# Genetic analysis of signal transduction pathways: The regulation of invasive growth and starch degradation in *Saccharomyces cerevisiae*

by

**Dewald van Dyk**

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*Supervisor:*
Prof. I.S. Pretorius

*Co-supervisor:*
Dr. F.F. Bauer

# 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.

# SUMMARY

Cells of the yeast *Saccharomyces cerevisiae* are able to change their morphological appearance in response to a variety of extracellular and intracellular signals. The processes involved in morphogenesis are well characterised in this organism, but the exact mechanism by which information emanating from the environment is integrated into the regulation of the actin cytoskeleton and the yeast cell cycle, is still not clearly understood. Considerable progress has, however, been made. The processes are investigated on various levels including: (i) the nature of the signals required to elicit a morphological adaptation, (ii) the mechanism by which these signals are perceived and transmitted to the nucleus for gene transcription regulation (signal transduction pathways), (iii) the role of the cytoskeleton, particularly actin, in morphogenesis, and (iv) the relationship between cell cycle regulators and factors required for alterations in cellular shape.

The focus of this study was on elements involved in the regulation of one of these morphological processes, pseudohyphal formation, in *S. cerevisiae*. During pseudohyphal differentiation normal oval yeast cells become elongated and mother and daughter cells stay attached after cytokinesis to give rise to filaments. These filaments are able to penetrate the growth substrate, a phenomenon referred to as invasive growth. Actin remodelling is a prerequisite for the formation of elongated cells during pseudohyphal development and invasive growth. Its main contribution to this event is the directing of vesicles, containing cell wall constituents and enzymes, to specific sites of cell wall growth at the cell periphery. In order to fulfil this cellular function, actin is regulated on several levels. Signal transduction pathways that are activated in response to external nutritional signals play important roles in the regulation of the actin cytoskeleton during pseudohyphal differentiation. For this reason a literature review was compiled to introduce various aspects of actin-structure, the regulation of this structure and the functions actin performs during morphogenesis. The connection between signal transduction elements involved in morphological processes and actin remodelling is also reviewed.

This study entailed the genetic analysis of numerous factors involved in the regulation of pseudohyphal differentiation, invasive growth and starch metabolism. Several transcriptional regulators playing a role in these phenomena were investigated. Apart from the transcription factors, which include Mss11p, Msn1p, Ste12p, Flo8p, Phd1p and Tec1p, additional elements ranging from transporters to G-proteins, were also investigated. Mutant strains deleted for one or more of these factors were constructed and tested to assess their abilities to form filaments that penetrate the growth substrate, and to utilise starch as a carbon source. Complex genetic relationships were observed for various combinations of these factors. Specifically, Flo8p, Msn1p and Ste12p were shown to act independently in controlling invasive growth and starch metabolism, suggesting that these factors are regulated by different signal transduction pathways. Mss11p, on the other

hand, was found to play an indispensable role and seems to act as a downstream factor of Msn1p, Flo8p, Ste12p and Tec1p. The exception to this is Phd1p, since multiple copies of *PHD1* partially suppress the effect of a *MSS11* deletion. The data suggests that Mss11p functions at the confluence of several signalling pathways controlling the transcriptional regulation of genes required for invasive growth and starch degradation.

Different nutritional signals were also found to differentially regulate specific signalling elements during the invasive growth response. For example, Tec1p requires Msn1p activity in response to growth on media containing a limited nitrogen source. This dependency, however, was absent when invasive growth was tested on glucose and starch media.

Evidence was also obtained that confirmed the transcriptional co-regulation of *MUC1* and *STA2*. *MUC1* encodes a mucin-like protein that is required for invasive growth and pseudohyphal differentiation, whereas *STA2* encodes a glucoamylase required for starch degradation. Unpublished results indicated that several transcriptional regulators of invasive growth also exert an effect on starch metabolism. The data generated during this study complemented and confirmed published results. It also contributed to the compilation of a more detailed model, integrating the numerous factors involved in these signalling processes.

# OPSOMMING

*Saccharomyces cerevisiae* gisselle beskik oor die vermoë om hul morfologiese voorkoms in respons tot 'n verskeidenheid van ekstrasellulêre en intrasellulêre seine te verander. Die prosesse betrokke by morfogenese is goed gekarakteriseerd in hierdie organisme, maar die presiese meganisme waardeur inligting vanuit die omgewing geïntegreer word in die regulering van die aktien-sitoskelet en die gisselsiklus, word nog nie ten volle verstaan nie. Aansienlike vordering in die verband is egter gemaak. Die prosesse word op verskeie vlakke ondersoek, insluitende: (i) die aard van die seine wat benodig word om 'n morfologiese aanpassing te inisïeer; (ii) die meganisme waardeur hierdie seine waargeneem en herlei word na die selkern vir die regulering van geen-transkripsie (seintransduksie paaie); (iii) die rol van die sitoskelet, spesifiek aktien, in morfogenese en (iv) die verhouding tussen selsiklusreguleerders en faktore wat benodig word vir verandering in selvorm.

