition of substrate in yeast cell extracts. Cell extracts of W303-1a cells expressing the reporter plasmid and either the ADH-LexA-Swi6 expressing plasmid or the ADH-LexA only control were divided into 15 equal samples. Lowery assays later determined that the aliquoted samples were in the linear range as described in the previous section. Luciferase substrate was added to each of the aliquots. Luciferase activity assays were then performed on each sample at 1min intervals for up to 8 min then at 2 min intervals for a further 12 min,
making the total time span of the experiment 20 min. Luciferase activity of the $\text{ADH}$-LexA-Swi6 expressing cells at the initial (zero) time point was $13 \times 10^5$ CPM, and remained at this level for 5 min. Thereafter, activity declined over the remaining 15 min (figure 4.4). Luciferase activity in the control cells remained low ($2 \times 10^5$ CPM) throughout the experiment. The results from this experiment indicates that the luciferase substrate is not depleted rapidly, so that multiple samples could be assayed in one experiment.

4.7 Luciferase activity is unstable in yeast

For the luciferase activity assays to be useful in a cell cycle experiment, I needed to confirm reports that the luciferase protein is unstable in yeast. If $\text{ADH}$-LexA-Swi6 dependent reporter gene activity is regulated in a similar fashion to genes under the control of SBF and MBF, then its activity would be expected to decrease in a population of cells which were arresting in early G1. This decrease in activity would only be detectable if the reporter gene protein product had a short half life in yeast. To test this possibility a mid-logarithmically growing population of W303-1a cells expressing the luciferase reporter plasmid and the plasmid expressing $\text{ADH}$-LexA-Swi6, were arrested at the G1/S transition point. The arrest was achieved by exposure to the mating pheromone $\alpha$-factor. A control population of cells expressing the reporter plasmid and the control plasmid expressing $\text{ADH}$-LexA was treated in the same manner. Cell samples were taken for luciferase assays throughout the holding period and frozen on dry ice at 15 min intervals for 2 h. Samples were also taken throughout the experiment to score percentage
In W303-1a cells expressing $\text{ADH-LexA-Swi6}$ and the $\text{lexA}_{op}$-luc reporter plasmid, there was a sharp reduction in luciferase activity after the addition of $\alpha$-factor (figure 4.5a). A successful G1 arrest is characterised by a population of unbudded cells (figure 4.5b).
of budding cells and to monitor cell cycle arrest. Figure 4.5a shows that after 15 min into the hold, luciferase activity of cells expressing ADH-LexA-Swi6 decreased 2 fold from 1.2 to 0.6. Activity decreased again after another 15 min to 0.4, 3 fold of the original zero time point. Luciferase activity remained at this level for a further 1.5 h to the end of the experiment. In contrast, the low level of luciferase activity in the control cells expressing ADH-LexA did not change throughout the 2 h holding treatment with α-factor. The budding profiles of both cultures demonstrated that the cells had arrested in G1 in response to α-factor (figure 4.5b). This experiment demonstrates that exposure to α-factor induces an immediate reduction in ADH-LexA-Swi6 dependent luciferase activity, therefore showing that luciferase protein is unstable in yeast, and suitable for use in cell cycle studies.

### 4.8 α-FACTOR SYNCHRONY SHOWS LUCIFERASE ACTIVITY TO FLUCTUATE

The previous section showed that the luciferase protein enzymatic activity might be a suitable reporter of ADH-LexA-Swi6 dependent transcription, therefore a cell synchrony experiment was performed. A mid-logarithmically growing culture of W303-1a cells carrying the reporter plasmid and the plasmid expressing ADH-LexA-Swi6 were treated with α-factor to arrest the cells in late G1 phase. During the arrest, cell samples were taken at hourly intervals during the 3 hr exposure to mating pheromone and frozen on dry ice before assaying for luciferase activity. Additionally, throughout the arrest samples were taken at 1/2 h intervals to score the percentage of budding to monitor the arrest. The
arrested culture was then released from the block and samples taken every 15 min to monitor luciferase activity and budding.

During the period of α-factor arrest, levels of luciferase activity decreased by 3 fold over the first 2 hr (figure 4.6). Over the next 1 hr of the arrest a 4 fold increase in luciferase activity was observed to 1.6. Upon release from the α-factor arrest, there was almost a 1.7 fold increase in luciferase activity over the first 45 min to 2.7. Levels of activity then declined 1.2 fold over the next 45 min to 2.3 before rising back to 2.7 after a further 30 min. The budding profile in figure 4.6 verified the cell culture divided synchronously over one cell cycle. Hence from this experiment and similar repeated experiments it would appear that ADH-LexA-Swi6 dependent reporter gene activity fluctuates in a cell cycle dependent manner.

4.9 DBF2-2 INDUCED SYNCHRONY DEMONSTRATES THAT LUCIFERASE ACTIVITY IS PERIODIC

To exclude the possibility that changes in ADH-LexA-Swi6 activity resulted from artefactual perturbations due to α-factor, a similar experiment was carried out, this time inducing synchrony by releasing cells from a dbf2-2-induced cell cycle arrest (Toyn and Johnston, 1993). Dbf2 is a mitotic kinase essential for exit from mitosis. At the restrictive temperature of 37°C, dbf2-2 mutant cells arrest at the end of mitosis, so that on return to the permissive temperature of 25°C, they undergo synchronous cell division. dbf2-2 cells growing mid-logarithmically expressing the reporter plasmid and the plasmid expressing ADH-LexA-Swi6 were arrested by incubation at 37°C. Cell samples were removed for luciferase assays and bud contents at 30 min intervals during the 2 h arrest
Figure 4.6

α-factor induced cell synchrony demonstrates that transcriptional activation by ADH-LexA-Swi6 is cell cycle regulated, as measured by luciferase activity.

Figure 4.7

Mitotic arrest induced cell cycle synchrony demonstrates that ADH-LexA-Swi6 activity fluctuates, as measured by luciferase activity.
and at 15 min intervals during the 1.5 h release from the block. Figure 4.7 shows luciferase activity decreasing 10 fold during the cell cycle block from 0.5 to 0.05, then increasing 24 fold to a peak 60 min after release to 1.2, before finally declining 3 fold to 0.4 over a further 30 min. Thus, like the α-factor induced synchrony experiment luciferase activity appeared to fluctuated through out the cell cycle. The analysis of the budding profile revealed little to confirm the synchrony of the culture because of the high fraction of small unbudding cells which accumulated during the arrest do not recover upon thermal shift down.

4.10 Northern analysis of a dbf2-2 induced synchronous culture showed Luciferase mRNA to fluctuate periodically

As expected the budding profile of the previous experiment revealed little to indicate how successful the temperature induced synchrony was. In an attempt to confirm synchrony samples taken from the experiment describe above were analysed by northern hybridisation with probes to mRNAs of known cell cycle synchrony. Probing of the northern membrane with a RNR1 probe, revealed a sharp peak in its expression as would be seen in a synchronously dividing culture. Striping and reprobing with H2B, a gene expressed shortly after RNR1 in the cell cycle, showed its expression to peak some 15 min after that of RNR1, thus demonstrating that the dbf2-2 induced synchrony had been a success. The membrane was stripped again and this time reprobed for luciferase mRNA. Northern analysis revealed that luciferase mRNA levels decreased 5.5 fold during the arrest period, then rose to a peak 45 min after the shift to permissive temperature, before
Figure 4.8

Northern analysis of dhf2-2 synchronised cells demonstrates that maximal luciferase mRNA expression peaks at the same time as RNR1. Additionally, the duration of the message exhibits kinetics similar to H2B.
declining to a minimum at 90 post shift (figure 4.8). The timing of this expression was compared to that of transcripts of known cell cycle periodicity, namely \textit{RNRI} and \textit{H2B}. Maximal luciferase expression occurred at the same time as the MBF-regulated \textit{RNRI} transcript. However, the onset and duration of luciferase expression exhibited kinetics more like the S phase expression of \textit{H2B}.

\textbf{4.11 DISCUSSION}

The experiments in this chapter examine whether transcriptional activation by LexA-Swi6 is subjected to cell cycle regulation. Before this could be attempted a suitable reporter system had to be developed where protein activity and message half lives were sufficiently short to reflect both increases and decreases in transcriptional activity.

Results from section 3.1 demonstrate that, whereas \textit{ADH-LexA-Swi6} can activate a \(\beta\)-galactosidase expression from a \textit{lexA}_{op}-\textit{lacZ} reporter gene, there was no \(\beta\)-galactosidase activity detectable in the \textit{ADH-LexA} control. However when I performed northern hybridisation on these samples, I was surprised to discover that a band corresponding to \textit{lacZ} mRNA as detectable in both samples. Thus even though there was no functional protein expressed in cells carrying the \textit{ADH-LexA} only control, spurious message was detected which contained \textit{lacZ} sequences. It was not determined if this was a phenomenon unique to the \textit{lacZ} sequence in the reporter construct, the \textit{lexA}_{op} or some other uncharacterised plasmid sequence. I was however able to determine that it was not unique to the plasmid based reporter system, for the same result occurred when I assayed for \textit{lacZ} mRNA in the reporter strain, CTY10-5d. This kind of reporter gene anomaly
was not the first our laboratory had encountered, present and past members of the division had also experienced difficulties using \textit{lacZ} reporter plasmids. The spurious transcripts seen during northern hybridisation could be a result of multiple starts upstream of the \textit{lacZ} sequence, all of which produced untranslatable mRNA. However, the curious nature of these problematic transcripts was not investigated further, and an alternative method of assaying reporter gene expression was devised.

