

**Mss11p mediated regulation  
of transcription,  
pseudohyphal differentiation  
and flocculation in  
*Saccharomyces cerevisiae*.**

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by

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## **DECLARATION**

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Jaco Franken



## SUMMARY

In all cellular systems the ability to alter cellular programs in response to extracellular cues is essential for survival. This involves the integration of signals triggered by membrane bound receptors in order to adjust the expression of target genes and enzyme activities and consequently phenotypic outcome. The yeast *Saccharomyces cerevisiae* has evolved several adaptations, such as, sporulation and pseudohyphal differentiation, in order to survive changes in the surrounding environment. Pseudohyphal differentiation and the related phenotype, invasive growth, are proposed to be adaptations that enable the yeast to forage for scarce nutrients or escape from a detrimental environment. This dimorphic transition is associated with a change from the normal "yeast" form to a pseudohyphal form, which involves changes in budding pattern, cell-cycle progression, cellular elongation, and cell-cell and cell-substrate adherence. The outcome of these changes is elongated cells, which bud in a unipolar fashion and do not separate after budding to form chains of cells referred to as pseudohyphae. These pseudohyphae are able to penetrate the surface of agar containing growth medium, a process referred to as invasive growth.

Nutrient-induced adaptations, such as pseudohyphal growth, have been extensively studied in *S. cerevisiae*, and several factors have been implicated in the regulation thereof, many of which are part of specific signalling pathways. The most clearly defined are the filamentous growth specific MAP kinase cascade and the Gpa2p-cAMP-PKA pathway. *MUC1/FLO11*, encoding a member of a family of cell wall associated proteins involved in cell-cell/cell-substrate adhesion, is regulated by these pathways and considered to be critical in the establishment of pseudohyphal differentiation and invasive growth. The promoter region of *MUC1/FLO11* represents one of the largest yeast promoters identified to date, with *cis*-acting elements present up to 2.4 kb upstream from the first coding triplet. The upstream regulatory region of *MUC1/FLO11* is almost identical to that of the *STA2* gene, which encodes an extracellular glucoamylase required for the utilisation of extracellular starch.



As suggested by the extent of homology between these two promoters, *MUC1/FLO11* and *STA2* are co-regulated to a large degree and both require the same transcription factors.

Mss11p plays a central role in the regulation of *MUC1/FLO11* and *STA2* and consequently starch metabolism and pseudohyphal differentiation. The regulation conferred by *MSS11* on the transcriptional levels of *MUC1/FLO11* and *STA2* also appears to be dependent on signals generated specifically in the presence of low nitrogen and glucose. Mss11p does not have significant homology to any other yeast protein, with the exception of limited homology to the transcriptional activator Flo8p. However, several distinctive domains have been identified in the *MSS11* gene product. Firstly, Mss11p contains poly-glutamine and poly-asparagine domains. It also contains a putative ATP- or GTP-binding domain (P-loop), commonly found in proteins such as kinases, ATPases or GTPases. Two short stretches close to the N-terminal, labelled H1 and H2, share significant homology to the transcriptional activator, Flo8p. Both the H2 domain and the extreme C-terminal of Mss11p are able to stimulate RNA polymerase II dependent transcription. Furthermore, the H1 domain together with the P-loop negatively regulates the activation potential of the H2 domain.

This study presents further insight into the functioning of Mss11p and the involvement of the separate activation and regulatory domains in mediating transcriptional activation and pseudohyphal differentiation in response to nutrient limitation. Genetic interactions between Mss11p and other factors involved in the regulation of pseudohyphal growth and starch degradation were revealed, and specific regions of Mss11p were shown to be required by these factors in order to achieve their required function. In addition, results obtained in this study implicate Mss11p in the regulation of Ca<sup>2+</sup>-dependent flocculation and suggest that the *FLO1* gene is also regulated by Mss11p in this capacity.



## OPSOMMING

Die vermoë om sellulêre programme in reaksie op ekstrasellulêre seine te verander, is 'n essensiële vereiste vir alle sellulêre sisteme. Dit behels die integrasie van seine gegeneer deur membraan-gebonde reseptore om ekspressie van teikengene en ensiemaktiwiteite sodanig aan te pas dat gewenste fenotipiese uitkomstebewerkstellig kan word. Die gis *Saccharomyces cerevisiae* het verskeie aanpassingsmeganismes ontwikkel, soos byvoorbeeld sporulasie en pseudohifevorming, om veranderinge in die omgewing te kan oorleef. Pseudohifevorming en die verwante fenotipe, penetrasiegroei, word beskou as aanpassings te wees wat die gis in staat stel om van 'n skadelike omgewing weg te kom, of dit in staat te stel om by skaars voedingstowwe uit te kom. Hierdie dimorfiese transisie word geassosieer met 'n verandering van die normale "gisvorm" tot pseudohifevorming wat veranderinge in die botpatroon, selsiklusprogressie, selverlenging, sel-sel en sel-substraat aanhegting behels. Die uitkoms van hierdie verandering is verlengde selle, wat unipolêr bot en nie van mekaar skei nie om sodoende kettings van selle te vorm en waarna verwys word as pseudohifes. Hierdie pseudohifes is ook in staat om die oppervlak van 'n agar bevattende groeimedium te penetreer, 'n proses waarna verwys word as penetrasiegroei.

Aanpassings soos pseudohifevorming is die afgelope dekade intensief nagevors, en verskeie faktore en seintransduksienetwerke is in die regulering daarvan geïmpliseer. Onder hierdie seintransduksienetwerke is die bes gedefiniëerde paaie die filamentasie-spesifieke MAP-kinasekaskade en die Gpa2p-cAMP-PKA pad. *MUC1/FLO11* kodeer vir 'n lid van 'n geenfamilie wat met sel-sel/sel-substraat aanhegting geassosieer word en dit word deur hierdie seintransduksie netwerke gereguleer. *MUC1/FLO11* word as essensieel vir pseudohife vorming beskou. *MUC1/FLO11* word gereguleer deur die grootste gispromoter wat tot op hede geïdentifiseer is, met *cis*-werkende elemente so ver as 2.4 kb stroom-op van ATG. Die *MUC1/FLO11* promoter is feitlik identies tot die van die *STA2*-geen, wat kodeer vir 'n ekstrasellulêre glukoamilase wat die gis in staat stel om ekstrasellulêre stysel te benut.



Weens die homologie tussen die twee promoters, word *MUC1/FLO11* en *STA2* tot 'n groot mate ge-koreguleer en beide benodig dieselfde transkripsiefaktore.

Mss11p speel 'n sentrale rol in die regulering van *MUC1/FLO11* en *STA2* en dus ook in die regulering van pseudohifevorming en styselmetabolisme. Die regulering wat deur Mss11p of *MUC1/FLO11* en *STA2* uitgeoefen word, blyk verder onderhewig te wees aan seine wat gegenerer word spesifiek in die teenwoordigheid van lae konsentrasies glukose en stikstof. Mss11p het nie betekenisvolle homologie met enige ander gisproteïen nie, behalwe vir beperkte homologie met die transkripsionele aktiveerder Flo8p. Verskeie onderskeidbare domeine is egter in die *MSS11* geenproduk teenwoordig. Eerstens, Mss11p bevat kenmerkende poliglutamien en poli-asparagien domeine. Verder bevat Mss11p ook 'n voorspelde ATP- of GTP-bindings domein (P-lus), wat algemeen in proteïene soos kinases, ATPasaes en GTPases voorkom. Twee kort areas naby die N-terminaal, aangedui as H1 en H2, het betekenisvolle homologie met die transkripsiefaktor Flo8p. Beide die H2 domein en die ekstreme C-terminaal van Mss11p is in staat om RNA polimerase II afhanklike transkripsie te stimuleer. Verder het die H1-domein in samewerking met die P-lus 'n negatiewe uitwerking op die aktiveringspotensiaal van die H2-domein.

Hierdie studie bied verdere insig tot die werking van Mss11p en die betrokkenheid van die verskeie aktiverings- en reguleringsdomeine by die bemiddelling van transkripsionele aktivering en pseudohifevorming in reaksie op beperking van voedingstowwe. Genetiese interaksies tussen Mss11p en ander faktore betrokke met die regulering van pseudohifevorming en styselafbraak is in hierdie studie aangetoon. Voorts is daar ook gewys dat spesifieke areas van Mss11p benodig word deur hierdie faktore om hulle biologiese funksie uit te oefen. Daar is ook 'n rol vir Mss11p in die regulering van  $\text{Ca}^{2+}$ -afhanklike flokkulasie aangetoon en daar is bewys dat die *FLO1* geen deur Mss11p benodig word om hierdie effek uit te oefen.

**This thesis is dedicated to Anel.  
Hierdie tesis is opgedra aan Anel.**

## **BIOGRAPHICAL SKETCH**

Jaco Franken was born in Bloemfontein, South Africa, on 30 October 1976. He matriculated at the DF Malan High School, Bellville, 1994. In 1995 he enrolled at Stellenbosch University and obtained a BSc degree in Biochemistry, Microbiology and Genetics in 1998. In 2000 he completed a BSc Hons degree in Wine Biotechnology at the Institute for Wine Biotechnology, University of Stellenbosch.

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## PREFACE

This thesis is presented as a compilation of five chapters. Each chapter is introduced separately and is written according to the style of the journal *Molecular Microbiology*. Supplemental information is provided in an appendix.

### Chapter 1

#### General Introduction and Project Aims

### Chapter 2

#### LITERATURE REVIEW

Chromatin and transcriptional regulation in *Saccharomyces cerevisiae*.

### Chapter 3

#### Research results

The effect of nucleosomal positioning on the transcriptional regulation of *MUC1/FLO11* and *STA2*.

### Chapter 4

#### Research Results

Mss11p mediated regulation of transcription, pseudohyphal differentiation and flocculation in *Saccharomyces cerevisiae*.

### Chapter 5

#### General Discussion and Conclusions

### Appendix

**Mss11p is a transcription factor regulating pseudohyphal differentiation, invasive growth and starch metabolism in *Saccharomyces cerevisiae* in response to nutrient availability. [Molecular Microbiology (2003) 47: 119-134]**

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Gagiano, M., Bester, M., Van Dyk, D., Franken, J., Bauer, F.F., and Pretorius, I.S. (2003) Mss11p is a transcription factor regulating pseudohyphal differentiation, invasive growth and starch metabolism in *Saccharomyces cerevisiae* in response to nutrient limitation. *Molecular Microbiology* 47: 119-134

## **CHAPTER 1**

# **INTRODUCTION AND PROJECT AIMS**



## 1.1 INTRODUCTION

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A characteristic common to all organisms is the ability to constantly coordinate metabolic activities with environmental changes. Communication with the environment is achieved through a number of pathways that receive and process signals from the external environment. Signals transmitted via individual pathways are integrated to adjust the expression of target genes and consequently discrete cellular functions. Many of the modules involved in the recognition and interpretation of signaling events are conserved from yeast to higher eukaryotes. Our understanding of the diversity of biochemical and functional interactions required to sustain eukaryotic life has been greatly enhanced by a variety of model organisms. One of the most important of these organisms has been the yeast *Saccharomyces cerevisiae*. The wide variety of molecular tools available for this organism, and the relative ease of manipulation, has made *S. cerevisiae* possibly the best understood of all cellular systems.

Several cellular responses have evolved in *S. cerevisiae* to adjust to changes in the surrounding environment. Among these is the ability of yeast to undergo pseudohyphal differentiation in conditions of carbon or nitrogen limitation. Pseudohyphal differentiation is described as a conversion from ovoid, "yeast" shaped cells, which bud in an axial (haploid) or bipolar (diploid) fashion, to elongated cells budding in a unipolar fashion. Daughter cells remain attached to the mother cells, resulting in the formation of chains of cells referred to as pseudohyphae. These pseudohyphae are able to grow away from the colony and also penetrate the surface of agar containing medium, which is referred to as invasive growth (for review see Kron, 1997; Madhani and Fink, 1998; Borges-Walmsley and Walmsley, 2000; Pan *et al.*, 2000; Bauer and Pretorius, 2001; Gancedo, 2001; Gagiano *et al.*, 2002). This adaptation is thought to provide a selective advantage, as it could facilitate foraging for scarce nutrients or allow escape from a limiting environment. The



transition from normal to pseudohyphal growth involves changes in budding pattern, cell-cycle progression, cellular elongation, and cell-cell and cell-substrate adherence. A variety of genes involved in the regulation of these cellular processes have been identified, most of which are part of signaling pathways. The most clearly defined pathways are the filamentous-growth-specific MAP kinase cascade, which acts upstream of the transcription factors Ste12p and Tec1p (Gimeno *et al.*, 1992; Liu *et al.*, 1993; Möscher *et al.*, 1996; Cook *et al.*, 1996, 1997; Madhani and Fink., 1997, 1998; Madhani *et al.*, 1997; Rupp *et al.*, 1999), and the Gpa2p-cAMP-PKA pathway, regulating the transcription factors Flo8p and Sfl1p (Ward *et al.*, 1995; Lorenz and Heitman., 1998; Roberts *et al.*, 1997; Roberstson and Fink, 1998; Möscher *et al.* 1996; Pan and Heitman, 1999; Rupp *et al.*, 1999; Lorenz *et al.*, 2000; Tamaki *et al.*, 2000). Numerous other factors have been identified for their involvement in the regulation of pseudohyphal differentiation and invasive growth and remain to be placed into the context of known or alternate pathways. Among these are Phd1p (Gimeno and Fink, 1994; Lorenz and Heitman, 1998), Sok2p (Ward *et al.*, 1995; Pan and Heitman, 1999), Elm1p (Blacketer *et al.*, 1993; Garret *et al.*, 1997; Koehler and Meyers, 1997), Rme1p (van Dyk *et al.*, 2003), Msn1p and Mss11p (Gagiano *et al.*, 1999a, b).

Pathways that are involved in the regulation of pseudohyphal differentiation and invasive growth regulate the expression of *MUC1/FLO11* (Rupp *et al.*, 1999), encoding a member of a family of cell wall associated proteins involved in cell-cell/cell-substrate adhesion (Guo *et al.*, 2000). Other members of this family of glycosyl-phosphatidylinositol (GPI)-linked cell surface glycoproteins include Fig2p and Aga1p, required for mating, and also Flo1p, Flo5p, Flo9p and Flo10p, which are involved in flocculation (Guo *et al.*, 2000). *MUC1/FLO11* was shown to be essential in the establishment of pseudohyphal differentiation and invasive growth. Deletion of the gene leads to a loss of the filamentous phenotype, whereas overexpression of *MUC1/FLO11* results in flocculation in liquid media and pseudohyphal differentiation and invasive growth on solid media (Lambrechts *et al.*, 1996; Lo and Dranginis., 1996, 1998; Guo *et al.*, 2000).



The promoter region of *MUC1/FLO11* is one of the largest yeast promoters identified to date, with *cis*-acting elements present up to 2.4 kb upstream of the first coding triplet (Gagiano *et al.*, 1999a; Rupp *et al.*, 1999). The upstream regulatory region of *MUC1/FLO11* is almost identical to that of the *STA2* gene, which encodes an extracellular glucoamylase required for the utilization of extracellular starch (Pretorius *et al.*, 1991; Vivier *et al.*, 1997; Gagiano *et al.*, 1999b). The homology extends over a sequence of more than 3.5 kb, with the only significant difference being the presence of two inserts of 20 and 64 bp in the *MUC1/FLO11* promoter. The high homology suggests that the promoters of the two genes are co-regulated. Expression analysis confirmed that they are co-regulated to a large degree and that both require the same activators (Gagiano *et al.*, 1999a). However, differences were observed when basal expression levels of these genes were compared (Gagiano *et al.*, 1999a, b).

Mss11p appears to play a central role in the regulation of starch metabolism and pseudohyphal differentiation. The presence of multiple copies of *MSS11* in the cell results in elevated transcriptional levels of both *MUC1/FLO11* and *STA2* and consequently an increase in flocculation, pseudohyphal differentiation, invasive growth and the cell's ability to utilize starch (Gagiano *et al.*, 1999a, b). Deletion of *MSS11* leads to complete loss of these phenotypes, which cannot be reversed by overexpression of any of the other related factors identified to date (Gagiano *et al.*, 1999a, b). The regulation conferred by *MSS11* on the transcriptional levels of *MUC1/FLO11* and *STA2* also appears to be dependent on signals generated specifically in the presence of low nitrogen and low glucose (Gagiano *et al.*, 2003).

Mss11p does not have significant homology to any other yeast protein, with the exception of limited homology to the transcriptional activator Flo8p (Gagiano *et al.*, 1999a). Several distinctive domains are found in the *MSS11* gene product. Firstly Mss11p contains exceptionally long poly-glutamine and poly-asparagine domains. It also contains a putative ATP- or GTP-binding domain (or p-loop), commonly found in proteins such as kinases, ATPases or GTPases (Saraste *et al.*, 1990). Two short stretches close to the N-terminal,



denoted H1 and H2, share significant homology to another transcriptional activator, Flo8p (Gagiano *et al.*, 2003).

With the use of a comprehensive set of systematic deletions from both the N- and C-termini, we have identified two separate activation domains capable of stimulating RNA polymerase II dependent transcription as well as areas that appear to be involved in regulation of Mss11p's activation capability (Gagiano *et al.*, 2003; this paper is included as an appendix in this thesis since it formed the background for work presented in Chapter 4). Firstly, both the H2 domain and the extreme C-terminus are able to activate a reporter gene when fused to the DNA-binding domain of Gal4p. Interestingly an area of 92 amino acids that immediately precedes the H2 domain, containing the H1 domain and the putative P-loop, appears to negatively regulate the activity of the H2 domain. In all cases, deletion of the H1-P-loop results in increased levels of transcription compared to constructs where this area is present. The effect of an H1-P-loop deletion is especially clear when combined with deletion of the C-terminal domain, suggesting that the apparent regulatory influence of the H1-P-loop is directed specifically towards the H2 domain.

Work performed during the course of this study intended to address two separate areas of interest. Firstly we were prompted to investigate the possibility of chromatin playing a dynamic role in the regulation of *MUC1/FLO11* and *STA2* transcription and the possible role of Mss11p in the mediation thereof. Several lines of evidence suggest that these two promoters could be governed by the stringent control offered by regulated nucleosomal positioning and modification. At the outset the complexity and size of the *MUC1/FLO11* and *STA2* promoters and the amount of factors and pathways involved in their regulation may suggest the presence of such a form of regulatory mechanism. Also of notice is that despite the considerable degree of homology between the two promoter regions, basal levels of *STA2* transcription are consistently higher than that of *MUC1/FLO11* (Gagiano *et al.*, 1999b). Genetic screens have also revealed that *STA2* requires the presence of *SNF2*, the ATPase subunit of the SWI/SNF ATP-dependent chromatin-remodeling complex (Yoshimoto and Yamashita, 1991; Yoshimoto *et al.*,



1991, 1992; Yamashita, 1993 Kuchin *et al.*, 1993). In addition, a role for the ISWI chromatin-remodeling complex in the regulation of invasive growth has recently also been revealed (Kent *et al.*, 2001). Furthermore, cAMP mediated repression of *MUC1/FLO11* requires the general repressor Tup1-Ssn6 (Conlan and Tzamarias, 2001). Finally, regulation of *FLO1*, also a FLO family member, takes place in an extensive chromatin domain regulated by the activities of the SWI/SNF co-activator and the Tup1-Ssn6 co-repressor, raising the possibility that similar mechanisms could be in effect in the regulation of *MUC/FLO11* (Fleming and Pennings, 2001). On account of the importance of this section, the topic of the literature study section was chosen to be the involvement of chromatin in transcriptional regulation in *Saccharomyces cerevisiae*.

However, consistent data could not be generated with regards to the nucleosome positioning on the *MUC1/FLO11* promoter. For this reason, we shifted the focus of this study by investigating the cellular and molecular functioning of *MSS11*. A selected subset of Mss11p truncations was used that would highlight the activity of the two activation domains (H2 and the C-terminal) as well as the apparent regulatory influence of the H1-P-loop. The Mss11p derivatives was also exploited to reveal any genetic interaction between Mss11p and other factors (*FLO8*, *STE12*, *TEC1*, *MESN1*, *PHD1* and *RME1*), considered as being important in the regulation of *MUC1/FLO11* and the establishment of pseudohyphal differentiation and invasive growth. In this manner we have established that Mss11p is responsive to carbon and nitrogen signaling and that regulation exerted by Mss11p in these conditions is dependent on the H2 domain, which is negatively regulated by the H1-P-loop. We also show that the C-terminal is essential in the regulation of pseudohyphal differentiation, invasive growth, starch degradation and flocculation by Mss11p. This could implicate the C-terminal in an indispensable function such as DNA binding, nuclear import, binding to other DNA associated proteins, or mediating the activities of other proteins or complexes that are required for transcriptional activation. It was also found that Mss11p requires the presence of *FLO1* to induce Ca<sup>2+</sup>-dependent flocculation.



## 1.2 PROJECT AIMS

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The following aims were set out in this project:

1. (i) to establish if chromatin is involved in the transcriptional regulation of *MUC1/FLO11* and *STA2*;  
(ii) to determine the influence of various growth conditions on nucleosomal positioning in the promoters of *MUC1/FLO11* and *STA2*;  
(iii) to establish a link between the factors involved in the regulation of these genes, and the state of chromatin in the regulatory regions;
  
2. (i) to further investigate the nutrient responsiveness of Mss11p;  
(ii) to analyse the roles played by the various domains of Mss11p in the regulation of transcription and pseudohyphal development ;  
(iii) to determine possible interactions between *MSS11* and other factors involved in the regulation of *MUC/FLO11*;  
(iv) to study the regulation of Ca<sup>2+</sup>-dependent flocculation by *MSS11*:

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**CHAPTER 2**

**LITERATURE REVIEW**

**CHROMATIN AND TRANSCRIPTIONAL  
REGULATION IN *SACCHAROMYCES  
CEREVISIAE***

## 2.1 INTRODUCTION

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The basic repeating structure of chromatin, the nucleosome, provides the framework for compaction of the genome and also plays a highly dynamic role in the regulation of transcription (for review see Horn and Peterson, 2002). The nucleosome consists of two copies of histones H2A, H2B, H3 and H4, around which ~ 147 bp of DNA are tightly wound. Chromatin not only functions by compacting the entire eukaryotic genome into the volume of a nucleus, but also plays a highly dynamic role in the regulation of transcription. In *Saccharomyces cerevisiae* nucleosome loss *in vivo* by depletion of histone H4 results in a significant increase or decrease in 15% or 10% of genes, respectively (Wyrick *et al.*, 1999), illustrating the requirement for the correct nucleosomal architecture in both gene activation and repression. In principle, regulating chromatin structure and accessibility could control all nuclear processes that need to gain access to the genome. By controlling access of DNA to regulatory factors, the transcriptional machinery and co-factors, the state of activation or repression of a gene can be tightly governed by the state of the encompassing chromatin. Two distinct classes of chromatin modifying activities regulate the accessibility of the DNA template to binding factors. ATP-dependent complexes move nucleosomes, thereby exposing or occluding DNA sequences, and can also create conformations where DNA is more accessible on the surface of the nucleosome (for a recent review see Peterson, 2002). The other class of complexes can covalently modify the N-terminal “tails” of core histones in a reversible fashion. Such modifications include acetylation, methylation, phosphorylation and ubiquitination (reviewed by Izuka and Smith, 2003). These modifications determine interactions of histones with DNA and other proteins, which in turn may regulate chromatin structure (Strahl and Allis, 2000).

Our understanding of the delicate interplay between chromatin structure and transcriptional regulation has been aided by the use of the powerful techniques available in yeast genetics (reviewed by Gregory, 2001). Many of



the characteristics of chromatin in higher eukaryotes are observed in *S. cerevisiae*. One of the main advantages of studying chromatin in yeast, apart from the ease of genetic manipulation, is the presence of only two copies of each of the genes encoding the core histones per haploid genome (compared to the estimated 110 copies of each of the histone genes in *Drosophila melanogaster*).

This chapter is a summary of our current knowledge concerning the involvement of chromatin in transcriptional regulation in the yeast *S. cerevisiae*. First of all, the structural components of chromatin and their involvement in transcriptional regulation will be discussed. In the second section, the components and functioning of multi-protein complexes that mediate changes in chromatin structure and accessibility, will be reviewed. Finally, the specific regulation established by coordination between these complexes and the transcriptional machinery will be considered.

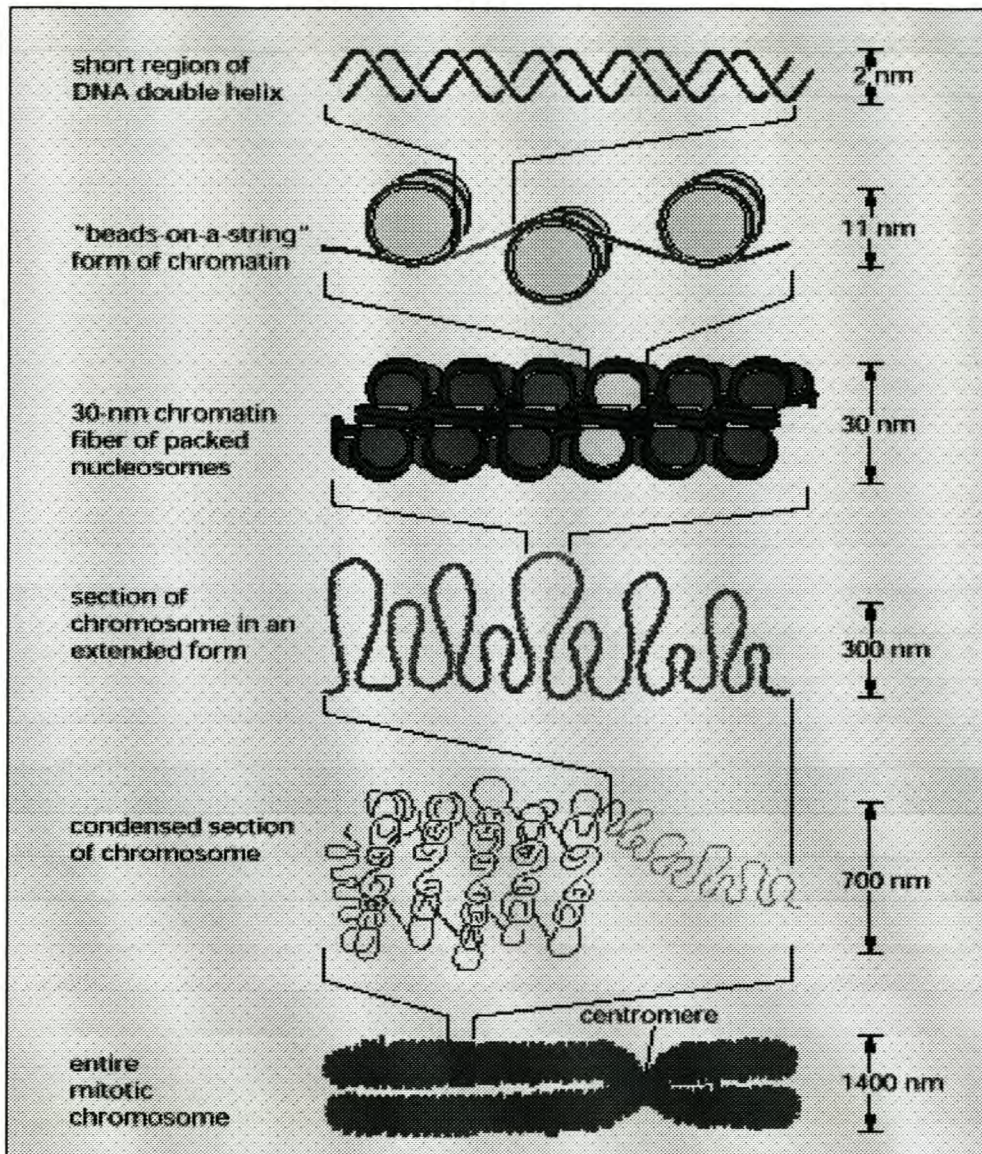
## **2.2 CHROMATIN STRUCTURE**

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### **2.2.1 THE NUCLEOSOME**

Chromatin is organised into a hierarchy of structures ranging from the basic repeating structure, the nucleosome core particle (NCP), to the condensed state observed in metaphase chromosomes (Figure 2.1). The nucleosome is composed of two copies of each of the four histone proteins H2A, H2B, H3 and H4 (Luger *et al.*, 1997; White *et al.*, 2001). Around this protein centre two tight superhelical turns of ~ 147 bp of DNA are wrapped. Histones H2A/H2B and H3/H4 dimerize through non-covalent interaction between the C-terminal histone fold domain (Arents and Moundrianakis, 1995; Luger *et al.*, 1997). The integrity of the nucleosome is further maintained by polar and hydrophobic interactions between H2B and H4 and between two molecules of H3 within the NCP. The repeating nucleosome cores, connected by a variable length of linker DNA, are further compacted into higher order assemblies of unknown architecture. In higher eukaryotes, addition of linker histone H1 further contributes to the stabilisation of the 30 nm chromatin fibre.



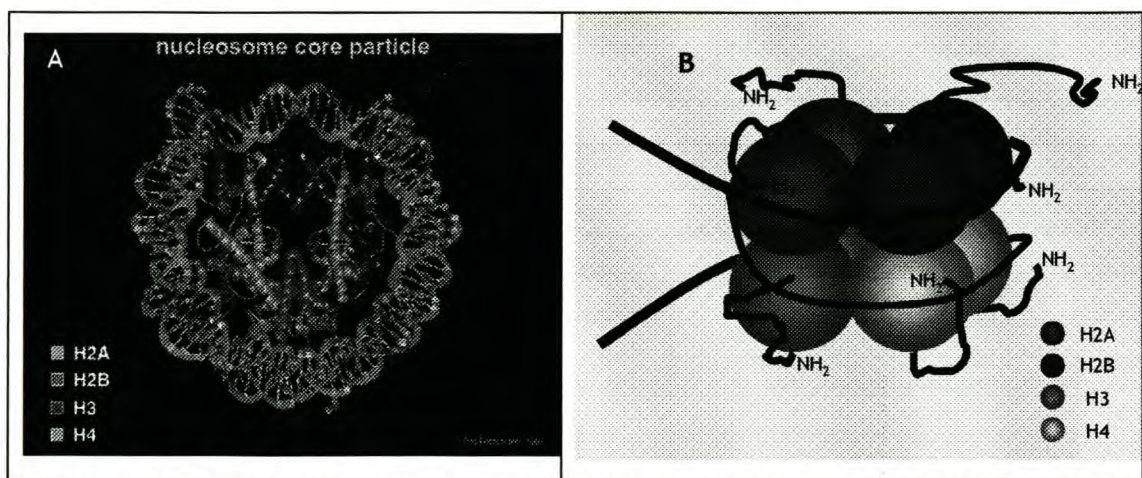


**Figure 2.1** Multiple levels of chromatin folding. DNA compaction occurs through a hierarchy of histone-dependent interactions and can be subdivided into primary, secondary and tertiary levels of structure. Strings of nucleosomes comprise the primary structural subunit. Formation of a 30-nm fibre through histone tail-mediated nucleosome-nucleosome interactions provides a secondary level of compaction, whereas tail-mediated interaction of individual fibres produces tertiary structures (such as condensed chromosomes). Tertiary structures observed in yeast, however, do not fold into the same level of compaction as in higher eukaryotes. Adapted from Alberts *et al.* (1998).