Hierdie navorsing fokus op elemente betrokke by die regulering van een van hierdie morfologiese prosesse in *S. cerevisiae*, naamlik pseudohife-vorming. Gedurende pseudohife-differensiëring neem tipiese ovaalvormige selle 'n verlengde voorkoms aan wat tot die vorming van filamente lei. Hierdie filamente is in staat om die groeisubstraat te penetreer, 'n verskynsel bekend as penetrasie-groei. Aktienherrangskikking is 'n voorvereiste vir die vorming van verlengde selle tydens pseudohife-ontwikkeling. Die hoofbydrae van aktien tot hierdie verskynsel is die oriëntering van uitskeidingsvesikels, wat selwandkomponente en ensieme bevat, na spesifieke areas van selwandgroei op die seloppervlak. Aktien word op verskeie vlakke gereguleer om hierdie sellulêre funksie te vervul. Seintransduksiepaaie wat geaktiveer word in respons tot ekstrasellulêre voedingsseine speel 'n belangrike rol in die regulering van die aktien-sitoskelet tydens pseudohife-differensiëring. Op grond hiervan is 'n literatuuroorsig saamgestel vir die bekendstelling van verskeie aspekte van aktienstruktuur, die regulering van hierdie strukture en die funksies wat deur aktien gedurende morfogenese vervul word. Die verband tussen seintransduksie-elemente betrokke by morfologiese prosesse en aktien herrangskikking word ook behandel.

Hierdie studie het die genetiese analisering van verskeie faktore betrokke by pseudohife-differensiëring, penetrasie-groei en styselmetabolisme, behels. Verskeie transkripsionele reguleerders wat 'n rol speel in hierdie prosesse was bestudeer. Buiten die transkripsiefaktore Mss11p, Msn1p, Ste12p, Flo8p, Phd1p en Tec1p, was addisionele faktore, wat gewissel het van transporters tot G-proteïene, ook ondersoek. Mutante-rasse met geendelesies vir een of meer van hierdie faktore is gekonstrueer en getoets om vas te stel hoe dit hul vermoë raak om penetrerende filamente te vorm, asook om te bepaal of stysel as koolstofbron gebruik kan word. Komplekse genetiese interaksies vir verskeie kombinasies van hierdie faktore is waargeneem. Dit was waargeneem dat Flo8p, Msn1p

en Ste12p onafhanklik funksioneer tydens die regulering van penetrasie-groei en styselmetabolisme, wat impliseer dat hierdie faktore deur verskillende seintransduksiepaaie gereguleer word. Mss11p word beskou as 'n onmisbare rolspeler in hierdie prosesse en dit kom voor asof hierdie protein as 'n stroom-af faktor is en vereis word vir die funksionering van Msn1p, Flo8p, Ste12p en Tec1p. Phd1p is egter 'n uitsondering, aangesien veelvuldige kopieë van *PHD1* die effek van 'n *MSS11*-delesie gedeeltelik oorkom. Die data impliseer dat Mss11p by die samevloei van verskeie seintransduksiepaaie, benodig vir die transkripsionele regulering van gene betrokke by penetrasie-groei en styselmetabolisme, funksioneer.

Dit was ook waargeneem dat verskillende voedingsseine die faktore betrokke by die penetrasie-groeirespons differensieel reguleer. Tec1p byvoorbeeld benodig Msn1p-aktiwitieit in respons tot groei op media met 'n beperkte stikstofbron. Hierdie afhanklike interaksie is egter afwesig wanneer penetrasie-groei bestudeer word op glukose- en styselmedia.

Resultate wat die gesamentlike transkripsionele regulering van *MUC1* en *STA2* bevestig, is ook verkry. *MUC1* kodeer vir 'n mukienagtige proteïen wat benodig word vir pseudohife-vorming en penetrasie-groei, terwyl *STA2* kodeer vir 'n glukoamilase essensieël vir styselafbraak. Ongepubliseerde resultate dui daarop dat verskeie transkripsionele reguleerders van penetrasie-groei ook 'n effek uitoefen op styselmetabolisme. Die data wat gegenereer is tydens hierdie studie komplementeer en bevestig reeds gepubliseerde resultate. Dit het ook bygedra tot die samestelling van 'n gedetaileerde model wat die verskillende faktore, betrokke by hierdie seintransduksieprosesse, integreer.

**This thesis is dedicated to my parents, Lianda and Surentia.**
**Hierdie tesis is opgedra aan my ouers, Lianda en Surentia.**

# BIOGRAPHICAL SKETCH

Dewald van Dyk was born in Tygerberg, South Africa, on 8 May 1975. He attended the Monte Vista Primary School and matriculated at the President High School in 1993. Dewald enrolled at the University of Stellenbosch in 1994 and obtained a B.Sc. degree in Biochemistry and Microbiology in 1996, and a B.Sc.Hons degree in Microbiology in 1997.

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# PREFACE

This thesis is presented as a compilation of four chapters. Each chapter is introduced separately and is written according to the style of the journal *Molecular Microbiology* and *Journal of Bacteriology* in which Chapter 3.2 and Appendix were published.

**Chapter 1** **General Introduction and Project Aims**

**Chapter 2** **Literature Review**
The role of actin in morphogenesis

**Chapter 3** **Research Results**
Genetic analysis of factors involved in invasive growth and starch metabolism
Msn1p/Mss10p, Mss11p and Muc1p/Flo11p are part of a signal transduction pathway downstream of Mep2p regulating invasive growth and pseudohyphal differentiation in *Saccharomyces cerevisiae*

**Chapter 4** **General Discussion and Conclusions**

**Appendix** **Research Results**
Divergent regulation of the evolutionary closely related promoters of the *Saccharomyces cerevisiae STA2* and *MUC1* genes