Assays for luciferase enzymatic activity proved very successful in quantitating \textit{ADH-LexA-Swi6} activity. Assays for protein activity were used because of the mRNA problems encountered using the \textit{lacZ} system, and both reporter systems have many common features. The periodic cell cycle studies using both luciferase protein activity and mRNA analysis, demonstrated that like SBF and MBF, activation of reporter gene expression by \textit{ADH-LexA-Swi6} was subjected to cell cycle dependent changes. Northern analysis is more accurate than enzymatic assays for it is a true reflection of gene expression. The periodic expression of a \textit{lexA_{op-luc}} reporter gene by \textit{LexA-Swi6} peaked at the same time as a \textit{bona fide} Start-specific gene, \textit{RNR1}. Genes such as \textit{RNR1} are controlled by MBF and their transcript levels are known to peak at the G1/S transition (Lowndes \textit{et al.}, 1991; Lowndes and Johnston, 1992). Although the maximal levels of transcriptional activation by \textit{LexA-Swi6} and the MBF and SBF complexes coincided, the kinetics of induction were not identical. In comparison to the MCB-driven \textit{RNR1} transcript, the accumulation of \textit{LexA-Swi6}-driven luciferase reporter transcripts was slightly delayed and somewhat more prolonged. Apart from the potential effects of differential message stability, it is also possible that Swi6 transactivation may be subject
to further modulation when acting within MBF and MBF complexes. This can be appreciated, for in these cell cycle experiments I was assaying for Swi6 activity whose DNA binding activity was not regulated in the same way as in SBF and MBF. Indeed, Swi4 binding to DNA exhibits cell cycle dependent changes (Harrington and Andrews, 1996; Koch et al., 1996). Similarly, MBF binding to DNA shows periodicity in gel retardation assays (Lowndes et al., 1991). Therefore Swi6 may be active into early S phase, but this activity is only detected once we assay for Swi6 activity and not SBF and MBF. The Swi6-artificial DNA binding domain argument could be applied to the reappearance of Swi6 dependent luciferase activity seen late into a α-factor arrest. This surge in activity could be a true reflection of a role for Swi6 earlier in the cell cycle than was previously thought. Alternatively there could be a role for Swi6 in a G1 arrest, with Swi6 being active in association with some other as yet undetermined proteins, or as an artefact now the binding of Swi6 to reporter gene promoter region is no longer regulated. However, the most likely explanation is that it could be the first molecular indicator that the cells in the culture are becoming insensitive to α-factor and beginning to escape from the arrest. Additionally, a minor peak in luciferase activity is also seen in the dbf2-2 experiment, although there is no such parallel increases seen in the transcript levels of luciferase mRNA. Whatever the reason for the pattern of Swi6 dependent reporter gene expression not being in total agreement with SBF and MBF dependent transcripts, it is important to remember that in these experiments I was assaying for Swi6 activity using an artificial DNA binding domain and not SBF and MBF activity.
CHAPTER FIVE

TRANSCRIPTIONAL ACTIVATION BY SWI6 REQUIRES THE G1 CYCLIN CLN3

5.1 INTRODUCTION

The results presented so far demonstrate that Swi6, if given a heterologous DNA binding domain, can activate reporter gene expression. Furthermore, \textit{ADH}-LexA-Swi6 activity displays cell cycle fluctuations. Prior to these observations, the regulation of Swi6-containing SBF and MBF complexes had been attributed to the Cln3 cyclin rather than the other G1 cyclins, Cln1 and Cln2 (Dirick et al., 1995; Stuart and Wittenberg, 1995; Tyers et al., 1993). Therefore, I went on to compare transcriptional activation by SBF, MBF and LexA-Swi6 both in wild type cells and in G1 cyclin mutant derivatives, to test whether Cln3 had a regulatory effect on the activity of these transcription factors.

5.2 MBF ACTIVITY IS DEPENDENT ON CLN3

The transcriptional activation properties of Swi6 in association with one of its known natural DNA binding partners, Mbp1, was monitored by expression of a \textit{lacZ} reporter gene driven by tandem MCB elements (Lowndes et al., 1991). Mid-logarithmically growing wild type, W303-1a cells and isogenic mutants lacking each of the G1 cyclins carrying the MCB-\textit{lacZ} reporter plasmid were assayed for \(\beta\)-galactosidase activity. As expected, reporter gene expression was high in wild type cells containing native MBF, at 1.9 \(\beta\)-galactosidase units (figure 5.1). In \textit{cln1} and \textit{cln2} cells, an increase in activity was
observed by 1.5 and 2.7 fold respectively. Conversely, in the cln3 strain there was a 4 fold decrease in activity relative to wild type cells. This result suggests that MBF activity is dependent upon Cln3, but not on Cln1 or Cln2. The experiment was repeated using a derivative reporter plasmid that carries mutated, inactivated MCB elements. However, no reporter activity was seen in any of the strains tested (figure 5.1) showing that the effects of the cln deletions were transmitted exclusively via the MCB elements in the reporter construct.

5.3 SBF activity is dependent on Cln3

A similar series of experiments examined the effects of the cln deletions on SBF activity. SBF activity was detected using a plasmid carrying four SBF binding sites upstream of a lacZ reporter gene. The experimental procedure used was identical to that of the previous experiment. As expected, reporter gene expression in the wild type strain was high with a β-galactosidase activity of 1.3 units, demonstrating that SBF is active in these cells (figure 5.2). In the cln1 and cln2 mutant strains, reporter activity was 2.2 and 2.15 fold above wild type. Conversely, activity in the cln3 mutant strain was decreased, levels being 5 fold less than wild type. Thus, like MBF, native SBF is dependent upon Cln3 for activity.

5.4 The need for a plasmid expressing low levels of LexA-Swi6

The previous sections demonstrate that SBF and MBF transcriptional activities are Cln3 dependent. It was therefore important to test if LexA-Swi6 activity is similarly Cln3-dependent. In order to test the possible effects the Cln3 protein may have on Swi6 it is
Figure 5.1
A MCB-lacZ reporter system was utilised to demonstrate that MBF activity is dependent on the G1 cyclin Cln3 and not Cln1 or Cln2. Additionally, the effects on MBF activity in the cln mutants is specific to the MCB elements. MCB$^\text{mut}$ = mutated MCB elements.

Figure 5.2
Using a SCB-lacZ reporter plasmid the activity of SBF was shown to be dependent on Cln3 and not Cln1 or Cln2 for optimal activity.
important to consider the mechanics by which this G1 cyclin may regulate Start. The Cln3-associated kinase activity is very low throughout the cell cycle and it is thought that subtle increases in Cln3 protein as cells increase in mass in G1 may be a regulatory factor in the execution of Start. If levels of Cln3 protein are a limiting factor in activating Swi6, then the levels of LexA-Swi6 would have to be kept at a minimum, so as not to quench all the Cln3 from the cell. The multicopy plasmid used up to now for investigating LexA-Swi6 activity is under the control of the strong ADH promoter (ADH-LexA-Swi6), and is unsuitable. A second plasmid, pTR240, was constructed with LexA-Swi6 expression under the control of the galactose inducible promoter and a copy number of one per cell. Hence, when a yeast strain supporting this plasmid is grown with glucose as a sole carbon source then expression of LexA-Swi6 can be kept to a minimum. Expression of LexA-Swi6 from this plasmid when grown on glucose as the sole carbon source shall be referred to as GLU-LexA-Swi6.

5.5 GLU-LexA-Swi6 is functional

It was important to determine if the GAL-LexA-Swi6 fusion construct was functional at the cellular level. The yeast strain, K2003 (Nasmyth and Dirick, 1991), which is deleted for swi6 and carries a swi4<sup>ts</sup> temperature sensitive mutation, was used in this functionality test. Cells are viable when grown at the permissive temperature of 25°C, whereas incubation at the restrictive temperature of 37°C results in lethality. K2003 was transformed with either the plasmid expressing GLU-LexA-Swi6 or the control plasmid expressing GLU-LexA. Transformants were streaked out on selective agar and grown at
25°C, demonstrating that both strains could grow at the permissive temperature. Upon re-streaking to 37°C, cells carrying the vector plasmid failed to proliferate. Cells expressing the GLU-LexA-Swi6 fusion protein were able to grow at the restrictive temperature, demonstrating that the Swi6 moiety of the LexA fusion protein could complement the mutational defects of the K2003 strain (figure 5.3).

5.6 GLU-LexA-Swi6 activity is dependent on CLN3

Transcriptional activation by the GLU-LexA-Swi6 fusion was investigated via β-galactosidase assays of mid-logarithmically growing yeast using the reporter strain CTY10-5d, and isogenic cln derivatives (figure 5.4). The level of reporter gene expression in all strains expressing the GLU-LexA control plasmid was very low as expected. Expression of GLU-LexA-Swi6 fusion in the wild type strain was 0.6 β-galactosidase units, a 12 fold increase over the control levels. The levels of reporter gene expression driven by GLU-LexA-Swi6 in the isogenic cln1, cln2 strains was higher than expression in the wild type strain, increasing 2.7 and 1.5 fold respectively. In contrast, comparison of GLU-LexA-Swi6 activity between wild type cells and the cln3 mutant, revealed GLU-LexA-Swi6 activity to be Cln3 dependent, as seen by the 3 fold decrease. Furthermore, the wild type level of GLU-LexA-Swi6 activity was restored to 0.6 β-galactosidase units in the cln3 mutant upon introducing a plasmid which carries a single copy of the CLN3 gene expressed under its own promoter (figure 5.4). To exclude the possibility that changes in GLU-LexA-Swi6 activity were due to varying levels of the fusion protein from strain to strain.
Figure 5.3
Plasmids expressing the GLU-LexA-Swi6 fusion protein was tested for functionality. GLU-LexA-Swi6 can rescue the temperature sensitive strain K2003 (Δswi6 swi4ts) at the restrictive temperature of 37°C.

Figure 5.4
GLU-LexA-Swi6 is dependent on Cln3 for optimal activation, as assayed via the 'one-hybrid' system using the reporter strain CTY10-5d.
strain, cell extracts were monitored by Western blot using a polyclonal αSwi6 antibody. GLU-LexA-Swi6 levels were constant in all strains (figure 5.5).

These experiments demonstrate that, like SBF and MBF, the transcriptional activation properties of GLU-LexA-Swi6 are dependent on Cln3, but not on Cln1 or Cln2.

5.7 The dependency of CLN3 is specific to SWI6

It could be argued that Cln3 is somehow involved in general transcription, hence in a strain deleted for CLN3 one would expect a reduction in activity by other transcription factors as well as SBF, MBF and GLU-LexA-Swi6. To dismiss this possibility, the transcriptional activation potential of two known non-yeast transcription factors were assayed. Plasmids expressing either an ADH-LexA-p53 or ADH-LexA-pE2F fusion protein were transformed into the reporter strain, CTY10-5d and isogenic cln mutants (figure 5.6). Wild type CTY10-5d cells expressing ADH-LexA-p53 gave β-galactosidase units of 11. Reporter gene expression in cln1, cln2 and cln3 cells showed little change when compared to wild type cells. There was a 1.35 fold increase in cln1 cells, a 1.1 fold decrease in cln2 cells and a no change in cln3 cells. Similarly, expression of ADH-LexA-E2F gave 21 β-galactosidase units. Reporter gene expression in cln1, cln2 and cln3 cells was similar to wild type cells. This experiment demonstrates that the effects Cln3 has on transcription activation are specific to Swi6 and not to general transcription.
Western blotting with Swi6 antibodies revealed that changes in GAL-LexA-Swi6 activity in cln mutants is not as a result of changes in its protein levels.