Our understanding of nucleosome and chromatin structure has been furthered by the determination of a variety of three dimensional nucleosome structures, including the yeast NCP (Figure 2.2; Luger *et al.*, 1997; Richmond *et al.*,



1988; White *et al.*, 2001; for review see Luger, 2003). Despite the distinct sequence divergence between histone proteins of *S. cerevisiae* and metazoans (Thatcher and Gorovsky, 1994; White *et al.*, 2001; Sullivan *et al.*, 2002; <http://genome.nhgri.nih.gov/histones>), the overall architecture of the histone octamer, as well as all of the residues that are involved in direct protein-DNA interactions, are unchanged between *S. cerevisiae* and *X. laevis* (White *et al.*, 2001), indicating that the general mechanism by which the histone octamer interacts with and distorts linear DNA into a tight superhelix is maintained between yeast and higher eukaryotes. Interestingly, changes were observed with regards to protein-protein interaction within the yeast nucleosome core particle. The most significant changes are located in the H2A L1 loops, involved in the interaction between H2A-H2B dimers. As this region is seemingly involved in holding the two gyres of the superhelix, even a subtle destabilization could have marked effects on overall nucleosome stability during transcription. This finding correlates with experimental evidence indicating significant destabilisation of yeast nucleosome core particles in mononucleosomes and in nucleosomal arrays, suggesting a less constrained structure compared to that of higher eukaryotes (Lee *et al.*, 1982; Morse *et al.*, 1987; Piniero *et al.*, 1991).

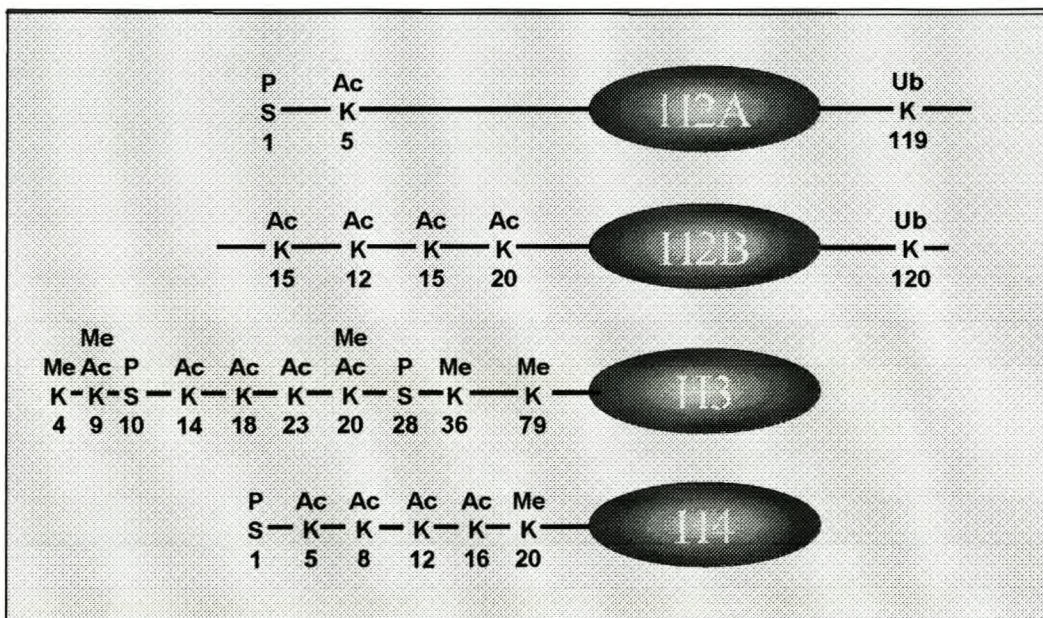


**Figure 2.2** Overall structure of the nucleosome core particle (NCP). (A) Front view of the NCP, viewed down the superhelical axis (Luger *et al.*, 1997). (B) Diagrammatic representation of the NCP, with histone tails extending from the structure



## 2.2.2 THE CORE HISTONES

Because of the central functional role played by the nucleosome, the core histones are among the slowest evolving, highest conserved proteins in eukaryotes. Characteristic of all eukaryotic core histones are the C-terminal core domain (referred to as the histone fold), and the amino terminal tail domain, which reaches outside the nucleosome and the wrapped DNA. The histone fold is involved in the formation of specific histone heterodimers and also determines the path of the DNA within the nucleosome. The unique DNA binding abilities of the histone fold motif has assured that it has remained conserved in a range of evolutionary diverse organisms such as archeal bacteria, fungi, insects, birds, amphibians and vertebrates (Luger, 2003; Sullivan *et al.*, 2002).



**Figure 2.3** The histone tails. The N-terminal histone tails are indicated as straight lines with lysine (K) and serine (S) residues indicated. Possible histone modifications are indicated: acetylation (Ac), methylation (Me), phosphorylation (P) and ubiquitination (Ub). Adapted from Pérez-Martín (1999).

The histone amino terminal represents a distinct functional domain referred to as the histone tail. The tail domain is highly basic and binds DNA *in vitro* and in native chromatin (Luger and Richmond, 1998). These domains are not required for assembling or maintaining the structure of the nucleosome core



and removal thereof results only in slight differences in hydrodynamic shape and stability of the nucleosome (Ausio *et al.*, 1989; Hayes *et al.*, 1991; Polach *et al.*, 2000; Schwarz *et al.*, 1996; Zheng and Hayes, 2003). However, the tail domains are essential for the folding of oligonucleosomal arrays into 30 nm chromatin fibres and are probably required for efficient assembly of fibres into higher order structures (Allan *et al.*, 1982; Garcia-Ramirez *et al.*, 1992). Histone tails undergo numerous post-translational modifications, which either directly or indirectly alter chromatin structure to facilitate transcriptional activation or repression (Figure 2.3; Hong *et al.*, 1993; Strahl and Allis, 2000; Jenuwein and Allis, 2001; Wu and Grunstein, 2000). Thus, modifications of these domains are an endpoint to many signaltransduction pathways directed to the nucleus (Cheung *et al.*, 2000).

Two types of genetic manipulations have been favoured to study the role played by histones in transcription: alterations of histone expression and mutations in the genes coding for the core histones. One of the earliest screens that linked chromatin to transcription in yeast was the study of mutations able to overcome the effect of Ty insertions in the promoter region of *LYS2* and *HIS4* (Simchem *et al.*, 1984; Winston *et al.*, 1984). SPT mutants (Suppressor of Ty) mutants included a group of genes involved in both chromatin structure and transcriptional regulation. Among them the *HTA1-HTB1* locus, which encodes for histones H2A and H2B, was identified (Winston *et al.*, 1984). By variation of either H2A-H2B or H3-H4 gene dosage *in vivo* it was found that the observed suppression of transcriptional defects was due to an imbalance in the syntheses of H2A-H2B relative to H3-H4 (Clark-Adams *et al.*, 1988; Norris *et al.*, 1988). In a more directed study, histone H4 depletion was investigated by placing the histone H4 gene under the control of the *GAL1* promoter in a strain not containing the native H4 genes (Kim *et al.*, 1988). When grown in the presence of glucose H4 was depleted. The induced nucleosome loss led to the activation of repressed genes like *PHO5*, *CYC1* and *GAL1*. The effect of nucleosomal loss on genome-wide transcription gives insightful perspectives on the role of the nucleosome in transcriptional regulation (Wyrick *et al.*, 1999). Expression is increased in only 15% and repressed in 10% of genes by more than three



fold, while 75% remain unaffected. This highlights the requirement of correct nucleosomal architecture in both gene activation and repression.

A second set of genes (the SWI/SNF genes) were obtained from genetic screens directed towards the analysis of the transcriptional regulation of the *HO* gene, encoding an endonuclease (required for mating type switching), and the *SUC2* gene, encoding an invertase (required for growth on sucrose and raffinose) (Stern *et al.*, 1984; Neigeborn *et al.*, 1984). Several genes were identified as activators of *HO* (referred to as *SWI* genes, for switching), and *SUC2* (referred to as *SNF* genes, for sucrose non-fermenting). A subsequent study of suppressors of defects in the SWI/SNF genes further strengthened a link to chromatin. A specific SIN mutation (for SWI/SNF-independent) was found to lie in the coding region of the histone H3 gene (Kruger *et al.*, 1995). Other histone SIN mutants have recently been identified (Fleming and Pennings, 2001). Histone SIN mutations are situated at the protein DNA binding interface of histones H3 and H4. These mutations could facilitate access of transcription factors to chromatin by destabilising interactions between the histone H3-H4 tetramer and the H2A-H2B dimer, by disrupting histone DNA interactions or by preventing higher order folding of chromatin. How the state of the nucleosome itself can be fine-tuned to achieve tightly regulated transcription at specific loci will be discussed in following sections.

### 2.2.3. THE LINKER HISTONE

Histone H1, encoded by the *HHO1* gene, associates with the DNA linking the core histone octamers and is therefore referred to as the linker histone. In higher eukaryotes the linker histone plays a vital role in stabilising and maintaining higher order chromatin structures and also participates in transcriptional repression (Layborn and Kadonaga, 1991). *S. cerevisiae* Hho1p has sequence homology to other known linker histones and was shown to interact with nucleosomes *in vitro* (Patterton *et al.*, 1998; Baxeavanis and Landsman, 1998). However, unlike the core histones, deletion of the *HHO1* gene does not result in any clear phenotypic effects. There is an



approximate stoichiometry of 1 molecule of Hho1p to 37 nucleosomes (compared to a 1:1 ratio observed in higher eukaryotes) (Freidkin and Katcoff, 2001), which is not surprising since there is also no observed difference between nucleosome spacing in *hho1Δ* cells compared to wild type. This could also explain why yeast chromatin does not fold into more dense and highly compacted chromatin such as found in higher eukaryotes (Figure 2.1). Recently a possible role for *HHO1* has been indicated in DNA repair through homologous recombination (Downs *et al.*, 2003).

#### **2.2.4. HISTONE VARIANTS**

Most histone modifications take place on their amino- and carboxy-terminal tails, presumably because these are on the chromatin surface and therefore accessible to enzymatic modification. Regions buried within the nucleosome may not be accessible to enzymatic activities; however, sequence variations have evolved in these regions, and histones differing in these regions are referred to as histone variants. Histone variants within any species are highly conserved, specialized histones that co-exist with the major histone types in the nucleus and have the potential to locally alter chromatin structure. In *S. cerevisiae* the histone H2A variants, H2A.X and H2A.Z, and also the centromere specific H3 variant, CenpA (encoded by *CSE4*), are present. H2A.X is associated with sites of DNA double strand breakage, where it is considered to mark such sites by securely anchoring into the surrounding chromatin (for review see Redon *et al.*, 2002). H2A.Z displays 60% homology to H2A and 90% between species. Mutagenic analysis has demonstrated that H2A.Z is essential for development in yeast (Jackson and Gorovsky, 2000). H2A.Z has been localised to yeast promoters and displays a redundant role with ATP-dependent chromatin remodeling complexes and interacts directly with the transcriptional machinery during gene expression (Santisteban *et al.*, 2000; Adam *et al.*, 2001). However, the functional dynamics of H2A.Z enrichment in active chromatin remains enigmatic as other studies describe H2A.Z deposition to have a repressive effect on gene expression (Dhillon and Kamakaka, 2000). More recently H2A.Z was shown to antagonize the Sir3p dependent propagation of repressive heterochromatin stretches from HMR, a



silent mating type locus in *S. cerevisiae* (Meneghini *et al.*, 2003). H2A.Z was also shown to be involved in derepression of telomere proximal genes (Meneghini *et al.*, 2003)

### 2.2.5 NON-HISTONE CHROMATIN ASSOCIATED PROTEINS

Selective association of non-histone proteins with the complex of DNA and histone can modify chromatin structure. Primary among these are the high mobility group (HMG) proteins. Based on amino acid sequence motifs, DNA binding characteristics and molecular mass, three types of HMG's can be distinguished: HMGB; HMGN, and HMGA (Bustin, 2001). Only members of the HMGB superfamily have been found in yeast, which is characterised by the presence of one or more copies of an 80 amino acid domain (the HMG box) responsible for DNA binding (Landsman and Bustin; 1993; Bustin, 2001). The HMGB superfamily is composed of two subfamilies specified by their abundance, function and DNA specificity. The HMG domain transcription factors, typified by the testis-determining factor *SRY*, are of low abundance, usually containing a single HMG box and bind site specifically to promoter regions of regulated genes. In contrast, the ubiquitous non-histone chromosomal proteins of HMGB, typified by *HMG-1/2*, are characterised by their moderate DNA binding affinity with minimal sequence specificity and recognition of pre-bent and modified DNA (for review see Bustin, 1999).

In yeast, *SIN1/SPT2* was initially identified in two different screens and characterised as a transcriptional repressor with properties related to HMG proteins (Roeder *et al.*, 1985; Kruger and Herskowitz, 1991). Further detailed analysis indicated that under certain conditions *SIN1* could act as a transcriptional activator, but more importantly a functional interaction between *SIN1* and the C-terminal domain of RNA polymerase II was shown (Peterson *et al.*, 1991). Genetic and biochemical evidence points to interaction between Sin1p and the SWI/SNF chromatin-remodeling complex (Pérez-Martín and Johnson, 1998). This could implicate a role for Sin1p as a regulatable bridge between the SWI/SNF complex and chromatin.



The *NHP6A* and *NHP6B* genes encode two highly identical, functionally redundant proteins which contain a single HMG box (Kolodrubetz and Burgum, 1990; Lu *et al.*, 1996). The Nhp6 proteins are highly abundant, with about 50 000 copies of Nhp6a and Nhp6b combined found per haploid nucleus (Paull *et al.*, 1996). Deletion of both genes leads to significant phenotypes including aberrant transcription, slow growth and temperature sensitivity (Sidorova and Breden, 1999; Yu *et al.*, 2003; Costigan *et al.*, 1994). Purified *NHP6* was shown to bind nucleosomes *in vitro* and subsequently recruits Spt16-Pob3 to form the SPN (Spt16-Pob3-Nhp6) complex that leads to increased nuclease sensitivity of the nucleosomes (Formosa *et al.*, 2001). Nhp6 also associates with the yeast CP complex, a 1:1 complex of Cdc68 and Pob3p (a structural homologue of the vertebrate FACT complex) and mediates CP-related phenotypes *in vivo* (Brewster *et al.*, 2001). Both the FACT and CP complexes are implicated in interaction with the transcription machinery and in the process of transcriptional elongation (Orphanides *et al.*, 1998). In the regulation of *HO* gene expression, Nhp6p functions in parallel with Gcn5p (the histone acetyl transferase of the SAGA complex) by promoting TBP (TATA-binding protein) binding, and hence stimulating transcription (Yu *et al.*, 2003).

### **2.3 NON-STRUCTURAL PROTEINS ASSOCIATED WITH CHROMATIN**

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Apart from the structural components of chromatin, several other proteins have been implicated in chromatin-mediated regulation of transcription. The following sections will discuss in more detail the complexes required for stringent regulation of transcription through the remodeling and modification of chromatin, as well as interactions between these complexes.

### **2.4 ATP-DEPENDENT CHROMATIN REMODELING**

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Although initial studies focused on nucleosomes, subsequent studies identified multi-protein complexes that controlled transcription by manipulating nucleosome structure and/or interactions. One class of chromatin remodeling factors is represented by of a family of ATP-dependent complexes that utilise ATP hydrolysis to enhance or suppress the accessibility of nucleosomal DNA.



This family is further subdivided into three subgroups based on their biochemical properties and the overall sequence similarities of their ATPase subunits: (i) the SWI/SNF group; (ii) the ISWI group and (iii) the Mi-2/CHD group (Boyer *et al.*, 2000). Each of the separate ATPase subunits associates and forms complexes with different additional proteins of which the exact role remains unclear (Figure 2.4). The function of these complexes is conserved in eukaryotes. In *S. cerevisiae* four ATPases have been purified as members of distinct complexes: SWI/SNF; RSC (remodels the structure of chromatin), and ISW1 and ISW2 (imitation switch). Most studies on chromatin remodeling complexes have focused on their roles in transcriptional regulation; there is however strong evidence suggesting roles in replication, DNA repair and recombination (for review see Kadam and Emerson, 2002). This section will only be concerned with the effect that these activities have on transcriptional regulation.

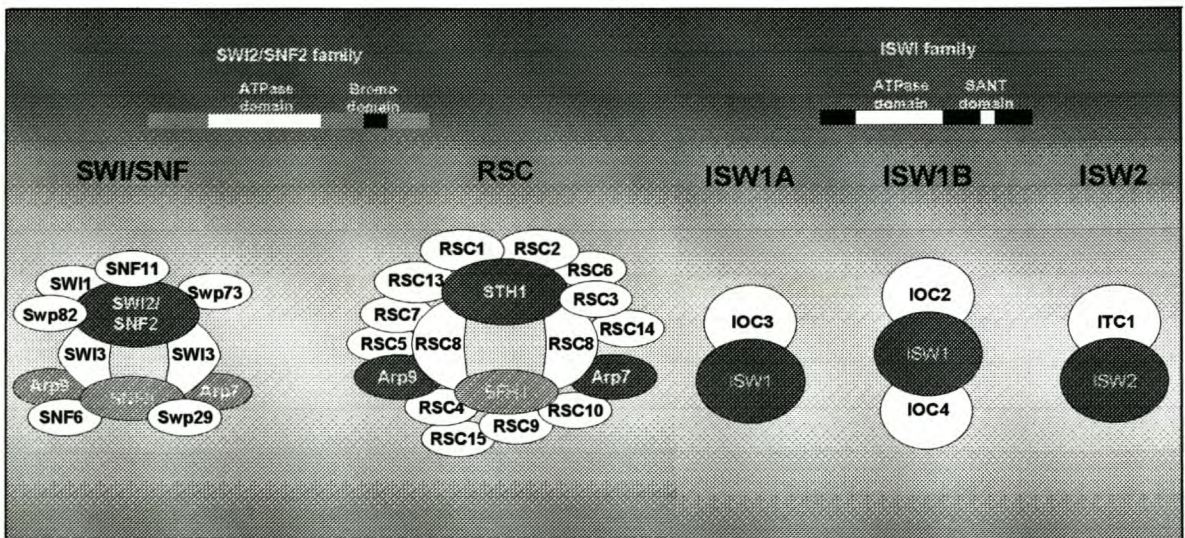


Figure 2.4 Yeast ATP-dependent remodeling complexes.

## 2.4.1. SWI/SNF AND ASSOCIATED COMPLEXES

### 2.4.1.1 SWI/SNF

SWI/SNF is a 2 MDa multisubunit complex that was first discovered in *S. cerevisiae* in two separate screens for altered gene expression (for review see Martens and Winston, 2003). SWI/SNF is highly conserved in eukaryotes with homologous complexes identified in *Drosophila* and humans. The SNF



(sucrose non-fermenting) genes were identified by mutations that caused aberrant expression of the *SUC2* gene, which is required for growth on sucrose and raffinose as carbon sources (Neigeborn and Carlson, 1984). Similarly the *SWI* (switching) genes were isolated as mutations that led to defective expression of the *HO* gene, which is required for mating type switching (Stern *et al.*, 1984).

SWI/SNF plays a well characterised role in transcriptional activation, with various well-documented examples of interactions on several promoters of genes such as *HO*, *SUC2*, *PHO5*, *PHO8* and *FLO1* (Fleming and Pennings, 2001; for review see Martens and Winston, 2003). Substantial evidence has also shown that SWI/SNF can repress transcription and recent studies have demonstrated that SWI/SNF directly contributes to *SER3* repression by altering chromatin structure (Martens and Winston, 2002). Therefore, differential use of the same complex may occur at promoters that are activated or repressed by SWI/SNF. Mutations in both *SWI* and *SNF* genes cause pleiotropic phenotypes, suggesting a global role for SWI/SNF in the regulation of gene expression. Genome wide expression studies however indicate that less than 5% of yeast genes require a functional SWI/SNF complex (Sudarsanam *et al.*, 2000). Furthermore SWI/SNF is estimated to be a relatively rare complex in yeast, with only 100-500 copies present per cell (Coté *et al.*, 1994; Peterson and Workman., 2000). These observations support a gene specific targeting of SWI/SNF to achieve regulated activation or suppression. Purified SWI/SNF was shown to interact with a variety of transcriptional activators such as Gcn4p, Swi5p, Gal4-VP16 and Gal4-AH (Natarajan *et al.*, 1999; Neely *et al.*, 1999; Neely *et al.*, 2002). These interactions are mediated by the acidic activation domain and are sensitive to mutations that compromise activation function. Three subunits of yeast SWI/SNF were recently shown to interact with a specific set of activators *in vitro* (Neely *et al.*, 2002). Interactions between specific SWI/SNF components and DNA-bound proteins may provide selectivity among SWI/SNF function. Similar to what is observed in gene activation, SWI/SNF mediated repression



seems to require its targeted recruitment by gene-specific repressors (Dimova *et al.*, 1999).

SWI/SNF complexes are comprised out of at least nine or more proteins including both conserved (core) and non-conserved components. The ATPase activity of Swi2p/Snf2p, the ATPase subunit of the yeast SWI/SNF complex, is stimulated equally well by free DNA or nucleosomal DNA and possesses remodeling activity. Genetic analysis indicates that most of the SWI/SNF subunits are required for function *in vivo*. Still, the exact biological roles of the other subunits remain to be clarified (Tsukiyama, 2002; Hirschhorn *et al.*, 1992; Martens and Winston, 2002). The Snf5p subunit seems to be required for remodeling *in vivo* since an  $\Delta snf5$  mutant is unable to remodel chromatin on the *SUC2* promoter, yet was proficient for SWI/SNF assembly and recruitment to the *SUC2* promoter (Geng *et al.*, 2001). As stated above, a subset of SWI/SNF subunits associates with DNA bound regulatory proteins, and three distinct subunits, Swi2p, Snf5p and Swi1p, have been shown to interact with a set of activators *in vitro* (Neely *et al.*, 2002).

All Swi2p/Snf2p ATPases contain a motif found in several transcription factors, referred to as the bromodomain, which can bind acetylated lysine residues in histone N-terminal tails (Jacobson *et al.*, 2000; 1999; Hassan *et al.*, 2002). Stable promoter occupancy by SWI/SNF requires either activator binding or acetylated histones. The bromodomain of Swi2p/Snf2p is required to maintain promoter occupancy *in vivo* and *in vitro* (Hassan *et al.*, 2001b; Hassan *et al.*, 2002). It was shown that the bromodomain of Swi2p/Snf2p is required for binding to the SWI/SNF dependent promoter of *SUC2* (Hassan *et al.*, 2002). Two other motifs also play important roles in the functioning of SWI/SNF remodeling complexes. Firstly, the DNA binding properties of SWI/SNF are very similar to proteins containing an HMG-box (Quinn *et al.*, 1996). Studies in *Drosophila* and human have demonstrated that mutants of the SWI/SNF-like complexes BAF and BRM, with lacking HMG domains, are defective in normal regulation (Chi *et al.*, 2002; Papoulas *et al.*, 2001). Whether this holds true for the yeast SWI/SNF complex remains to be



investigated. Secondly, the SANT domain was recently described as a motif present in several eukaryotic transcriptional regulators. The SANT motif is essential for the *in vivo* functions of three yeast proteins, Swi3p (SWI/SNF subunit), Ada2p (SAGA subunit) and Rsc8p (RSC subunit). Deletion of these motifs resulted in mutant phenotypes similar to defective SWI/SNF, SAGA and RSC complexes (Boyer *et al.*, 2002). These observations are consistent with a general role for the SANT domains in functional interactions with histone N-terminal tails; a possible role in recruitment or catalytic activity can, however, not be excluded.

#### **2.4.1.2 THE SWI2/SNF2-LIKE ATPASE CONTAINING COMPLEX, RSC**

In addition to SWI/SNF, the prototypical ATP-dependent chromatin-remodeling complex, the SWI/SNF subfamily also includes the yeast RSC complex. RSC (remodels the structure of chromatin) is an abundant, 15 subunit chromatin remodeling complex of *S. cerevisiae* (Cairns *et al.*, 1996). Unlike SWI/SNF, RSC is essential for viability, making genetic analysis more difficult than for SWI/SNF.

Whole genome analysis of two components of RSC, Rsc3p and Rsc30p, which appear to play a role in the targeting or regulation of the complex, reveals different effects on the regulation of ribosomal protein genes, cell wall integrity and the nitrogen discrimination pathway (Angus-Hill *et al.*, 2001). Like SWI/SNF, RSC appears to be involved in activation and repression. This is further confirmed by two genome-wide localization studies, where RSC localized to both repressed and activated promoters (Ng *et al.*, 2002b; Damelin *et al.*, 2002). There are, however, differences between results obtained by the two studies, making it difficult to predict a specific cellular pathway(s) to which the complex seems responsive. Interestingly, the RSC complex is generally recruited to RNA Pol III promoters and specifically recruited to RNA Pol II promoters by transcription activators or repressors (Ng *et al.*, 2002b).



## 2.4.2 ISWI ATPASE CONTAINING COMPLEXES

The second group of ATP-dependent chromatin remodeling complexes contain the ISWI (imitation switch) protein as the ATPase subunit. Isw1p and Isw2p, two ISWI-related proteins, were identified in yeast based on their sequence homology to the ATPase domain of *Drosophila* ISWI (Tsukiyama *et al.*, 1999). The ISWI-containing complexes are smaller and contain fewer subunits than their SWI/SNF counterparts and unlike SWI/SNF the ISWI complexes are generally associated with maintaining transcriptional repression. Isw2p associates with Itc1p to form a heterodimer (Gelbart *et al.*, 2001). Isw1p co-purifies with three other proteins referred to as Ioc2p, Ioc3p and Ioc4p, and coexists as two separable complexes in yeast: Isw1a (comprising of Isw1p and Ioc3p) and Isw1b (comprising of Isw1p, Ioc2p and Ioc4p) (Vary *et al.*, 2003).

Of the two complexes, ISW2 has received most research attention. Deletion of ISW2 leads to the derepression of several meiosis specific genes under normal growth conditions (Goldmark *et al.*, 2000). It was found that repression of the meiotic *REC104* promoter involves the targeting of the ISW2 complex to the promoter through direct interaction with the sequence-specific Ume6p repressor. Ume6p requires the ISW2 complex to establish a repressive chromatin structure, which is further stabilised through deacetylation by the SIN3-RPD3 histone deacetylase complex. Additional data suggests that SIN3-RPD3 and ISW2 complexes pool resources to repress the transcription of Ume6p-dependent and some Ume6-independent genes and that the ISW2 complex functions by creating DNaseI-inaccessible chromatin structures at the promoters of many of these genes (Fazzio *et al.*, 2001). Both subunits of the ISW2 complex (Isw2p and Itc1p) are required for interaction with nucleosomal arrays, as well as for stimulation of ATPase and remodeling activities *in vitro*. In addition, *isw2* and *itc1* deletion mutants have virtually identical phenotypes (Gelbart *et al.*, 2001).

Until recently, Isw1p was considered to be the ATPase subunit of a single four-protein complex. However, two complexes, ISW1a and ISW1b, have



overlapping functions in the transcriptional regulation of some genes, yet distinct functions at others (Vary *et al.*, 2003). The effects of deletion of either *ISW1* or genes encoding associated subunits were, however, subtle, with not more than a two fold variation in transcription. Possible explanations for the small effect could be that, like the ISW2 complex, ISW1 also functions in parallel with factor(s) such as SIN3-RPD3, which could soften effects observed when deleting only *ISW1*. Another explanation could be that the ISW1 complexes regulate nuclear processes other than transcription, since it has been shown that ISW1 does affect chromatin at several genes *in vivo* without altering transcription levels (Kent *et al.*, 2001)

### **2.4.3 MECHANISM OF ATP-DEPENDENT REMODELING**

Understanding the mechanism of ATP-dependent remodeling has been a major area of investigation. Biochemical analysis has demonstrated several different outcomes of ATP-dependent remodeling *in vitro*, however little is known about the outcomes of remodeling *in vivo*. Outcomes observed through biochemical assays include nucleosome sliding, octamer transfer to another DNA molecule, dinucleosome formation and alteration of nucleosome structure.

The most tangible mechanism for catalysing DNA exposure on a chromatin template entails “sliding” of DNA with respect to the histone octamer (Meerseman *et al.*, 1992). Sliding involves identical amounts of movement of the entry and exit points of the DNA in the same direction, resulting in an octamer that is repositioned. This would end up leaving DNA originally associated with the histone octamer to be non-nucleosomal. Analysis of starting and ending positions of nucleosomes on defined fragments of DNA indicates that SWI/SNF shifts nucleosomes to new positions *in cis* (on the same strand of DNA) (Whitehouse *et al.*, 1999). SWI/SNF action on nucleosomal arrays blocked certain restriction enzyme sites in linker regions, suggesting the SWI/SNF repositioned nucleosomes over these previously accessible sites (Jaskeliof *et al.*, 2000). Visualisation of nucleosomal arrays by atomic force microscopy before and after remodeling by human SWI/SNF



association at a different site on the nucleosome will result in a bulge or loop which could be transmitted around the octamer. SWI/SNF, as well as other remodeling enzymes, are capable of generating superhelical torsion (Havas *et al.*, 2000). The possibility that SWI/SNF remodeling creates a bulge or loop that is propagated around the nucleosome is strongly supported by in-depth biochemical studies of the remodelled state (Narlikar *et al.*, 2001). Such findings are consistent with the idea of a wave of accessible DNA caused by translocation induced topological stress.