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# CHAPTER 1

# General Introduction and Project Aims

# GENERAL INTRODUCTION AND PROJECT AIMS

## 1.1 INTRODUCTION

In *Saccharomyces cerevisiae*, pseudohyphal differentiation and invasive growth are related cellular processes occurring in response to the limited availability of nutrients, in particular carbon and nitrogen sources. In the presence of limited amounts of these nutrients, yeast cells undergo profound molecular, physiological and morphological changes leading to pseudohyphal and invasive growth. On the molecular level the activities of numerous proteins are modulated while the transcription of several genes is activated or repressed. Physiologically, the cell cycle is reprogrammed to co-ordinate alterations in cellular morphology and specific stages of the cell cycle. The morphological changes include a transition from round cells to elongated cells, alterations in budding pattern and incomplete separation after budding, resulting in filaments of elongated cells. These filaments are comprised of daughter cells that stay attached to the mother cells, providing the filament with a septated appearance generally referred to as pseudohyphae (Gimeno *et al.*, 1992). Haploid and diploid cells are able to develop filaments, but cell elongation in the case of haploid cells is less prominent. The physiological relevance of these phenomena appears to be linked to nutrient scavenging, since filament formation enables cells to penetrate their immediate surroundings in search for nutrient sources. A complex network of signal transduction pathways governs this cellular response to limited availability of nutrients.

Signal transduction pathways provide a relay system through which signals, emanating from the environment, are transmitted to the nucleus. Receptor-mediated perception of the exogenous nutritional signal leads to the activation of signalling modules which exert their effects on several levels, modifying transcription, enzymatic activities and protein-protein interactions. In the case of pseudohyphal differentiation, the targets of these signalling pathways include proteins and genes that are i) required for the morphogenetic aspects of filamentous growth, e.g. the bud-site selection proteins, polarity establishment factors, the actin cytoskeleton and its regulators, ii) required for the cell cycle-mediated timing of these events, e.g. the cell cycle regulators, and iii) general downstream factors, e.g. the genes encoding substrate adhesion molecules and glucoamylases. The complexity of the overall filamentous growth event is underlined by the wide array of factors required for its establishment. Although participating elements are still being identified, considerable progress has been made in the unravelling of the underlying events.

Two distinct pathways controlling both pseudohyphal development and invasive growth, have been identified and were subsequently characterised (**Figure 1.1**) (Liu *et al.*, 1993; Mösch *et al.*, 1997; Robertson and Fink, 1998; Pan and Heitman, 1999). However, several lines of evidence support the existence of additional pathways. The best-characterised pathway contains several components of the pheromone-induced

MAP kinase cascade (Liu *et al.*, 1993). Activation of the filamentous growth MAP kinase pathway requires Ras2p, a small G-protein (Gimeno *et al.*, 1992). A receptor acting as a regulator of Ras2p has not been identified to date. Ras2p activity results in the activation of Cdc42p (Mösch *et al.*, 1996), which in turn interacts with cell growth polarity establishment factors, including Cdc24p and Bem1p, as well as with the mammalian $p65^{PAK}$ homologue, Ste20p (Leeuw *et al.*, 1995, Whiteway *et al.*, 1995; Leberer *et al.*, 1997). Upon the activation of Ste20p the signal is transmitted through a MAP kinase cascade which consists of Ste11p, Ste7p and Kss1p. In its unphosphorylated state, Kss1p interacts with Ste12p, thereby acting as an inhibitor of Ste12p function (Cook *et al.*, 1997; Madhani *et al.*, 1997; Madhani and Fink, 1997). Dig1p and Dig2p also regulate Ste12p activity in a similar manner, since a phosphorylation step is required before these proteins release, and as a result, activate Ste12p (Cook *et al.*, 1997). Dissociation of Kss1p from the transcriptional activator Ste12p, enables the latter to associate with Tec1p (Gavrias *et al.*, 1996) to form a heterodimeric complex that exerts a regulatory effect on the transcription of *MUC1*/*FLO11*. The latter gene was independently shown to be required for filamentous growth by Lambrechts *et al.* (1996a) and Lo and Dranginis (1998). *MUC1* is also involved in the formation of cell aggregates, in a process referred to as flocculation (**Figure 1.1**) (Lo and Dranginis, 1998).

Apart from the elements of the MAP kinase pathway, cAMP levels also exert a regulatory effect on filamentous growth (Lorenz and Heitman, 1997). This pathway is controlled by several proteins including Gpr1p, Mep2p, Gpa2p and Ras2p. Gpr1p, a G-protein-coupled receptor homologue, is a putative candidate for a glucose sensor regulating the cAMP-pathway (Yun *et al.*, 1998). Mep2p was identified as a membrane-bound receptor molecule that controls filamentous growth in response to ammonium-specific signals (Lorenz and Heitman, 1998a). The gene product of *GPA2*, a homologue to $\alpha$-subunits of heterotrimeric G-proteins, serves as a target for both Mep2p- and Gpr1p-dependent activation (Lorenz and Heitman, 1998a, Xue *et al.*, 1998). Together with Ras2p, Gpa2p regulates the activity of adenylyl cyclase, which is required for the conversion of ATP into cyclic-AMP. In turn, cAMP interacts with the regulatory subunit, Bcy1p, of the protein kinase A PKA complex, to stimulate the dissociation and subsequent activation of the catalytic subunits, Tpk1p-3p. Until recently the three Tpks were considered as being redundant, but work conducted by two independent groups revealed that the Tpk2p catalytic subunit plays a positive role in the activation of filamentous growth, while Tpk1p and Tpk3p play negative roles. (Robertson and Fink, 1998; Pan and Heitman, 1999). Tpk2p is required for the activation of Flo8p, a transcriptional regulator of flocculation genes, and the inactivation of Sfl1p, an inhibitor of these genes. Taken together, it appears that both pathways converge on the *MUC1*-promoter (Rupp *et al.*, 1999).