The transcriptional activation of ADH-LexA-p53 and ADH-LexA-E2F was assayed in CTY10-5d and isogenic cln mutants, showing that the dependency for Cln3 was specific to Swi6.
5.8. DISCUSSION

From experiments in this chapter, a parallel can be seen between transcriptional activation driven by LexA-Swi6 and gene expression stimulated by MBF and SBF, in all cases Cln3 was required for optimal activity. The need for Cln3 for expression of MCB-lacZ and SCB-lacZ reporter genes is in agreement with earlier reports that the activities of MBF and SBF are Cln3-dependent (Stuart and Wittenberg, 1995; Tyers et al., 1993). In fact the patterns of gene activation observed in the cln mutants was the same for SBF, MBF or GLU-LexA-Swi6 activity. This consistency, whether looking at Swi6 in conjunction with its two natural DNA binding partners or with an artificial LexA DNA binding domain, suggests that the effect the G1 cyclins have on the overall transcriptional activity of Swi6 is the same, independently of the presence of Swi4 or Mbp1.

Although Cln3 is the usual initiator of SBF and MBF activity, loss of Cln3 is not lethal and some transactivation by SBF, MBF and GLU-LexA-Swi6 persists (Dirick et al., 1995; Stuart and Wittenberg, 1995; Tyers et al., 1993). Quantitation of the residual level of this activation is problematic because, compared to wild type cells, all three cln mutants have extended G1 phases (Hadwiger et al., 1989; Nash et al., 1988). As argued by Stuart and Wittenberg (1995), a prolonged G1/S would give greater opportunity for activity by SBF and MBF, as well as by GLU-LexA-Swi6, and so would account for the apparent increase in gene expression in cln1 and cln2 mutants compared to wild type cells. But even though cln3 mutants also have an extended G1, they still displayed reduced levels of gene expression whether driven by GLU-LexA-Swi6 or by SBF and MBF. Thus, a comparison of cln3 with cln1 or cln2 mutants would be more appropriate.
and this shows that transcriptional activation by GLu-LexA-Swi6 in the absence of Cln3 is reduced by at least 90%. Under these conditions, viability relies on the presence of either Cln1 or Cln2 (Cross, 1990; Richardson et al., 1992). However, the mechanism by which Cln1 and Cln2 fulfil this vital function differs from that of Cln3. It is not known if the residual 10% of Swi6 activity in a cln3 mutant is basal activity or a reflection of some other as yet uncharacterised activator of Start.
CHAPTER SIX

INVESTIGATING THE ACTIVATION PROPERTIES OF CLN3

6.1 INTRODUCTION

The published evidence suggests that both Swi6 and Cln3 play crucial roles in gene expression at Start. These findings, coupled with the observation that Glu-LexA-Swi6 transcriptional activity was Cln3 dependent (chapter 5) suggested that the proteins could possibly act in concert in a transcriptional activation complex. To investigate this possibility further, the transcriptional activation properties of Cln3 were explored, utilising a ‘one-hybrid’ strategy.

6.2 LEXA-CLN3 IS A VERY WEAK TRANSCRIPTIONAL ACTIVATOR

The Cln3 coding sequence was cloned into the ‘one-hybrid’ vector, pTR27, to produce a plasmid which expresses a LexA-Cln3 fusion protein under the control of the yeast ADH promoter (ADH-LexA-Cln3). The transcriptional activation potential of this construct was assayed in the reporter strain, CTY10-5d. lexAop-lacZ, reporter gene expression was quantitated in cells expressing either the ADH-LexA-Cln3 fusion or the ADH-LexA control. Cells expressing the ADH-LexA-Cln3 fusion resulted in a β-galactosidase activity of 0.1 units, only a 2 fold increase over the ADH-LexA control. This minimal increase in reporter gene expression can be appreciated in figure 6.1, which compares the transcription activation potential of ADH-LexA-Swi6 and ADH-LexA-Swi4 with that of ADH-LexA-Cln3.
Figure 6.1
The transcriptional activation by ADH-LexA-Cln3 is very poor in comparison to ADH-LexA-Swi6, as assayed in the reporter strain CTY 10-5d.

Figure 6.2
ADH-LexA-Cln3 with PEST sequence deleted can activate transcription of a reporter gene. The activity of ADH-LexA-Cln3ΔPEST is dependent on the cyclin box.
6.3 **Stable LexA-Cln3 is a Potent Transcriptional Activator**

Endogenous cyclin protein levels have a high rate of turnover *in vivo* (Salama *et al.*, 1994; Wittenberg *et al.*, 1990) and Cln3 is an especially unstable protein (Cross and Blake, 1993). The carboxyl-terminal (C-term) domains of G1 cyclins contain sequences rich in Pro, Glu (and Asp), Ser, and Thr, so-called PEST motifs, which have been envisaged as targets for the rapid degradation of these and other unstable proteins. In an attempt to stabilise the ADH-LexA-Cln3 protein, the PEST sequences were deleted from the C-terminus of Cln3, resulting in a ADH-LexA-Cln3 fusion with only the first 447 N-terminal amino acid residues (ADH-LexA-Cln3ΔPEST) (figure 6.3). Expression of ADH-LexA-Cln3ΔPEST in the CTY10-5d reporter strain resulted in a 33 fold increase in reporter gene expression over the of ADH-LexA control and a 16.5 fold increase over wild type ADH-LexA-Cln3 (figure 6.2).

6.4 **LexA-Cln3ΔPEST Activity is Dependent on the Cyclin Box**

The Cdc28 cyclin-dependent protein kinase interacts through the N-terminal segment of Cln3, which contains the cyclin box, a region conserved among different cyclins. The cyclin box region I and II was deleted from the ADH-LexA-Cln3ΔPEST construct to determine if this motif is essential for ‘one-hybrid’ Cln3-dependent reporter gene expression. 166 N-terminal amino acids of Cln3 were deleted in the ADH-LexA-Cln3ΔPEST fusion resulting in the removal of region I and II of the three cyclin box domains (figure 6.3). Removal of the cyclin box from ADH-LexA-Cln3ΔPEST resulted in the total loss of reporter gene activation, with β-galactosidase activity falling almost 34
Figure 6.3

Line drawing of LexA-Cln3 deletion constructs used to demonstrate that ADH-LexA-Cln3APEST is a transcriptional activator, and that this activity is dependent on the cyclin box.
fold to background \( \text{ADH-LexA} \) control levels (figure 6.2). Hence, loss of the cyclin box in the \( \text{ADH-LexA-Cln3} \Delta \text{PEST} \) expressing plasmid resulted a dramatic decrease in transcriptional activation.

### 6.5 \( \text{LEXA-CLN3} \) IS DEPENDENT UPON \( \text{SWI6} \) FOR TRANSCRIPTIONAL ACTIVATION

The observation that \( \text{GLU-LexA-Swi6} \) activity is dependent on Cln3, and that \( \text{ADH-LexA-Cln3} \Delta \text{PEST} \) can activate transcription, together with evidence that both proteins play a key role in gene expression at Start, prompted an investigation of whether \( \text{ADH-LexA-Cln3} \Delta \text{PEST} \) activity was Swi6 dependent. An interdependent relationship between Swi6 and Cln3 might be expected if the two proteins were associated in a common complex.

Out of the three \( \text{ADH-LexA-Cln3} \) constructs, only \( \text{ADH-LexA-Cln3} \Delta \text{PEST} \) can activate transcription, so this activation potential in wild type CTY10-5d reporter cells was compared against a \( \text{swi6} \) isogenic mutant (figure 6.4a). Reporter gene expression decreased more than 8 fold from 1.6 units. Figure 6.4a also shows no change in the already minimal reporter gene expression driven by \( \text{ADH-LexA-Cln3} \Delta \text{APESTAC-box} \) in the \( \text{swi6} \) mutant compared to wild type cells. Furthermore, assays for two non-yeast transcription factors, \( \text{ADH-LexA-p53} \), \( \text{ADH-LexA-E2F} \), showed the reduction of \( \text{ADH-LexA-Cln3} \Delta \text{PEST} \) in the \( \text{swi6} \) strain to be specific to Cln3 (figure 6.4b).

Reported gene expression driven by \( \text{ADH-LexA-p53} \) and \( \text{ADH-LexA-E2F} \) are 11 and 21 \( \beta \)-galactosidase units in wild type CTY10-5d. Assays for p53 and E2F activity in \( \text{swi6} \) mutant cells demonstrated only minor changes in activity when compared to wild type cells, with \( \beta \)-galactosidase units falling 1.15 and 1.2 fold respectively.
**Figure 6.4**
The ability of stabilised ADH-LexA-Cln3 to activate reporter gene expression is dependent on Swi6.

**Figure 6.4a**
The activity of two non-yeast transcription factors is not greatly reduced in a swi6 mutant.

**Figure 6.5**
ADH-LexA-Cln3 and ADH-LexA-Cln3ΔPEST are functional at a cellular level, as seen by their ability to rescue a triple *cln* mutant. The control and the cyclin box mutant cannot rescue the strain.
6.6 Functionality Tests on LexA-Cln3 Constructs

In addition to testing the transcriptional activation potency of the three ADH-LexA-Cln3 constructs, they were also tested for functionality by their ability to rescue a triple cln mutant. The yeast strain BF411-2C is deleted for CLN1 and CLN2, with CLN3 under the control of the galactose inducible promoter. Culture on glucose-containing agar results in lethality, but when cultured on galactose as the sole carbon source the stain is viable, as a result of the GAL-induced expression of CLN3. However, expression of a functional Cln protein from a plasmid is sufficient to restore viability to the strain when grown on glucose-containing media. Strain BF411-2C was transformed with plasmids expressing one of the three ADH-LexA-Cln3 constructs. In addition, a control strain expressing the negative, ADH-LexA was tested. After transformation and selection on galactose agar, the triple cln mutants carrying the test plasmids were streaked onto agar containing galactose or glucose as the sole carbon source. Figure 6.5 shows that all strains could grow on the galactose plates, whereas only the strains containing ADH-LexA-Cln3, ADH-LexA-Cln3APEST grow on glucose plates. Conversely the strain containing the negative control and the ADH-LexA-Cln3APESTAC-box failed to proliferate on glucose plates.