Twisting of DNA could however be a natural consequence of DNA translocation and therefore not be the sole mechanism of SWI/SNF remodeling (Aoyagi *et al.*, 2002). In a recent paper, RSC, the Swi2p/Snf2p homologue, was tested for ATP-dependent DNA translocation activity. Mechanical disruption of nucleosome-DNA interaction was found to be the more likely mechanism for displacement rather than the propagation of a conformational change such as twisting (Saha *et al.*, 2002). Two investigations by Lorch and colleagues suggest that RSC binds at the entry/exit site on a nucleosome (Lorch *et al.*, 1998; Lorch *et al.* 2001). These results would be fitting a model where RSC assumes a fixed position at the DNA entry/exit site on the nucleosome, and its translocation activity causes a break in DNA histone contacts, generating a wave of DNA that propagates around the nucleosome (Saha *et al.*, 2002). Similarly, Aoyagi and colleagues (2002), predict a "loop-recapture" model for human SWI/SNF remodeling, in which a DNA bulge is transmitted around the nucleosome in a wave like manner. These models are consistent with previous findings that indicated that SWI/SNF remodeling requires changes in DNA topology (Gavin *et al.*, 2001; Guyon *et al.*, 2001). Also the introduction of DNA nicks, a modification that will block transmission of twist in DNA, does not inhibit the remodeling capacity of human SWI/SNF or RSC (Saha *et al.*, 2002; Aoyagi *et al.*, 2002).

In contrast to the SWI/SNF family, all of the data available for the ISWI-based complexes are consistent with sliding of the DNA being the main mechanism (Hamiche *et al.*, 1999; Aalfs *et al.*, 2001; Langst and Becker, 2001). It is therefore possible that SWI/SNF and ISWI-based complexes expose



also showed clear changes in nucleosomal distribution (Schnitzler *et al.*, 2001).

Sliding does, however, not explain how substantial tracts of DNA can be made accessible in regions of tightly spaced nucleosomes. Mechanisms that could expose DNA sequences within the boundaries of the histone octamer would provide a means by which DNA could be exposed in regions of tightly packed nucleosomes without a requirement for repositioning. This hypothesis is supported by several observations. Firstly SWI/SNF increases the sensitivity of DNase and restriction enzyme of DNA sites within a mononucleosome (Kingston and Narlikar, 1999). A sliding model cannot explain this observation since mononucleosomes do not have flanking regions to facilitate a sliding action. Secondly, another form of remodelled chromatin has been characterised as having many of the properties expected for a dinucleosome. (Lorch *et al.*, 1998; Schintzler *et al.*, 2001). Such species could be generated as the result of the association of nucleosomes that have been moved to the end of DNA fragments leaving DNA binding sites on the octamer surface exposed. Similar structures might also be generated during the transfer of a histone octamer from one chromatin fibre to another (Lorch *et al.*, 1999; Phelan *et al.*, 1999).

The high-resolution structure of the nucleosome core particle provides insight into how DNA might be moved over the surface of nucleosomes (Luger *et al.*, 1997). Unequal lengths of DNA are wrapped on either side of the nucleosome, resulting in the underwinding of DNA over a 10bp region on the shorter DNA half. Application of torsion to DNA, by remodeling activity that is also tethered to a histone component, could result in the alteration of DNA twist on the surface of a nucleosome. In a topologically closed system this twist can be partitioned between twist and wrythe, which will change the trajectory of the helical axis of the DNA duplex, causing the lifting of a DNA off the octamer surface. The propagation of this wrythe over the surface of a nucleosome would result in the movement of DNA over the surface of a nucleosome by diffusion. This could have the effect of pushing the DNA off the nucleosome. Once histone DNA interaction has been disrupted, re-



nucleosomal DNA by different mechanisms. The biological relevance of this divergence is not presently clear.

## **2.5 CHEMICAL MODIFICATION OF CHROMATIN**

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The N-terminal ends of the core histones are the predominant targets for an assortment of covalent modifications such as acetylation, phosphorylation and methylation. These modifications are thought to affect chromatin through two distinct mechanisms (Berger, 2002; Wu and Grunstein, 2000). Firstly, nearly all modifications alter the electrostatic charge of the histone and this, in principle, could change the structural properties of the histone DNA binding capacity. Secondly, modifications could create binding surfaces for protein recognition modules and thus recruit specific functional complexes to their required sites of action. Examples of such modules have been identified, such as the bromodomain (recognising acetylated lysines) and the chromodomain (recognising methylated lysines). The potential specificity of these signal/recognition interactions provoked Strahl and Allis (Strahl and Allis, 2000) to propose the “histone code” hypothesis by which specific combinatorial sets of histone modification signals can dictate the recruitment of particular trans-acting factors to accomplish specific functions (Strahl and Allis, 2000; Turner, 2000). The many possible combinations of different histone modifications are staggering and the challenge for the future is to systematically dissect the functional relationship between these modifications.

### **2.5.1 HISTONE ACETYLATION**

Histone acetylation and deacetylation of conserved lysine residues in histone tails have long been linked to transcriptional activity (Allfrey *et al.*, 1964). In general, hyperacetylated histones are associated with transcriptionally active chromatin and hypoacetylated histones with transcriptionally inactive chromatin (Ebenharter and Becker, 2002; Grunstein, 1997). Acetylation of histones H3 and H4 counteracts the tendency of nucleosomal fibres to fold into highly compact structures *in vitro*. Acetylated nucleosomes are also more accessible to interacting proteins *in vivo* as is illustrated by increased accessibility of DNA to DNaseI, restriction enzymes and transcription factors



(Lee *et al.*, 1993; Anderson *et al.*, 2001; Sewack *et al.*, 2001). This might be caused in part by the lowered positive charge of acetylated N-termini and a consequent lowered stability of interaction with DNA. Histone acetylation can also decrease compaction of nucleosomal arrays by disrupting internucleosomal interactions established through the histone tails (Tse *et al.*, 1998). An alternative option is that acetylated residues in different combinations can be involved in the recruitment of additional transcription factors such as proposed by the histone code hypothesis. In yeast the ground state of chromatin is characterised by a mixture of untargeted histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities (Vogelauer *et al.*, 2000). Targeted modification occurs against this background, which allows a rapid return to the initial state of acetylation when targeting is removed.

### **2.5.1.1 HISTONE ACETYLTRANSFERASES**

Histone acetyltransferases (HATs) are separated into two groups, type A nuclear HATs and type B, cytoplasmic HATs (Roth *et al.*, 2001). The nuclear regulatory complexes, which mainly contain type A HATs, will be of significance to this discussion. Three families of type A HATs have been identified, all of which share a highly conserved Acetyl-CoA binding site. Only two of these families are represented in yeast. Several of these HATs have been shown to have activities directed to histones *in vitro*, but seem to always act as part of multi-subunit complexes *in vivo* (Table 2.1). The different complexes have different subunit compositions and different histone specificities, and appear to be involved in distinct biological functions (Roth *et al.*, 2001).

The first family of HATs is the GNAT superfamily (Gcn5-related N-acetyltransferases), which includes proteins involved with, or linked to, transcriptional initiation (Gcn5p), elongation (Elp3p), histone deposition and telomeric silencing (Hat1p). *GCN5* was initially identified in a genetic screen designed to isolate mutants unable to grow under conditions of amino acid limitation (Georgakopoulos and Thireos, 1992; Penn *et al.*, 1983). A second



screen for mutants reversing toxicity caused by overproduction of Gal4-VP16 also delivered *Gcn5* along with two other genes, *ADA2* and *ADA3* (Berger *et al.*, 1992; Piña *et al.*, 1993). Further genetic and biochemical analysis revealed that Gcn5p, Ada2p and Ad3ap are the constituting elements of the ADA (alteration/deficiency in activation) complex (Marcus *et al.*, 1994; Candau *et al.*, 1996). A biochemical search for native complexes able to acetylate nucleosomes *in vitro* led to the isolation of two high molecular mass complexes of 0.8 and 1.8MDa, respectively, both complexes contain Gcn5p, Ada2p and Ada3p (Grant *et al.*, 1997). The larger of the two complexes turned out to contain Spt proteins (Spt20p, Spt3p, Spt8p and Spt7p) and is referred to as SAGA (Spt/Ada/Gcn5 acetyltransferase). Both complexes contain Gcn5p as the catalytic acetyltransferase subunit. The relationship between these two complexes is not clear. It is possible that the smaller complex is simply a subcomplex of the larger SAGA complex. Alternatively each complex could represent distinct HAT complexes with unique cellular functions. Gcn5p, like several transcription associated factors, also contains a bromodomain that allows for the preferential recognition of histone tails acetylated at specific residues (Owen *et al.*, 2000; Dhalluin *et al.*, 1999). Gcn5p is able to stabilise SWI/SNF promoter binding, an interaction that seems to be mediated by the bromodomain (Syntichaki *et al.*, 2000; Hassan *et al.* 2001). Like SWI/SNF, SAGA has also been shown to interact directly with a variety of transcriptional activators, including Gcn4p, Swi5p, Gal4-VP16 and Gal4-AH (Krebs *et al.*, 1998; Neely *et al.*, 1999; Natarajan *et al.*, 1999; Yudkovsky *et al.*, 1999).

The MYST family of histone acetyltransferases is named after the founding members *MOZ* (a human oncogene), Ybf2p/Sas3p, Sas2p and Tip60p. The yeast homologue of *MOZ* is Sas3p, the catalytic subunit of the histone H3-specific HAT complex, NuA3 (nucleosomal acetyltransferase of histone H3) (John *et al.*, 2000). This complex is predicted to function in transcriptional elongation and replication and in transcriptional repression of the yeast silent mating type loci (Reifsnyder *et al.*, 1996; John *et al.*, 2000). Esa1p, an essential protein in yeast, is also a MYST family member that predominantly modifies histones H4 and H2B. Esa1p represents the acetyltransferase



activity of the NuA4 (nucleosomal acetyltransferase of histone H4) HAT complex (Allard *et al.*, 1999). Esa1p acetylates histones H2A and H4 and can be recruited by activation domains *in vitro* to stimulate transcription from chromatin templates in an acetyl co-enzyme A dependent manner (Vignalli *et al.*, 2000; Ikeda *et al.*, 1999). The *in vivo* targets of Esa1p and thus NuA4 have been identified as ribosomal protein promoters, to which these complexes are specifically recruited, possibly through the general DNA binding factors Rap1p and Abf1p (Reid *et al.*, 2000).

The different functions of HAT complexes are likely caused by differences in the amino acid residues in different histones that are acetylated, and differences in targeting to specific regions in the genome. Each complex contains a specific set of non-HAT subunits, which might interact with different sequence specific activators that target the complexes to distinct areas of regulation. The subunits may also differentially modulate HAT activity, since it has been shown that Gcn5p-containing complexes have different substrate specificities than isolated Gcn5p (Brownell *et al.*, 1996; Grant *et al.*, 1999)

**Table 2.1** Constituents of yeast type A histone acetyltransfer complexes. Adapted from Roth *et al.*, 2001).

GNAT superfamily		MYST superfamily	
ADA	SAGA	NuA3	NuA4
Gcn5p	Gcn5p	Sas3p	Esa1p
Ada2p	Ada1p		
Ada3p	Ada2p		
	Ada3p		
	Ada5p/Spt20p	Spt16p	
	Spt3p		
	Spt7p		
	Spt8p		
	Tra1p		Tra1p
	TAFII90	TAFII30	
	TAFII61/68		
	TAFII60		
	TAFII23/25		
	TAFII17/20		
	Sin4p		
Ahc1p			Act3p/ARP
			Act1p
			Epl1p
			Eaf3p



Johnson, 2000). It was demonstrated that *TUP1* represses gene activity *in vivo* in part through its utilisation of HDA1 to deacetylate histones H3 and H2B. This deacetylation occurs at a localised region containing the TATA element adjacent to the Tup1p recruitment site at the *ENA1* and *STE6* loci (Wu *et al.*, 2001a).

Two complexes containing a member of class III HDACs, Sir2p, have been identified and are known to be involved in heterochromatin silencing at silent mating type loci, telomeres and ribosomal DNA (Moazed *et al.*, 2001). Specialised regions of heterochromatin such as these are transcriptionally inactive, hypo-acetylated regions (Ekwall *et al.*, 1997). Sir2p has been shown to have NAD-dependent HDAC activity (Imai *et al.*, 2000; Landry *et al.*, 2000; Smith *et al.*, 2000).

## 2.5.2 HISTONE METHYLATION

Although at present, less is known about histone methylation compared to acetylation, it is a very active research area. Methylation of histones is divided into two categories, targeting either arginine or lysine residues (see Kouzarides., 2002 for review). In *S. cerevisiae*, methylation of arginines in histones has not been described, but the N-terminal tail of histone H3 is methylated at lysines 4, 36 and 79. The SET domain-containing enzymes mediate histone lysine methylation. The SET domain is an evolutionary conserved domain of 130 amino acids named for its presence in Su(var) 3-9 (suppressor of position effect variegation), E(z) (enhancer of zest) and Trx (trithorax) (Stassen *et al.*, 1995; Jenuwein *et al.*, 1998). In *S. cerevisiae* there are only 7 SET domain gene sequences, compared to more than 70 that are present in mammals (Jenuwein., 2001)

Set1p is responsible for the methylation of lysine 4 of histone H3 *in vitro* and disruption of the *SET1* gene leads to absence of lysine 4 methylation of histone H3 *in vivo* (Briggs *et al.*, 2001; Krogan *et al.*, 2003b; Nagy *et al.*, 2002; Boa *et al.*, 2003). Set1p methylation was initially considered to be of importance in rDNA silencing and normal cell growth (Briggs *et al.*, 2001; Bryk



### 2.5.1.2 HISTONE DEACETYLASE COMPLEXES

The background of histone acetylation established by the activities of the HAT complexes is balanced out by the activities of histone deacetylase complexes (HDACs), which are involved in the creation of localised regions of repressed chromatin. HDACs identified to date are ordered into three main classes based on sequence similarity (Khochbin *et al* 2001; Khochbin and Kao, 2001). Class I deacetylases are similar to yeast Rpd3p and include yeast Hos1p and Hos2p and the mammalian HDAC1-3 (Knupfler and Eisenman, 1999; Rundlett *et al.*, 1996). Rpd3p forms a complex with Sin3p and Sap3p (referred to as the Rpd3-Sin3 HDAC complex) that is recruited to DNA by the Ume6p transcriptional repressor (Kadosh and Struhl, 1998; Zhang *et al.*, 1998; Fazio *et al.*, 2001). Genomic profiles of genes upregulated by *RPD3* deletion correspond to genes that fluctuate with cell-cycle periodicity (Bernstein *et al.*, 2000). Although Rpd3p regulates meiosis genes via Ume6p, its influence on cell-cycle dependent genes appears to be distinct. The Rpd3p-Sin3p complex has recently been linked to repression of ribosomal gene expression in response to nutrient limitation (Rohde and Cardenas, 2003). In this case a dynamic interplay between Rpd3-Sin3 and the Esa1 HAT complex regulates the expression status in response the nutritional environment.

Class II histone deacetylases share similarity to the yeast Hda1p, a putative catalytic subunit, and include the mammalian *HDAC4*, *HDAC5* and *HDAC6*. However little is known about the complexes that include these deacetylases (Grozinger *et al.*, 1996). Yeast Hda1p was first identified as a component of a complex containing four subunits: Hda1p, Hda3p and two peptides that are thought to be posttranslationally modified variants of Hda2p (Carmen *et al.*, 1996). Subsequent analysis revealed that the HDA1 complex is composed of a Hda1p homodimer and an Hda2p-Hda3p heterodimer, and that interaction between the two dimers is essential for the activity of the HDA1 complex (Wu *et al.*, 2001b). A role was reported for HDA1 in *TUP1* mediated repression (Wu *et al.*, 2001a). *TUP1* is a general yeast repressor that affects pathways involved in mating, DNA repair and oxygen and glucose utilisation (Edmondson *et al.*, 1996; Huang *et al.*, 1998; Wahi *et al.*, 1998; Smith and



telomeres, mating type loci and rDNA, implying a direct role for Dot1p in telomeric silencing. Furthermore binding of Sir2p and Sir3p at silent loci is decreased in a *dot1* mutant (Ng *et al.*, 2002a).

### 2.5.3 OTHER MODIFICATIONS

A potentially vast number of histone modifications within the unstructured tails and the structured C-termini of histones may prove to be involved in several nuclear processes. These include phosphorylation, ubiquitination and ADP-ribosylation events.

Phosphorylation of histones H1 and H3, for example, is known to play important roles in transcriptional regulation and mitosis (Cheung *et al.*, 2000). In *S. cerevisiae* the Snf1p kinase has been identified as a transcriptionally linked histone kinase (Lo *et al.*, 2001), and Snf1p was shown to coregulate transcription of the *INO1* gene. It is probable that, similar to HATs and the SWI/SNF complexes, histone kinases may be recruited to specific promoters as coactivators.

Also, the C-terminus of histone H2B is ubiquitinated in a *Rad6p*-dependent manner, and loss of this ubiquitination leads to defects in mitosis and meiosis (Robzyk *et al.*, 2001). Two different groups reported that ubiquitination of histone H2B is required for *Set1p*-dependent methylation of H3 K4 (Sun *et al.*, 2002; Dover *et al.*, 2002). These data support the emerging paradigm (such as the histone code model) that complex interactions between different modification activities are required in the regulation of various processes.

## 2.6 REGULATION OF GENE EXPRESSION THROUGH CHROMATIN

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In order to understand how a eukaryotic cell selects between gene expression or repression, it is essential to understand how the chromatin-modifying complexes, described above, interact with other components of the transcription machinery in a coordinated manner. The transitions between highly condensed chromatin structures and decondensed chromatin are not



*et al.*, 2002). In contrast to these observations, global analysis of modification status revealed that lysine 4 methylation frequently occurs in coding regions to facilitate transcription (Bernstein *et al.*, 2002), and is required for effective transcription of 80% of yeast genes (Boa *et al.*, 2003). Also lysine 4 methylation has been linked to transcriptional elongation (Krogan *et al.*, 2003). In mammalian systems the activity of the K4 histone 3 methyltransferase *SET9/SET7* is also associated with positive regulation (Lachner and Jenuwein., 2002). How methylation can be associated with both activation and repression became clear only recently. A study using antibodies discriminating between di- and tri-methylated H3 lysine 4 revealed that trimethylation is specific for the active state of transcription, whereas dimethylated lysine 4 is present in both active and repressed genes (Santos-Rosa *et al.*, 2003). Thus the number of methyl groups on a modified lysine residue, in addition to the particular residue modified, appears to play an important role in the functional consequences of histone methylation.

Lysine 36 methylation of H3 is mediated by Set2p. Although Set2-LexA could repress transcription of the lacZ reporter when merged to a heterologous promoter, and *SET2* is necessary for repression of basal levels of *GAL4* transcription, the *in vivo* role of Set2p remains a puzzle (Strahl *et al.*, 2002; Landry *et al.*, 2003). In particular, two independent studies suggest that Set2p, through association with the elongating form of RNA polymerase II, plays an important role in transcription elongation (Li *et al.*, 2002; Schaft *et al.*, 2002).

One of the key discoveries in histone methylation during the past year is Dot1p (disruptor of telomeric silencing-1), a novel histone methyl transferase (HMT) that is involved in silencing at telomeres, mating type loci and rDNA (Van Leeuwen *et al.*, 2002; Ng *et al.*, 2002a; Lacoste *et al.*, 2002; Krogan *et al.*, 2003a). HMTs have until recently been characterised by the SET domain, which is lacking in Dot1p. Furthermore, unlike other HMTs, Dot1p does not target lysine residues in the histone tail, but methylates histone H3 on lysine79, which resides within the core domain (Lacoste *et al.*, 2002; Van Leeuwen *et al.*, 2002). Mutation of lysine 79 displays a defect in silencing at



well understood; therefore this discussion will focus on the regulation of target genes once they are in a decondensed state.

While the functional interactions between the various complexes that regulate transcription require further intense study, the emerging picture is that they are able to act in many different orders and can assist in each other's function. There is no specific indication that ATP-remodeling, covalent modification of histones, binding by regulatory factors or the transcription machinery takes place in any particular order. The only requirement appears to be that at an appropriate stage a properly structured template be attained.

### **2.6.1 TARGETING OF ACTIVITIES**

Genome-wide expression studies have revealed that SWI/SNF, and Gcn5p containing HAT complexes are involved in the expression of 6% and 5%, respectively, of all yeast genes (Holstege *et al.*, 1998; Sudarsanam *et al.*, 2000). Accumulating evidence suggests that this specificity may be explained by targeting of these activities to specific areas by sequence specific activators/repressor that directly bind to ATP-dependent complexes and chemical modifiers. This could have three functional consequences with regard to the modification of the rate of remodeling. Firstly, contacts between the activator/repressor could increase the affinity of a specific complex for a particular region of DNA. This, in turn, would increase overall activity by increasing the local concentration of the complex. Secondly, targeting by sequence specific factors could also increase the rate at which a complex binds to the chromatin template, if the binding event is rate limiting. Finally interactions with specific DNA binding factors could directly regulate the activity of the complex.

SWI/SNF has been shown to interact with several promoter binding transcription factors (Natarajan *et al.*, 1999; Neely *et al.*, 1999; Yudkovsky *et al.*, 1999; Hassan *et al.*, 2001a). Although the molecular details of association between SWI/SNF and transcription activators remain largely unclear, specific features of activation domains seem to be of importance. A study of the



chimeric Gal4-VP16 acidic transcription activator has indicated that targeting is reduced or eliminated by mutations that disrupt its acidic residues (Hassan *et al.*, 2001a; Peterson and Workman, 2000). Nuclear HAT complexes and co-activators with intrinsic HAT activity have also been found to interact with transcriptional activators (Roth *et al.*, 2001). As described for SWI/SNF, it has been demonstrated that transcription activators containing acidic domains can directly interact with SAGA and NuA4 and target these complexes to specific promoters (Hassan *et al.*, 2001a; Peterson and Workman, 2000). The interaction between these complexes and acidic activators was shown to be mediated by Tra1p, a common subunit of SAGA and NuA4 (Brown *et al.*, 2000).

In contrast, *Isw2p* is targeted to promoters of early mitotic genes by the transcriptional repressor *Ume6p* (Goldmark *et al.*, 2000). The recruitment of *Isw2p* leads to the formation of an inaccessible chromatin structure proximal to the *Ume6p* binding site, and consequently represses gene expression. *Sin3-Rpd3* is one of the most extensively studied histone deacetylase complexes to date. This complex has also been shown to be targeted to specific promoters by the same transcriptional repressor, *Ume6p*, resulting in local histone deacetylation and transcriptional repression (Fazio *et al.*, 2001). Furthermore, the transcriptional corepressors *Hir1p* and *Hir2p* can recruit SWI/SNF to a responsive promoter (Dimova *et al.*, 1999).

These targeting mechanisms likely serve to initiate a cascade of events at a given promoter that results in local alteration of chromatin structure to facilitate formation of an active or a repressed state. The requirement for specific remodeling/modification activities may vary for each particular gene. The precise order in which complexes function at specific genes depends on the nature of the promoter, the complement of transcription factors present and the chromatin structure at the promoter. On the *HO* promoter, the recruitment of ATP-dependent chromatin remodelers precedes that of HAT complexes (Cosma *et al.*, 1999; Krebs *et al.*, 1999). On the other hand, activation of the inducible *GAL1* promoter during interphase requires *Gcn5p*, but not SWI/SNF. Induction of this gene in late mitosis, however, requires



both SWI/SNF and Gcn5p, indicating that ATP-dependent remodeling complexes are required to assist HAT-dependent gene expression when chromatin is highly condensed (Krebs *et al.*, 2000).

## 2.6.2 COLLABORATION BETWEEN COMPLEXES

There is strong evidence to support the idea that ATP-dependent remodelers and covalent modifiers work together to facilitate gene expression. A functional link between ATP-dependent remodeling and HAT complexes was first suggested by genetic studies in yeast (Pollard and Peterson, 1997; Roberts and Winston, 1997). Mutations in subunits of the SAGA complex (excluding Gcn5p) were lethal in combination with mutations in the SWI/SNF complex, though none of the single mutants showed any severe growth defects, indicating a synergistic interaction between these two complexes. Genetic studies and genome-wide analysis suggest that Isw2p and Sin3p-Rpd3p, despite having unique biochemical activities, function synergistically to regulate gene expression (Goldmark *et al.*, 2000; Fazio *et al.*, 2001).

Two studies raise the possibility that acetyltransferase complexes might stabilise the interaction of SWI/SNF with the chromatin template. Work performed using an altered *PHO5* promoter suggested that the Gcn5p-containing SAGA complex might serve dual functions (Syntichaki *et al.*, 2000). The catalytic Gcn5p subunit firstly acetylates a promoter region, and subsequently the bromodomain of Gcn5p is proposed to stabilise the binding of SWI/SNF to the acetylated nucleosomes. Biochemical analysis provides a slightly different picture in support of the basic notion that acetyltransferases can stabilise the binding of SWI/SNF. In an *in vitro* system it was shown that SWI/SNF preferentially binds to acetylated nucleosomes, suggesting that acetylation stabilizes SWI/SNF association (Hassan *et al.*, 2001b).

Similar to the effects of sequence specific activators, two types of complexes could assist each other in various ways. Direct interaction between ATP-dependent remodelers and chromatin modifiers could increase their affinity for the chromatin template. Such interaction could also affect the activities of the



separate complexes. Finally, alteration of the chromatin template by one complex could render a more suitable substrate for another complex. For example, remodeling of nucleosomes by ATP-dependent activities could increase the accessibility of the N-terminal tails to chemical modification. Alternatively, ATP-dependent remodelers might bind more efficiently, or dissociate more slowly, from nucleosomes modified at specific positions.

### 2.6.3 SYNCHRONISING WITH THE TRANSCRIPTIONAL MACHINERY

Some transcription factors and regulatory complexes can bind to chromatin prior to recruitment of chromatin-modifying complexes and are capable of altering chromatin structure. Biochemical studies have shown that binding of activators such as Gal4p can displace nucleosomes in *cis* and in *trans* to create nucleosome-free areas (Workman and Kingston., 1992). Elongation by RNA polymerase can also cause changes in chromatin structure and nucleosomal positioning (Lee and Garrard., 1991). ATP-dependent remodeling complexes can increase binding by gene specific activators and components of the preinitiation complex, such as TBP (Burns and Peterson., 1997; Côté *et al.*, 1994; Imbalanzo *et al.*, 1994). Acetylation of chromatin can also increase binding of transcription factors (Sewack *et al.*, 2001). Both HAT complexes and ATP-dependent remodeling complexes can significantly increase the rate of overall transcription from nucleosomal templates in defined *in vitro* transcription systems (Ikeda *et al.*, 1999; Neely *et al.*, 1999). Thus components of the general transcription machinery can assist in remodeling and chromatin-remodeling complexes can increase function of the transcription machinery. Thus, the idea that there is no compulsory order of action for ATP-dependent remodeling and chemical modification can be extended to state that there is no compulsory order of action for chromatin modifying complexes and complexes in the general transcription machinery.

It appears that these very specific requirements for a specific complex on a given promoter reflect the particular pathway that has evolved to function on that specific promoter, not a requirement that is general for the



activator/repressor that is involved, or for other promoters. The only requirement is that the structure of the template and association of appropriate components of the general transcription machinery be reached in a timely fashion. As a consequence, participants can assist each other in order to locate the most practical solution to achieve tightly regulated control of transcription.

## 2.7 CONCLUSION

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The regulation of gene expression and consequently cellular programmes and phenotypic outcome requires the implementation of strikingly delicate processes involving collaboration between various complexes and factors. The past decade has seen a dramatic expansion in our perception of mechanisms involved in the coordination of transcriptional regulation. The use of the well-defined genetic system available through yeast genetics, complimented with an expanding range of biochemical implementations, allowed the unravelling of not only the structural and functional properties of the complexes involved, but also the assessment of the effect of these separate complexes and factors on genome-wide regulation of transcription.

The mechanisms by which this regulation is achieved are the keys that control regulatory networks in eukaryotes. Emerging hypotheses such as the histone code model provide an exciting range of future studies aiming to generate a comprehensive picture of events leading to transcription repression or induction. Future research to dissect the convergence of the variety of modification and remodeling activities available to the eukaryotic cell should lead to a dramatic new view of chromatin function.

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## **CHAPTER 3**

# **RESEARCH RESULTS**

## **THE EFFECT OF NUCLEOSOMAL POSITIONING ON THE TRANSCRIPTIONAL REGULATION OF *MUC1/FLO11* AND *STA2***



### 3.1 INTRODUCTION

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Regulation of gene expression/repression is governed by various extra- and intra-cellular cues that need to be perceived and appropriately integrated in order to adjust cellular responses. The interface between chromatin and regulatory factors presents an extremely efficient checkpoint where transcriptional regulation can be adjusted with remarkable precision. The involvement of chromatin structure and nucleosomal positioning in transcriptional regulation has been extensively characterised during the past decade (for review see Chapter 2).

We were urged to investigate the possibility of chromatin playing a dynamic role in the regulation of *MUC/FLO11* and *STA2* transcription and the possible involvement of Mss11p in the mediation thereof. Several lines of evidence suggest that these two promoters could be controlled in part through nucleosomal positioning and modification. Firstly, both the complexity and untypically large size of the *MUC1/FLO11* and *STA2* promoters suggested chromatin as a potential regulatory mechanism. Secondly, despite the considerable degree of homology between the two promoter sequences basal levels of *STA2* transcription are constantly higher than that of *MUC1/FLO11* (Gagiano *et al.*, 1999). Genetic screens have revealed that *STA2* expression requires the presence of *SNF2*, the ATPase subunit of the SW2/SNF2 ATP-dependent chromatin remodelling complex (Yoshimoto and Yamashita, 1991; Yoshimoto *et al.*, 1991, 1992; Yamashita, 1993; Kuchin *et al.*, 1993). A role for the *ISW2* chromatin-remodelling complex in the regulation of invasive growth has recently also been revealed (Kent *et al.*, 2001). Furthermore, cAMP mediated repression of *MUC1/FLO11* requires the general repressor *TUP1-SSN6* (Conlan and Tzamarias, 2001). Finally, regulation of *FLO1*, also a FLO family member, takes place in an extensive chromatin domain regulated by the activities of the SWI/SNF co-activator and the TUP1-SSN6 co-repressor, raising the possibility that similar mechanisms could be in effect in the regulation of *MUC/FLO11* (Fleming and Pennings, 2001).

### 3.2 MATERIALS AND METHODS

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All methodologies involving manipulation of DNA, yeast strains and culture conditions were executed as described in Chapter 4. Yeast strains and their relevant genotypes



are listed in Table 3.1. Plasmids used in this study are listed in Table 3.2. The components of the yeast media used in this section are specified in Table 3.3.