The Ste MAP kinase- and the cAMP-signalling pathways are not solely responsible for the activation of transcriptional regulators of *MUC1*. Several factors have been identified that seem to act independently of these two pathways, but are

still important for filamentous growth. These factors include *PHD1*, *ASH1, MSN1, MSS11* and *ELM1*, some of which have been shown to be cell cycle regulated (Gimeno 1994; Chandarlapaty, 1998; Edgington 1998; Gagiano *et al.*, 1999a). Furthermore, genetic screens have led to the identification of a wide range of genes that are still not placed in context of the known elements involved in filamentous growth (Mösch and Fink, 1997; Lorenz and Heitman, 1998b). Extensive research is therefore still required before a comprehensive picture of all the elements involved in filamentous growth can be drawn.

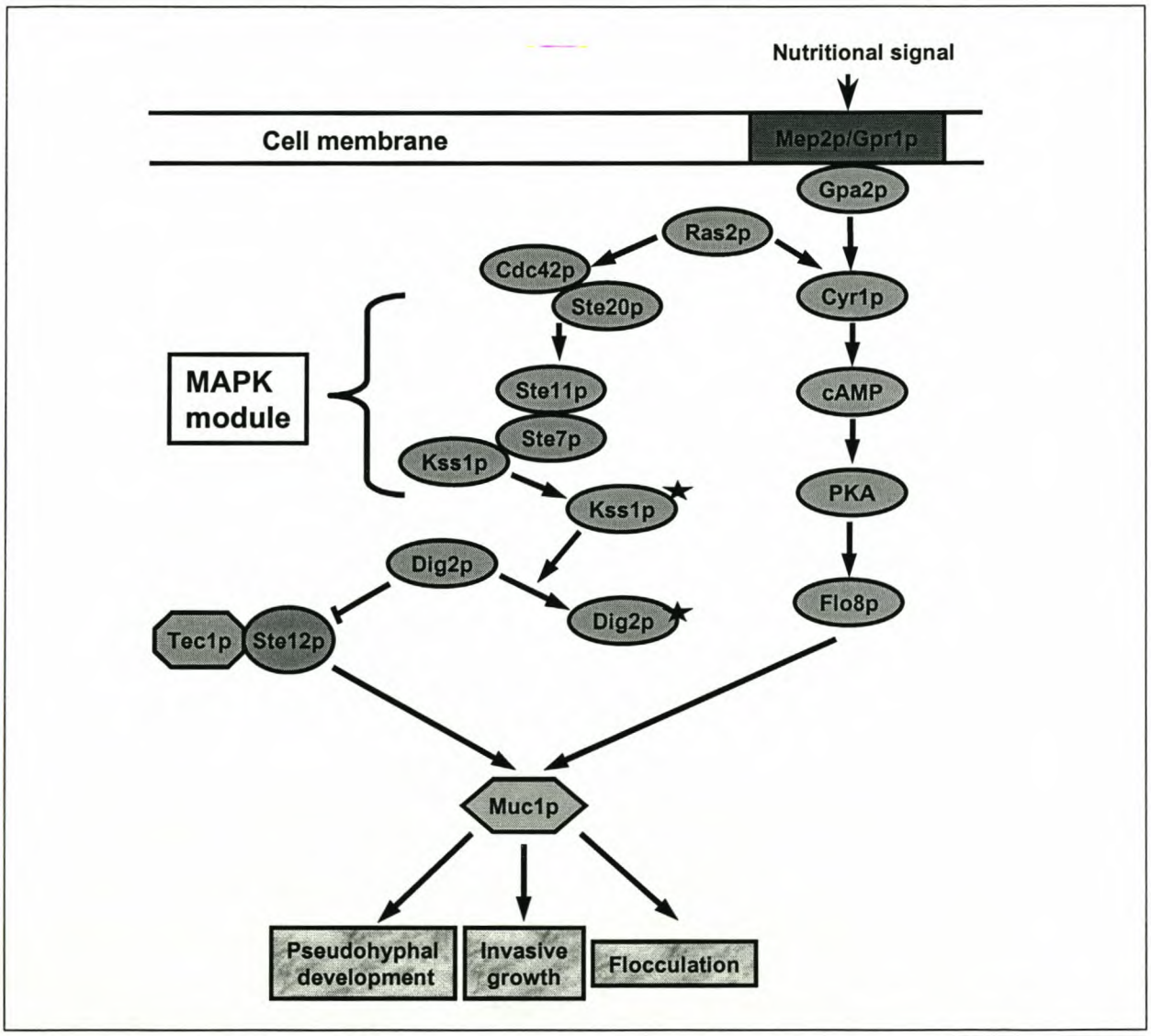


**Figure 1.1** A model representing elements of two signal transduction pathways involved in pseudohyphal differentiation. *MUC1* transcription results in filamentous growth and flocculation.