6.7 Discussion

Within the field of yeast cell cycle studies, it has been assumed that Swi6 and Cln3 play pivotal roles in the G1/S transition. Indeed there are numerous publications supporting this hypothesis. Furthermore, my recent experiments suggest that Swi6 and Cln3 may be
interdependent for the activation of gene expression. Results in this chapter show that while Cln3 can not substantially activate gene expression in a ‘one-hybrid’ assay, a PEST deletion mutant of Cln3 can. Hence it would appear that Cln3, once stabilised can act as a transcription factor.

The PEST sequences located in the C-terminal region of Cln3 act as a signal for Cdc28-dependent, ubiquitin-dependent degradation (Yaglom et al., 1995). Therefore if the PEST sequence is removed, resulting in the stabilisation of Cln3, there is an increase in overall Cln3 protein levels and in the associated kinase activity. It is possible that this increase in associated kinase activity is what results in LexA-Cln3 driven reporter gene expression. If simply increasing the associated kinase activity of Cln3 is all that is needed to activate gene expression in this ‘one-hybrid’ system then, then over-expressing LexA-Cln3 could conceivably result in an increase in gene transcription. However the LexA-Cln3 construct used in these experiments is carried on a multicopy plasmid and is under the control of the high expression promoter ADH1, but no increase in reporter gene expression is observed over the negative control. Therefore the mechanisms by which LexA-Cln3 activates could be independent of associated kinase activity, or there could be a critical level of this kinase needed for activation which is only reached when Cln3 is stabilised by the deletion of the PEST sequences and expressed at a high copy number.

It was demonstrated that the transcriptional activation potential of LexA-Cln3ΔPEST is totally dependent on the cyclin box region of Cln3. The cyclin box reflects a structural motif required for interaction with and activation of Cdc28 (Morgan, 1996).
Loss of this region in LexA-Cln3ΔPEST would therefore result in the loss of Cdc28 association and its kinase activity. This would suggest that at least Cdc28 association is necessary if not essential for the ‘one-hybrid’ activity demonstrated by LexA-Cln3ΔPEST. However, it is important to remember that the LexA-Cln3ΔPESTΔC-box construct used in this study only contains a fraction of the wild type LexA-Cln3 protein and therefore could lead to the Cln3 protein becoming unstructured. A more detailed study of point mutations of both the PEST and cyclin box regions is needed.

In addition to LexA-Cln3ΔPEST activity being dependent on the cyclin box, the absence of Swi6 from the cell results in a dramatic fall in reporter gene activity. From the previous chapter which demonstrated that LexA-Swi6 activity is dependent on Cln3, it could be argued that Swi6 some how recruits Cln3 to the promoter, where it activates Swi6 dependent transcription. However this can not be the case for if Cln3 is given its own means of contacting the promoter region, by means of an artificial DNA binding domain then Swi6 function would be redundant in a ‘one-hybrid’ assay. However, the presence of Swi6 is absolutely required for Cln3’s gene transcription properties. The realisation that both proteins need each other’s presence for optimal activation suggest some kind of dependence, which in turn suggest that the two proteins may be components of a common complex, which in wild type cells is needed for the execution of Start specific gene transcription.
CHAPTER SEVEN

CLN3 INTERACTION WITH SBF IS PRIMARILY THROUGH SWI6

7.1 INTRODUCTION

The interdependency of Swi6 and Cln3, the prominent roles these two proteins have at Start, and the genetic evidence that these two proteins may be components of the same regulatory process suggested that Swi6 and Cln3 might physically interact with each other. Possible \textit{in vivo} interactions between Swi6 and Cln3 were investigated using a two-hybrid approach (Chien et al., 1991; Fields and Song, 1989) (figure 7.1). In the two-hybrid assay, one protein is fused to a DNA binding protein. This DNA binding chimera specifically recognises a DNA motif upstream of a reporter gene. For a two-hybrid assay, it is important that this protein is not itself a transcriptional activator and so would be unable to activate transcription alone. A second protein is then fused to a transcriptional activation domain, but because this protein cannot specifically recognise the DNA motif upstream of the reporter gene there is again no transcriptional activation. If the two proteins interact \textit{in vivo}, then the first DNA binding hybrid can recruit the second transcriptionally active chimera to the DNA binding site and activate the reporter gene.
Figure 7.1
The 'two-hybrid' system. a) A hybrid consisting of the bacterial LexA DNA-binding protein (LexA\textsubscript{BD}) fused to protein X is localised to the \textit{lexA}op upstream of the \textit{lacZ} reporter gene. It is unable to activate transcription because it lacks a transcriptional activation domain. b) A hybrid consisting of an activation domain (Gal4\textsubscript{act}) fused protein Y is unable to localise to the upstream region of the reporter gene. c) A protein-protein interaction between X and Y brings the activation domain and the DNA binding domain into close proximity, enabling reporter gene transactivation.
7.2 \textbf{ADH-LEXA-SWI6Δ345 INTERACTS WITH GAL4\textsc{act}-CLN3}

Because of the native transcriptional activation properties of wild type \textit{ADH}-LexA-Swi6 it is unsuitable for a two-hybrid test. However, transposition mutants of the \textit{ADH}-LexA-Swi6 expressing plasmid, pTR28 have been identified which express truncated \textit{ADH}-LexA-Swi6 proteins which are unable to activate transcription (Sedgwick \textit{et al.}, 1998). pΔ345 expresses a fusion protein truncated at Swi6 residue 345 (\textit{ADH}-LexA-Swi6Δ345).

Structural sequences for the three G1 cyclins were fused in-frame downstream of the sequence encoding the Gal4 activation domain (Gal4\textsc{act}) in the plasmid pACT (Durfee \textit{et al.}, 1993). The reporter strain CTY10-5d expressing \textit{ADH}-LexA-Swi6Δ345 and one of the Gal4\textsc{act}-Cln fusions or the Gal4\textsc{act} control were assayed for β-galactosidase activity in mid-logarithmically growing cultures (figure 7.2). Like control cells expressing \textit{ADH}-LexA-Swi6Δ345 and Gal4\textsc{act}, no increase in reporter gene activity was seen in cells expressing \textit{ADH}-LexA-Swi6Δ345 and either Gal4\textsc{act}-Cln1 or Gal4\textsc{act}-Cln2. The β-galactosidase activity in these strains remained at background 0.06 units. In cells co-expressing \textit{ADH}-LexA-Swi6Δ345 and Gal4\textsc{act}-Cln3, there was a 33 fold increase in reporter gene expression in comparison to the control cells expressing \textit{ADH}-LexA-Swi6Δ345 and Gal4\textsc{act}. This experiment suggests no interaction occurs between Swi6Δ345 and Cln1 or Cln2. Conversely, a very strong \textit{in vivo} interaction between Swi6Δ345 and Cln3 can be seen. Furthermore, a domain for Cln3 interaction appears to be present in the Swi6Δ345 construct.
**Figure 7.2**
A two-hybrid assay demonstrates that a C-terminal deletion of Swi6 interacts with Cln3 and not Cln1 or Cln2.

**Figure 7.3**
Western blotting with αGal4act antibodies shows that the cyclin constructs are expressed.

**Figure 7.4**
Although full length ADH-LexA-Swi6 has intrinsic transcriptional activation properties a two-hybrid interaction can be seen between Swi6 and Cln3 but not Cln1 or Cln2.
7.3 **Gal4act-Clns are functionally expressed**

It could be argued that the \textit{ADH-LexA-Swi6Δ345} demonstrated no interaction with 

Gal4act-Cln1 and Gal4act-Cln2 because these Gal4act-Cln proteins were not expressed or not functional. Extracts from W303-1a cells expressing either Gal4act-Cln1, Gal4act-Cln2, Gal4act-Cln3 or the Gal4act control, growing in mid-logarithmic phase were monitored by western blotting using a polyclonal \(\alpha\text{Gal4act}\) antibody. Figure 7.3 demonstrates that Gal4act-Cln1, Gal4act-Cln2 and Gal4act-Cln3 were expressed in these cells. In addition to western analysis, W303-1a cells expressing any of the Gal4act-Cln expressing plasmids or the Gal4act control were scored for relative cell size using a FACS machine. Table 7.1 below demonstrates that expression of all three Gal4act-Cln fusions reduced the overall size of cells as a result of accelerating Start, in the same way as multicopy Cln1, Cln2 and Cln3 (Lew \textit{et al.}, 1992; Nash \textit{et al.}, 1988; Tyers \textit{et al.}, 1993).

Thus all three Gal4act-Cln proteins used in the two-hybrid assays in section 7.2 are functional even though only Gal4act-Cln3 generates a positive response in interaction assays with \textit{ADH-LexA-Swi6Δ345}.

**Table 7.1**

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>Geometric-mean cell size</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303-1a pACTI</td>
<td>190</td>
</tr>
<tr>
<td>W303-1a pACTI-Cln1</td>
<td>155</td>
</tr>
<tr>
<td>W303-1a pACTI-Cln2</td>
<td>172</td>
</tr>
<tr>
<td>W303-1a pACTI-Cln3</td>
<td>150</td>
</tr>
</tbody>
</table>
7.4 Full length LexA-Swi6 interacts with Gal4act-Cln3

Although full length ADH-LexA-Swi6 is not ideally suited for a two-hybrid assay because of its intrinsic activation properties, it was still possible to detect an additional increase in reporter gene expression when ADH-LexA-Swi6 was co-expressed with Gal4act-Cln3. The control strain expressing ADH-LexA-Swi6 and Gal4act gave β-galactosidase activity of 1.1 demonstrating again the intrinsic gene activation properties of ADH-LexA-Swi6 (figure 7.4). In cells co-expressing ADH-LexA-Swi6 and either Gal4act-Cln1 or Gal4act-Cln2, there was no change in reporter gene expression. However, in cells expressing ADH-LexA-Swi6 and Gal4act-Cln3, β-galactosidase activity rose 3 fold over that of the ADH-LexA-Swi6 and Gal4act control. This experiment demonstrates that a two hybrid association between wild type ADH-LexA-Swi6 and Gal4act-Cln3 can be seen above the intrinsic activation properties of ADH-LexA-Swi6.