**Table 3.1** The yeast strains used in this study

Strain	Relevant genotype	Reference
ISP20	<i>MATa leu2 thr1 trp1 ura3</i>	This Laboratory
ISP20 $\Delta$ <i>mss11</i>	<i>MATa leu2 thr1 trp1 ura3 mss11::LEU2</i>	Gagiano <i>et al.</i> , 1999b

**Table 3.2** List of constructs used in this study

Plasmid	Genotype	Reference
YEpLac195-PMUC1	2 $\mu$ <i>URA3 PMUC1</i>	This Laboratory
YEpLac195-PSTA2	2 $\mu$ <i>URA3 PSTA2</i>	This Laboratory

### 3.2.1 NUCLEOSOMAL MAPPING

The assay performed to assess nucleosome positioning on the *MUC1/FLO11* and *STA2* promoters are similar to the methodology described by Ryan *et al.* (1999). The yeast strains ISP20 was used for the determination of the nucleosomal positioning on both the *MUC1/FLO11* and *STA2* promoter regions since it is well characterised for its ability to form pseudohyphae and metabolise extracellular starch (Gagiano *et al.*, 1999b). Plasmids bearing a 3541 bp region upstream of the *MUC1/FLO11* ATG and 3457bp upstream of *STA2* ATG were transformed by the lithium acetate method. Transformants were streaked out on selective SCD medium and incubated at 30°C. A single colony was inoculated into 5 ml of selective medium and grown overnight, 100 $\mu$ l of this culture was transferred to 100 ml of either SCD or SCGE medium containing the required amino acids and grown to and OD<sub>600</sub> of 1.0.

The culture was pelleted by 5 min centrifugation at 2500 rpm and resuspended in 20 ml of 1 M sorbitol. Following a washing step the culture was again suspended in 1 M sorbitol along with 500  $\mu$ l of Zymolyase 100T (10 mg/ml) (ICN) and incubated at 30°C to allow spheroplast formation. Spheroplast formation was monitored by a decrease in OD<sub>600</sub> when comparing a sample of cells not treated with Zymolyase to the sample undergoing treatment that has been resuspended in water. Cells were considered to be well spheroplasted when absorbance reached approximately 20% of the untreated sample, which in general was after 30 min of treatment.



Speroplasts were harvested by 5 min centrifugation at 3000 rpm, washed once in 1M sorbitol and resuspended in 800µl of freshly made buffer A (1 M sorbitol, 5 mM CaCl<sub>2</sub>, 10 mM Tris.Cl (pH7.4), 5 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 1 mM β-mercaptoethanol, 0.5 mM spermidine). Aliquots of 100 µl was dispensed into four separate microcentrifuge tubes kept on ice and gently mixed with 100 µl of freshly prepared buffer B (same as buffer A with the addition of NP40 to a final concentration of 0.1%). The remaining 400 µl was placed in a microcentrifuge tube and gently mixed with 400 µl of buffer B to be used as free-DNA samples (DNA unbound by histones and other proteins).

To the four prepared samples, micrococcal nuclease (ICN) was added to final concentrations of 100, 50, 25 and 12.5 U/ml and incubated for exactly 5 minutes at 37°C. The reaction was quenched by adding 20 µl of 250 mM EDTA and 5% SDS. All four samples and the free-DNA sample were treated with proteinase-K to a final concentration of 0.56 mg/ml for one hour at 37°C. After a phenol: chloroform: isoamylalcohol (25:24:1) (PCI) extraction, 20 µl of RNase A (10 mg/ml) was added, and the samples were incubated at 37°C for one hour. After two PCI extractions, DNA was precipitated by adding 25 µl of 7.5M NH<sub>4</sub>CH<sub>3</sub>COO and 500 µl of 98% ethanol and incubation for 15 min at -80°C. The precipitated DNA was collected by centrifugation for 10 min at 12 000 rpm, washed once with 70%, dried and dissolved in 200 µl of water (treated samples) or 400 µl for the free-DNA sample. Aliquots of 100 µl were taken from the free-DNA sample, placed in four separate microcentrifuge tubes and mixed with 100 µl of buffer B. Micrococcal nuclease was added to the final concentrations of 10, 5, 2.5 and 1.25 U/ml and incubated at 37°C for five minutes. The reactions were quenched by addition of 20 µl of 250 mM EDTA and 5% SDS. DNA extraction was repeated as described above, and the free-DNA samples were dissolved in 100 µl of water each.

A 20 µl aliquot from each sample was run on a 1% agarose gel, and appropriately digested samples identified and digested with *Hind*III, cutting 23 bp downstream of both the *MUC1/FLO11* and *STA2* translation start codons. Following incubation at 37°C for 12 h, the entire sample was precipitated, dissolved in 20 µl of TE buffer (pH 7.5) and run on a 1.5% agarose gel (20 x 25 cm) in TAE for 16 hours at 40 V, with buffer re-circulation. Southern blot analysis was performed as described by Sambrook *et al.* (1989). A probe homologous to an area stretching 23 bp



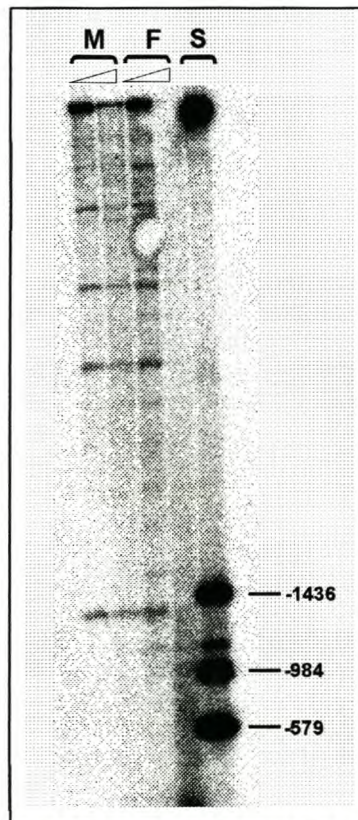
downstream to 123 bp upstream of *MUC1/FLO11* and *STA2* was used for mapping the chromatin structure of the two respective promoter regions by indirect end labelling.

**Table 3.3** The components of the different yeast media used in this work.

Media	Nitrogen source	Carbon source
SCD	1.7% yeast nitrogen base, 40 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2% glucose
SCGE	1.7% yeast nitrogen base, 40 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2% glycerol, 3% ethanol

### 3.3 RESULTS AND DISCUSSION

#### 3.3.1 NUCLEOSOME POSITIONING IN THE REGULATORY REGIONS OF *MUC1/FLO11* AND *STA2*



**Figure 3.1** Nucleosome mapping of the *MUC1* promoter by micrococcal nuclease digestion, followed by indirect-end labelling. The standard (S) indicates positions upstream of the *MUC1* ATG. No apparent difference is observed between the treated samples (M) and the free-DNA control (F).

Preliminary results seemed to indicate that there is no structured array of nucleosomes present on the *MUC1* promoter under repressed conditions (SCD). We were, however, unable to confirm this result due to various technical difficulties. A



different picture might be available when different conditions are taken into account. Unfortunately, attempts to assay nucleosomal positioning in derepressed conditions (SCGE) were flawed by an inability to generate suitable spheroplasts from cells grown in glycerol/ethanol containing media. This is likely due to the severely reduced growth rate in SCGE compared to cultures grown in SCD, which could result in the slower forming cell wall to be more resistant to Zymolyase treatment. In a separate approach, assays were performed in an  $\Delta mss11$  strain, since transcription levels are similar to levels observed in repressed conditions. Unfortunately, due to time constraints and various technical difficulties, we were unable to generate answers in relation to the involvement of chromatin in the regulation of the genes concerned and also in the identifying factors involved in the possible regulation thereof. Future efforts will be focussed on the successful standardisation of techniques necessary for the investigation of chromatin structure on the regulatory regions of *MUC1/FLO11* and *STA2*. Also mutants such as  $\Delta mss11$ ,  $\Delta flo8$  and  $\Delta tec1$ , where basal transcription of both genes are reduced, will be included in future studies.

### 3.4 REFERENCES

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## **CHAPTER 4**

# **RESEARCH RESULTS**

## ***MSS11* MEDIATED REGULATION OF TRANSCRIPTION, PSEUDOHYPHAL DIFFERENTIATION AND FLOCCULATION**



## 4.1 SUMMARY

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Pseudohyphal differentiation refers to the morphogenic shift that is triggered by the limited availability of nutrients, most notably by carbon and nitrogen limitation. In response to these signals, a multitude of regulators control the expression of the cell surface flocculin, Muc1p/Flo11p, which was shown to be critical for both pseudohyphal differentiation and invasive growth. These factors include Ste12p, Tec1p (functioning downstream of the filamentous growth MAPK cascade), Flo8p (downstream of the cAMP-PKA pathway) and numerous other proteins that have not been placed in the context of known signalling pathways, for instance Mss11p, Msn1p, Phd1p and Rme1p. Epistasis analysis suggests that Mss11p plays a decisive role in the regulation of pseudohyphal differentiation and invasive growth. *MSS11*, when overexpressed, was shown to activate *MUC1/FLO11* and the co-regulated *STA2* gene, which encodes an extracellular glucoamylase. A detailed molecular analysis led to the identification of specific domains of Mss11p required to confer transcriptional activation as well as regions involved in the regulation of the activation potential of these domains. To gain further insight into the cellular function of Mss11p, we investigated the effect of carbon and nitrogen induced signalling events on the functioning of these separate domains. In addition the requirement of specific fragments of Mss11p in the regulation of filamentous growth, starch degradation and flocculation was explored. We also present evidence of genetic interaction between Mss11p and other factors involved in the regulation of *MUC1/FLO11* expression.

## 4.2 INTRODUCTION

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Cells respond to changes in the physical and chemical properties of the environment. The decision by cells to execute an appropriate response to a specific stimulus is critical for viability. In response to limited availability of nutrients, cells of the yeast *Saccharomyces cerevisiae* undergo a transition from normal single budding ovoid cells to a filamentous form, characterised by elongated cells budding in a unipolar fashion, with daughter cells remaining attached to the mother cells producing chains of cells referred to as pseudohyphae. These filaments are able to penetrate the agar



beneath the colony, a process referred to as invasive growth. This cellular adaptation could provide a selective advantage, as it may facilitate foraging for scarce or limited nutrients (for review see Kron., 1997; Madhani and Fink., 1998; Borges-Walmsley and Walmsley., 2000; Pan *et al.*, 2000; Bauer and Pretorius., 2001; Gancedo., 2001; Gagiano *et al.*, 2002). The wide range of tools available in yeast genetics has allowed the identification of a large number of genes involved in the switch from unicellular to pseudohyphal growth. Many of these genes form part of distinct signalling cascades, of which the Gpa2p-cAMP-PKA pathway, regulating the transcription factors Flo8p and Sfl1p (Ward *et al.*, 1995; Lorenz and Heitman, 1998; Roberts *et al.*, 1997; Roberstson and Fink, 1998; Mösch *et al* 1996; Pan and Heitman, 1999; Rupp *et al.*, 1999; Lorenz *et al.*, 2000; Tamaki *et al.*, 2000), and the filamentation specific MAP kinase cascade, functioning upstream of the transcription factors Ste12p and Tec1p, are most comprehensively defined (Gimeno *et al.*, 1992; Liu *et al.*, 1993; Mösch *et al.*, 1996; Cook *et al.*, 1996, 1997; Madhani and Fink, 1997, 1998; Madhani *et al.*, 1997; Rupp *et al.*, 1999). Numerous other factors have been identified for their involvement in the regulation of pseudohyphal differentiation and invasive growth and remain to be placed into the context of known or alternate pathways. Among these are Phd1p (Gimeno and Fink, 1994; Lorenz and Heitman, 1998), Sok2 (Ward *et al.*, 1995; Pan and Heitman, 1999), Elm1p (Blacketer *et al.*, 1993; Garret *et al.*, 1997; Koehler and Meyers., 1997), Rme1p (van Dyk *et al.*, 2003), Msn1p and Mss11p (Gagiano *et al.*, 1999a, b).

Pathways involved in the regulation of pseudohyphal differentiation and invasive growth regulate the expression of *MUC1/FLO11* (Rupp *et al.*, 1999), a member of a family of cell wall associated proteins involved in cell-cell and cell-substrate adhesion (Guo *et al.*, 2000). Other members of this family of glycosyl-phosphatidylinositol (GPI)-linked cell surface glycoproteins include Fig2p and Aga1p, which are involved in mating, and also Flo1p, Flo5p, Flo9p and Flo10p which are involved in flocculation (Guo *et al.*, 2000). *MUC1/FLO11* was shown to be critically involved in the establishment of pseudohyphal differentiation and invasive growth, with deletion of the gene leading to a loss of filamentous phenotype and overexpression resulting in flocculation in liquid media and pseudohyphal differentiation and invasive growth on solid media (Lambrechts *et al.*, 1996a; Lo and Dranginis., 1996, 1998; Guo *et al.*, 2000).

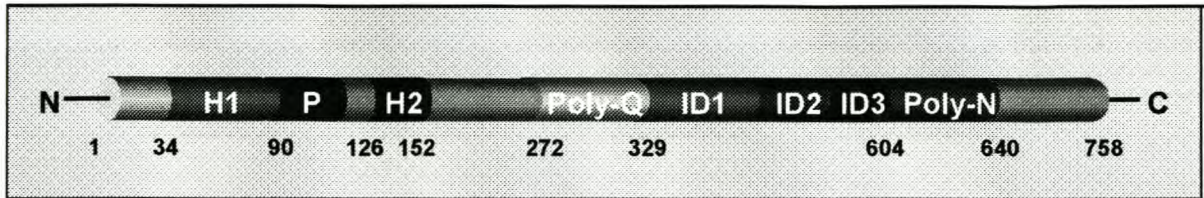


The promoter region of *MUC1/FLO11* represents the largest yeast promoter identified to date, with *cis*-acting elements present up to 2.4 kb upstream from the first coding triplet (Gagiano *et al.*, 1999a; Rupp *et al.*, 1999). The upstream regulatory region of *MUC1/FLO11* is almost identical to that of the *STA2* gene, which encodes for an extracellular glucoamylase that is required for the utilisation of extracellular starch (Pretorius *et al.*, 1991; Vivier *et al.*, 1997; Gagiano *et al.*, 1999b). The homology extends over more than 3.5 kb with the only significant difference being the presence of two inserts of 20 and 64 bp in the *MUC1/FLO11* promoter. The great extent of homology suggests that the promoters of the two genes are co-regulated. Expression analysis confirmed that they both require the same transcription factors. Discrepancies are however observed when basal expression levels of these genes are compared (Gagiano *et al.*, 1999a, b).

Mss11p appears to play a central role in the regulation of starch metabolism and pseudohyphal differentiation. The presence of multiple copies of *MSS11* in the cell results in elevated transcriptional levels of both *MUC1/FLO11* and *STA2*. The increased levels lead to various phenotypes, including an increase in flocculation, pseudohyphal differentiation, invasive growth and the cells ability to utilise starch (Gagiano *et al.*, 1999a, b). Deletion of *MSS11* leads to complete loss of these phenotypes, which cannot be reversed by overexpression of any of the other related factors identified to date (Gagiano *et al.*, 1999a, b). The regulation conferred by *MSS11* on the transcriptional levels of *MUC1/FLO11* and *STA2* also appears to be regulated by signals generated specifically in the presence of low nitrogen and glucose (Gagiano *et al.*, 2003).

Mss11p does not show significant homology to any other yeast protein, with the exception of limited homology to the transcriptional activator Flo8p (Gagiano *et al.*, 1999a). Several distinctive domains are, however, represented in the *MSS11* gene product. Firstly, Mss11p contains relatively large poly-glutamine and poly-asparagine domains. It also contains a putative ATP- or GTP-binding domain, commonly found in proteins such as kinases, ATPases or GTPases (Saraste *et al.*, 1990). Two short stretches close to the N-terminal, labelled H1 and H2, share significant homology to Flo8p (Figure 4.1).





**Figure 4.1** A diagrammatic representation of Mss11p, illustrating the position and length of the various domains. H1 and H2 indicate the two domains that share homology with the *S. cerevisiae* transcription factor, Flo8p. P represents the putative ATP- or GTP- binding domain (P-loop). Poly-Q and Poly-N indicates the poly glutamine and poly asparagine domains respectively. The large domain between Poly-Q and Poly-N has no known or predicted structural features or homology to any protein identified to date. For the purpose of functional analysis this area was subdivided into three domains (referred to as interdomain regions) indicated by ID1, ID2 and ID3

With the use of a comprehensive set of systematic deletions from both the N- and C-termini, two separate activation domains capable of stimulating RNA polymerase II dependent transcription as well as areas that appear to be involved in regulation of Mss11p's activation capability have been identified (Gagiano *et al.*, 2003). Firstly, both the H2 domain and the extreme C-terminus are able to activate a reporter gene when fused to the DNA-binding domain of Gal4p. Interestingly, an area of 92 amino acids that immediately precedes the H2 domain, containing the H1 domain and the putative P-loop, seems to negatively regulate the activity of the H2 domain. In all cases deletion of the H1-P-loop results in increased levels of transcription compared to constructs where this area is present. The effect of an H1-P-loop deletion is especially clear when combined with deletion of the C-terminal domain, suggesting that the regulatory influence of the H1-P-loop is directed specifically towards the H2 domain (Gagiano *et al.*, 2003).

This study was concerned with further investigating the cellular and molecular functioning of *MSS11*. For this purpose, we selected a subset of Mss11p truncations (Table 4.1) that would highlight the activity of the two activation domains (H2 and the C-terminal) and also the apparent regulatory influence of the H1-P-loop. The Mss11p derivatives would also be exploited to reveal any genetic interaction between Mss11p and other factors (Flo8p, Ste12p, Tec1p, Msn1p, Phd1p and Rme1p) regulating *MUC1/FLO11*. In this manner we have established that Mss11p



(S288C), standard laboratory strains that are unable to flocculate because of a naturally occurring mutation in *FLO8* (Liu *et al.*, 1996), were used for the determination of flocculation. Strain PJ69-4A is commonly used in the analysis of two-hybrid interactions and was generously provided by P. James (James *et al.*, 1996)

The carbon and nitrogen sources used in the preparation of the different yeast media are listed in Table 4.3. The yeast nitrogen base that was used did not contain any amino acids or nitrogen source (Becton Dickinson). All synthetic media were supplemented with the specific amino acids required to fulfil the auxotrophic demands of each specific strain or transformant. Amino acids were obtained from Sigma-Aldrich and added according to recommended concentrations (Sherman *et al.*, 1991; Ausubel *et al.*, 1994). Solid media contained 2% agar (Becton Dickinson).

**Table 4.3** The components of the different yeast media used in this work.

Media	Nitrogen source	Carbon source
SCD	1.7% yeast nitrogen base, 40 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2% glucose
SCLD	1.7% yeast nitrogen base, 40 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.1% glucose
SLAD	1.7% yeast nitrogen base, 20 μM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2% glucose








#### 4.3.2 YEAST STRAIN CONSTRUCTION

Yeast strains of the ISP15 background bearing double gene disruptions of *MSS11* in combination with *FLO8*, *STE12*, *TEC1*, and *MSN1*, were generously provided by Dewald van Dyk (this laboratory) along with the disruption cassettes  $\Delta tec1::LEU2$ ,  $\Delta phd1::LEU2$ ,  $\Delta flo8::LEU2$  and  $\Delta rme1::URA3 \Delta mss11::URA3$ . These cassettes along with the existing disruption cassette  $\Delta ste12::URA3$  (Gagiano *et al.*, 1999a), were used to disrupt the open reading frames of *FLO8*, *STE12*, *TEC1*, *MSN1*, *PHD1* and *RME1* in the two-hybrid strain PJ69-4A by means of homologous recombination and integration (Ausubel *et al.*, 1994). The open reading of frame (ORF) of *RME1* was also disrupted in *ISP15Δmss11* as well as the ORF of *MSS11* in *ISP15Δphd1*. Disruptions were confirmed by the polymerase chain reaction using the primers listed in Table 4.4.



is responsive to carbon and nitrogen signalling and that regulation exerted by Mss11p under these conditions is dependent on the H2 domain, which is negatively regulated by the H1-P-loop. We also show that the C-terminal is essential for the regulation of pseudohyphal differentiation, invasive growth and flocculation by Mss11p. This could implicate the C-terminal in various indispensable functions such as DNA binding, nuclear import, binding to other DNA associated proteins or mediating the activities of other proteins or complexes that are required for transcriptional activation. We also show that Mss11p requires the presence of Flo1p to induce Ca<sup>2+</sup>-dependent flocculation.

**Table 4.1** Description of the selected truncations used in this study

Truncation	Description
OF-OR 	<i>MSS11</i> <sub>1-758</sub>
OF-ID2R 	<i>MSS11</i> <sub>1-551</sub>
H2F-OR 	<i>MSS11</i> <sub>146-758</sub>
H2F-ID2R 	<i>MSS11</i> <sub>146-551</sub>
H2F-QR 	<i>MSS11</i> <sub>146-272</sub>
PH2F-OR 	<i>MSS11</i> <sub>169-758</sub>
ID3F-OR 	<i>MSS11</i> <sub>504-758</sub>

## 4.3 MATERIALS AND METHODS

### 4.3.1 YEAST STRAINS, GENETIC METHODS AND MEDIA

Standard molecular, genetic and yeast techniques were used throughout this study (Sherman *et al.*, 1991; Ausubel *et al.*, 1994). Yeast transformations were performed using the lithium acetate method (Ausubel *et al.*, 1994). The yeast strains used in this study, along with the relevant genotypes, are listed in Table 4.2. The strains ISP15, ISP20 and  $\Sigma$ 1278b have been used extensively for the characterisation of invasive growth and pseudohyphal development (Gimeno *et al.*, 1992; Lambrechts *et al.*, 1996a, b; Webber *et al.*, 1997; Gagiano *et al.*, 1999a, b). FY23 and BY4742



### 4.3.3 PLASMID CONSTRUCTION

Standard procedures for isolation and manipulation of DNA were used throughout this study (Ausubel *et al.*, 1994). Restriction enzymes, T4 DNA-ligase and Expand Hi-Fidelity polymerase used in enzymatic manipulation of DNA were obtained from Roche Diagnostics (Randburg, South Africa) and used according to instructions provided by the supplier. *Escherichia coli* DH5 $\alpha$  (Gibco BRL/Life Technologies) was used for the propagation of all plasmids and was grown in Luria-Bertani (LB) broth at 37°C. All *E. coli* transformations and the isolation of DNA were done according to Ausubel *et al.* (1994). All constructs used in this study (Table 4.5) were verified by restriction enzyme analysis.

**Table 4.2** The yeast strains used in this study

Strain	Relevant genotype	Reference
BY4742	<i>MAT<math>\alpha</math> his3 leu2 lys2 ura3</i>	Euroscarf deletion library
BY4742 $\Delta$ <i>mss11</i>	<i>MAT<math>\alpha</math> his3 leu2 lys2 ura3 mss11::KanMX4</i>	Euroscarf deletion library
BY4742 $\Delta$ <i>muc1</i>	<i>MAT<math>\alpha</math> his3 leu2 lys2 ura3 muc1::KanMX4</i>	Euroscarf deletion library
BY4742 $\Delta$ <i>flo1</i>	<i>MAT<math>\alpha</math> his3 leu2 lys2 ura3 flo1::KanMX4</i>	Euroscarf deletion library
BY4742 $\Delta$ <i>flo10</i>	<i>MAT<math>\alpha</math> his3 leu2 lys2 ura3 flo10::KanMX4</i>	Euroscarf deletion library
ISP15	<i>MAT<math>\alpha</math> STA2 his3 leu2 trp1 thr1 ura3</i>	Lambrechts <i>et al.</i> , 1996
ISP15 $\Delta$ <i>mss11</i>	<i>MAT<math>\alpha</math> STA2 his3 leu2 trp1 thr1 ura3 mss11::LEU2</i>	Webber <i>et al.</i> , 1997
ISP15 $\Delta$ <i>mss11</i> $\Delta$ <i>flo8</i>	<i>MAT<math>\alpha</math> STA2 his3 leu2 trp1 thr1 ura3 mss11::LEU2 flo8::URA3</i>	This laboratory
ISP15 $\Delta$ <i>mss11</i> $\Delta$ <i>ste12</i>	<i>MAT<math>\alpha</math> STA2 his3 leu2 trp1 thr1 ura3 mss11::LEU2 ste12::URA3</i>	This laboratory
ISP15 $\Delta$ <i>mss11</i> $\Delta$ <i>tec1</i>	<i>MAT<math>\alpha</math> STA2 his3 leu2 trp1 thr1 ura3 mss11::LEU2 tec1::URA3</i>	This laboratory
ISP15 $\Delta$ <i>mss11</i> $\Delta$ <i>msn1</i>	<i>MAT<math>\alpha</math> STA2 his3 leu2 trp1 thr1 ura3 mss11::LEU2 msn1::URA3</i>	Webber <i>et al.</i> , 1997
ISP15 $\Delta$ <i>mss11</i> $\Delta$ <i>phd1</i>	<i>MAT<math>\alpha</math> STA2 his3 leu2 trp1 thr1 ura3 mss11::URA3 phd1::LEU2</i>	This study
ISP15 $\Delta$ <i>mss11</i> $\Delta$ <i>rme1</i>	<i>MAT<math>\alpha</math> STA2 his3 leu2 trp1 thr1 ura3 mss11::LEU2 rme1::URA3</i>	This study



**Table 4.2** The yeast strains used in this study (continued)

Strain	Relevant genotype	Reference
Σ1287b	<i>MAT<math>\alpha</math> ura3-52 trp::hisG leu2::hisG his3::hisG</i>	H.U. Mosch
Σ1278bΔ <i>mss11</i>	<i>MAT<math>\alpha</math> ura3-52 trp::hisG leu2::hisG his3::hisG mss11::LEU2</i>	Gagiano <i>et al.</i> , 2003
Σ1278bΔ <i>mss11</i> Δ <i>muc1</i> ::LacZ	<i>MAT<math>\alpha</math> ura3-52 trp::hisG leu2::hisG his3::hisG mss11::LEU2 muc1::LacZ-HIS3</i>	Gagiano <i>et al.</i> , 2003
PJ69-4A	<i>MAT<math>\alpha</math> his3 trp1 leu2 ura3 gal4 gal80 LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i>	James <i>et al.</i> , 1996
PJ69-4AΔ <i>flo8</i>	<i>MAT<math>\alpha</math> his3 trp1 leu2 ura3 gal4 gal80 LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ flo8::LEU2</i>	This study
PJ69-4AΔ <i>ste12</i>	<i>MAT<math>\alpha</math> his3 trp1 leu2 ura3 gal4 gal80 LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ ste12::URA3</i>	This study
PJ69-4AΔ <i>tec1</i>	<i>MAT<math>\alpha</math> his3 trp1 leu2 ura3 gal4 gal80 LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ tec1::LEU2</i>	This study
PJ69-4AΔ <i>msn1</i>	<i>MAT<math>\alpha</math> his3 trp1 leu2 ura3 gal4 gal80 LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ msn1::URA3</i>	This study
PJ69-4AΔ <i>phd1</i>	<i>MAT<math>\alpha</math> his3 trp1 leu2 ura3 gal4 gal80 LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ phd1::LEU2</i>	This study
PJ69-4AΔ <i>rme1</i>	<i>MAT<math>\alpha</math> his3 trp1 leu2 ura3 gal4 gal80 LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ rme1::URA3</i>	This study
FY23Δ <i>mss11</i>	<i>MAT<math>\alpha</math> leu2 trp1 ura3 mss11::LEU2</i>	Gagiano <i>et al.</i> , 1999a

Primers MSS11-PF and MSS11-PR were used to amplify the promoter region of *MSS11*, with YEpLac112-*MSS11* (Gagiano *et al.*, 1999a) as template. The reverse primer was designed with an *EcoRI* site after the start codon. This fragment was digested with *EcoRI* and *ScaI* and inserted into YCpLac22 (Gietz and Sugino, 1988) digested at unique sites with *NarI*, followed by blunt-end generation by Klenow enzyme, and *EcoRI*. The *MSS11* terminator region was amplified with the primers MSS11-TF and MSS11-TR (Table 4.4) also using YEpLac112-*MSS11* as template. The forward primer was designed to contain a *SaII* restriction site directly 5' to the stop codon, and the reverse primer was designed to contain a *HindIII* site for cloning of the fragment into unique *SaII* and *HindIII* sites of plasmid Ycplac22. The resulting plasmid, YCpLac22-*MSS11*exp, therefore contained the full-length *MSS11* promoter, start codon, stop codon and terminator region, as well as unique *EcoRI* and *SaII* sites for the insertion of the various *MSS11* fragments. These fragments were



subcloned from the existing YEpLac112 constructs used in Gagiano *et al.* (2003). All constructs were verified by restriction enzyme analysis and sequenced to assure that no mutations were introduced through PCR.

#### 4.3.4 INVASIVE GROWTH PLATE ASSAYS

For the monitoring of invasive growth and pseudohyphal development strains of the ISP15 and  $\Sigma$ 1278b genetic backgrounds were transformed with Yeplac112 bearing the truncated versions of *MSS11* and with the unmodified vector, Yeplac112, as negative control. Five days after transformation three independent colonies were inoculated into 5 ml of SCD medium containing only the amino acids required to sustain growth. At an optical density ( $OD_{600}$ ) of 1.0, 15  $\mu$ l was spotted onto solid SCD, SCLD and SLAD agar plates (see Table 4.2 for media components). After 4 and 8 days cells were washed off the surface of the plates with a gloved finger under running water. Only cells that have grown invasively into the surface of the plate remain attached to the plate.