*S. cerevisiae* strains that possess one or more of the *STA1-3* genes, encoding for three isomeric glucoamylases, are able to degrade starch and can therefore utilise starch as an alternative carbon source. Expression of the *STA*-encoding genes is also under the control of transcriptional regulators acting on the promoter of *MUC1* (Gagiano *et al.*, 1999a, 1999b). The promoters of *MUC1* and *STA1-3* genes, the largest promoters identified in yeast to date (>3kb), are 99% homologous. Two genes

cloned for their ability to promote starch degradation when overexpressed, Msn1p/Mss10p and Mss11p (Lambrechts *et al.*, 1996b, Webber *et al.*, 1997), were also identified as transcriptional activators of *MUC1* (Gagiano *et al.*, 1999a, Lorenz and Heitman, 1998b). In addition, Ste12p and Flo8p were shown to activate the transcription of *STA2*, implying that the Ste MAP kinase and the cAMP-dependent pathways function in concert to regulate starch utilisation and filamentous growth in budding yeast (Gagiano *et al.*, 1999a, 1999b).

## 1.2 PROJECT AIMS

Lambrechts *et al.* (1996a) showed that Mss10p regulates the transcription of *MUC1* and *STA2* and that the promoters of these genes are 99% homologous. Since *MUC1* was shown to be required for invasive growth and pseudohyphal differentiation these data suggested co-regulation between starch degradation, invasive growth and pseudohyphal formation.

The specific aims and approaches of this study were as follows:

(i) ascertaining whether Mss11p is involved in invasive growth;
(ii) determining the genetic relationship between Mss10p and Mss11p by means of epistasis analysis;
(iii) identifying genetic links between Mss10p, Mss11p and factors involved in invasive growth, e.g. components of the MAPK-module;
(iv) establishing the extent of co-regulation between *MUC1* and *STA2* by cross-examining factors required for invasive growth and starch degradation;
(v) assessing the effects of different carbon and nitrogen sources on the ability of mutants to grow invasively.

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# CHAPTER 2

## Literature Review

## The role of Actin in Morphogenesis

# LITERATURE REVIEW

## 2.1 INTRODUCTION

Cellular morphogenesis is a focal point of studies world-wide. Morphogenetic events are central to a wide range of cellular processes, which include i) cell propagation, ii) cellular differentiation during embryogenesis iii) cell migration, and iv) polarised growth. Most organisms are able to modify cell morphology, either as a result of genetic programming during embryogenesis, or in response to specific external signals. Changes in cellular morphology are common in lower and higher eukaryotes. *S. cerevisiae* cells are able to adopt different morphological forms depending on the cellular process to which the cells are committed. Alterations in cellular morphology include (i) bud-formation for reproductive purposes, (ii) mating-projection formation required for mating, as well as (iii) the formation of elongated cells during pseudohyphal growth.

All these processes are the result of well orchestrated events in which the remodelling of the cytoskeleton plays a fundamental role. Actin and actin-binding proteins act in concert with additional cytoskeletal structures, e.g. tubulin and intermediate filaments, to constitute the cytoskeleton, a protein structure that provides an intracellular framework around which cellular components are organised. The cytoskeleton therefore contributes to, and is largely responsible for, the morphological appearance of a cell. Actin in particular plays an important role in the establishment of polarised growth in yeast. It is involved in numerous cellular processes, which include (i) the directed vesicular transport and polarised secretion of cell wall constituents and enzymes to sites of active cell growth, (ii) organelle segregation and (iii) cytokinesis. In order to facilitate growth at specific sites at the cell periphery, actin structures are constantly redistributed during the yeast cell cycle. Polarised actin distribution is most prominent during bud emergence, mating-projection formation and cell elongation, a process involved in the development of pseudohyphae. In other organisms, actin has additional functions which are absent in yeast, motile functions in particular. As oppose to yeast cells, muscle cells use actin-myosin complexes for force generation that enable these cells to contract. Furthermore, *Dictyostelium discoideum*, a unicellular organism, directs its movement towards cAMP gradients, a process that requires force generation which is provided by rapid actin assembly and disassembly.

Actin distribution, assembly and disassembly are regulated by a wide array of factors in all organisms. In *S. cerevisiae* several signal transduction pathway components exert a regulatory effect on actin assembly and distribution. Signalling modules are activated in response to endogenous signals, e.g. elements signalling the positions of the cell cycle, as well as exogenous signals, e.g. mating-pheromone and nutritional information. Provided that the cellular response to these signals involves polarised growth, actin is assembled into larger structures, which include

actin filaments and cables, and localised to sites where cell growth will commence. Although progress has been made in our overall understanding of the events leading to morphological changes in response to internal or external cues, extensive investigations are still required to resolve the persisting questions surrounding the signalling of information *via* signal transduction pathways to the actin cytoskeleton.

Morphogenesis, in general, is the result of a wide range of cellular events which include the perception of initialising signals, signal transmission, gene transcription, cell cycle reprogramming, actin cytoskeleton remodelling, vesicle transport and cell wall construction. The focal point of the opening section of this literature review is the structure of the actin cytoskeleton, which is mainly based on studies conducted on higher eukaryotic organisms. The focus in the following sections is shifted towards the regulatory and functional aspects of the actin cytoskeleton, as derived from elaborate studies on the yeast *S. cerevisiae*. Finally, factors involved in the transmission of exogenous and endogenous signals to the actin cytoskeleton and regulators thereof, are discussed. The latter section includes the role of signal transduction elements and the yeast cell cycle in this event.

## 2.2 ACTIN CYTOSKELETON: STRUCTURE AND CONSTITUENTS

Numerous factors interact to form a sub-cellular network of filaments known as the actin cytoskeleton. Actin filaments form the bulk of this structure. The polymerisation of actin into microfilaments and its distribution is dependent on a multitude of actin-interacting proteins, themselves governed by signals originating either internally or externally. In order to create a model integrating the variety of signal inputs and the effects on actin remodelling, as well as the subsequent events resulting from these modifications, it is necessary to discuss the structure of actin itself.