7.5 Deletion analysis of LexA-Swi6 interactions with Gal4act-Cln3

The previous two-hybrid experiments demonstrate interactions between Cln3 and either wild type Swi6 or a N-terminal portion of Swi6. Figure 7.5 shows further deletion derivatives of ADH-LexA-Swi6 and their interactions with Gal4act-Cln3. ADH-LexA-Swi6Δ145 comprises 145 N-terminal residues of Swi6 as a result of transposition of pTR28 with TnHIS3, and is expressed from pΔ145 (Sedwick et al., 1998). ADH-LexA-Swi6ΔNsiI is an internal deletion derivative where 103 N-terminal and 74 C-terminal residues of Swi6 are fused in frame by deletion of an internal NsiI fragment, and is expressed from pTR29. These additional derivatives of ADH-LexA-Swi6 were tested for
Figure 7.5

Deletion derivatives of ADH-LexA-Swi6 reveal a Cln3 interaction domain between Swi6 residue 145-345, as assayed in a 'two hybrid' with Gal4act-Cln3.
Figure 7.6
Deletions of AC0-LexA-Swi6 identifies a Cln3 interaction domain (CID) between residue 145 and 345 of the Swi6 protein.

Figure 7.7
In comparison to LexA-Swi6, LexA-Swi4 does not interact with G1 the cyclins, as assayed by 'two-hybrid' in CTY 10-5d.
two-hybrid interaction with Gal4act-Cln3. The previous section showed that wild type
ADH-LexA-Swi6 and ADH-LexA-Swi6Δ345 interact with Gal4act-Cln3 giving β-
galactosidase units of 3.3 and 2.6 respectively. Co-expression of Gal4act-Cln3 with
either ADH-LexA-Swi6Δ145 or ADH-LexA-Swi6ΔNsil resulted in a total loss of in vivo
two-hybrid interaction with β-galactosidase activity falling to that of the ADH-LexA
control (figure 7.6). Together these data on Swi6 and Cln3 interaction suggests that the
Swi6 region required for Cln3 interaction resides somewhere between residue 145 and
345. This region of Swi6 was termed CID (Cln3 Interaction Domain) and is presented in
a line drawing of Swi6 in figure 7.5.

7.6 TWO-HYBRID ASSAY BETWEEN SWI4 AND G1 CYCLINS

The two-hybrid assay was used to test if Swi4 and the G1 cyclins interact in vivo. The
reason for this test was two fold. Firstly, it was important to examine whether
interactions occur between other components of SBF and the G1 cyclins. Secondly, it
was important to test another protein for Gal4act-Cln3 interaction to ensure that Gal4act-
Cln3 does not interact with any protein fused to LexA. ADH-LexA-Swi4 has been
previously demonstrated to be a poor activator of reporter gene transcription (section
3.7). CTY10-5d was used to detect any possible two-hybrid interaction between ADH-
LexA-Swi4 and Gal4act-G1 cyclin hybrids, reporter gene expression was compared to
levels expressed from the ADH-LexA-Swi6 and Gal4act-Cln3 interaction. Figure 7.7 shows
that compared to the ADH-LexA-Swi4 and Gal4act negative control, there was little
additional activation by ADH-LexA-Swi4 with the Gal4act-Clns.
Over the years there has been an accumulation of evidence which has suggested pivotal roles for Swi6 and Cln3 in the execution of Start. Together with my experimental results, these observations suggest that these two proteins could be members of the same regulatory complex. The ‘two-hybrid’ test results described in this chapter indicate that Swi6 and Cln3 do appear to interact in this \textit{in vivo} assay. This apparent ‘two-hybrid’ interaction could be one of two possibilities. Firstly it could be a true reflection of an interaction between Swi6 and Cln3. Secondly, as it is known from chapter 5, Cln3 is needed for optimal transcriptional activation by Swi6, and the over-expression of Gal4act-Cln3 could be increasing the ‘one-hybrid’ activation properties of LexA-Swi6. However a direct interaction between Swi6 and Cln3 is very probable for members of our division have further corroborative, \textit{in vitro} GST pull down data, and \textit{in vivo} immunoprecipitation data. In addition, an interaction between Swi6 and Cln3 can be seen using a novel assay, which takes advantage of the SOS recruitment system in yeast (Aronheim \textit{et al}., 1997). It would be interesting to see if the over-expression of Cln3 in the absence of Gal4act had any effect on LexA-Swi6 reporter gene expression. However, Cln3 may be increasing the transcriptional activation properties of Swi6 through a direct physical interaction, therefore it would be difficult to separate the two properties.

A known binding partner of Swi6, Swi4, did not interact with any G1 cyclins under the same ‘two hybrid’ conditions. This is rather surprising, for if the three proteins were all components of the same complex, one might expect a positive result with Swi4 and Cln3 if the Swi6 protein could act to ‘bridge’ the two molecules. A complex
containing both Swi4 and Cln3 may however be possible, but undetectable using a ‘two-
hybrid’ assay. The proteins may interact directly or indirectly, at a weak but functional
level that would not show up in a ‘two-hybrid’ assay. Furthermore, the temporal nature
of an interaction may mean that in a population of mid-logarithmic cells, there may be
insufficient time to detect an interaction in a two-hybrid assay. There could of course be
multiple complexes in the cell, which have Swi6 as a component, that may be mutually
exclusive. Because LexA-Cln3ΔPEST can act as a transcription initiator, this would
suggest that Cln3 may have a role in stimulating gene expression by being in close
proximity to the transcriptional initiation complex. We already know that this is how
Swi4/Mbp1 behave, since these two proteins harbour the specific DNA binding domains
of SBF and MBF. For this reason, there could be multiple transcription complexes in the
promoter regions of genes at or around the time of Start. For example active and inactive
transcription complexes, containing Swi6 may have either Swi4, Mbp1, Cln3 or other
proteins in their numbers. A detailed study of protein complexes formed on SCB and
MCB elements could prove interesting.

In addition to demonstrating that Cln3 and Swi6 interact via a two hybrid
approach, it was possible using deletion derivatives of Swi6, to identify a putative Cln3
interaction domain. This ‘Cln3 Interaction Domain’ CID, is between residue 145-345 on
Swi6. The ability to identify a region/domain for interaction is reassuring in support for a
direct physical interaction between the two proteins. It would be interesting to study
further deletions and point mutations of Swi6 to characterise this interaction domain.
Furthermore, similar Cln3 constructs would be informative. Indeed, such deletions have
been constructed and will soon be investigated.
CHAPTER EIGHT

FURTHER ANALYSIS OF SWI6 AND CLN3 INTERDEPENDENCY

8.1 INTRODUCTION

Results from the previous section investigating the ‘two-hybrid’ interaction between Swi6 and Cln3 utilised a ADH-LexA-Swi6 deletion derivative containing only 345 N-terminal Swi6 residues. ADH-LexA-Swi6Δ345 comprises Swi6 residues up to the end of the first ankyrin repeat motif and is transcriptionally inactive partly because of the loss of a C terminal transcriptional activation domain. However, work by colleagues had demonstrated that if a further 36 residues are deleted from the C-terminus of ADH-LexA-Swi6Δ345, then the fusion protein regains transcriptional activation. Thus, the removal of the remaining ankyrin repeat revealed a second putative transcriptional activation domain of Swi6. The full characterisation of Swi6 is under extensive research by members of our group and this chapter only investigates the activation properties of the N-terminal activation domain in terms of Cln3 dependency.

8.2 SWI6 ACTIVATION DOMAIN 1 IS CLN3 DEPENDENT FOR TRANSCRIPTIONAL ACTIVATION

The N-terminal putative activation domain of Swi6 has been analysed further by members of our laboratory, revealing that Swi6 residues 188-270 are sufficient to activate lexAop-lacZ reporter expression. As full length wild type Swi6 activity is Cln3 dependent in a
One-hybrid' assays using the reporter strain CTY10-5d and isogenic G1 cyclin mutants shows that transcriptional activation region 1 of Swi6 (GLU-LexA-TAR1) to be totally Cln3 dependent. Cln2 is also required for optimal activity.
one-hybrid assay, it was of interest to determine if this transcriptional activation region (TAR1) was also Cln3 dependent for transcriptional activity. To test this a GLU-LexA-TAR1 fusion was constructed and its activity assayed in the same manner as the wild type GLU-LexA-Swi6 in chapter 5. Expression of GLU-LexA-TAR1 in wild type reporter cells demonstrated that this construct was 1.4 fold more transcriptionally active than wild type GLU-LexA-Swi6, with β-galactosidase activity of almost 0.85 (figure 8.1).

Expression of GLU-LexA-TAR1 in the cln1, cln2 and cln3 mutant cells resulted in a 1.1, 4.2 and 17 fold decreases in reporter gene expression. These reductions seen in figure 8.1, clearly demonstrate that in the absence of Cln3 the transcriptional activity of GLU-LexA-TAR1 is reduced to that of background GLU-LexA negative control levels. Although the transcriptional activity of GLU-LexA-TAR1 is Cln3 dependent, there is also a appreciable reduction observed in the absence of Cln2.

8.3 Functional dependence of Cln3 on Swi6

Cln3 activity was next tested for its reliance on Swi6 at a cellular level. Over-expression of Cln3, like Cln1 and Cln2 accelerates Start in wild type cells resulting in cells budding at a smaller cell size than usual (Lew et al., 1992; Richardson et al., 1989). Conversely, deletion of any single cyclin gene increases cell size as a result of a delay in Start (Hadwiger et al., 1989; Nash et al., 1988). In a swi6 mutant there is also a marked delay in Start and a corresponding increase in cell size. My work has suggested that Cln3 activates Swi6 and consequently the transcription factors SBF and MBF, which in turn activate CLN1 and CLN2 expression leading to the onset of Start. It would therefore be
predicted that in the absence of Swi6, over-expression of Cln1 and Cln2 will still affect
cell size at Start whereas over-expression of Cln3 would have no affect. To test this
prediction, the coding sequences of Cln1, Cln2 and Cln3 were cloned into the vector
pSD.06A (Dalton and Treisman, 1992) to produce the plasmids pGAL-Cln1, pGAL-Cln2
and pGAL-Cln3 respectively. During the course of plasmid construction the VP16
activation domain normally associated with this plasmid was deleted. pSD.06A
expressed the G1 cyclins from the galactose inducable CYC1/GAL1 promoter. Wild type,
W303-1a and the swi6 isogenic mutant were transformed with either pGAL-Cln1, pGAL-
Cln2, pGAL-Cln3 or the control plasmid pSD.06A. Transformants were selected and
grown to mid-logarithmic phase with galactose as the sole carbon source before cell
volume was scored. In comparison to the expression of the control vector in wild type
W303-1a cells, over-expression of Cln3, like Cln1 and Cln2 resulted in a reduction of cell
volume compared to cells expressing the control, from 41fl to 21, 20 and 23 respectively
(table 8.1). In swi6 mutant cells expressing Cln3, an increase in cell volume was observed,
rising from 63fl in the vector control to 154fl in cell over-expressing Cln3. Over-
expression of Cln1 and Cln2 in swi6 mutant yeast resulted in multiple cell morphology
defects. These morphological changes were so extreme that meaningful assays of cell
volume were not possible. Changes included the development of multinucleate branched
cells and budded cells with no nucleus.