**Table 4.4** List of primers used in this study

Primer	Sequence
FLO8-F	5'-ACTGGGTACCATGGGTCCAACAGT-3'
FLO8-R	5'-CCGGAACAAACCTTTAGCAATTGCG-3'
STE12-F	5'-CACAGCATTCTTTTCGGAG-3'
STE12-R	5'-AATCTCGCTTTTTCTGGTGG-3'
TEC1-F	5'-CCGGAATTCAAACAAGCTCAGGAGCTGGACTCC-3'
TEC1-R	5'-CCGGAATTCGCATGGCGCTAGAGAAGTTTC-3'
MSN1-F	5'-CACCTACAAAGCGTTGATGG-3'
MSN1-R	5'-GTTGTTGGCTGACTTCTGAG-3'
PHD1-F	5'-GGCCTATCCACGCCAATTTA-3'
PHD1-R	5'-TCGAGCTTTGAGCGCAGAGT-3'
RME1-F	5'-GTTTGGACAGGGATAGTGGGTA-3'
RME1-R	5'-CGTGGTGCCATATTCACG-3'
MSS11-F	5'-ATCTGTCGACCTTAAAACCTATTAACAACAAAAAGTGTTTC-3'
MSS11-R	5'-GATCCAGCTTTGGCCAGATAGCTTGCTTAC-3'
MSS11-TF	5'-ATCTGTCGACCTTAAAACCTATTAACAACAAAAAGTGTTTC-3'
MSS11-TR	5'-GATCAAGCTTTGGCCAGATAGCTTGCTTAC-3'



### 4.3.5 FLOCCULATION ASSAY

The extent of Ca<sup>2+</sup>-dependent flocculation was determined by the addition of EDTA (pH8.0) to a 5 ml culture to a final concentration of 30 mM (Stratford, 1992). After cells were dispersed by vigorous agitation (10 sec vortex at maximum speed), 100 µl was immediately removed and added to 900 µl of a 20 mM EDTA (pH 8.0) solution. The optical density (OD<sub>600</sub>) of this mixture was taken and used as measurement A. To induce Ca<sup>2+</sup>-dependent flocculation 1 ml of the initial culture was harvested by quick centrifugation (5 sec 12000 rpm) and resuspended in 1 ml of 5 mM CaCl. The cells were resuspended (10 sec vortex at maximum setting) and left undisturbed for 60 seconds. A second spectrophotometric measurement (measurement B) was performed as described above on a 100 µl sample carefully taken from just below the meniscus. The percentage of Ca<sup>2+</sup>-induced flocculation was calculated using the following formula (standard deviation ranged between 10% and 20%).

$$\% \text{Flocculation} = \left[ \frac{A - B}{A} \right] \times 100$$

**Table 4.5** List of constructs and vectors used in this study

Plasmid	Genotype	Reference
YEpLac112	2µ <i>TRP1</i>	Gietz and Sugino, 1988
YEpLac112-MSS11-OF-OR	2µ <i>TRP1 MSS11</i> <sub>1-758</sub>	This laboratory
YEpLac112-MSS11-OF-ID2R	2µ <i>TRP1 MSS11</i> <sub>1-551</sub>	This laboratory
YEpLac112-MSS11-H2F-OR	2µ <i>TRP1 MSS11</i> <sub>146-758</sub>	This laboratory
YEpLac112-MSS11-H2F-ID2R	2µ <i>TRP1 MSS11</i> <sub>146-551</sub>	This laboratory
YEpLac112-MSS11-H2FQR	2µ <i>TRP1 MSS11</i> <sub>146-272</sub>	This laboratory
YEpLac112-MSS11-PH2F-OR	2µ <i>TRP1 MSS11</i> <sub>169-758</sub>	This laboratory
YEpLac112-MSS11-ID3F-OR	2µ <i>TRP1 MSS11</i> <sub>504-758</sub>	This laboratory
pGBD-C2	2µ <i>TRP1 GAL4</i> <sub>1-147</sub>	James <i>et al.</i> , 1996
pGBD-C2-MSS11-OF-OR	2µ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>1-758</sub>	Gagiano <i>et al.</i> , 2003
pGBD-C2-MSS11-OF-ID2R	2µ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>1-551</sub>	Gagiano <i>et al.</i> , 2003
pGBD-C2-MSS11-H2F-OR	2µ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>146-758</sub>	Gagiano <i>et al.</i> , 2003
pGBD-C2-MSS11-H2F-ID2R	2µ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>146-551</sub>	Gagiano <i>et al.</i> , 2003
pGBD-C2-MSS11-H2FQR	2µ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>146-272</sub>	Gagiano <i>et al.</i> , 2003
pGBD-C2-MSS11-PH2F-OR	2µ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>169-758</sub>	Gagiano <i>et al.</i> , 2003



**Table 4.5** List of constructs and vectors used in this study (continued)

Plasmid	Genotype	Reference
pGBD-C2-MSS11-ID3F-OR	2 $\mu$ TRP1 GAL4 <sub>1-147</sub> MSS11 <sub>504-758</sub>	Gagiano <i>et al.</i> , 2003
YCpLac22	CEN4 TRP1	Gietz and Sugino, 1988
YCpLac22-MSS11-OF-OR	CEN4 TRP1 MSS11 <sub>1-758</sub>	This study
YCpLac22-MSS11-OF-ID2R	CEN4 TRP1 MSS11 <sub>1-551</sub>	This study
YCpLac22-MSS11-H2F-OR	CEN4 TRP1 MSS11 <sub>146-758</sub>	This study
YCpLac22-MSS11-H2F-ID2R	CEN4 TRP1 MSS11 <sub>146-551</sub>	This study
YCpLac22-MSS11-H2FQR	CEN4 TRP1 MSS11 <sub>146-272</sub>	This study
YCpLac22-MSS11-PH2F-OR	CEN4 TRP1 MSS11 <sub>169-758</sub>	This study
YCpLac22-MSS11-ID3F-OR	CEN4 TRP1 MSS11 <sub>504-758</sub>	This study
YEplac195	2 $\mu$ URA3	Gietz and Sugino, 1988
YEplac195-MSS11	2 $\mu$ URA3 MSS11	This laboratory
YEplac195-FLO8	2 $\mu$ URA3 FLO8	This laboratory
YEplac195-STE12	2 $\mu$ URA3 STE12	This laboratory
YEplac195-TEC1	2 $\mu$ URA3 TEC1	This laboratory
YEplac195-MSN1	2 $\mu$ URA3 MSN1	This laboratory
YEplac195-PHD1	2 $\mu$ URA3 PHD1	This laboratory
YEplac195-RME1	2 $\mu$ URA3 RME1	This laboratory
PMSS11- $\Delta$	$\Delta$ mss11::LEU2	Webber <i>et al.</i> , 1997
p $\Delta$ flo8	$\Delta$ flo8::LEU2	This laboratory
p $\Delta$ ste12	$\Delta$ ste12::URA3	Gagiano <i>et al.</i> , 1999a
p $\Delta$ tec1	$\Delta$ tec1::LEU2	This laboratory
p $\Delta$ msn1	$\Delta$ msn1::URA3	Webber <i>et al.</i> , 1997
p $\Delta$ phd1	$\Delta$ phd1::LEU2	This laboratory
p $\Delta$ rme1	$\Delta$ rme1::URA3	This laboratory

#### 4.3.6 $\beta$ -GALACTOSIDASE LIQUID AND PLATE ASSAYS

Strains containing the *lacZ* reporter gene (under control of either the *GAL7* or *MUC1* promoters) were transformed, and three independent colonies from each transformation were grown in 5 ml of selective SCD medium to an OD<sub>600</sub> of 1.0. A 5ml culture of selective medium (SCD, SCLD or SLAD) was inoculated from each of these pre-cultures to an OD<sub>600</sub> of 0.05. These cultures were incubated at 30°C with agitation and grown to an OD<sub>600</sub> of 1.0.  $\beta$ -Galactosidase assays were performed as described by Ausubel *et al.* (1994). Assays were performed on all three transformants (n=3) and the mean activity was calculated. At least two independent



sets of transformation were assayed. The standard deviation did not exceed 15% and was usually less than 8%. Differences between assay values were calculated at a  $p < 0.005$  level using the student paired t-test.

For the plate assays, yeast strains and mutants were transformed with the required constructs. Three colonies from each transformation were inoculated into 5ml of selective SCD medium and grown to an  $OD_{600}$  of 1.0. From each of these starter cultures, 15  $\mu$ l was spotted on solid SCD, SCLD and SLAD agar plates. These plates contained X-gal (40 mg/l), added according to Ausubel *et al* (1994), which allowed for a rough assessment of the level of expression of the *lacZ* reporter gene (either *MUC1-lacZ* or *GAL7-lacZ*) in the strains containing the different sets of constructs and mutants.

## 4.4 RESULTS

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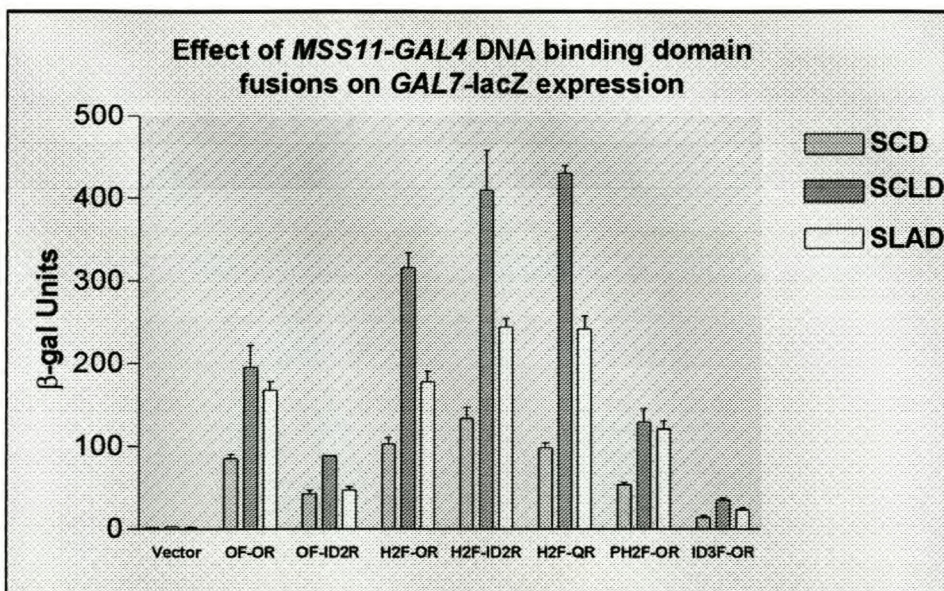
### 4.4.1 *MSS11* IS RESPONSIVE TO NUTRIENT SIGNALLING

In order to characterise the ability of Mss11p to activate transcription, a subset of truncation constructs that had previously been described as resulting in clearly distinct activation patterns, was selected (Gagiano *et al.*, 2003; Table 4.1). These particular truncations were expressed under control of the constitutively active *ADH1* promoter and fused to the Gal4p DNA-binding domain. To assess the response to carbon and nitrogen limitation, the *MSS11-GAL4* DNA-binding domain fusion truncations were transformed into the two-hybrid strain PJ69-4A, which contains an integrated reporter gene, *lacZ*, under control of the *GAL7* promoter. These transformants were subjected to  $\beta$ -Galactosidase liquid assays.

The effect of the various truncations fused to the *GAL4* DNA-binding domain on the expression of the reporter gene (*GAL7-lacZ*) is presented in Figure 4.2. In SCD, containing sufficient carbon and nitrogen, *MSS11-GAL4* overexpression leads to an almost 40-fold induction when compared to the negative control, where only the Gal4p DNA-binding domain is expressed. In conditions of glucose and nitrogen limitation (SCLD; SLAD), a further increase is observed in the ability of Mss11p to stimulate transcription. There is however a slight, but significant increase in basal

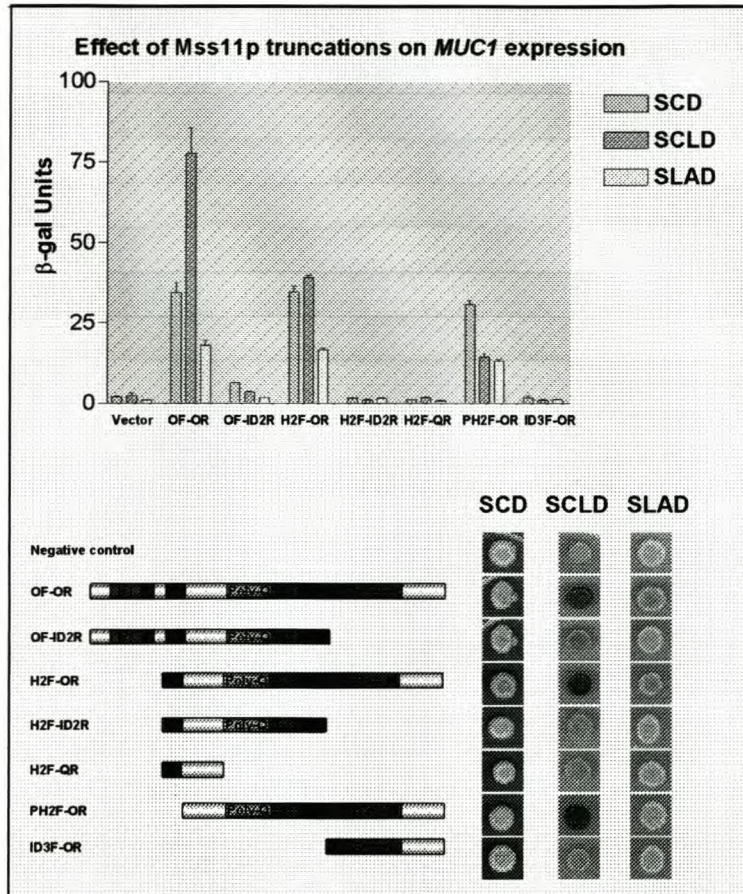


levels of *GAL7* transcription when comparing expression in SCD to SCLD (*GAL7* expression in SCD vs SCLD  $p = 0.02$ ). This could result in a spill over of activity when comparing the effect of the truncations in different growth conditions. In low nitrogen, however, the *Mss11p* fusion constructs result in a significant increase (vector vs constructs in SLAD, in all cases  $p < 0.05$ ) in reporter gene activity, without any induction being observed in the control strains (vector in SCD vs SLAD,  $p = 0.3$ ). Removal of the H1-P-loop results in a surge in activation, which is especially striking in the presence of limited glucose. This surge in activity is lost upon the removal of the H2 domain (construct PH2F-OR). From these observations it appears that both carbon and nitrogen signalling affect *Mss11p*. This is especially clear when the H1-P-loop is removed, suggesting that the H1-P-loop negatively regulates the H2 domain specifically with regards to limited glucose availability. Since these *Mss11p* derivatives are expressed from the *ADH1* constitutive promoter, which is unaffected by nutrient availability, the variations observed in transcriptional activation could be the result of signalling events affecting the activation potential of *MSS11*. It should, however, be taken into account that the varying lengths of the truncations could affect the efficiency of transcription or translation, and could thereby alter the ability of a specific fragment to activate transcription when compared to a shorter or longer fragment.



**Figure 4.2** Levels of reporter gene (*GAL7-lacZ*) activity in the presence of different *MSS11* fusion proteins in liquid media (SCD, SCLD and SLAD), as measured by  $\beta$ -Galactosidase activity. Standard deviation is indicated with error bars.





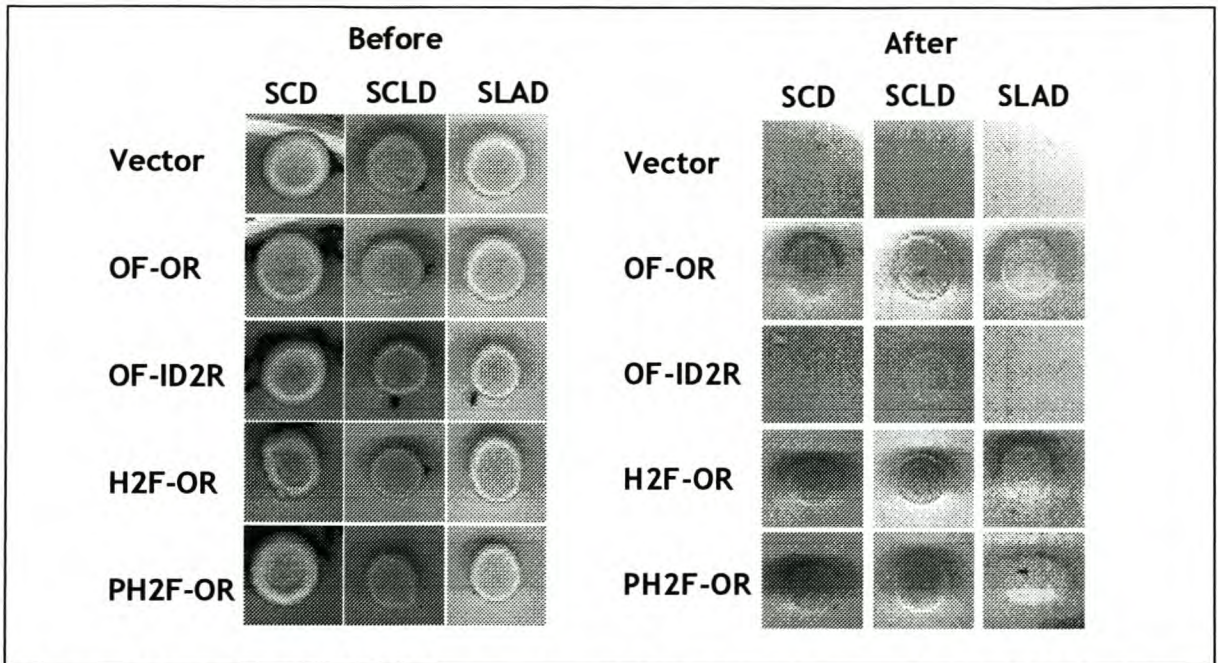
**Figure 4.3** Effect of the various fragments of *MSS11* on *muc1::lacZ* reporter gene activity in various liquid media (SCD, SCLD and SLAD), as measured by  $\beta$ -Galactosidase activity. Reporter activity in the plate assays corresponds to the intensity of colony colouration in the photographs. Standard deviation is indicated with error bars.

#### 4.4.2 REGULATION OF *MUC1/FLO11* EXPRESSION AND OF PSEUDOHYPHAL GROWTH BY *MSS11*

To gain further insight into the cellular functioning of *MSS11* with regards to the regulation of filamentous growth, the *MSS11* derivatives (Table 4.1) based on 2 $\mu$  plasmids under the control of the native *MSS11* promoter and terminator were transformed into the yeast strains  $\Sigma 1278b\Delta mss11$  and  $\Sigma 1278b\Delta mss11\Delta muc1::lacZ$ . These truncations were not fused to a DNA-binding domain, and could therefore provide clues to areas of Mss11p that would be able to function in a similar capacity. The open reading frame of *MSS11* was deleted in these strains to remove any effect that its presence might have on our interpretation (Gagiano *et al.*, 2003). In strain  $\Sigma 1278b\Delta mss11\Delta muc1::lacZ$  the ORF of *MUC1/FLO11* was replaced by the *lacZ* reporter gene (Gagiano *et al.*, 2003). This strain was used for quantification of



*MUC1/FLO11* expression and also serves as  $\Delta msc1$  mutant. The transformed strains were spotted on SCD, SCLD, and SLAD plates (with and without X-gal) and strain  $\Sigma 1278b\Delta mss11\Delta muc1::lacZ$  was also used in liquid  $\beta$ -Galactosidase assays.



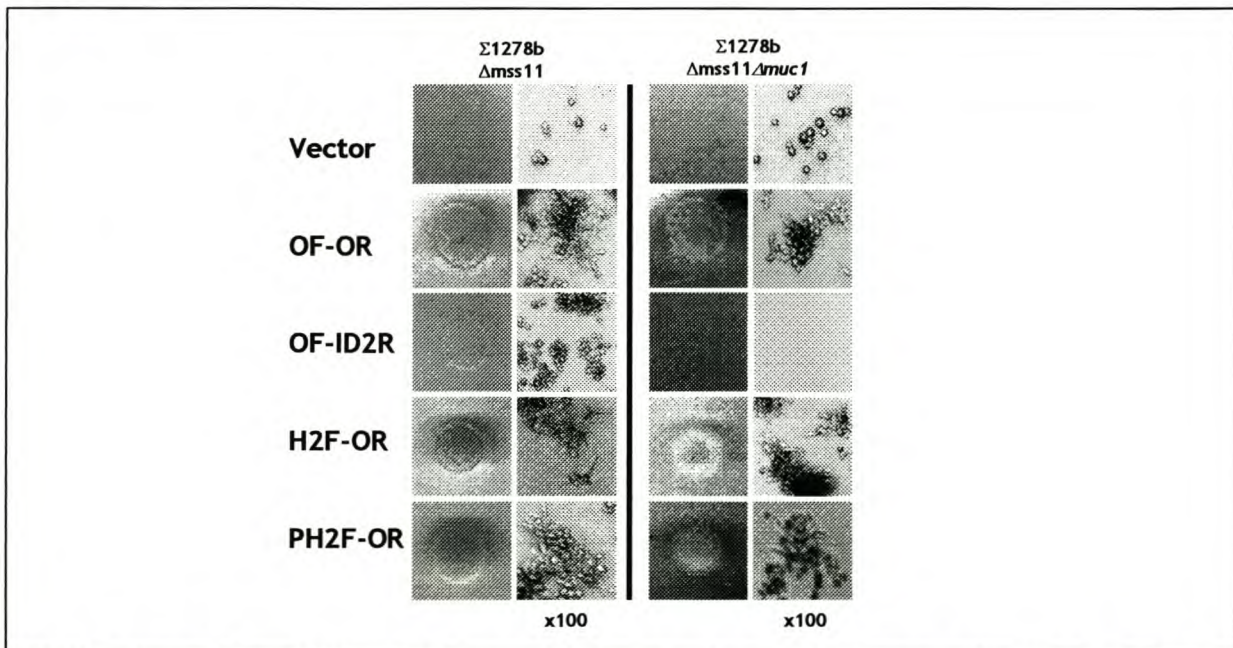
**Figure 4.4** Ability of *MSS11* fragments to induce invasive growth in a  $\Sigma 1278b\Delta mss11$  genetic background. No difference in growth was observed between colonies before the plates were washed. All of the selected truncations were assayed for invasive growth; only the fragments able to induce invasive growth are shown.

Liquid assays show that carbon limitation results in a significant increase in activation of *MUC1/FLO11* transcription by *MSS11* (Figure 4.3; Negative control vs OF-OR,  $p = 0.01$ ). Deletion of the H1-P-loop (construct H2F-OR) results in a decrease of *MUC1/FLO11* activity conferred by *MSS11*. Deletion of the H2 domain results in a further decrease, indicating that these domains are required for the mediation of carbon signalling on the *MUC1/FLO11* promoter by *MSS11*. Limited availability of nitrogen, however, results in a lowered basal level of *MUC1/FLO11* transcription (*MUC1* expression in SCD vs SLAD,  $p = 0.02$ ; Figure 4.3). This reduction remains apparent in the presence of multiple copies of full-length *MSS11* and also in the case of constructs H2F-OR and PH2F-OR. Yet, on SLAD plates containing X-gal, multiple copies of *MSS11* result in an increase in *MUC1/FLO11* transcription, indicated by blue colouration, that is absent in nitrogen rich SCD (Figure 4.3). This is most prominent at the centre of the spotted colony, where nutrients are expected to be more exhausted than at the periphery. This would expose cells to different conditions than those encountered in liquid media (where cells are dispersed and



cells are dispersed and exposed to similar concentrations of nutrients). These results do not provide an absolute reflection of Mss11p's responsiveness to nutrient signalling, since it should also be taken into account that *MUC1/FLO11* transcription is regulated by various signalling events generated in response to nutrient availability.

Assays to quantify *lacZ* expression from the *MUC1/FLO11* promoter reveal interesting differences when compared to induction of *GAL7-lacZ* by the selected truncations fused to the Gal4p DNA-binding domain (Figures 4.2 and 4.3). Of special interest is the ability of the truncated Mss11p versions that are unfused to a DNA-binding domain to regulate *MUC1/FLO11* expression. In all cases where the C-terminal has been removed there is a reduced ability to activate. This is especially clear when comparing the effect of construct H2F-ID2R, where the fusion construct results in elevated levels of *GAL7-lacZ* expression but the unfused version is unable to stimulate transcription from the *MUC1/FLO11* promoter. These results suggest an important role for the C-terminal in delivering Mss11p to where its function is required. Also, construct PH2F-OR, where the H1-P-loop and the H2 domains are removed, is able to induce *MUC1/FLO11* transcription. This confirms that the C-terminal contains sequences that can facilitate transcription at the *MUC1/FLO11* promoter.



**Figure 4.5** Comparison between constructs able to induce invasive growth in  $\Sigma 1278b \Delta mss11$  and  $\Sigma 1278b \Delta mss11 \Delta muc1$  on SCLD plates. Cell morphology photos were



taken at 100x magnification on the plates after the cells were washed of, with the use of bright field microscopy.

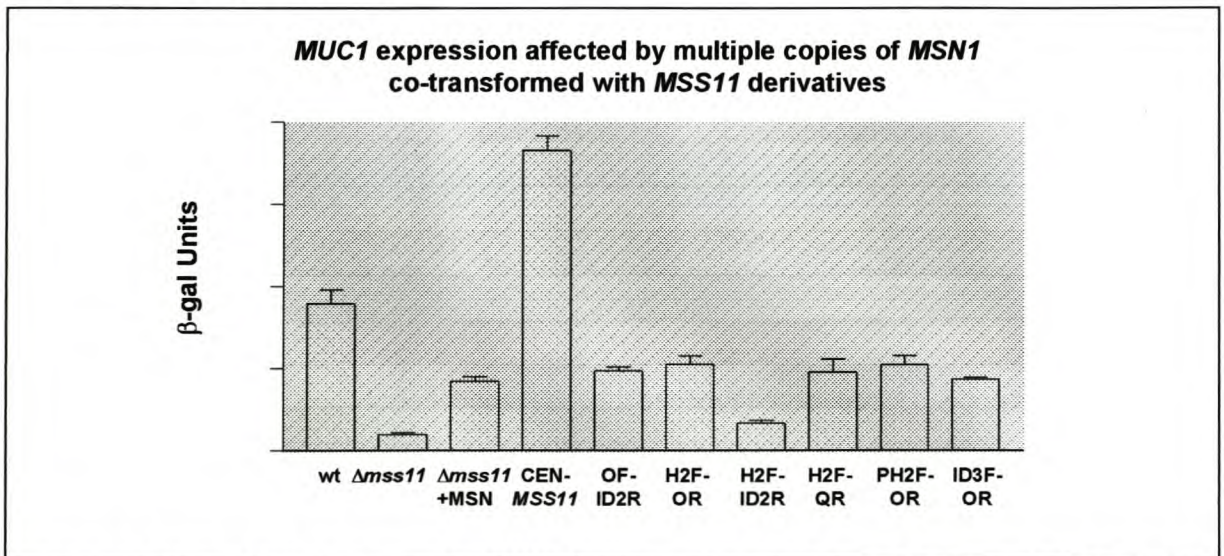
As suggested by the *MUC1/FLO11* expression values, only constructs containing the C-terminal domain are able to induce invasive growth and starch degradation. The only exception is construct OF-ID2R, lacking only the C-terminal, which is able to generate slight invasion on media with limited glucose (Figure 4.4). When comparing the cell-morphology of the different transformants it is evident that invasion is accompanied by cell-elongation except in the case of OF-ID2R, where cells are invading as clumps of round cells (Figure 4.5). The ability of OF-ID2R to induce invasive growth is lost in a  $\Delta muc1/flo11$  strain, unlike the other constructs that still allows for invasive growth and cellular elongation in the absence of *MUC1/FLO11* (Figure 4.5). Thus, OF-ID2R seems to be only affecting *MUC1/FLO11*, and not the other target(s) of *MSS11*, as *MUC1/FLO11* is not involved in cell-elongation but only in cell-substrate adherence (Palecek *et al.*, 2000).

#### **4.4.3 GENETIC INTERACTIONS BETWEEN FACTORS REGULATING PSEUDOHYPHAL DIFFERENTIATION AND *MSS11*.**

Two separate approaches were implemented to identify possible interactions between *MSS11* and factors involved in the regulation of pseudohyphal differentiation and invasive growth (*FLO8*, *STE12*, *TEC1*, *MSN1*, *PHD1* and *RME1*). To probe if any of these factors are required by *MSS11* or the truncated derivatives of *MSS11* in the regulation of invasive growth, multi-copy plasmids bearing *MSS11* and the selected truncations under the regulation of the native *MSS11* promoter and terminator were transformed into ISP15 $\Delta mss11$ . These constructs were also transformed into the same strain with deletions of the genes encoding the various factors,  $\Delta ste12$ ;  $\Delta tec1$ ;  $\Delta flo8$ ;  $\Delta msn1$ ;  $\Delta phd1$  and  $\Delta rme$  respectively. *MSS11* was deleted in these strains to remove its effect on the appearance of invasive phenotype. These transformants were spotted onto SCD, SCLD and SLAD plates to assay for invasive growth. No difference was observed when comparing the transformants with the strains containing additional deletions of *FLO8*, *STE12*, *TEC1*, *MSN1*, *PHD1*, and *RME1* (results not shown). Therefore, none of these factors had an effect on *Mss11p*'s involvement in the regulation of invasive growth.



To investigate possible interaction with regards to transcriptional activation, *MSS11* and the truncated ORFs fused to the Gal4p DNA-binding domain under control of the constitutive *ADH1* promoter was transformed into the two-hybrid strain PJ69-4A and also into the same strain bearing deletions of the *FLO8*, *STE12*, *TEC1*, *MSN1*, *PHD1* and *RME1* genes respectively. Transformants were spotted onto SCD, SCLD and SLAD plates containing X-gal in order to monitor changes activation from the *GAL7-lacZ* reporter. Again, no difference was detectable when comparing the PJ69-4A transformants to the strains bearing deletions of the mentioned factors (results not shown). Therefore, Mss11p does not require the presence of any of the factors in order to stimulate transcription.

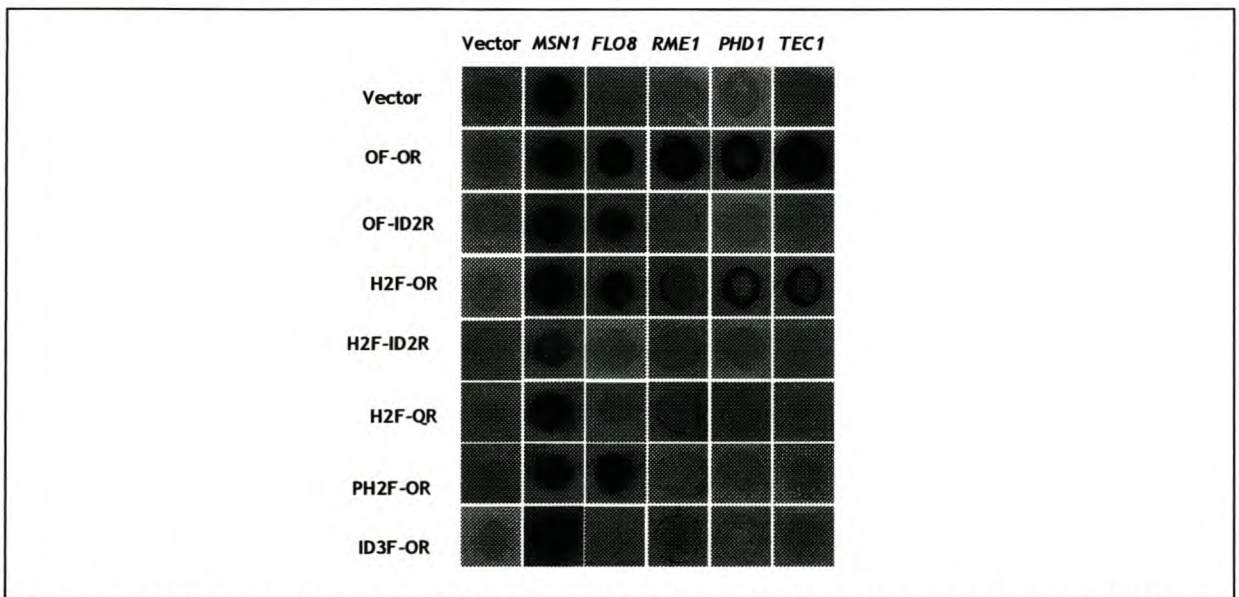


**Figure 4.6** Quantitative values of *MUC1-lacZ* reporter gene activity as affected by multiple copies of *MSN1* combined with the various fragments of *MSS11* expressed from centromeric plasmids. Standard deviation is indicated with error bars.