### 2.2.1 THE STRUCTURE OF ACTIN

Depending on its regulation, actin either exists as single monomeric units, or assembled actin polymers. The actin polymers or microfilaments are in turn organised into larger and more complex structures that include actin cables and actin cortical patches.

#### 2.2.1.1 ACTIN MONOMER

One of the most remarkable aspects of actin is the high extent of conservation of the amino acid sequence amongst species. This feature enables researchers to extrapolate the results obtained from a model organism, e.g. *Dictyostelium discoideum*, to higher and more complex eukaryotic organisms. The actin of the slime mold, *Physarum polycephalum*, exhibits 92% amino acid sequence homology with mammalian skeletal muscle actin and 95% homology with mammalian nonmuscle actins (Korn, 1982). Moreover, the single conventional actin isoform from

*S. cerevisiae* shares 86% amino acid sequence homology with known mammalian actins (Amberg 1998). In *S. cerevisiae* the essential *ACT1* gene (Gallwitz and Sures, 1980) encodes for the only conventional actin found in cytoskeletal structures such as microfilaments, cytoplasmic cables and subcortical dots (Kilmartin and Adams; 1984; Mulholland *et al.*, 1994). It is one of the predominant cytoplasmic protein types found in *S. cerevisiae*.

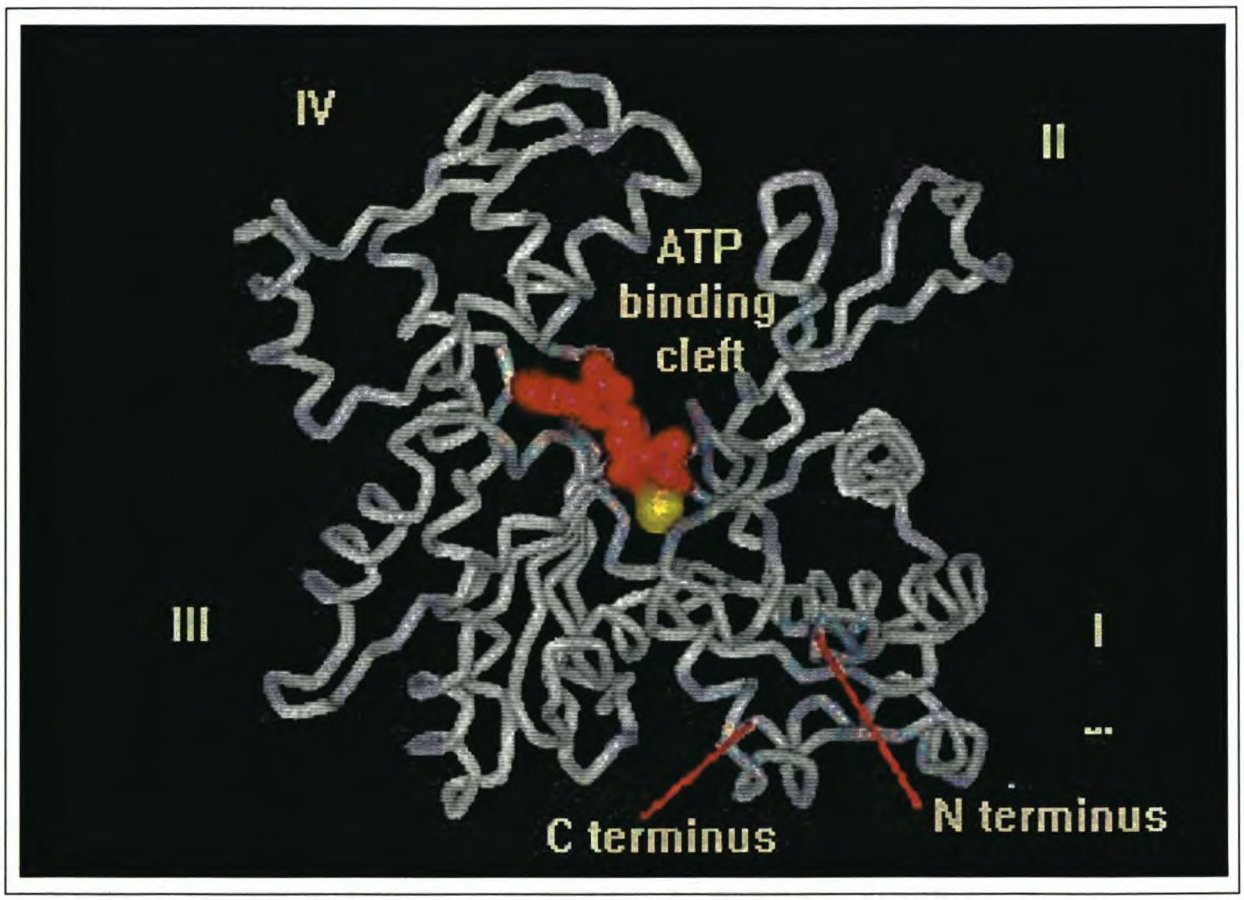


**Figure 2.1** A structural model for monomeric actin associated with adenosine tri-phosphate (red) and calcium (yellow). The binding-sites are located in the actin-fold. I, II, III, IV indicate the four domains of the actin monomer (adapted from Lodish *et al.*, 1995).