Thus in the absence of SWI6 over-expression of Cln3 is unable to accelerate Start,
suggesting that Cln3’s ability to drive the cell cycle is dependent on the presence of Swi6.
Figure 8.2
Comparison of the transcriptional activation region 1 (TAR1) and the putative Cln3 interaction domain (CID) of Swi6.
Furthermore, the balance of gene expression at Start appears to be highly disrupted in the absence of SWI6 when Cln1 or Cln2 are over-expressed.

Table 8.1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cell Volume (fl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303-1a pSD.06</td>
<td>41 +/-11*</td>
</tr>
<tr>
<td>W303-1a pgal-Cln1</td>
<td>20 +/-11*</td>
</tr>
<tr>
<td>W303-1a pgal-Cln2</td>
<td>23 +/-12*</td>
</tr>
<tr>
<td>W303-1a pgal-Cln3</td>
<td>21 +/-11*</td>
</tr>
<tr>
<td>swi6 pSD.06</td>
<td>63 +/-21*</td>
</tr>
<tr>
<td>swi6 pgal-Cln1</td>
<td>N/A</td>
</tr>
<tr>
<td>swi6 pgal-Cln2</td>
<td>N/A</td>
</tr>
<tr>
<td>swi6 pgal-Cln3</td>
<td>154 +/-64*</td>
</tr>
</tbody>
</table>

* denotes standard deviation.

8.4 DISCUSSION

The TAR1 domain which is dependent upon Cln3 for its activity falls within the CID region of Swi6 which is necessary for Cln3 interaction (figure 8.2). The obvious interpretation of this data would strongly suggest that it is this region of Swi6 which can interact with Cln3 and influence its activation potential. It is important to note the activation potential of ADH-LexA-TAR1 is reduced to background levels in a cln3 strain, whereas there is only a 4 fold reduction of activation driven by wild type full length ADH-LexA-Swi6. This may be because Cln3’s effect on Swi6 activity is exclusively reserved to the TAR1 region, with other possible factors influencing activity of the full length molecule, and its second putative activation domain.
In addition to a reduction of LexA-TAR1 activity in a cln3 mutant, there is also a marked reduction in the cln2 mutant. This observation is difficult to account for, but may reflect some additional regulatory role for Cln2 on the activation potential of the TAR1 region, which is masked when assaying for gene transcription using the full length Swi6 molecule. Conversely the effect that the cln2 mutants has on TAR1 activity may be artifactual for the same reason, that is only a small portion of Swi6 is used in these assays, therefore removing any possible effects the full length molecule may have on other regulatory molecules.

Experimental results on cell size and morphology in wild type and swi6 mutant cells, over-expressing the G1 cyclins strongly suggest that Cln3 and not Cln1 or Cln2 is an upstream activating factor of Swi6 at Start. The surprising increase in cell size in a swi6 mutant when Cln3 is over-expressed could be the result of a checkpoint further delaying the onset of Start as the balance of regulatory proteins is greatly disrupted. This disruption of the balance of Start-inducing proteins could help explain the aberrant cell morphology defects of swi6 mutant cells over-expressing Cln1 and Cln2. Cells are elongated with budding and cell wall defects. Some cells had multiple bud sites and nuclear segregation defects. These phenotypes could be as result of some aspects or pathways of Start being accelerated or promoted with others being delayed or repressed. Similar phenotypes in wild type cells over-expressing Cln1 and Cln2 have been observed (Lew and Reed, 1993; Barral et al., 1995). The appearance of more than one type of morphology defect in swi6 mutant cells over-expressing Cln1 and Cln2 could be a result
of different levels of Cln1 and Cln2 proteins expressed from the 2µ plasmid (Rose and Broach, 1990; Barral et al., 1995).

A study of Start specific genes in these cells has been planned. This includes analysis of mRNA levels of DNA synthesis, budding and cell wall genes in these different strains. In conclusion, the results in this chapter identify a region of Swi6 which can activate reporter gene expression which is totally Cln3 dependent, and partially Cln2 dependent. Furthermore this activation domain corresponds with a putative Cln3 interaction domain. Additionally, Cln3 can no longer accelerate Start in the absence of Swi6.
CHAPTER NINE

BCK2 AND ITS EFFECT ON TRANSCRIPTION

9.1 INTRODUCTION

Our present understanding of G1/S gene expression envisages that many genes expressed at Start are regulated by the SBF and MBF transcription factors via SCB and MCB elements located in their promoters. Swi6 is a common component of both transcription factors and my recent work has demonstrated that it is this protein that holds the key to periodic gene transcription at this stage in the cell cycle. The G1 cyclin, Cln3, also has a crucial role in gene expression at Start. However, the whole picture of G1/S gene transcription remains unresolved, and there do appear to be alternative mechanisms which can drive gene transcription in the absence of either Swi6 or Cln3, some of these are discussed in the chapter 1. From reviewing possible alternative mechanisms of gene expression at Start, one gene, BCK2, is of particular interest. Not only has Bck2 been implicated in the G1 cyclin gene expression, but it was also isolated from screen by its ability to rescue the G1 arrest of a triple cln mutant. This apparent ability to bypass Cln requirement, and the reduction in viability as a result of Bck2 loss in either cln3 or swi6 backgrounds suggests that Bck2 may play an important role at Start in the yeast cell cycle.
A MCB-lacZ reporter system was utilised to demonstrate that MBF activity is dependent on Bck2 for optimal activity.

Using a SCB-lacZ reporter plasmid the activity of SBF was shown to be dependent on Bck2.
9.2 SBF AND MBF ACTIVATION IS BCK2 DEPENDENT

A role for Bck2 in the expression of CLN1 and CLN2 has been demonstrated (Di Como et al., 1995). In cells over-expressing Bck2, there was an increase in CLN1 and CLN2 transcription, furthermore, this elevation in CLN1/2 RNA synthesis was Swi6-dependent. I therefore tested if SBF and MBF activity was Bck2 dependent. Wild type W303-1a and bck2 mutant cells expressing the MCB-lacZ reporter plasmid were assayed for β-galactosidase activity as an indication of MBF activity (figure 9.1a). In comparison to wild type cells, MCB-lacZ reporter activity decreased by 2 fold in bck2 cells, from 2.0 to 1.0 β-galactosidase units. A second series of experiments examined the effects of the bck2 deletion on SBF activity. SBF activity was detected with a SCB-lacZ reporter plasmid in wild type W303-1a and a bck2 mutant (figure 9.1b) β-galactosidase activity in the bck2 mutant showed a 3 fold decrease to activity in comparison to wild type, with activity falling from 1.2 to 0.4 β-galactosidase units. Together, assays for native SBF and MBF activity demonstrate a partial Bck2 dependency.

9.3 LEXA-SWI6 ACTIVITY IS DEPENDENT ON BCK2

Activity of the GLU-LexA-Swi6 fusion protein was next assayed in the reporter strain, CTY10-5d, and an isogenic bck2 derivative. Yeast carrying either the GLU-LexA-Swi6 expressing plasmid or the GLU-LexA expressing control plasmid were assayed for β-galactosidase activity (figure 9.2). The level of reporter gene expression in both
Figure 9.2

GLU-LexA-Swi6 is dependent on Bck2 for optimal activation, as assayed via the 'one-hybrid' system using the reporter strain CTY10-5d. The re-introduction of Bck2 restores LexA-Swi6 activity.

Figure 9.3

The Bck2 expressing plasmid can rescue the lethality of a triple cln mutant. Plasmid pTR476 expresses Bck2 under its own promoter and has the ability to rescue the strain BF305-15d no.21 (triple cln mutant) when grown on glucose. The positive control plasmid expressing Cln2 under the strong S. pombe ADH promoter can also rescue the triple cln mutant. The strain fails to grow on glucose when expressing the vector only control.
strains expressing the GLU-LexA control plasmid very low as expected. Expression of GLU-LexA-Swi6 fusion in the bck2 mutant showed a 6 fold decrease in reporter activity in comparison to wild type cells expressing GLU-LexA-Swi6.

To test the reduction in GLU-LexA-Swi6 activity was specific to BCK2, a plasmid expressing a single copy of Bck2 under its own promoter was reintroduced into bck2 mutant cells expressing GLU-LexA-Swi6. The plasmid expressing Bck2 was shown to be functional by its ability to rescue a triple cln mutant (figure 9.3). The reintroduction of a single copy of Bck2 resulted in the restoration of reporter gene activity, comparable to wild type levels. Furthermore, β-galactosidase activity in wild type cells expressing GLU-LexA-Swi6 and the Bck2 expressing plasmid increased reporter gene activity 1.8 fold over cells expressing GLU-LexA-Swi6 and a vector control.

9.4 The TAR1 region of Swi6 is dependent on Bck2 for activity

Experiments from chapter 8 revealed that minimal activation domain 1 of Swi6 (GLU-LexA-TAR1) was Cln3-dependent for transcriptional activation. It was therefore of interest to determine if this GLU-LexA-TAR1 construct was also dependent upon Bck2 for transcriptional activation. Wild type reporter cells, CTY10-5d, and the isogenic bck2 mutant were transformed with either a plasmid expressing the GLU-LexA-TAR1 construct or the control plasmid expressing GLU-LexA (figure 9.4). In comparison to GLU-LexA-TAR1 expression in wild type CTY10-5d cells, expression of GLU-LexA-TAR1 in the bck2 mutant resulted in a 8.5 fold decrease in reporter gene expression. This experiment
demonstrates that like full length GLU-LexA-Swi6, GLU-LexA-TAR1 is dependent upon Bck2 for activation of reporter gene expression.

9.5 LexA-Cln3ΔPEST Activity is not Dependent on Bck2

From previous chapters a potential interdependency between Swi6 and Cln3 was revealed. Furthermore, with Swi6, MBF and SBF being dependent on Bck2 for transcriptional activity, it was of interest to investigate if the absence of BCK2 had any effect on the transcriptional activation properties of Cln3. Because of the minimal activation properties of ADH-LexA-Cln3, the PEST deletion, ADH-LexA-Cln3ΔPEST, was used in this experiment.