An alternative approach was taken in order to determine if *FLO8*, *TEC1*, *MSN1*, *PHD1* and *RME1* require specific fragments of Mss11p in order to activate *MUC1/FLO11* transcription. The factors were expressed from multi-copy plasmids using their own promoters and terminators and were co-transformed with *MSS11* and the various truncations on centromeric plasmids, expressed from the native *MSS11* promoter and terminator into  $\Sigma 1278b \Delta mss11 \Delta muc1::lacZ$ . Transformants were spotted onto SCLD plates containing X-gal and also used in liquid  $\beta$ -Galactosidase assays (in SCLD) to monitor reporter gene activity. The reasoning behind this strategy is that deletion of *MSS11* results in a reduction of *MUC1/FLO11*



expression (wt vs  $\Delta mss11$ ,  $p = 0.04$ ; Figure 4.6) that cannot effectively be reinstated by multiple copies of any of the mentioned factors, except for *MSN1* that is able to activate *MUC1/FLO11* in the absence of *MSS11*, but at a significantly reduced level ( $\Delta mss11$  vs  $\Delta mss11 + MSN1$ ,  $p = 0.05$ ; Figure 4.6; Gagiano *et al.*, 1999b). Expressing *MSS11* from a centromeric plasmid does however compensate for the loss of the deleted genomic copy, when co-expressed with the mentioned factors on multi-copy plasmids (Figure 4.7). Therefore, co-expressing the various *Mss11p* truncations together with the selected factors could reveal possible interactions between these proteins. *STE12* was not included in this study, since overexpression thereof does not have any significant effect on *MUC1/FLO11* expression. This is most likely due to a block of *Ste12p* activity by the negative regulators, *Dig1p* and *Dig2p*, which needs to be relieved by activation of the MAP kinase cascade (Cook *et al.*, 1996; Bardwell *et al.*, 1998)



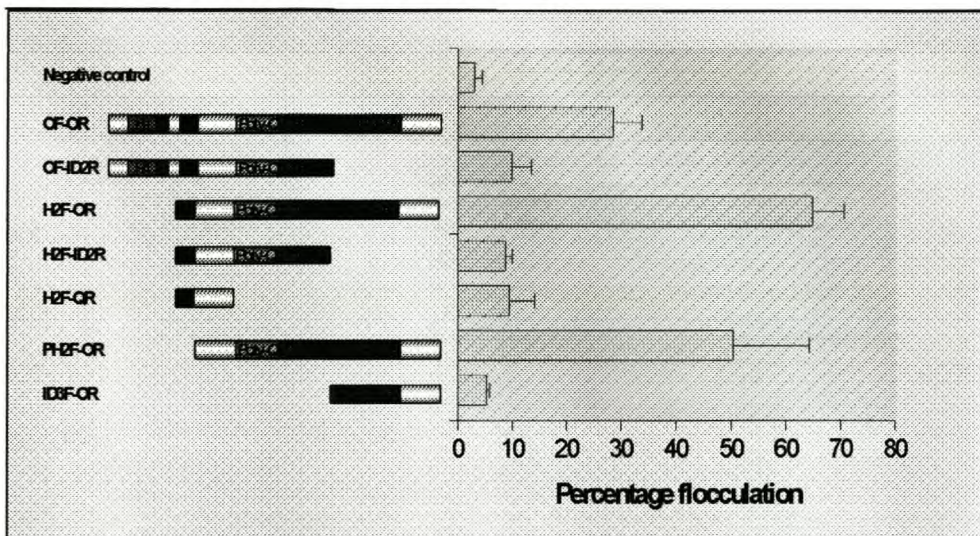
**Figure 4.7** The effect of multiple copies of *FLO8*, *TEC1*, *MSN1*, *PHD1* and *RME1* in combination with the various fragments of *MSS11* expressed from centromeric plasmids in  $\Sigma 1278b \Delta mss11 \Delta muc1::lacZ$ . *MUC1-lacZ* activity is represented by the intensity of colony colouration.

The presence of *MSS11* and the different *MSS11* fragments on centromeric plasmids alone is not sufficient to stimulate *MUC1/FLO11* transcription (Figure 4.7). Induction is only observed in combination with multiple copies of the separate factors. In the case of *MSN1* overexpression, activity was noticeable after three days and saturated after four days. For all the other factors (*FLO8*, *TEC1*, *PHD1*



and *RME1*), activation was apparent only after 12 days. Also no activity was observed in liquid assays performed in SCLD, except for *MSN1*, suggesting that the observed activation by *FLO8*, *TEC1*, *PHD1* and *RME1* is not due to a low glucose signal but a more severe state of cellular stress.

As with all the factors, *MSN1* activates most significantly in combination with the full-length version of *MSS11* (construct OF-OR) (Figure 4.7). Deletion of any fraction of *MSS11* leads to reduced levels of activation in the presence of *MSN1*. Of specific interest is construct H2F-ID2R, where the C-terminal and the H1-P-loop is removed, which appears to interfere with the functioning of *MSN1*. Intriguingly *PHD1* and *TEC1* behave similarly in response to truncations of *Mss11p*. In both cases the H2 domain and the C-terminal is required by *PHD1* and *TEC1* in order to induce *MUC1/FLO11* transcription, deletion of either results in loss of activation. Furthermore, *FLO8* shares the same requirement of *MSS11* in order to activate *MUC1/FLO11*, where deletion of the *MSS11* C-terminal results in a loss of activation by *FLO8*. Of specific interest is the ability of *FLO8* in combination with construct PH2F-OR to activate *MUC1/FLO11*. Since none of the other factors are able to enhance *MUC1/FLO11* transcription in the presence of this fragment, a specific requirement for *MSS11* by *FLO8* seems evident.

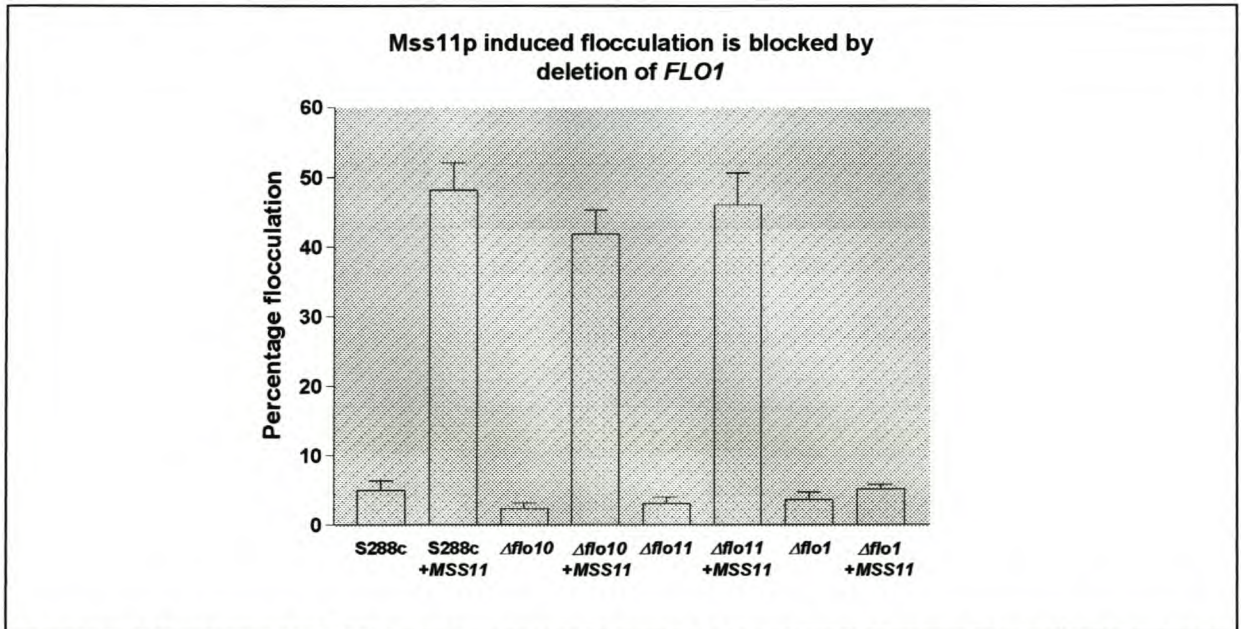


**Figure 4.8** Ability of truncated versions of *MSS11* to induce  $\text{Ca}^{2+}$ -dependent flocculation in *FY23Δmss11*.  $\text{Ca}^{2+}$  induced flocculation calculated as a percentage of induced versus uninduced values. The presence of the *Mss11p* C-terminal is essential in the establishment of *MSS11* mediated flocculation via activation sequences mainly concentrated in the H2 domain. Standard deviation is indicated with error bars.



#### 4.4.4 *MSS11* REGULATES $\text{Ca}^{2+}$ -DEPENDENT FLOCCULATION

In order to investigate the ability of *MSS11* to act as a regulator of flocculation, strains of the S288C genetic background (FY23 and BY4742), were transformed with multi-copy plasmids bearing the various *MSS11* truncations under control of the native *MSS11* promoter and terminator. To establish possible targets of *MSS11* in the regulation of flocculation, YepLac195-*MSS11* was transformed into strains with single deletions of *FLO1*, *FLO10* and *MUC1/FLO11* respectively. S288C was chosen for its known inability to flocculate due to a nonsense mutation in the *FLO8* gene (Liu *et al.*, 1996),  $\text{Ca}^{2+}$ -dependent flocculation was calculated using an assay in which the controlled addition of  $\text{Ca}^{2+}$  after binding of all bivalent ions in the growth medium was bound by EDTA. Percentage of flocculation was calculated as described in section 4.3.5.



**Figure 4.9** The effect of multiple copies of *MSS11* on the induction of  $\text{Ca}^{2+}$ -dependent flocculation in strains bearing single deletions of central flocculation genes reveals that a functional copy of *FLO1* is required by *MSS11*. Standard deviation is indicated with error bars.

Despite the absence of *FLO8* in the FY23 genetic background *MSS11* is able to induce flocculation (Figure 4.8). This is especially clear when the regulatory H1-P-loop is removed. Of interest is the resemblance between the induction of *MUC1/FLO11* transcription by the truncated versions of Mss11p (Figure 4.3), and the ability of these truncations to stimulate  $\text{Ca}^{2+}$ -dependent flocculation (Figure 4.8).



Similar to the regulation of *MUC1/FLO11* transcription, removal of the C-terminal leads to a reduction in Mss11p's ability to induce flocculation, strengthening the essential role played by the C-terminal in Mss11p's cellular function.

The level of flocculation attained by the presence of multiple copies of *MSS11* does not correlate with that reported for overexpression of *MUC1/FLO11* (Guo *et al.*, 2000). Furthermore, *MSS11* is able to effectively induce flocculation in a  $\Delta muc1$  strain (Figure 4.9), suggesting that another member of the FLO family might be targeted by *MSS11*. A possible target seemed to be *FLO10*, as overexpression thereof was shown to induce invasive growth in the absence of *MUC1/FLO11* (Guo *et al.*, 2000). However, *MSS11* is still able to establish flocculation in a  $\Delta flo10$  strain (Figure 4.9) and preliminary results indicate that *MSS11* also induces invasive growth in a  $\Delta flo10\Delta flo11$  double mutant (results not shown). On the other hand, the presence of *FLO1* is required for *MSS11* to enhance flocculation and deletion thereof abolishes the ability of *MSS11* to do so (Figure 4.9). Of future interest will be to investigate the effect Mss11p on *FLO1* transcription levels, and also to establish if Mss11p is able to induce invasive growth through *FLO1*.

#### 4.5 DISCUSSION

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In this study we present an analysis of Mss11p and identify specific interactions between Mss11p and other factors required for the activation of *MUC1/FLO11*. In order to study the activation potential of the separate domains of Mss11p, a heterologous system was used in which fragments of Mss11p were fused to the Gal4p DNA-binding domain, and transcriptional activation measured from an integrated *GAL7-lacZ* reporter gene. In response to both glucose and nitrogen limitation a clear surge in activation is noted, which is enhanced by the removal of the H1 domain and the putative P-loop. Removal of the C-terminus in combination with the H1-P-loop leads to a further increase in transcriptional activation, suggesting that the regulatory effect enforced by the H1-P-loop is directed specifically towards the H2 domain. Signalling associated with carbon limitation appears to have a dominant effect with regards to the activation function of the H2 domain, this could, however, also be effected by bias generated within the heterologous system in different media conditions. Since these fusion proteins are expressed from the



constitutive *ADH1* promoter, which is unaffected by nutrient signalling, the activation function of Mss11p is likely to be regulated by signalling events in response to nutrient limitation. This could either be by directly affecting Mss11p or an interacting factor(s). It is, however, also possible that the efficiency of either transcription or translation could be affected by the varying lengths of the *MSS11* truncation constructs, and this would therefore alter the ability of a specific truncation to induce transcription when compared to a longer or shorter version.

To date Mss11p has only been shown to be involved in regulating *MUC1/FLO11* and *STA2* expression and the associated phenotypes of filamentous growth and starch degradation. Other targets for Mss11p do however exist, since over-expression of *MSS11* allows weak invasion in a *muc1/flo11* strain. In addition, *MSS11* also induces cellular elongation, which is not effected by *MUC1/FLO11*. We provide evidence that *MSS11* harboured on a multi-copy plasmid strongly induces  $\text{Ca}^{2+}$ -dependent flocculation. Furthermore we show that this achieved in the absence of *FLO8*, *FLO10* and *MUC1/FLO11*, but does require the presence of *FLO1*. Expressing *MSS11* without the C-terminal domain results in an inability to induce *MUC1/FLO11* transcription, invasion and flocculation. Removal of the C-terminal does, however, not affect the ability of the H2 domain to activate transcription when fused to the Gal4p DNA-binding domain, which implicates a critical role for the C-terminal in delivering *MSS11* to where its function is required. Possible options include involvement of the C-terminal in nuclear import, DNA-binding or binding to DNA-associated factors.

The regulation of *MUC1/FLO11* transcription, transcription in general, invasive growth and starch degradation via *MSS11*, or any fragment of *MSS11*, does not require any of the factors involved in the activation of *MUC1/FLO11* (*FLO8*, *STE12*, *TEC1*, *MSN1*, *PHD1* and *RME1*). Co-expression of *FLO8*, *TEC1*, *MSN1*, *PHD1* and *RME1* in combination with selected fragments of *MSS11* reveals that these factors do however show specific dependencies towards particular regions of *MSS11*. Deletion of *MSS11* causes a loss of *MUC1/FLO11* transcriptional activity that cannot be re-established by multiple copies of any of the factors central to *MUC1/FLO11* activation, except for *MSN1*, which induces *MUC1/FLO11* at a significantly reduced level in the absence of *MSS11*. For all the factors optimal activation of *MUC1/FLO11*



requires the presence of full-length *MSS11*. Deletion of any region of *MSS11* results in a decrease in activation by *MSN1*. Interestingly, expressing *MSN1* together with an *MSS11* derivative of which the C-terminal and the H1-P-loop has been removed interferes with the capacity of *MSN1* to induce *MUC1/FLO11* transcription. This suggests a direct interaction with either *MSN1* or elements required for its functioning at the *MUC1/FLO11* promoter. Further biochemical studies are required to clarify at which exact point this interference occurs. *FLO8* displays a unique dependence on *MSS11*, and is only able to activate *MUC1/FLO11* in combination with fragments of *MSS11* that are able to stimulate activation when present on multi-copy plasmids. *FLO8*, unlike other factors, is able to induce *MUC1/FLO11* transcription when the H1-P-loop as well as the H2 domain are removed, indicating a genetic link between the functioning of *MSS11* and *FLO8*. *PHD1* and *TEC1* require the presence of both the C-terminal and the H2 domain; if either domain is absent, both factors are unable to activate *MUC1/FLO11* transcription. Although these results provides interesting insight into possible interactions between *MSS11* and *FLO8*, *TEC1*, *MSN1*, *RME1* and *PHD1* more substantial data is required to clarify the capacity in which *MSS11* functions inside the cell.

Future efforts will focus on identification of proteins interacting with Mss11p and also identification of other target genes affected by Mss11p.

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**CHAPTER 5**

**GENERAL DISCUSSION  
AND CONCLUSION**



## 5.1 CONCLUDING REMARKS

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The regulation of pseudohyphal differentiation and invasive growth has been comprehensively researched over the past decade. Various signalling pathways and downstream transcription factors have been implicated in the regulation of these phenotypes. The activity of these signalling cascades converge to regulate the expression of the *MUC1/FLO11* gene, encoding a member of a family of cell wall associated proteins connected with cell-cell/cell-substrate adhesion (Rupp *et al.*, 1999; Guo *et al.*, 2000). *MUC1/FLO11* expression is to a large extent co-regulated with that of the *STA2* gene, encoding an extra-cellular glucoamylase (Gagiano *et al.*, 1999b). Both genes are regulated by extremely large promoter regions, with regulatory elements present up to 2.4 kb upstream from the first coding triplet. The regulation of *MUC1/FLO11* and *STA2* presents us with an excellent model system in which to unravel the complex mechanisms involved in the regulation of eukaryotic transcription.

The transcriptional activator Mss11p plays a central role in the regulation of *MUC1/FLO11* and *STA2*, and consequently pseudohyphal growth and starch metabolism in response to carbon and nitrogen limitation (Gagiano *et al.*, 1999a, b; Gagiano *et al.*, 2003). All other factors considered to be critically involved in the activation of *MUC1/FLO11* and *STA2* are dependent on *MSS11* in order to achieve their required function. Mss11p does not have significant homology to any yeast protein, with the exception of limited homology to the transcriptional activator Flo8p (Gagiano *et al.*, 1999a). Several distinct domains are, however, present in the *MSS11* gene product. Firstly Mss11p contains distinguishing poly-glutamine and poly-asparagine domains. It also contains a putative ATP- or GTP-binding domain, commonly found in proteins such as kinases, ATPases or GTPases (Saraste *et al.*, 1990). Two short stretches close to the N-terminal, labelled H1 and H2, share significant homology to Flo8p.

Molecular analysis of Mss11p identified two separate domains that are able to stimulate RNA polymerase II dependent transcription. Firstly, both the H2 domain and the extreme C-terminus are able to activate a reporter gene when fused to the DNA-binding domain of Gal4p. Interestingly an area of 92 amino acids that immediately precedes the H2 domain, containing the H1 domain and the putative P-loop, seems to negatively regulate the activity of the H2 domain. In all cases deletion



of the H1-P-loop results in increased levels of transcription compared to constructs where this area is present. The effect of an H1-P-loop deletion is especially clear when combined with deletion of the C-terminal domain, suggesting that the apparent regulatory influence of the H1-P-loop is directed specifically towards the H2 domain.

This study also presents further insight into the functioning of Mss11p and the involvement of the separate activation and regulatory domains in mediating transcriptional activation. Mss11p is responsive to carbon and nitrogen limitation, and the limited availability of glucose does appear to have an overriding effect on Mss11p function. Transcriptional activation by Mss11p in response to glucose limitation is achieved through the activity of the H2 domain, which is negatively regulated by the H1-P-loop. Furthermore, the C-terminal is crucially required for the activation of *MUC1/FLO11* by Mss11p; deletion thereof completely abolishes the ability of Mss11p to stimulate transcription. However when the C-terminal deleted version of Mss11p is fused to the Gal4p DNA-binding domain, this fragment is able to activate transcription of a *PGAL7-lacZ* reporter gene. This could implicate a role for the C-terminal in delivering Mss11p to where its function is required. Deletion of the C-terminal domain could therefore affect processes such as nuclear import, DNA binding, binding to DNA associated factors or interaction with a factor(s) or a complex required for transcriptional activation of the genes in question.

Additionally, we propose a role for Mss11p in the regulation of Ca<sup>2+</sup>-dependent flocculation. Similar to the induction of *MUC1/FLO11* and invasive growth, the C-terminal is required for the induction of flocculation. We also present evidence that Mss11p requires the presence of *FLO1* in order to stimulate flocculation. Future efforts will include assessment of Mss11p's effect on the transcriptional regulation of *FLO1*.

Deletion of *MSS11* causes a decrease in *MUC1/FLO11* transcriptional activity that cannot be re-established by multiple copies of any of the factors central to *MUC1/FLO11* activation (*FLO8*, *STE12*, *TEC1*, *MSN1*, *PHD1* and *RME1*). We specifically indicate that *PHD1* and *TEC1* require both the C-terminal and the H2 domain in order to activate *MUC1/FLO11*. *FLO8* is only able to activate *MUC1/FLO11* in the presence of Mss11p derivatives that are able to activate *MUC1/FLO11* when they are present in multiple copies. Also, unlike the other



factors, Flo8p is able to induce *MUC1/FLO11* transcription when the H1-P-loop and the H2 domains are removed, indicating a genetic link between the functioning of *MSS11* and *FLO8*. Interestingly, expressing *MSN1* together with an *MSS11* derivative of which the C-terminal and the H1-P-loop has been removed inhibits the capacity of *MSN1* to induce *MUC1/FLO11* transcription. This fragment is able to induce transcription when fused to the Gal4p DNA-binding domain and also induces *MUC1/FLO11* expression when only the H1-P-loop is removed and not the C-terminal. The interference of this fragment could be due to interaction with a factor or complex involved in *MUC1/FLO11* stimulation/potentialiation that is required by *MSN1* (and possibly other factors), but in the absence of the C-terminus this activity is not correctly targeted. Although these results provides interesting insight into possible interactions between *MSS11* and *FLO8*, *TEC1*, *MSN1*, *RME1* and *PHD1*, respectively, more data is required to clarify the capacity in which *MSS11* functions in the regulation of pseudohyphal differentiation, invasive growth and regulation of *MUC1/FLO11*.

The dependence of many different factors on Mss11p could involve Mss11p as part of a complex that assists transcription factors in activating genes in response to specific nutritional signals. Considering the amount of genetic evidence that points to *MUC1/FLO11* and *STA2* transcription being repressed by the state of chromatin over their promoters (Yoshimoto and Yamashita., 1991; Yoshimoto *et al.*, 1991, 1992; Yamashita., 1993 Kuchin *et al.*, 1993; Kent *et al.*, 2001; Conlan and Tzamarias., 2001), a role for Mss11p in a capacity that would reduce the repressive effect of chromatin, seems possible. We set out to describe the chromatin structure on the promoter of *MUC1/FLO11* and *STA2* under repressed and induced conditions in addition to mutants of chromatin complexes and *MSS11*. We could however not achieve this goal within the time constraints of this study.

Future work will focus on the effect of *MSS11* overproduction and deletion on genome-wide transcription, in order to allocate other targets of Mss11p. Also of interest will be to establish possible biochemical interactions with other proteins and/or DNA.



## 5.2 REFERENCES

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## **APPENDIX**

**Mss11p is a transcription factor regulating  
pseudohyphal differentiation, invasive growth  
and  
starch metabolism in  
*Saccharomyces cerevisiae*  
in  
response to nutrient availability**

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# Mss11p is a transcription factor regulating pseudohyphal differentiation, invasive growth and starch metabolism in *Saccharomyces cerevisiae* in response to nutrient availability

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## Summary

In *Saccharomyces cerevisiae*, the cell surface protein, Muc1p, was shown to be critical for invasive growth and pseudohyphal differentiation. The transcription of *MUC1* and of the co-regulated *STA2* glucoamylase gene is controlled by the interplay of a multitude of regulators, including Ste12p, Tec1p, Flo8p, Msn1p and Mss11p. Genetic analysis suggests that Mss11p plays an essential role in this regulatory process and that it functions at the convergence of at least two signalling cascades, the filamentous growth MAPK cascade and the cAMP-PKA pathway. Despite this central role in the control of filamentous growth and starch metabolism, the exact molecular function of Mss11p is unknown. We subjected Mss11p to a detailed molecular analysis and report here on its role in transcriptional regulation, as well as on the identification of specific domains required to confer transcriptional activation in response to nutritional signals. We show that Mss11p contains two independent transactivation domains, one of which is a highly conserved sequence that is found in several proteins with unidentified function in mammalian and invertebrate organisms. We also identify conserved amino acids that are required for the activation function.

## Introduction

Upon nutrient limitation, cells of the yeast *Saccharomyces cerevisiae* undergo a transition from ovoid cells, which bud in an axial (haploid) or bipolar (diploid) fashion, to elongated cells that bud in a unipolar fashion. The daughter

cells remain attached to the mother cells, which results in chains of cells referred to as pseudohyphae. These filaments can grow invasively into the agar and away from the colony and are hypothesized to be an adaptation of yeast cells to search for nutrient-rich substrates (reviewed by Kron, 1997; Madhani and Fink, 1998; Borges-Walmsley and Walmsley, 2000; Pan *et al.*, 2000; Bauer and Pretorius, 2001; Gancedo, 2001). A large number of genes that play a role in this adaptation to changing environmental conditions have been isolated, and most were shown to participate in distinct signalling cascades that regulate the dimorphic switch from yeast to hyphal form. The best characterized of these signalling pathways are the invasive growth MAP kinase cascade (Liu *et al.*, 1993; Cook *et al.*, 1996; 1997; Möscher *et al.*, 1996; Madhani *et al.*, 1997) and the Gpa2p-cAMP-PKA pathway (Ward *et al.*, 1995; Roberts *et al.*, 1997; Robertson and Fink, 1998; Pan and Heitman, 1999; Rupp *et al.*, 1999; Lorenz *et al.*, 2000; Tamaki *et al.*, 2000; Gagiano *et al.*, 2002). In addition to the components of these established regulatory cascades, several other factors have also been identified for their roles in regulating pseudohyphal and invasive growth. These include Phd1p (Gimeno and Fink, 1994; Lorenz and Heitman, 1998), Sok2p (Ward *et al.*, 1995; Pan and Heitman, 1999), Elm1p (Blacketer *et al.*, 1993; Garret, 1997; Koehler and Myers, 1997), Msn1p and Mss11p (Gagiano *et al.*, 1999a,b), but these factors have not been placed in the context of known signal transduction pathways, have not been characterized sufficiently or seem to function through alternative pathways.

*MUC1* (also known as *FLO11*) is a member of the adhesin- or flocculin-encoding genes and is regulated by the signalling pathways that determine filamentous growth (Guo *et al.*, 2000). It encodes a large, cell wall-associated, glycosylphosphatidylinositol (GPI)-anchored threonine/serine-rich protein with structural resemblance to mammalian mucins and yeast flocculins (Lambrechts *et al.*, 1996a; Lo and Dranginis, 1996). Deletion analyses demonstrated that *MUC1* is critical for pseudohyphal differentiation and invasive growth and that overexpression of this gene results in flocculating yeast strains in liquid media and pseudohyphal/invasive growth on solid media

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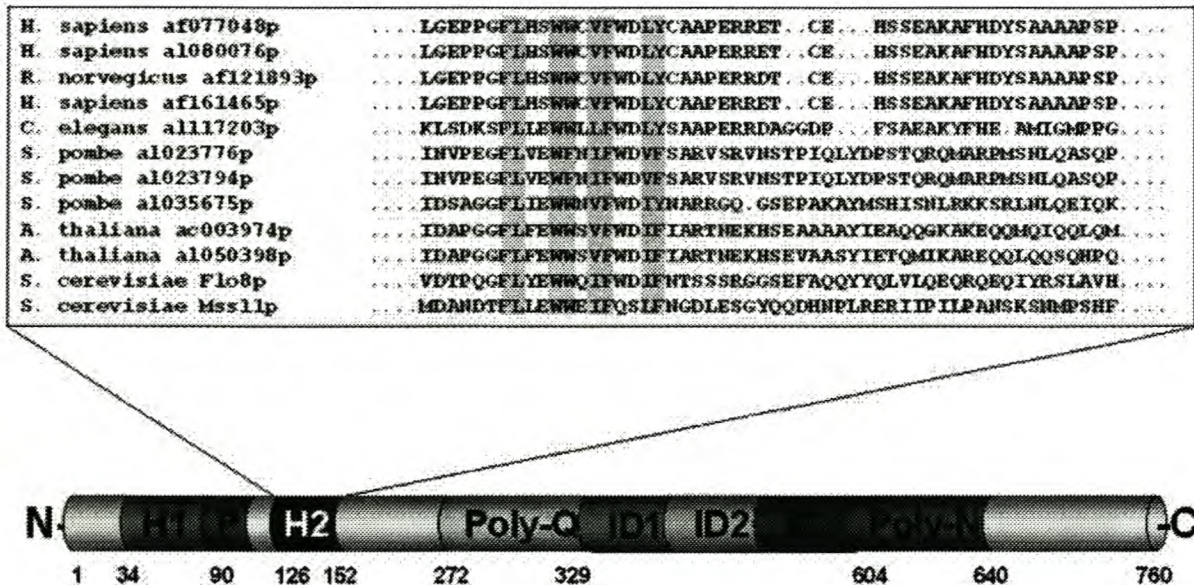
(Lambrechts *et al.*, 1996a; Lo and Dranginis, 1996; 1998; Guo *et al.*, 2000).

The upstream regulatory region of *MUC1* is one of the largest yeast promoters identified to date, and areas as far as 2.4 kb upstream of the transcription start site have been shown to be required for the regulation of *MUC1* expression (Gagiano *et al.*, 1999a; Rupp *et al.*, 1999). The data suggest that most, if not all, the previously mentioned signalling pathways and regulatory proteins converge on this promoter to regulate invasive growth and pseudohyphal differentiation, making this gene the most relevant target of this complex regulatory network. The entire *MUC1* upstream region is almost identical to that of the *STA2* gene (Gagiano *et al.*, 1999b), which is present only in some *S. cerevisiae* strains and codes for an extracellular glucoamylase that enables the yeast cell to use starch as a carbon source (reviewed by Pretorius *et al.*, 1991; Vivier *et al.*, 1997; Gagiano *et al.*, 2002).

Of the regulatory proteins mentioned above, Mss11p appears to play one of the most central roles in regulating filamentous growth and starch metabolism. When the *MSS11* gene is present on a multiple copy plasmid, strong invasive and pseudohyphal growth is observed in all the strains tested, including those with single or multiple deletions of genes encoding other factors that activate *MUC1* and *STA2* transcription (Gagiano *et al.*, 1999a,b). On the

other hand, the deletion of the *MSS11* locus results in the complete absence of these phenotypes, which cannot be suppressed efficiently by overexpressing any of the factors identified to date, including Ste12p, Flo8p and Msn1p (Gagiano *et al.*, 1999a,b).