Monomeric actin units consist of 375 amino acid residues with a total molecular weight of 43 kDa. Kabsch *et al.* (1990) proposed a model for monomeric or globular actin (G-actin) in which the protein consists of two major domains. Although fairly similar in size, the two domains are referred to as the large and small domains of actin, due to historic reasons. These two domains are each divided into two subdomains, which in turn consist of α-helices and β-sheets (Kabsch *et al.*, 1990) (**Figure 2.1**). Structural analysis of rabbit skeletal muscle actin complexed with bovine pancreatic deoxyribonucleasel (DNaseI) by means of X-ray crystallography revealed a binding site for both ATP and the divalent metal cation, calcium, in the core of the actin molecule. Upon protein folding, the actin polypeptide adopts a cleft-like structure at its centre that is referred to as the actin fold (reviewed by Kabsch and Holmes, 1995). Its structural appearance is attributed to the organisation of the two larger domains during the folding process. In addition to salt bridges, binding of ATP

and the divalent metal ion to the respective binding sites in the actin fold is believed to link the four subdomains, thereby adding conformational stability to the rather flexible protein (Kabsch *et al.*, 1990). Structurally, the molecule can further be divided into loops with external binding sites that allow a single actin molecule to interact with other actin monomers, myosin or other proteins. Specific residues in the actin sequence were also identified to play a role in the interaction between actin and phalloidin, tropomyosin, troponin I, profilin, α-actinin, gelsolin segment-1 and other actin binding proteins (Kabsch *et al.*, 1990). In addition to the actin-DNaseI complex, the three-dimensional structures of actin-forming complexes with gelsolin segment-1 (McLaughlin *et al.*, 1993) and profilin (Schutt *et al.*, 1993) have been resolved. Actin's ability to associate with numerous proteins underlines the complexity of its regulation and the diversity of its binding properties, which ultimately contribute to the dynamic structural properties of the actin cytoskeleton.

### 2.2.1.2 ACTIN POLYMER

The various cellular functions of actin *in vivo* are not attributed to single monomeric units, *per se*, but rather to the ability of G-actin to polymerise into functional filaments (F-actin). Polymer formation can be induced *in vitro* by the addition of salts to samples containing pure monomeric actin molecules (Korn, 1982). The sequential association of single actin units by means of non-covalent bonds results in the formation of fibrous actin, which in turn interacts with various actin-binding proteins to form microfilaments. Some of the activity of actin-associated proteins is regulated by signals initialised at the cell periphery and is therefore probably due to external cues emanating from the environment (Kabsch and Holmes, 1995). Specialised actin-binding proteins govern the polymerisation process *in vivo*. The exact process of monomer incorporation during filament formation is discussed in more detail as part of the polymer elongation section.

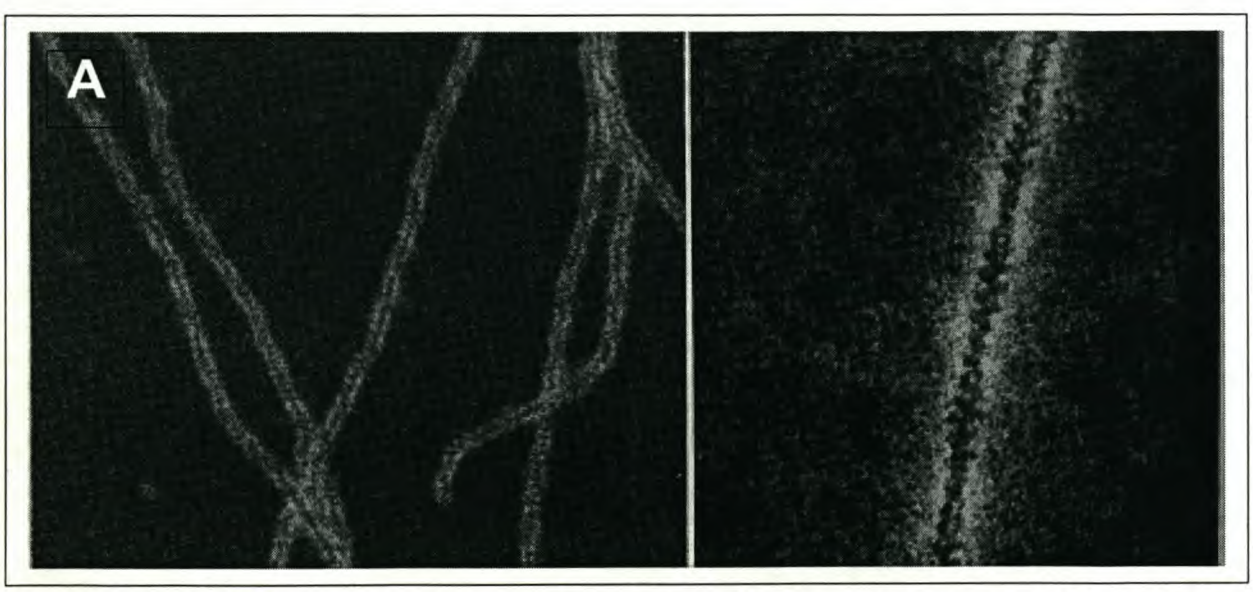


**Figure 2.2** Electron microscopy images of negatively stained F-actin filaments at (A) 200 000X and (B) 500 000X magnification (Bremer *et al.*, 1994).