The activity of the ADH-LexA-Cln3ΔPEST fusion protein was assayed in the wild type, CTY10-5d reporter strain, and the isogenic bck2 mutant (figure 9.5). Reporter gene expression driven by ADH-LexA-Cln3ΔPEST in the bck2 mutant was comparable to that of wild type cells with only a 1.1 fold decrease in activity, from 1.6 to 1.45 β-galactosidase units. This experiment demonstrates that the transcriptional activity of ADH-LexA-Cln3ΔPEST is not dependent upon Bck2.

9.6 Reduction in Transcriptional Activation in a Bck2 Mutant is not Specific to Swi6

The reduction in GLU-LexA-Swi6 activity in a cln3 mutant was shown to be specific to Swi6 as determined by quantitating the activity of non yeast transcription factors in the absence of Cln3. A similar experiment revealed that the dependency of transcriptional activation in a bck2 mutant is not as clear as the Cln3 dependency (figure 9.6).
Figure 9.4

GLU-LexA-TAR1 is dependent on Bck2 for optimal activation, as assayed via the 'one-hybrid' system.

Figure 9.5

The ability of ADH-LexA-Cln3ΔPEST to activate reporter gene expression is not dependent on Bck2.
ADH-LexA-p53 and ADH-LexA-E2F stimulate reporter gene expression in the CTY10-5d strain over 9 and 17 times that of ADH-LexA-Swi6 with β-galactosidase values of 11 and 21 respectively. ADH-LexA-SLN1 (a Xenopus transcription factor) dependent reporter gene expression is comparable to that of ADH-LexA-Swi6, with a β-galactosidase value of 1.5. When compared to wild type cells, levels of reporter gene expression driven by ADH-LexA-p53 and ADH-LexA-E2F are dramatically reduced by 4 and 5 fold respectively in bck2 mutant cells. This result would give the impression that like GLU-LexA-Swi6, GLU-LexA-TAR1, SBF and MBF activity, there is also a reduction in the two other non yeast transcription factors tested.

In contrast to p53 and E2F, reporter gene expression driven by ADH-LexA-SLN1 showed no change in bck2 cells compared to wild type. Hence an analysis of the Bck2 dependency of lexAop-lacZ reporter gene expression driven by different transcription factors gave different results. While there was a reduction in gene expression driven by Swi6, TAR1, p53 and E2F in a bck2 mutant compared to wild type cells, no such reduction in expression when assaying for Cln3APEST or SLN1 activity.

Thus the reduction in transcriptional activation in a bck2 mutant is not specific to LexA-Swi6. Furthermore, from these experiment it can been seen that Bck2’s effect on transcriptional activation is not an effect of the ADH promoter activity.

9.7 ADH-LexA-BCK2 CAN ACTIVATE TRANSCRIPTION

The discovery that in the absence of Bck2 there is a loss in transcription activation of two out of three unrelated transcription factors suggests that Bck2 may have a more general
'One-hybrid' assays in the reporter strain CTY 10-5d and an isogenic \( bck2 \) mutant reveal \( \text{ADH-LexA-p53} \) and \( \text{ADH-LexA-E2F} \) activity to be Bck2 dependent. Conversely, the transcriptional activity of \( \text{ADIi-LexA-SLNI} \) is not Bck2 dependent.

In a 'one-hybrid' assay \( \text{ADH-LexA-Bck2} \) can activate transcription of the reporter strain CTY 10-5d to a similar extent as \( \text{ADH-LexA-Swi6} \).
role in transcriptional activation. Therefore a ‘one-hybrid’ approach was used to test the activation potential of Bck2. The Bck2-coding sequence was cloned into pTR27 resulting in the expression of an ADH-LexA-Bck2 fusion protein. The transcriptional activation potential of this fusion protein was compared to ADH-LexA-Swi6 in the reporter strain CTY10-5d (figure 9.7). Expression of ADH-LexA-Bck2 resulted in β-galactosidase activity of 1.0, which is comparable to ADH-LexA-Swi6 dependent reporter gene activation of 1.2 β-galactosidase units. Thus, using the same reporter system, ADH-LexA-Bck2 can effectively activate reporter gene expression almost to the same extent as ADH-LexA-Swi6.

Additionally, figure 9.8 shows the ADH-LexA-Bck2 construct to be functional at a cellular level, by its ability to rescue the triple cln mutant by the same method as described in section 6.7.

9.8 TRANSCRIPTIONAL ACTIVATION BY ADH-LexA-Bck2 IS DEPENDENT ON CLN3 BUT NOT SWI6

The data presented in earlier sections revealed that GLU-LexA-Swi6 transcriptional activity is dependent on the G1 cyclin Cln3. Furthermore, I also demonstrated that ADH-LexA-Cln3ΔPEST activity to be dependent on Swi6. The interdependency between Swi6 and Cln3, together with data suggesting a role for Bck2 at Start, prompted an investigation into ADH-LexA-Bck2 activation in different null backgrounds. The ADH-LexA-Bck2 expressing plasmid was assayed for transcriptional activity in CTY10-5d and isogenic cln1, cln2, cln3 and swi6 mutants. Figure 9.9 shows only slight differences in reporter gene activity in swi6, cln1 and cln2 strains when compared to wild type, with a 1.2 fold
Figure 9.8
The ADH-LexA-Bck2 expressing plasmid (pTR432) can rescue the lethality of a triple *cln* mutant. The positive control plasmid expressing ADH-LexA-Cln3 in the same vector can also rescue the triple *cln* mutant. The strain fails to grow on glucose when expressing the ADH-LexA- vector only control.

Figure 9.9
A 'one-hybrid' assay demonstrates that ADH-LexA-Bck2 is dependent on Cln3 for optimal transcriptional activity. Conversely, Cln1, Cln2 and Swi6 are not needed for the transcriptional activity of ADH-LexA-Bck2.
increase and a 1.4 and 1.25 fold decrease respectively. Assays of \textit{ADH-LexA-Bck2} activity in a \textit{cln3} mutant revealed a 5 fold decrease in reporter activity when compared to wild type cells. This experiment demonstrates \textit{ADH-LexA-Bck2} activity to be \textit{Cln3} dependent but not dependent on Swi6, Cln1 or Cln2.

\textbf{9.9 Discussion}

Initial experiments by others suggested a role for Bck2 in the expression of Start specific genes, including Cln1 and Cln2. Furthermore, Bck2’s influence on this expression was shown to be at least, in part, SBF dependent. Using a different approach to other investigators, my experiments confirm that SBF, MBF and Swi6 activities are Bck2 dependent. The obvious interpretation would be that Bck2 can influence this expression even though it may not be a major player in G1/S gene expression. However, the picture is complicated by the finding that other non-yeast transcription factors also appear to be Bck2-dependent in their ability to activate transcription. The reduction of LexA-Swi6 driven transcription in a \textit{bck2} mutant and the increase in activation in cells over-expressing Bck2 may help explain why Bck2 was isolated in several different genetic screens. The screen by which Bck2 was first isolated was by its ability to suppress mutations of the \textit{PKC} pathway. This suppression may be as a result in an increase in expression of genes needed to produce cell wall components. Indeed, some genes downstream of the \textit{PKC} pathway contain SCB elements within their promoter regions, so it may be possible that Bck2 could enhance the expression of these genes in a SBF dependent manner. This, however, is not a totally satisfactory explanation. If this were the method by which Bck2 bypassed the PCK pathway, then over-expression of Swi6
and Swi4 would also result in a rescue, which is not the case (Epstein and Cross, 1994). Alternatively Bck2 may influence the transcription of cell wall genes in an SBF independent manner or in concert with SBF. The method by which Bck2 bypasses the PKC pathway may of course be cell wall gene independent and is presently unresolved.

A second screen by which Bck2 was isolated stems from its ability to rescue the G1 arrest of a triple cln mutant (Epstein and Cross, 1994). This apparent ability to bypass Cln requirement, and the reduction in viability as a result of Bck2 loss in either a cln3 or swi6 background again suggests that Bck2 may play an important role at Start. One of the possible mechanisms by which Bck2 could bypass the requirement for G1 cyclins may be to increase the activity or efficiency of proteins which drive this G1/S gene transcription. Indeed, I have shown that in the absence of Bck2, the transcriptional activation potential of SBF, MBF, Swi6 and Swi6-TAR1 are reduced. Furthermore, the activity of Swi6 is increased in the presence of one extra copy of Bck2. Increasing SBF- and MBF-dependent transcription is an overly simplified hypothesis, for over-expression of components of these transcription factors does not restore viability to the triple cln mutant. It is possible that Bck2 may be affecting other aspects of Start which are distinct from SBF and MBF dependent gene transcription. cln bypass may be achieved by increasing the expression of other cyclin-like genes which SBF and MBF can not achieve alone.

Like the interdependency demonstrated between Swi6 and Cln3, the transcriptional activation by Swi6 is Bck2-dependent and Bck2 transcriptional activity is Cln3 dependent. The picture is however far from clear because Bck2 transcriptional
activity is not Swi6 dependent, nor is Cln3 activity Bck2 dependent. Figure 9.10 shows a simple diagram representing the dependency between Swi6, Cln3 and Bck2 determined by ‘one-hybrid’ assays.

In conclusion, it would appear that Bck2 has a place in gene expression at Start, but its role is not necessary specific, and may play an almost subsidiary semi-specific role in other aspects of gene expression. This semi-specific ‘co-activator’ theory would help explain why Bck2 was isolated in different screens and why it appears to affect gene expression driven by SBF and MBF and other transcription factors.
Figure 9.10

A simple representation of interdependency between Swi6, Cln3 and Bck2. In this simple model Cln3 appears to be the key player, stimulating the activities of both Swi6 and Bck2. Swi6 activity is in turn dependent on both Cln3 and Bck2. This simple model is derived from 'one-hybrid' experiments in null mutant backgrounds.
CHAPTER TEN

GENERAL DISCUSSION

10.1 GENERAL DISCUSSION

The overall aim of my research was to gain a greater understanding of the regulation of gene expression during the G1 to S phase of the yeast cell cycle. To this end I demonstrated that the Swi6 protein is responsible for the cell cycle dependent transcriptional activation by the SBF and MBF transcription factors. The mechanisms by which Swi6 regulates these periodic transcription factors is still unclear, but it would appear that from my work that the G1 cyclin, Cln3, is needed for optimal transcriptional activation. The involvement of Cln3 in gene transcription is supported, in part, by demonstrating that in ‘one-hybrid’ assays, LexA-Cln3ΔPEST is a transcriptional activator which relies on the presence of Swi6 just as LexA-Swi6 activity relies on Cln3. The involvement of Cln3 suggests a phosphorylation mechanism for stimulation of transcriptional activity of Swi6. However, when two putative Cdc28 phosphorylation sites were mutated in Swi6 there was no change in the transcriptional activation potential. Additionally, Sidorova and Breeden reported that changes in Swi6 phosphorylation did not correspond with peak transcriptional activation. A direct investigation of Cdc28 involvement was not explored in this thesis, and it is possible that Cdc28 may have a role
in Swi6 transcriptional activation. Further planned experiments will hopefully reveal more information into the role of Cdc28 in relation to Swi6 activity.