Despite a clear role in regulating filamentous growth and starch metabolism, the exact molecular function of Mss11p is unknown. Although it was shown to regulate the expression of *MUC1* and *STA2* at a transcriptional level (Webber *et al.*, 1997; Gagiano *et al.*, 1999a,b), and that this activation occurs via specific areas within the *MUC1* and *STA2* promoters (Gagiano *et al.*, 1999a), it is unclear whether it confers this activation directly, i.e. by acting as a transcription activator, or indirectly, i.e. by interacting with or favouring the recruitment of other transcription factors for example. Mss11p has no significant sequence homology to any yeast protein, with the exception of some limited homology to the Flo8p transcription activator (Gagiano *et al.*, 1999a). Mss11p, however, contains several distinctive domains (Fig. 1), including (i) a poly glutamine and (ii) a poly asparagine domain, which are similar to, but significantly larger than, similar domains observed in the repressor Ssn6p. It also contains (iii) a putative ATP- or GTP-binding domain, commonly found in ATP- or GTP-binding proteins such as the kinases, ATPases or GTPases (Saraste *et al.*, 1990); and (iv) two



**Fig. 1.** A diagrammatic representation of Mss11p that illustrates the position and length of the different domains. Domains H1 and H2 represent the domains with homology to *S. cerevisiae* Flo8p. The alignment of the second homology domain, H2, with Flo8p and proteins of unknown function from other organisms is shown. The first homology domain, H1, has no significant homology to any protein besides Flo8p. The putative ATP/GTP-binding domain (P-loop) is represented by a P. The poly glutamine and poly asparagine domains are indicated by poly Q and poly N respectively. The large domain between the poly glutamine and poly asparagine domains has no known or predicted structural features or homology to any protein identified to date. It was subdivided into three smaller domains for the functional analysis. These smaller domains were named interdomain regions 1, 2 and 3 and are indicated by ID1, ID2 and ID3 on the diagram.



short stretches (labelled H1 and H2) of amino acids with significant homology to Flo8p. The functional relevance and significance of all these domains has not yet been investigated. Furthermore, *Mss11p* has only been implicated in the regulation of *MUC1* and *STA2* transcription and, therefore, it is unknown whether any other target genes exist or whether *Mss11p* also plays a role in cellular processes other than filamentous growth or starch metabolism.

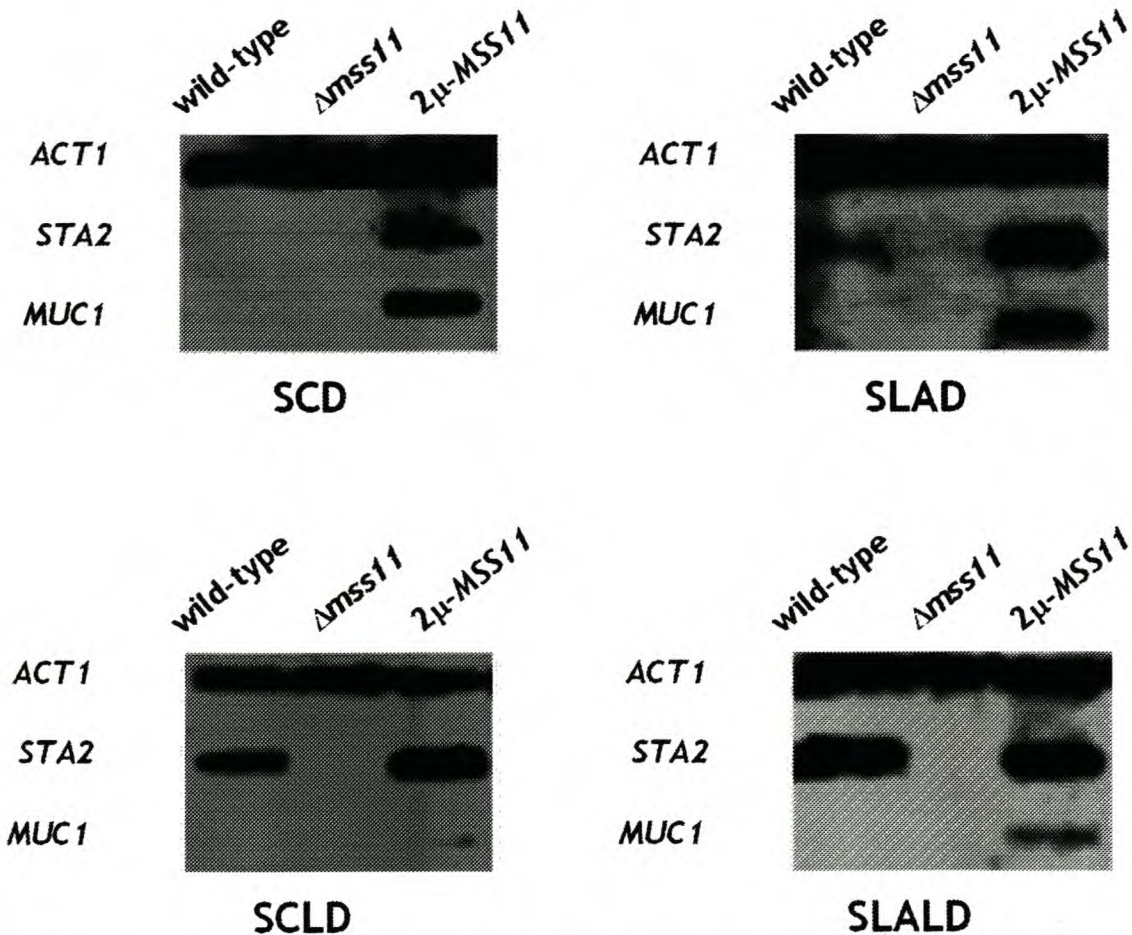
In this paper, we show that *Mss11p* can activate transcription directly. The data suggest that *Mss11p* is regulated on a post-translational level in response to specific nutritional signals and that the N-terminal domain is responsible, at least in part, for this regulation. Furthermore, we delineate activation domains in *Mss11p* by means of domain mapping and show that these domains are sufficient for the activation of a reporter gene, as well as of the *MUC1* and *STA2* genes. We also identify specific amino acids that are involved in transcriptional activation

and define a domain that is highly conserved in several mammalian and invertebrate proteins of unknown function.

## Results

### *Mss11p* regulates *MUC1* and *STA2* mRNA levels

To determine the transcription levels of *MUC1* and *STA2* under different nutritional conditions and to determine the effect of multiple copies of *MSS11* or the deletion of *MSS11*, we isolated RNA from cells grown in synthetic media containing high (2%) or low (0.1%) concentrations of glucose as carbon source and  $(\text{NH}_4)_2\text{SO}_4$  as nitrogen source. The wild-type strain, ISP15, was transformed with the 2  $\mu$  plasmid bearing *MSS11*, YEplac112-*MSS11* or the unmodified vector, YEplac112, as negative control. The effect of a deletion of *MSS11* was assessed using strain ISP15 $\Delta$ *mss11* transformed with the unmodified vector, YEplac112. The results are presented in Fig. 2.



**Fig. 2.** Northern blot analysis on the effect of single and multiple copies of *MSS11*, as well as the deletion thereof, on the transcript levels of *STA2* and *MUC1* under different nutritional conditions. The concentrations and components of the different media are described in detail in Table 2.



No mRNA from either *STA2* or *MUC1* could be detected in the  $\Delta mss11$  strain, confirming the essential role played by Mss11p in the transcription of these genes. In the wild-type strain, *MUC1* transcription levels were also undetectable in all media tested, including under nitrogen and carbon limitation. However, the transcription levels of *STA2* in the wild-type background were clearly detectable in all media except the synthetic medium containing 2% glucose (SCD). This discrepancy in the transcription levels of the two genes with almost identical regulatory regions has been described before and can be attributed, at least in part, to the presence of two inserts in the promoter region of *MUC1* that are absent from that of *STA2* (Gagiano et al., 1999a).

The data clearly show that both nitrogen and carbon limitations result in increased transcription of *STA2*. Although no *STA2* transcript was detectable in a medium with high glucose and ammonium concentration (SCD), the *STA2* mRNA signal intensity reached 17%, 100% and 100% of that of the actin signal when the cells were grown in SLAD (nitrogen limited), SCLD (glucose limited) and SLALD (glucose and ammonium limited) respectively. The presence of multiple copies of *MSS11* increased the transcription levels of *STA2* significantly in SCD, SLAD and SCLD, reaching 50% (up from non-detectable in the wild type), 66% (up from 17%) and 149% (up from 100%) of the actin signal intensity respectively. However, the presence of the multiple copy vector alone did not result in a further increase in the *STA2* transcription levels in SLALD medium.

Although the absolute transcription levels varied in the different media, the presence of *MSS11* in multiple copies increased the transcription of *MUC1* significantly under all conditions. The signal intensity of *MUC1* reached 55%, 32%, 36% and 18% of that of the actin signal in SCD, SLAD, SCLD and SLALD respectively. Contrary to what was observed for *STA2*, multiple copies of *MSS11* therefore resulted in a significant increase in *MUC1* mRNA levels in SLALD. However, the nutritional conditions continued to exert control over the relative expression levels of *MUC1* and *STA2* in the presence of multiple copies of *MSS11*. In particular, and contrary to what was observed for *STA2*, the expression of *MUC1* was high in media containing elevated (2%) glucose concentrations (SCD, SLAD). This observation can be explained by findings from other groups, which identified *MUC1* as being downstream of the Gpr1p-Gpa2p glucose receptor that senses high glucose concentrations and transmits the signal to *MUC1* via changes in intracellular cAMP levels (Lorenz et al., 2000). This pathway was shown to require glucose as well as complex media for sustained activation (Colombo et al., 1998), and a key component of the pathway, the Sch9p protein kinase, was shown to regulate

*MUC1* transcription in response to cAMP levels (Lorenz et al., 2000).

*Mss11p is a transcription activator that regulates MUC1 and STA2 differentially in response to nutritional conditions*

The Northern blot analysis clearly demonstrates that Mss11p mediates the expression levels of *MUC1* and *STA2*. However, it does not allow assessment of whether (i) Mss11p plays a direct role as a transcription activator or acts at a different level; and (ii) whether Mss11p is required for transmitting the nutritional information.

In order to clarify the first question, we exploited the modular characteristic of transcription factors (reviewed by Triezenberg, 1995) to identify the domain(s) in Mss11p that would be required for the transcriptional activation of the target genes. A series of fusions were created between the open reading frames (ORFs) of *MSS11* and the *GAL4* transcription factor, of which the activation domain was deleted. The constructs included the full-length *MSS11* ORF, as well as sequential deletions thereof, which were transformed into a strain containing an integrated reporter gene, *lacZ*, under the control of the *GAL7* promoter. This promoter contains binding sites for the Gal4p transcription activator and, if Mss11p contained transcription activation domains, the fusion protein would mediate the transcriptional activation of the reporter gene (James et al., 1996). Both liquid and plate  $\beta$ -galactosidase assays were used to identify such activation domains. The results of these assays are presented in Figs 3 and 4.

Figure 3 indicates that, relative to the vector containing only the Gal4p DNA-binding domain as a negative control, the fusion protein with full-length Mss11p results in an  $\approx$  40-fold increase in reporter gene activity in liquid SCD medium. This result clearly suggests that Mss11p can either activate transcription directly or recruit other factors required for activation.

The fusions of the *MSS11-GAL4* DNA-binding domain gene are under the control of the constitutively active *ADH1* promoter, resulting in high levels of transcription under all nutritional conditions. If transcriptional activation by Mss11p is constitutive, the fusion protein can be expected to result in high levels of  $\beta$ -galactosidase on all media. The differences in  $\beta$ -galactosidase levels observed on the different media (Fig. 4) would therefore suggest that the ability of Mss11p to activate transcription is regulated at a post-transcriptional level in response to specific nutritional conditions. Indeed,  $\beta$ -galactosidase activity is weakest on SCD medium, in which carbon and nitrogen are in sufficient supply, while being induced significantly on the three nutrient-limited media, SLAD, SCLD and



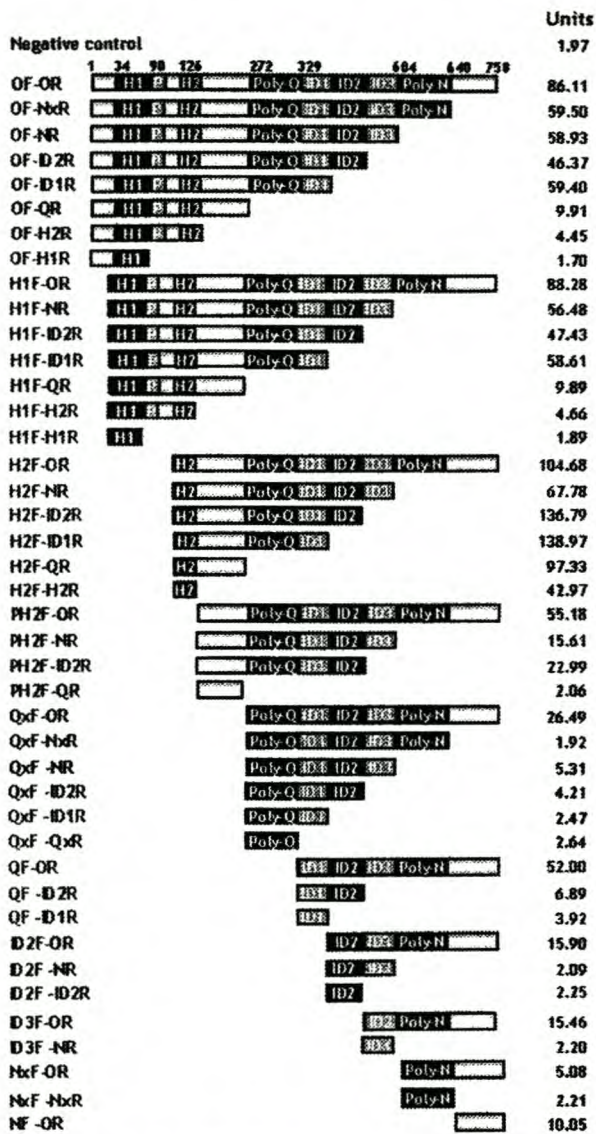


Fig. 3. Identification of *Mss11p* as a transcriptional activator and the identification of specific activation domains. The different *Mss11p* fragments fused to Gal4p are represented diagrammatically, and the levels of reporter gene activity conferred in liquid SCLD media by each, as measured by  $\beta$ -galactosidase activity, are indicated next to the relevant construct.

SLALD. This would suggest that *Mss11p*-dependent activation occurs directly as a response to nutrient limitation. Interestingly, the variation in expression levels conferred by the *Mss11p*-Gal4p fusion protein on the reporter gene (Fig. 4) correlates well with the observed differences in *STA2* mRNA levels in the presence of multiple copies of *MSS11* (Fig. 2). However, the same does not hold true for the *MUC1* mRNA levels detected in the Northern blots. The data therefore again suggest that other factors make

a contribution to the regulation of this gene in the presence of high levels of glucose.

*The conserved H2 domain and the C-terminus are required for the transcriptional activation function of Mss11p*

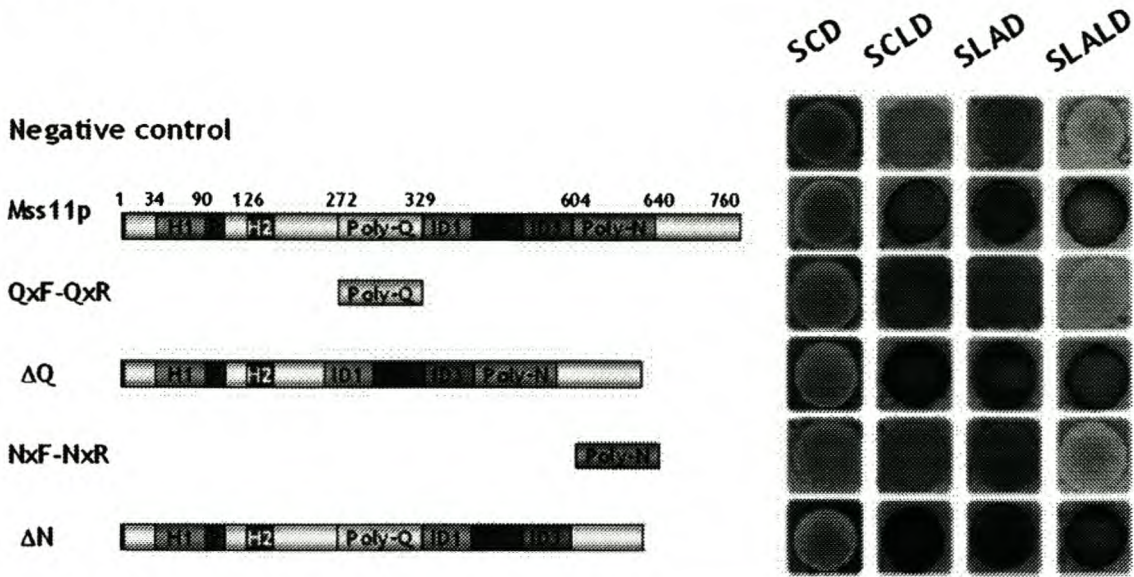
The systematic deletions from both the N- and C-termini to identify the domains specifically required for the activation function are presented in Fig. 3.

The data suggest that there are two areas in *Mss11p* that are required for transcriptional activation. All constructs in which the extreme C-terminal end of the protein (amino acids 640–720) is deleted show significantly reduced transcriptional activation of the reporter gene, independent of corresponding sequential deletions from the N-terminal side of the protein. Furthermore, the C-terminal domain on its own (construct NF-OR) confers significant activation to the reporter gene, suggesting that it is able to interact with the transcription machinery and to promote transcription.

However, the constructs in which the C-terminal sections of the protein are deleted continue to generate significant, although reduced, levels of  $\beta$ -galactosidase activity, and activation only ceases once the 272 N-terminal amino acids are deleted concomitantly with the C-terminal section (construct QxF-NxR). The data clearly show that a second activation domain is located in a region bordered by amino acids 126 and 272 (construct H2F-QR), which confers levels of  $\beta$ -galactosidase activity similar to those observed with full-length *Mss11p*. Within this section of the protein, the 26 amino acids that constitute the H2 domain (construct H2F-H2R), which corresponds to one of the two short stretches of *Mss11p* with homology to Flo8p, confer strong transcriptional activation, corresponding to 50% of the values obtained for full-length *Mss11p*, whereas the domain composed of amino acids 152–272 is unable to activate the reporter gene. This suggests that the 26-amino-acid H2 domain acts as a transcription activation domain. This is further supported by the fact that the deletion of this domain leads in all cases to a significant drop in transcription activation by the remaining sections of the protein.

It is interesting to note that activation by *Mss11p* appears to be negatively regulated by the domain composed of amino acids 34–126, which immediately precedes the H2 domain and contains the second short stretch of amino acids with significant homology to Flo8p, H1, as well as a putative P-loop. All constructs containing the H1-P-loop domain show significantly lower activation levels than corresponding constructs in which the sequence has been deleted (see for example H1F-ID2R compared with H2F-ID2R), whereas the truncated ver-





**Fig. 4.** Effect of variable nutrient availability on the ability of the Mss11p–Gal4p fusion protein to activate transcription and relevance of the Mss11p poly glutamine (poly Q) and poly asparagine (poly N) domains. The ability of the different Mss11p fragments, fused to the Gal4p DNA-binding domain, to activate the *PGAL7-lacZ* reporter system was assessed in strain pJ69-4A. The Mss11p domains fused to Gal4p are represented diagrammatically, and the levels of reporter gene activity conferred by each are represented by the intensity of the colonies in the photographs. The constituents of the media used are listed in Table 2.

sions of Mss11p with deletions of these amino acids result in the highest levels of activation observed. These levels are significantly higher than those achieved with the construct containing full-length Mss11p. This negative effect of the domain appears particularly clearly when the C-terminal activation domain has been deleted (see for example H1F-QR compared with H2F-QR), suggesting that the H1-P-loop sequence may particularly influence activation by the H2-associated domain.

#### *Analysis of the poly glutamine and poly asparagine domains of Mss11p*

Glutamine-rich domains have been identified as the activation domains of transcription factors in a number of organisms, ranging in complexity from yeast (e.g. Mcm1p) to humans (e.g. Oct1 and Oct2) (Johnson *et al.*, 1993). The difference between these prototypical glutamine-rich activation domains and the poly glutamine domain of Mss11p, however, is the dispersion of hydrophobic amino acids, such as leucine, valine and phenylalanine, between the glutamine residues (Johnson *et al.*, 1993; Triezenberg, 1995), which were shown to be critical for the activation function of the transcription factors (Gill *et al.*, 1994). A poly glutamine domain significantly shorter than that of Mss11p (12 glutamine residues) was also identified in the yeast protein, Pgd1, a component of the mediator complex between transcriptional activators and the RNA polymerase II complex, but its exact role has yet to be

investigated (Brohl *et al.*, 1994; Gustafsson *et al.*, 1998; Myers *et al.*, 1998).

The function of the poly asparagine domains in proteins is unknown at this stage. Asparagine-rich domains have been described for only two other *S. cerevisiae* proteins. *S. cerevisiae* Azf1p and Swb1p include short, asparagine-rich domains (Schmalix and Bandlow, 1994; Stein *et al.*, 1998), but the relationship between the function and the presence of these domains has not been investigated.

The data presented in Fig. 3 clearly demonstrate that both the poly glutamine and the poly asparagine domains (constructs QxF-QxR and NxF-NxR respectively) are unable to activate transcription when fused to the Gal4p DNA-binding domain (Fig. 3), whereas their deletion appears not significantly to affect transcriptional activation by the remains of the truncated proteins (Fig. 4).

As repetitive coding sequences, commonly referred to as trinucleotide repeats, are known to vary in size in mammalian genomes, and recombination between these sequences may cause neurodegenerative diseases such as Huntington's disease and Friedrich's ataxia in humans (reviewed by Jakupciak and Wells, 2000; Shimohata *et al.*, 2001), we investigated the size of the Mss11p poly glutamine and poly asparagine domains in different strains. The corresponding *MSS11*-encoding sequences were polymerase chain reaction (PCR) amplified from strains ISP15, ISP52, FY23 (S288C) and W303, and the resulting fragments were sequenced. There was no differ-



ence in either the size or the specific sequences between the different fragments obtained from these strains from different genetic backgrounds (results not shown).

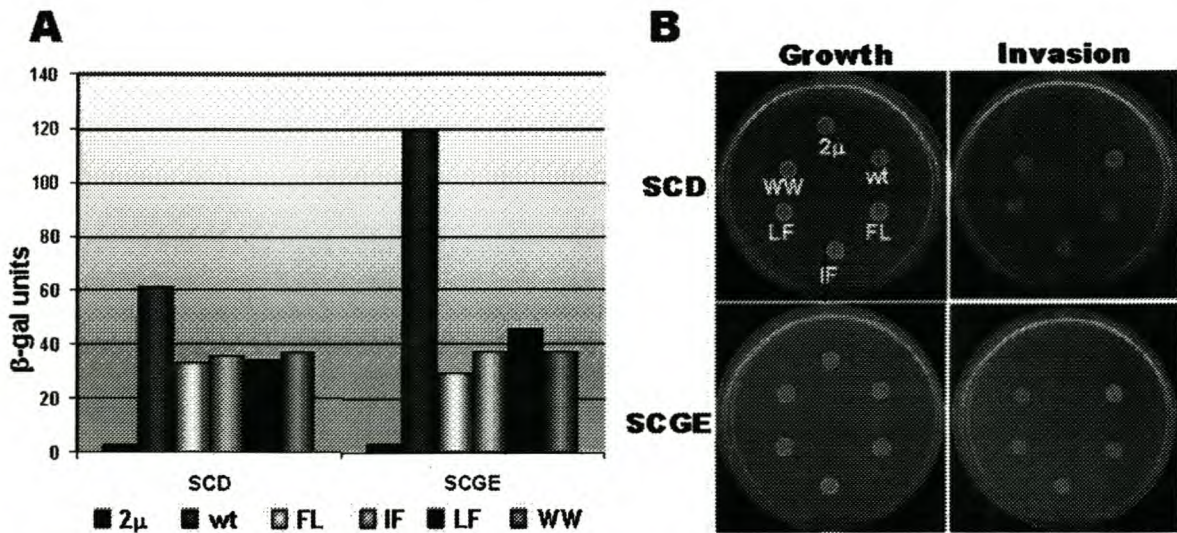
*Specific amino acids in the conserved H2 domain are critical for the Mss11p transcriptional activation function*

As the H2 domain was shown to be required for the activation function of *Mss11p* and also to be able to stimulate transcription of the *PGAL7-lacZ* reporter gene when fused to the Gal4p DNA-binding domain, we investigated whether the conserved amino acids identified in H2 (Fig. 1) are required for the activation function. We specifically targeted the conserved amino acid pairs Phe-133–Leu-134, Trp-137–Trp-138, Ile-140–Phe-141 and Leu-144–Phe-145 (Fig. 1). All these amino acids were mutated to glycine and alanine respectively (Table 4, Fig. 5). The effects of the mutations on the ability of *Mss11p* to stimulate transcription of the reporter genes were assessed through  $\beta$ -galactosidase liquid assays in strain  $\Sigma 1278b\Delta mss11\Delta muc1::lacZ$  (Fig. 5A), whereas invasive growth was assessed in strain  $\Sigma 1278b\Delta mss11$  (Fig. 5B).

From the results, it is clear that all the conserved pairs of amino acids are required for full activation, as all the mutations significantly reduced the ability of *Mss11p* to activate the *lacZ* reporter gene. For cells grown in SCD

medium,  $\beta$ -galactosidase values were reduced by  $\approx 50\%$  in all the mutants in comparison with those obtained for the wild-type protein. This reduction is similar to what is observed when the entire N-terminal domain of *Mss11p*, including H2, is deleted (Fig. 3), suggesting that the remaining activation may result from transcriptional activation by the C-terminal domain and further strengthening the hypothesis that *Mss11p* indeed contains two independent activation domains. Furthermore, although the expression of wild-type *Mss11p* results in increased transcriptional activation on SCGE medium compared with SCD medium, the mutated *Mss11p* proteins result in similar activation on both media. This suggests that activation by the H2 domain is responsible, at least in part, for the glucose-dependent regulation of gene expression by *Mss11p*.

The reduced ability of the mutated forms of *Mss11p* to activate the reporter gene correlates well with their ability to induce invasive growth (Fig. 5B). On both SCD and SCGE media, the  $\Delta mss11$  strains transformed by vectors encoding the H2-mutated versions of *Mss11p* all display phenotypes that are intermediate between the strong invasiveness of the strain transformed with wild-type *MSS11* and the absence of invasion of the strain transformed with the vector alone. These data clearly demonstrate a direct correlation between the ability of *Mss11p* to activate transcription and the regulation of invasive growth.



**Fig. 5.** Identification of critical amino acids in the H2 activation domain of *Mss11p*. FL (Phe–Leu), IF (Ile–Phe), LF (Leu–Phe) and WW (Trp–Trp) indicate the pairs of amino acids within the H2 domain that were mutated in the corresponding version of *Mss11p*. All pairs were changed to GA (Gly–Ala).

**A.** Impact of the mutations in the H2 domain on the ability of *Mss11p* to activate the chromosomal copy of *MUC1* in the  $\Sigma 1278b\Delta mss11\Delta muc1::lacZ$  strain (*lacZ* integrated in chromosomal copy of *MUC1*) in media containing fermentable and non-fermentable carbon sources.

**B.** Ability of multiple copy plasmids encoding either the wild-type (wt) or H2 point mutation forms of *Mss11p* to induce invasive growth in a  $\Sigma 1278b\Delta mss11$  strain. The invasiveness of each strain correlates perfectly with the level of transcriptional activation.



The length and composition in amino acids of the *Mss11p* poly glutamine domain are reminiscent of the poly glutamine stretches found in mammalian proteins, such as Huntington and frataxin. The poly glutamine domains of these two (and several other) mammalian proteins are notorious, as recombination events in the repetitive coding sequences, commonly referred to as trinucleotide repeats, cause neurodegenerative diseases such as Huntington's disease and Friedrich's ataxia (reviewed by Jakupciak and Wells, 2000; Shimohata *et al.*, 2001).

We could not detect any variation in the size of these sequences in four *S. cerevisiae* strains of different genetic background. This does not preclude the possibility that such variation may exist, and feral strains of *S. cerevisiae* in particular should be investigated.

The genetic evidence presented to date suggests that *Mss11p*, like *Pgd1p*, could also have a role as a transcriptional mediator. The results of the epistasis analyses involving *MSS11* demonstrated that all the other transcription factors required for the transcriptional activation of *MUC1* and *STA2*, i.e. *Ste12p*, *Mss10p* and *Flo8p*, also require *Mss11p* for their activation function (Gagiano *et al.*, 1999a,b). These results could also be interpreted as evidence that *Mss11p* is the most downstream component of each of the different signal transduction cascades represented by these transcription factors. However, the fact that *Flo8p*, *Ste12p* and *Msn1p* were all identified as DNA-binding transcription factors (Estruch and Carlson, 1990; Madhani and Fink, 1997; Kobayashi *et al.*, 1999) makes this explanation highly unlikely and points strongly towards *Mss11p* facilitating the transcriptional activation function of these transcription factors at the *MUC1* and *STA2* promoters.

The strong activation in response to specific nutritional signals suggests a direct role for *Mss11p* as a transcriptional activator, but the dependency of three structurally dissimilar and unrelated transcription factors, *Flo8p*, *Msn1p* and *Ste12p*, on *Mss11p* is difficult to reconcile with such a role. It is therefore possible that *Mss11p* is part of a complex that assists transcription factors in activating genes in response to specific signals. Considering the amount of genetic evidence that points towards *MUC1* and *STA2* transcription being repressed by the state of the chromatin over their promoters (Inui *et al.*, 1989; Okimoto *et al.*, 1989; Yoshimoto and Yamashita, 1991; Yoshimoto *et al.*, 1991; 1992; Kuchin *et al.*, 1993; Yamashita, 1993; Park *et al.*, 1999), a role for *Mss11p* in a complex that reduces this repressive effect, such as a histone acetyltransferase complex (reviewed by Sterner and Berger, 2000), seems possible. Removing or releasing the chromatin barrier over the *STA2* and *MUC1* promoters in response to specific nutritional signals could therefore result in the observed activation, as it would make the promoter accessible to *Flo8p*, *Msn1p* and *Ste12p*, as well

as to other transcription factors. It was demonstrated recently that the transcription of the *PGL1* gene is regulated by the same signalling elements that regulate the transcription of *MUC1* in conditions conducive for filamentous growth (Madhani *et al.*, 1999; Gognies *et al.*, 2001). The *PGL1* gene encodes an endopolygalacturonase that enables the yeast cell to hydrolyse pectin. These observations would suggest that the co-regulation of filamentous growth and starch metabolism should be extended to include polysaccharide degradation in general.