Possible roles for Cdc28 activity at Start can be envisaged. Cdc28 activity might be needed for the formation of the SBF and MBF complexes, just as the fission yeast Cdc28 homologue, Cdc2, is needed for MBF complex formation. However, mutation of a single Cdc2 consensus site of Cdc10, the fission yeast Swi6 counterpart, which results in the mimicking of a phosphorylated Cdc10, leads to the formation of the Cdc10/Res1 complex, but does not rescue the G1 defect of a cdc2 mutant (Connolly et al., 1997). This indicates that the promotion of Cdc10/Res1 complex formation is not the only function performed by Cdc2 in the execution of Start. Furthermore, my experiments suggest that complex formation is not the only potential function for Cdc28, since in the one-hybrid experiments the Swi6 fusion protein has an artificial DNA binding domain and hence no need for Swi4 or Mbp1.

I did however uncover a potential link between Cln3’s ability to activate gene transcription and its dependency on Cdc28. The transcriptional activation potential of LexA-Cln3 was lost if the cyclin box, the region needed for Cdc28 association, was deleted. This suggests that Cln3 dependent reporter gene transcription and Cdc28 association are not mutually exclusive. The role of Cdc28 in Swi6 and Cln3 activation requires direct examination. Perhaps by observing reporter gene activity driven in a ‘one-hybrid’ assay utilising reciprocal shift experiments. It is however important to remember that in deleting the PEST and the cyclin-box regions I and II of Cln3, the overall structure of the protein could be grossly affected.

137
The involvement of Cdc28 in SBF and MBF gene expression at Start is inescapable, and it is possible that Cdc28 may have alternative targets other than the known components of SBF/MBF, with Swi6/Cln3 functioning to bring Cdc28 and its potential substrates together. For example, a component of the basal or activated transcription complex may be the target. Indeed, there recently have been clues of a tentative link between the yeast Taf145 and Swi6 proteins. Taf145 is a subunit of the TFIID transcription apparatus and has an essential role in the expression of Start-specific genes at the G1/S phase transition. Furthermore, taf145 mutants display a cdc-like G1 arrest phenotype due to reductions in G1 cyclin expression. This would strongly suggest a connection between the transcription factors controlling gene expression at Start and a component of the transcription initiation complex, and would be the first link between a member of the transcriptional apparatus and a cell cycle specific activator in yeast.

How Swi6 and its associated factors influence gene expression, possibly via Taf145 is an interesting but unanswered question. Physical association between either Swi6 or Cln3 with Taf145 or additional components of the basal transcription apparatus might be predicted in a recruitment model for transcriptional activation as envisaged by Patashne and Gann (1997). Interestingly members of our group have found histone acetyltransferase activity in immunoprecipitates of Swi6 from crude cell extracts. Histone remodelling is another possible means of transcriptional activation and yeast Taf145 and the human homologue, TAFn250, have demonstrated histone acetyltransferase activity (Mizzen et al., 1996). Although these experiments are
preliminary, they are very encouraging and it remains to be shown whether the activities of Swi6 and Taf145 are connected.

The physical interaction between Swi6 and Cln3 may present a possible mechanism for Cdc28 recruitment to the transcription complex leading to possible interactions with Taf145 or its associated factors. Alternatively, the significance of the Swi6/Cln3 interaction may be to activate gene expression independently of Cdc28 involvement. The notion that cyclins can have other roles in addition to activating CDKs has some support. Mammalian cyclin D1 augments gene expression regulated by the oestrogen receptor transcription factor (Zwijsen et al., 1997; Neuman et al., 1997). This occurs independently of binding to a CDK partner and, instead, cyclin D1 associates directly with the oestrogen receptor protein to increase binding of the receptor to the estrogen response element, thereby increasing transcriptional activation. The binding of Cln3 to Swi6 has been demonstrated using several independent techniques, including conventional ‘two-hybrid’ assays, the SOS recruitment system (SRS) assay, co-immunoprecipitation, and in vitro binding assays between affinity purified Swi6 and in vitro transcribed and translated Cln1, 2 and 3. Similarly Puc1, the single Cln-type cyclin in fission yeast associates in vivo with Cdc10 (Caligiuri et al., 1997). Cdc2, the homologue of Cdc28 protein kinase, is not required for this interaction, and the requirement, if any, for Cdc2 in the early stages of G1 transcription in S. pombe has been questioned (Baum et al., 1997).

The interaction of Swi6 and Cln3 was investigated further revealing that the Cln3 binding occurs within the N-terminal activation domain of Swi6. The means by which
Cln3 and Swi6 influence each other is unclear but it is possible that the two proteins act as co-activators, possibly leading to conformational changes in one or both of the proteins. I believe the binding of Cln3 to the N-terminal activation domain of Swi6 is highly significant, for other members of our research team have isolated a novel Swi6 binding protein, which appears to bind to the CID region of Swi6 in two-hybrid assays. Furthermore, its only sequence homology to known proteins is a putative cyclin box.

Interdepartmental collaboration with the Division of Protein Structure is determining the functional and structural organisation of Swi6. X ray crystallographic imaging shows that the N-terminal transcriptional activator domain is largely unstructured and is wrapped over the rigid and highly structured ankyrin repeats which comprises a transcriptional antagonism domain.

A link between ankyrin domains, gene transcription, repression and cyclin kinases’ has already been partially explored in budding yeast. Pho81 is a CDK inhibitor and contains six copies of the ankyrin repeat. Under certain conditions this protein inhibits the Pho85 kinase and its cyclin partner, Pho80. The importance of the ankyrin repeats within Pho81 can be appreciated, for alone they are sufficient to inhibit Pho80-Pho85 kinase activity (Ogawa et al., 1993; Schneider et al., 1994). Repression of the Pho4 transcription factor is mediated by complex formation with the Pho80 cyclin (Jayaraman et al., 1994). Interestingly the ankyrin containing CDK inhibitor, Pho81 appears to bind to the cyclin component of the CDK molecule, just as Swi6 binds to Cln3. In the Pho gene regulation system, although the Pho81 ankyrin containing CDK inhibitor and the Pho4 transcription factor are distinct molecules, they both interact with
each other (Hirst et al., 1994) and are in turn regulate and are regulated by a cyclin kinase molecule. Parallels between the Pho and Swi6 system of gene regulation can be drawn, with the possibility that within the Swi6 molecule there are multiple domains which specify distinct functions, like transcription initiation and transcription repression.

Indeed, a structural analysis of Swi6 together with X ray crystallographic studies reveals several distinct domains, connected by flexible linkers (Sedgwick et al., 1998).

The study of Swi6 as a transcription factor is an exciting but sometimes confusing field. It is somewhat paradoxical that Swi6 and Cln3 have pivotal roles in the initiation of Start, yet neither gene is essential. It would therefore appear that there are other mechanisms for promoting gene expression at Start, namely the expression of CLN1 and CLN2. Although this in itself is an interesting field of study and there are known proteins which can stimulate G1 cyclin expression, an attempt to summarise this area is beyond the scope of this thesis. I did, however, begin work on characterising one such gene, BCK2, and its relationship to G1 gene expression. Indeed, Bck2 has been shown by others to affect the expression of CLN2, likewise I have demonstrated that lack of Bck2 results in reduced SBF, MBF and Swi6 activities as well as activities of other non-yeast transcription factors. In addition to affecting the transcription factors which regulate G1 cyclin expression, a single extra copy of Bck2 can restore the viability of a triple cln mutant strain. The confusing and sometimes contradictory results obtained from these initial studies of Bck2 suggest that it may have multiple roles or functions in gene expression or have some semi-general activity common to several distinct transcriptional activators, which could be the reason why BCK2 was isolated in different suppressor
screens. Bck2 could be an effector which could be involved in the regulation of a diverse array of genes in a similar way as Pho2, which can specifically recruit Pho4 transcription factor to DNA and also enhance its activity, as well as acting in concert with a number of other gene transcription processes (Barbaric et al., 1998). Although a role for Bck2 in G1-specific gene expression remains to be determined, there is no doubt that it has some influence at this control point.

In conclusion, the mechanism of gene expression at Start has developed rapidly in budding yeast over the past 10 years, with Swi6 and Cln3 being perceived as key players in this regulation. The Swi6 protein has been extensively studied due to its apparent multifunctional nature. It is a transcriptional activator and repressor, and its activity is affected by a number of proteins. It not only has a direct role in G1 gene expression under favourable conditions, but appears to play a role in genes induced under stress. Furthermore, SBF has recently been shown to act in concert with the protein kinase C (PKC1) MAP kinase pathway in the regulation of cell wall integrity (Igual et al., 1996; Madden et al., 1997). For these and other reasons the Swi6 protein and associated factors continue to be interesting subjects for studies of the mechanism whereby both intra- and extracellular signals are transduced, via MAP kinase and cell-cycle kinase/cyclin complexes, to the transcriptional machinery.
APPENDIX 1

PLASMIDS MAPS
ADH-LexA vector
ADH-LexA-Swi6 plasmid
GLU/GAL-LexA-Swi6 plasmid
*lexAop*-luciferase reporter plasmid
CLN1 expression plasmid
CLN2 expression plasmid
CLN3 expression plasmid
**BCK2 genomic clone**

**BCK2 ORF**

**pTR476**

10440 bps

**LEU2**

8000

**ampR**

10000

**BCK2 clone**

2000
ADH-LexA-Bck2 plasmid
REFERENCES


Toyn, J.H. and Johnston, L.H. 1993. Spol2 is a limiting factor that interacts with the cell cycle protein kinases Dbf2 and Dbf20, which are involved in mitotic chromatid disjunction. *Genetics*, 135: 963-971.