It does not appear that *Mss11p* plays a role in regulating the transcription of other members of the adhesin and flocculin gene family. A microarray analysis to identify target genes of *Mss11p*, other than *MUC1* and *STA2*, revealed that *Mss11p* is very specific in regulating the transcription of *MUC1* and *STA2* and failed to identify other genes with significantly increased transcription in the presence of multiple copies of *MSS11* (results not shown).

Future efforts will focus on the identification of proteins that interact with *Mss11p* to identify a more precise role for *Mss11p* in regulating *MUC1* and *STA2* transcription.

## Experimental procedures

### *Yeast strains, genetic methods and media*

The yeast strains used in this study, along with the relevant genotypes, are listed in Table 1. Strain ISP15 has the ability to degrade starch and to grow invasively. The strains ISP15 and  $\Sigma 1278b$  have been used extensively for the characterization of invasive growth and pseudohyphal differentiation (Gimeno *et al.*, 1992; Lambrechts *et al.*, 1996a,b; Webber *et al.*, 1997; Gagiano *et al.*, 1999a,b). Strain pJ69-4A is commonly used in the analysis of two-hybrid interactions and was generously provided by P. James (James *et al.*, 1996). In strains *ISP15* $\Delta$ *muc1::lacZ* and  $\Sigma 1278b$  $\Delta$ *mss11* $\Delta$ *muc1::lacZ*, the ORF of *MUC1* has been replaced by the ORF of the reporter gene *lacZ* fused to the *S. cerevisiae* *HIS3* gene, which is used as a selection marker.

The carbon and nitrogen sources used in the preparation of the different yeast media are listed in Table 2. The yeast nitrogen base that was used did not contain any amino acids or nitrogen source (Becton Dickinson). All synthetic media were supplemented with the specific amino acids required to fulfil the auxotrophic demands of each strain. Amino acids were obtained from Sigma-Aldrich and were added according to the recommended concentrations (Sherman *et al.*, 1991; Ausubel *et al.*, 1994). The solid media contained 2% agar (Becton Dickinson).

Standard molecular genetic and yeast techniques were used throughout this work (Sherman *et al.*, 1991; Ausubel *et al.*, 1994). Yeast transformations were performed using the lithium acetate method (Ausubel *et al.*, 1994).

### *Plasmid construction*

Standard procedures for the isolation and manipulation of



*The putative ATP- and/or GTP-binding domain of Mss11p appears not to affect transcriptional activation*

The analysis of transcriptional activation by truncated forms of Mss11p suggested a possible regulatory function for the domain containing the H1 domain together with the putative P-loop (Gly-88–Ser-89–Ala-90–Ser-91–Gly92–Gly-93–Lys-94–Thr-95–Ser-96), an ATP- and/or GTP-binding sequence. To test whether the P-loop regulated the activation function of Mss11p, we mutated two of the critical amino acids, Gly-93 and Lys-94, to Ala-93 and Arg-94 respectively. We tested the ability of the P-loop-mutated allele of *MSS11* to confer transcriptional activation in strains *ISP15Δmuc1::lacZ* and  $\Sigma$ 1278b, in either a wild-type or a  $\Delta$ *mss11* genetic background. We also fused the *MSS11* ORF, carrying the P-loop mutations, to the *GAL4* fragment encoding the DNA-binding domain to assess whether the encoded protein would be able to activate the *PGAL7-lacZ* reporter gene in strain pJ69-4A on different media.

In no case could we observe significant differences between the ability of the wild type and the mutated versions of Mss11p to activate transcription (data not shown). The data suggest that the putative P-loop is either non-functional or that it regulates other aspects of Mss11p function.

**Discussion**

In this paper, we present a molecular analysis of Mss11p, a transcriptional regulator of the *MUC1* and *STA2* genes of *S. cerevisiae*. As a regulator of these two genes, it is also a major regulator of the ability of *S. cerevisiae* to form pseudohyphae, grow invasively and metabolize starch (Webber *et al.*, 1997; Lorenz and Heitman, 1998; Gagiano *et al.*, 1999a,b). The correlation between Mss11p levels, *MUC1* and *STA2* transcription and these phenotypes is well established (Webber *et al.*, 1997; Lorenz and Heitman, 1998; Gagiano *et al.*, 1999a,b). However, the impact of specific nutritional signals on this relationship has never been assessed properly. Here, we show, through Northern analyses and reporter gene expression analysis in different media, that Mss11p relates the effect of nutritional signals, specifically the glucose and nitrogen limitation signals, to the transcription of *MUC1* and *STA2*. These observations reaffirm previous observations on the co-regulation of *MUC1* and *STA2* and, consequently, on the co-regulation of the filamentous growth and starch metabolism phenotypes. The results also suggest that the effects of the different nutritional conditions on *MUC1* and *STA2* transcription are transmitted via Mss11p.

The molecular analysis of Mss11p presents conclusive evidence that Mss11p acts as a transcriptional acti-

vator. This activation function is regulated on a post-transcriptional level, as the activation efficiency is clearly dependent on the amount of nutrients present in the media, even if Mss11p is fused to the Gal4p DNA-binding domain and expressed from a heterologous promoter that is not regulated by nutrient availability. Furthermore, the regulation of the transcriptional activation is not dependent on the binding of Mss11p to its target, either DNA or other proteins, as the Mss11p fusions to the Gal4p-binding domain result in a similar pattern of transcriptional regulation to that exerted by the native protein on *MUC1* and *STA2*. The molecular dissection of Mss11p identified two activation domains, one of which, named H2, appears to be highly conserved among several proteins of unknown function. Changes in individual amino acids within this domain significantly reduce the ability of Mss11p to activate transcription. This reduction is accompanied by a correspondingly reduced ability to induce invasive growth, confirming that the ability of Mss11p to activate transcription is directly correlated with the effects of Mss11p overexpression and deletion on invasive and pseudohyphal differentiation. Interestingly, the data strongly suggest that the H2 domain could be responsible for transmitting a carbon source-dependent signal, as the mutant versions no longer lead to higher *MUC1* transcription in media containing non-fermentable carbon sources. The domain therefore plays a central role in the control of Mss11p-dependent activation. The identification of this conserved domain may provide useful insights into the role of the proteins of unknown function listed in Fig. 1.

The data presented here also suggest that the N-terminal domain of Mss11p is involved in the regulation of the activation function. Mutations resulting in the deletion of the H1- and P-loop-containing domain result in increased transcription. This suggests that ATP or GTP binding may be involved in the regulation of Mss11p activity. It is possible that the observed regulation results from an autoregulatory function, similar to what is observed in the Snf1p protein kinase, for example, where a regulatory domain inhibits the function of the catalytic domain in repressive conditions (Carlson, 1998; 1999).

Furthermore, we show that the unique poly glutamine and poly asparagine domains are not required for the activation function of Mss11p. The role of these domains therefore remains to be identified. Although significantly smaller, a poly glutamine domain has been identified in only one other *S. cerevisiae* protein, namely Pgd1p (Brohl *et al.*, 1994). Pgd1p was shown to be a component of the mediator complex between transcriptional activators and the RNA polymerase II complex (Gustafsson *et al.*, 1998; Myers *et al.*, 1998). Considering that Pgd1p functions in a multicomponent protein complex, it is possible that the poly glutamine domain has a structural role or that it is required for protein–protein interactions.



**Table 3.** A list of primers used to generate the different truncations and deletions of *Mss11p* for expression under its own promoter and for fusion to the Gal4p DNA-binding domain (also included are the primers used to mutate the putative ATP/GTP-binding domain and the putative activation domain, H2).

Primer name	Position relative to ORF	Sequence
MSS11-P-F	-581 to -600	5'-ACAGGGCGCAATCAGCTACC-3'
MSS11-P-R	+3 to -21	5'-cgtgaattcCATATCTTTATCATGCACCTTTT-3'
MSS11-T-F	+2275 to +2304	5'-atctgtcgacCTTAAACCTATTAACAACAAAAAGTGTTC-3'
MSS11-T-R	+2717 to +2736	5'-gatcaagcttTGCCAGATAGCTTGCTTAC-3'
MSS11-OF	+4 to +30	5'-atcgaattcGATAACACGACCAATTAATACAAAT-3'
MSS11-OR	+2250 to +2274	5'-gcaggtcgacaGCTATCCATTAGATCAGGAGAAAAG-3'
MSS11-H1F	+103 to +126	5'-gatcgaattcTTTGATGCGGATTCTCGAGTTTTTC-3'
MSS11-H1R	+254 to +276	5'-tcaggtcgacaACCCGAAGCAGATCCGTTTATTC-3'
MSS11-H2F	+376 to +396	5'-gatcgaattcCTGATGGACGCTAATGACACG-3'
MSS11-H2R	+421 to +444	5'-tcaggtcgacaGTCTCCATTGAACAATGATTGAAA-3'
MSS11-PH2F	+442 to 465	5'-atggaattcGACCTAGAATCTGGGTACCAACAG-3'
MSS11-QF	+988 to +1011	5'-atcgaattcCACCGTATCCTATTGTCAACCA-3'
MSS11-QR	+794 to +816	5'-caggtcgacaTGCTGGTGATTGCAATCATTGA-3'
MSS11-QxR	+817 to +837	5'-atggaattcCAGCCCAGCAATCATCTCAA-3'
MSS11-QxR	+961 to +984	5'-gcaggtcgacaTTGCTGCTGTTGATGTTGCTG-3'
MSS11-ID1R	+1240 to +1260	5'-gatgtcgacaTTGCTGTAGTCTTGCTGCTG-3'
MSS11-ID2F	+1240 to +1260	5'-gatgaattcCAGCAGCAAGCACTACAGCAA-3'
MSS11-ID2R	+1510 to +1530	5'-gatgtcgacaTAATTGCTGGTTAGCCGCCAT-3'
MSS11-ID3F	+1510 to +1530	5'-gatgaattcATGCGGCTAACCAAGCAATTA-3'
MSS11-NF	+1921 to +1944	5'-atggaattcACCCACAGTATCACACCATCA-3'
MSS11-NR	+1789 to +1812	5'-caggtcgacaAGGCAAAGGAAAGACGGAGGTAGA-3'
MSS11-NxF	+1810 to +1839	5'-atggaattcCCTAACAATAACAATAACAACAACA-3'
MSS11-NxR	+1897 to +1926	5'-gcaggtcgacaGGGTGATTATTACTATTATTATTATT-3'
MSS11-QReco	+796 to +816	5'-atcgaattcTGCTGGTGATTGCAATCATT-3'
MSS11-NReco	+1789 to +1811	5'-atcgaattcGCAAAGGAAAGACGGAGGTAGA-3'
MSS11-PloopF	+247 to +288	5'-TTATCTAGAATAAACGGATCTGCTTCGGGT <b>GCGAGA</b> ACTAGC-3'
MSS11-WW-F	+409 to +432	5'-gaa <b>GCCGGC</b> AAATTTTCAATCATTG-3'
MSS11-WW-R	+391 to +414	5'-ttc <b>GCCGGC</b> TCCAGTAAAAACGTGTG-3'
MSS11-IF-F	+418 to +441	5'-gaa <b>GCCGGC</b> CAATCATTGTTCAATGGA-3'
MSS11-IF-R	+400 to 423	5'-ttg <b>GCCGGC</b> TCCACCATTCCAGTAA-3'
MSS11-FL-F	+403 to +417	5'-acg <b>GCCGGC</b> TGGAATGGTGGGAAAT-3'
MSS11-FL-R	+379 to +402	5'-cag <b>GCCGGC</b> CGTGTCCATTAGCGTCCAT-3'
MSS11-LF-F	+436 to +453	5'-tca <b>GCCGGC</b> CAATGGAGACCTAGAATCT-3'
MSS11-LF-R	+412 to +435	5'-att <b>GCCGGC</b> TGATTGAAAATTTCCA-3'

The different restriction sites generated and used for cloning purposes are indicated in underlined text. An additional nucleotide (a), indicated in italics, was inserted into the reverse primers to maintain the reading frame when ligating fragments into plasmids pGBD-C2 and YEplac112-*MSS11exp*. Specific nucleotide changes to introduce mutations in *MSS11* are indicated in bold text. *MSS11* sequences are given in capital letters. The positions relative to the ORF are given, considering the ATG as position +1 to +3 and the last nucleotide of the non-coding upstream region as position -1.

cloned as *EcoRI*-*Sall* fragments into the unique *EcoRI* and *Sall* sites of plasmid pGBD-C2 (James *et al.*, 1996). The resulting plasmids are listed in Table 5.

#### Site-directed mutagenesis

ATP- and GTP-binding proteins from a number of different organisms have been shown to carry a glycine-rich motif known as the P-loop, which is required for the binding of ATP and/or GTP and generally critical for the function of the protein (reviewed by Saraste *et al.*, 1990). The consensus sequence of this domain was determined as Gly-1-X-2-X-3-X-4-X-5-Gly-6-Lys-7-Ser/Thr-8 by mutation analysis of a common sequence found in myosin and many other nucleotide-binding enzymes (Saraste *et al.*, 1990). Mutation analyses of a very large number of ATP- and GTP-binding proteins suggested that the critical amino acids are indeed Gly-1, Gly-6 and Lys-7 (invariant), as well as Ser-8, which can be replaced only with a functionally equivalent Thr (Saraste *et al.*, 1990). The putative P-loop (Gagiano *et al.*,

1999a; Fig. 1) of *Mss11p* was eliminated by mutating amino acids that were shown to be critical for its function (Saraste *et al.*, 1990), namely a glycine at position 93 and a lysine at position 94 to alanine and arginine respectively. This was achieved by designing a forward primer that contained the desired nucleotide changes. The primer was extended to span a native *XbaI* site in the *MSS11* ORF that would aid in the cloning of the fragment. This primer, MSS11-PloopF, was used together with the reverse primer, MSS11-OR, to generate a fragment that contained the desired sequence alterations. The fragment was digested with *XbaI* and *Sall* before ligation into plasmid MSS11-OF-OR, in which the corresponding fragment had been removed. The construct was sequenced to verify that the correct alterations were made and that no additional mutations were introduced through PCR.

A small stretch of amino acids in *Mss11p* was shown to have some homology to a similar sized domain in Flo8p (Gagiano *et al.*, 1999a). This domain, dubbed H2, was subsequently found to be conserved between a number of eukaryotic proteins of unknown function. An alignment of the



**Table 1.** The yeast strains used in this study.

Strain	Genotype	Source or reference
ISP15	MATa STA2 his3 thr1 trp1 leu2 ura3	Gagiano et al. (1999a)
ISP15Δmss11	MATa STA2 his3 thr1 trp1 leu2 ura3 Δmss11::LEU2	Gagiano et al. (1999a)
ISP15Δmuc1::lacZ	MATa STA2 his3 thr1 trp1 leu2 ura3 Δmuc1::lacZ-HIS3	This study
Σ1278b	MATα ura3-52 trp1::hisG leu2::hisG his3::hisG	H. U. Mösch
Σ1278bΔmss11	MATα ura3-52 trp1::hisG leu2::hisG his3::hisG mss11::LEU2	This study
Σ1278bΔmss11Δmuc1::lacZ	MATα ura3-52 trp1::hisG leu2::hisG his3::hisG mss11::LEU2 muc1::lacZ-HIS3	This study
PJ69-4A	MATa his3 trp1 leu2 ura3 gal4 gal80 LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ	James et al. (1996)

DNA were used throughout this study (Ausubel et al., 1994). All restriction enzymes, T4 DNA ligase and Expand Hi-Fidelity polymerase used in the enzymatic manipulation of DNA were obtained from Roche Diagnostics and were used according to the specifications of the supplier. Most PCR fragments generated by the PCR technique for this work were first cloned into the plasmid pGEM-T of the pGEM-T PCR cloning kit, supplied by Promega. *Escherichia coli* DH5α (Gibco BRL/Life Technologies) was used for the propagation of all plasmids and was grown in Luria–Bertani (LB) broth at 37°C. All *E. coli* transformations and the isolation of DNA were done according to Ausubel et al. (1994).

The potential functional domains in *Mss11p* have been described previously (Gagiano et al., 1999a). The relative sizes and positions of these domains are illustrated in Fig. 1. To identify the functional relevance of these domains, a series of plasmids was constructed that encode versions of *Mss11p* that are either systematically shortened from the C- or N-terminal ends or without specific, internal domains. The 2 μ plasmid, YEplac112 (Gietz and Sugino, 1986), was used to construct a base plasmid containing the promoter, start codon, stop codon and terminator region of *MSS11*. The resulting plasmid, YEplac112-MSS11exp, was used for all expression purposes.

The promoter region of *MSS11* was PCR amplified using primers MSS11-PF and MSS11-PR, together with plasmid YEplac112-MSS11 (Gagiano et al., 1999a) as template. The reverse primer, MSS11-PR, was designed to contain an *EcoRI* site after the *MSS11* start codon. This fragment was digested with *EcoRI* and *ScaI* and inserted into the unique *EcoRI* and *HindIII* sites of plasmid YEplac112. The terminator region was PCR amplified using primers MSS11-TF and MSS11-TR, together with YEplac112-MSS11 as template. The forward primer, MSS11-TF, was designed to contain a *SaI* restriction site immediately 5' to the stop codon, and the reverse primer was designed to contain a *HindIII* restriction site for cloning the fragment into the unique *SaI* and *HindIII* sites of plasmid YEplac112. The resulting plasmid,

YEplac112-MSS11exp, therefore contained the full-length *MSS11* promoter, start codon, stop codon and terminator region, as well as unique *EcoRI* and *SaI* sites for the insertion of the different *MSS11* ORF fragments.

Different combinations of the primers listed in Table 3 were used to generate the truncated ORF fragments by means of PCR. Plasmid YEplac112-MSS11 was used as a template in all PCRs. All forward primers were designed to contain an *EcoRI* restriction site, and all reverse primers were designed to contain a *SaI* restriction site for cloning the different fragments in frame into plasmid YEplac112-MSS11exp, which is described above. The resulting plasmids are listed in Table 4. All plasmids were sequenced to verify that the expected deletions were correct and that no mutations were introduced through PCR.

The poly glutamine and poly asparagine domains were deleted by replacement with an *EcoRI* restriction site. Primer MSS11-OF was used in a PCR, together with primer MSS11-QReco, which is designed to contain an in frame *EcoRI* site. Plasmid YEplac112-MSS11 was used as template to generate a fragment stretching from the ATG initiation codon of *MSS11* to before the poly glutamine domain, ending in an *EcoRI* site. This fragment was digested with *EcoRI*, ligated into plasmid YEplac112-MSS11-QF-OR and then opened with *EcoRI* to generate an *MSS11* ORF, in which an *EcoRI* site replaced the area encoding the poly glutamine domain. The correct orientation was selected through restriction analysis, and the construct was sequenced for confirmation. The poly asparagine domain was deleted through a similar strategy, using YEplac112-MSS11 as template and primers MSS11-NReco and MSS11-OF in a PCR. This fragment was digested with *EcoRI* and ligated into plasmid YEplac112-MSS11-NF-OR, then opened with *EcoRI* to generate an *MSS11* ORF in which an *EcoRI* site replaced the area encoding the poly asparagine domain.

To fuse *Mss11p*, as well as the different truncated and mutated derivatives, to the Gal4p DNA-binding domain, the fragments were excised from YEplac112-MSS11exp and

**Table 2.** The components of the different yeast media used in this work.

Media	Nitrogen source	Carbon source (glucose)
YPD	1% yeast extract, 2% peptone	2%
YPLD	1% yeast extract, 2% peptone	0.1%
SCD	1.7% yeast nitrogen base, 40 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2%
SCLD	1.7% yeast nitrogen base, 40 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.1%
SLAD	1.7% yeast nitrogen base, 20 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2%
SLALD	1.7% yeast nitrogen base, 20 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.1%



**Table 4.** The list of plasmids used in this work.

Plasmid	Relevant genotype	Source/reference
PPMUC1- <i>lacZ</i>	<i>CEN4 URA3 P MUC1-lacZ</i>	Gagiano <i>et al.</i> (1999a)
PPSTA2- <i>lacZ</i>	<i>CEN4 URA3 P STA2-lacZ</i>	Gagiano <i>et al.</i> (1999a)
YEplac112	2 $\mu$ <i>TRP1</i>	Gietz and Sugino (1988)
YEplac112-MSS11	2 $\mu$ <i>TRP1 MSS11</i>	Gagiano <i>et al.</i> (1999a)
YEplac112-MSS11exp	2 $\mu$ <i>TRP1 PMSS11 TMSS11</i>	This work
YEplac112-MSS11-OF-OR	2 $\mu$ <i>TRP1 MSS11</i> <sub>1-758</sub>	This work
YEplac112-MSS11-Pmut	2 $\mu$ <i>TRP1 MSS11</i> <sub>1-758</sub> ; G93→A; K94→R	This work
YEplac112-MSS11-ΔQ	2 $\mu$ <i>TRP1 MSS11</i> <sub>1-758</sub> ; Δ272-329	This work
YEplac112-MSS11-ΔN	2 $\mu$ <i>TRP1 MSS11</i> <sub>1-758</sub> ; Δ605-640	This work
YEplac112-MSS11-FL	2 $\mu$ <i>TRP1 MSS11</i> <sub>1-758</sub> F133→G; L134→A	This work
YEplac112-MSS11-WW	2 $\mu$ <i>TRP1 MSS11</i> <sub>1-758</sub> W137→G; W138→A	This work
YEplac112-MSS11-IF	2 $\mu$ <i>TRP1 MSS11</i> <sub>1-758</sub> I140→G; F141→A	This work
YEplac112-MSS11-LF	2 $\mu$ <i>TRP1 MSS11</i> <sub>1-758</sub> L144→G; F145→A	This work

For the plasmids carrying *MSS11* fragments, the encoded area is indicated in subscript, giving the first and last amino acids of the Mss11p derivative encoded by the respective insert.

relevant protein sequences, with the conserved amino acids highlighted, is shown in Fig. 1. To establish whether these amino acids contribute to the functioning of Mss11p, the amino acids pairs, i.e. the isoleucine and phenylalanine, the phenylalanine and leucine, the leucine and phenylalanine, as well as the two tryptophans, were all mutated to glycine and alanine respectively. This was achieved through a PCR-based mutagenesis strategy. Forward and reverse primers containing the desired nucleotide changes were designed and, by changing the nucleotides to code for glycine and alanine, a unique *Cfr10I* restriction site was introduced. Using YEplac112-MSS11 as template, the different forward primers were used together with primer MSS11-OR, whereas the reverse primers were used together with primer MSS11-OF to generate fragments that contain the desired mutations. The smaller fragments, generated using primer MSS11-OF with the reverse primers, were digested with *EcoRI* and *Cfr10I*. The larger fragments, which were generated using the forward primers together with primer MSS11-OR, were digested with *Cfr10I* and *SalI*. The fragments were ligated in the necessary combinations, together with YEplac112-MSS11exp that had been digested with *EcoRI* and *SalI*, to form full-length *MSS11* fragments containing the desired mutations.

#### RNA isolation and Northern analysis

Colonies were inoculated from the selective media into 5 ml of liquid SCD medium and grown to an optical density, measured at 600 nm (OD<sub>600</sub>), of ≈1 to serve as starter cultures. These starter cultures were used to inoculate 50 ml flasks of media containing varying concentrations and types of nitrogen and carbon sources (Table 2). All media were inoculated to an initial OD<sub>600</sub> of 0.05 and incubated on a rotary shaker to reach a final OD<sub>600</sub> of 1.0. Total RNA from each strain was isolated and separated on a 1.2% formaldehyde agarose gel, using the Bio101 FastRNA RedKit according to the specifications of the supplier.

The RNA was transferred and fixed onto Hybond-N nylon membranes (Amersham Pharmacia Biotech), according to the specifications of the manufacturer. *ACT1*, *MUC1* and *STA2* transcripts were detected using gene-specific probes

prepared with the DIG PCR labelling kit (Roche Diagnostics) according to the specifications of the manufacturer. Hybridizations were done at 42°C for 16 h in standard formaldehyde buffer containing 50% formamide.

Densitometric analysis of the results was carried out using ALPHAIMAGER software version 5.5 (Alpha Innotec).

#### β-Galactosidase liquid and plate assays

Strains containing the *lacZ* reporter gene were transformed, and three independent colonies from each transformation were grown in 5 ml of selective SCD medium to an OD<sub>600</sub> of 1.0. From each of these starter cultures, a 5 ml culture of SCD was inoculated to an OD<sub>600</sub> of 0.05 and incubated to grow at 30°C to an OD<sub>600</sub> of 1.0. β-Galactosidase assays were performed as described by Ausubel *et al.* (1994). Assays were performed on all three transformants and, in each case, the mean value is presented. The standard deviation did not exceed 15% and was usually <8%.

For the plate assays, the strains were transformed with the different deletion and mutation constructs and with the unmodified vector, YEplac112, as negative control. Three colonies from each transformation were grown in 5 ml of selective SCD medium to an OD<sub>600</sub> of 1.0. From each of these starter cultures, 15  $\mu$ l was dropped onto solid YPD, YPLD, SCD, SCLD, SLAD and SLALD agar plates (see Table 2 for media components). These plates also contained Xgal, added according to Ausubel *et al.* (1994), for the optical assessment of the activity conferred by the different Mss11p derivatives on the transcription levels of the reporter genes.

#### Computer-aided analyses and homology searches

Homology searches with Mss11p were done using the WWW-based BLASTP function (Altschul *et al.*, 1997). Optimized sequence alignments between the Mss11p domains and the domains of the proteins identified through BLASTP (Fig. 1) were done using the BESTFIT and PILEUP functions of the GCG Wisconsin package. Access to the software was



**Table 5.** The list of plasmids used to identify the activation domains of *Mss11p*.

Plasmid	Relevant genotype	Source/reference
pGBD-C2	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub>	James <i>et al.</i> (1996)
pGBD-C2-MSS11-OF-OR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>1-758</sub>	This work
pGBD-C2-MSS11-OF-NxR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>1-640</sub>	This work
pGBD-C2-MSS11-OF-NR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>1-604</sub>	This work
pGBD-C2-MSS11-OF-ID2R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>1-511</sub>	This work
pGBD-C2-MSS11-OF-ID1R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>1-420</sub>	This work
pGBD-C2-MSS11-OF-QR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>1-272</sub>	This work
pGBD-C2-MSS11-OF-H2R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>1-168</sub>	This work
pGBD-C2-MSS11-OF-H1R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>1-112</sub>	This work
pGBD-C2-MSS11-H1F-OR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>35-758</sub>	This work
pGBD-C2-MSS11-H1F-NR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>35-604</sub>	This work
pGBD-C2-MSS11-H1F-ID2R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>35-511</sub>	This work
pGBD-C2-MSS11-H1F-ID1R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>35-420</sub>	This work
pGBD-C2-MSS11-H1F-QR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>35-272</sub>	This work
pGBD-C2-MSS11-H1F-H2R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>35-168</sub>	This work
pGBD-C2-MSS11-H1F-H1R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>35-112</sub>	This work
pGBD-C2-MSS11-H2F-OR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>146-758</sub>	This work
pGBD-C2-MSS11-H2F-NR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>146-604</sub>	This work
pGBD-C2-MSS11-H2F-ID2R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>146-511</sub>	This work
pGBD-C2-MSS11-H2F-ID1R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>146-420</sub>	This work
pGBD-C2-MSS11-H2F-QR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>146-272</sub>	This work
pGBD-C2-MSS11-H2F-H2R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>146-168</sub>	This work
pGBD-C2-MSS11-H2F-H1R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>169-758</sub>	This work
pGBD-C2-MSS11-PH2F-OR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>169-604</sub>	This work
pGBD-C2-MSS11-PH2F-NR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>169-511</sub>	This work
pGBD-C2-MSS11-PH2F-ID2R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>169-272</sub>	This work
pGBD-C2-MSS11-PH2F-QR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>274-758</sub>	This work
pGBD-C2-MSS11-QxF-OR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>274-640</sub>	This work
pGBD-C2-MSS11-QxF-NxR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>274-604</sub>	This work
pGBD-C2-MSS11-QxF-NR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>274-604</sub>	This work
pGBD-C2-MSS11-QxF-ID2R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>274-511</sub>	This work
pGBD-C2-MSS11-QxF-ID1R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>274-420</sub>	This work
pGBD-C2-MSS11-QxF-QxR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>274-329</sub>	This work
pGBD-C2-MSS11-QF-OR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>330-758</sub>	This work
pGBD-C2-MSS11-QF-ID2R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>330-511</sub>	This work
pGBD-C2-MSS11-QF-ID1R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>330-420</sub>	This work
pGBD-C2-MSS11-ID2F-OR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>414-758</sub>	This work
pGBD-C2-MSS11-ID2F-NR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>414-604</sub>	This work
pGBD-C2-MSS11-ID2F-ID2R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>414-511</sub>	This work
pGBD-C2-MSS11-ID2F-ID1R	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>504-758</sub>	This work
pGBD-C2-MSS11-ID3F-OR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>504-604</sub>	This work
pGBD-C2-MSS11-ID3F-NR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>605-758</sub>	This work
pGBD-C2-MSS11-NxF-OR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>605-640</sub>	This work
pGBD-C2-MSS11-NxF-NxR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>605-640</sub>	This work
pGBD-C2-MSS11-NF-OR	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>641-758</sub>	This work
pGBD-C2-MSS11-AP	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>1-758</sub> ; G93→A; K94→R	This work
pGBD-C2-MSS11-ΔQ	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>1-758</sub> ; D272→329	This work
pGBD-C2-MSS11-ΔN	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>1-758</sub> ; D605→640	This work
pGBD-C2-MSS11-FL	2 m <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>1-758</sub> F133→G; L134→A	This work
pGBD-C2-MSS11-WW	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>1-758</sub> W137→G; W138→A	This work
pGBD-C2-MSS11-IF	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>1-758</sub> I140→G; F141→A	This work
pGBD-C2-MSS11-LF	2 $\mu$ <i>TRP1 GAL4</i> <sub>1-147</sub> <i>MSS11</i> <sub>1-758</sub> L144→G; F145→A	This work

For the plasmids carrying *MSS11* fragments, the encoded area is indicated in subscript, giving the first and last amino acids of the *Mss11p* derivative encoded by the respective insert. The amino acids comprising the Gal4p DNA-binding domain are indicated in the same manner.

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