Earlier attempts to resolve the structure of fibrous actin involved the measuring of electron microscopy images, among other techniques (**Figure 2.2A** and **B**) (reviewed by Korn, 1982). When viewed by electron microscopy, the F-actin filament appeared as a two-start, double-stranded, right-hand helix with a total of 13 monomers between the crossover points of the two strands (**Figure 2.3A**). In other words, the filament appears to be formed by two long-pitch helical strands of monomeric actin subunits with the same polarity. Alternatively, the same structure could be viewed as a one-start single-stranded, left-hand helix (so-called "genetic" helix), with 2.16 subunits per turn and a repeat of 36 nm (**Figure 2.3B**). The subunits in both strands are positioned in an axial fashion with regard to each other (Bremer *et al.*, 1994). Interestingly, the interaction between subunits of a single strand is approximately three times stronger than the inter-subunit contact between two subunits of adjacent strands. As a result of the varying subunit affinities, the two long-pitch helical strands can be displaced as single units along the axis of the filament by a process known as "lateral slipping" (Bremer *et al.*, 1991). In addition to the mentioned structural appearances of actin filaments, electron microscopy has also served a valuable purpose in the assessment of the polarity of monomeric subunits within the filament. Myosin head fragments interact with actin subunits in a fashion that resembles an arrowhead pattern. The respective interactions between actin subunits and myosin head fragments are all directed to one pole of the filament, suggesting that monomer incorporation is orientated. The two opposite ends of a filament are referred to as the barbed- and pointed-end, respectively (Korn, 1982; Sohn and Goldschmidt-Clermont, 1994).

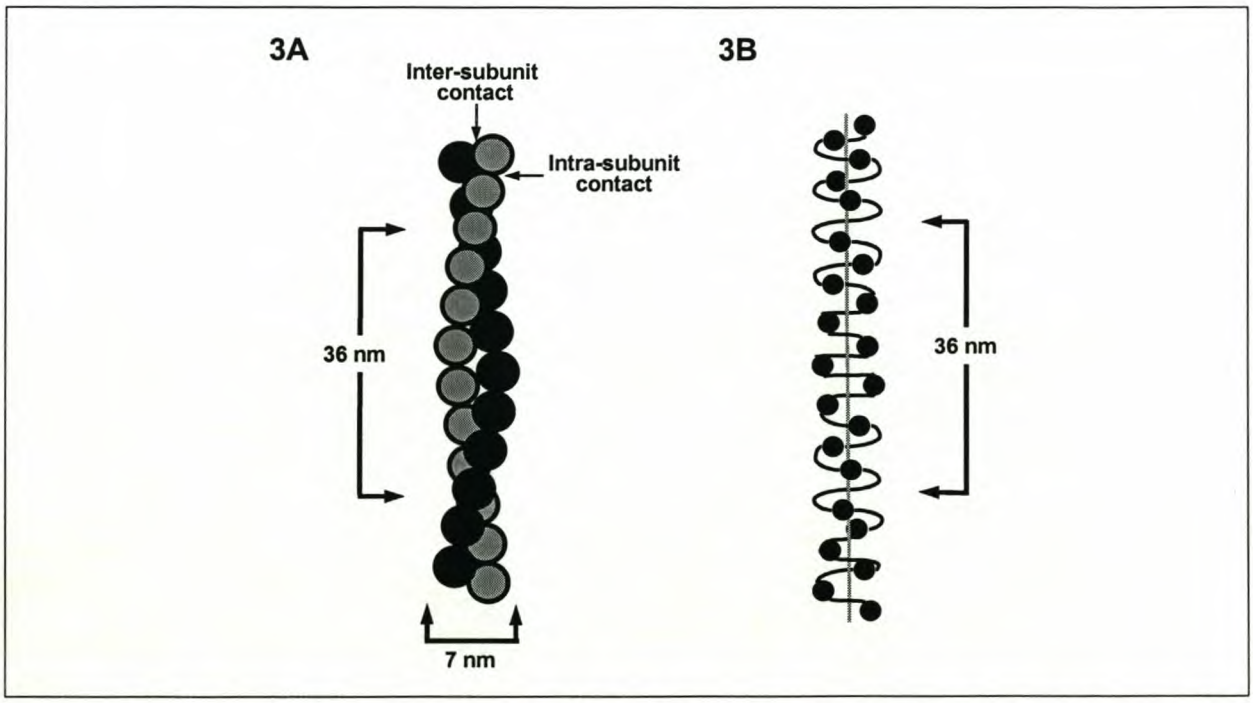


**Figure 2.3** Diagrams illustrating the two different interpretations of actin filament structure. (A) The filament viewed as a double-stranded helix with crossover points separated by 36 nm. (B) An alternative representation depicting the filament as a single-start or genetic helix (Adapted from Korn, 1982).

Holmes and co-workers (1990) have identified several amino acid residues that are involved in the interaction between two different actin monomers during the elongation process of F-actin. The authors' attempt to elucidate the orientation by which monomers are incorporated in the actin filament, was based on the assumption that actin complexed with DNaseI mimics actin-actin interaction. Differences between actin-DNaseI and actin-actin complexes seemed negligible and the former complex was therefore ideal for deciphering the orientation of a monomer with respect to the actin helix. An atomic model for F-actin was constructed with the use of an X-ray fiber diagram from oriented gels of F-actin and the known model for monomeric actin (**Figure 2.4A** and **B**). Data obtained during the course of the investigation resulted in valuable information on the interactions between monomeric actin and F-actin. Furthermore, it led to the observation that F-actin formation requires a specific monomer orientation during the addition of new actin subunits onto the end of the growing filament (Holmes *et al.*, 1990). More recently, image reconstruction studies on F-actin in association with myosin (Rayment *et al.*, 1993) and tropomyosin (Lorenz *et al.*, 1995) resulted in the identification of domains within the actin filament that are required for these specific interactions.

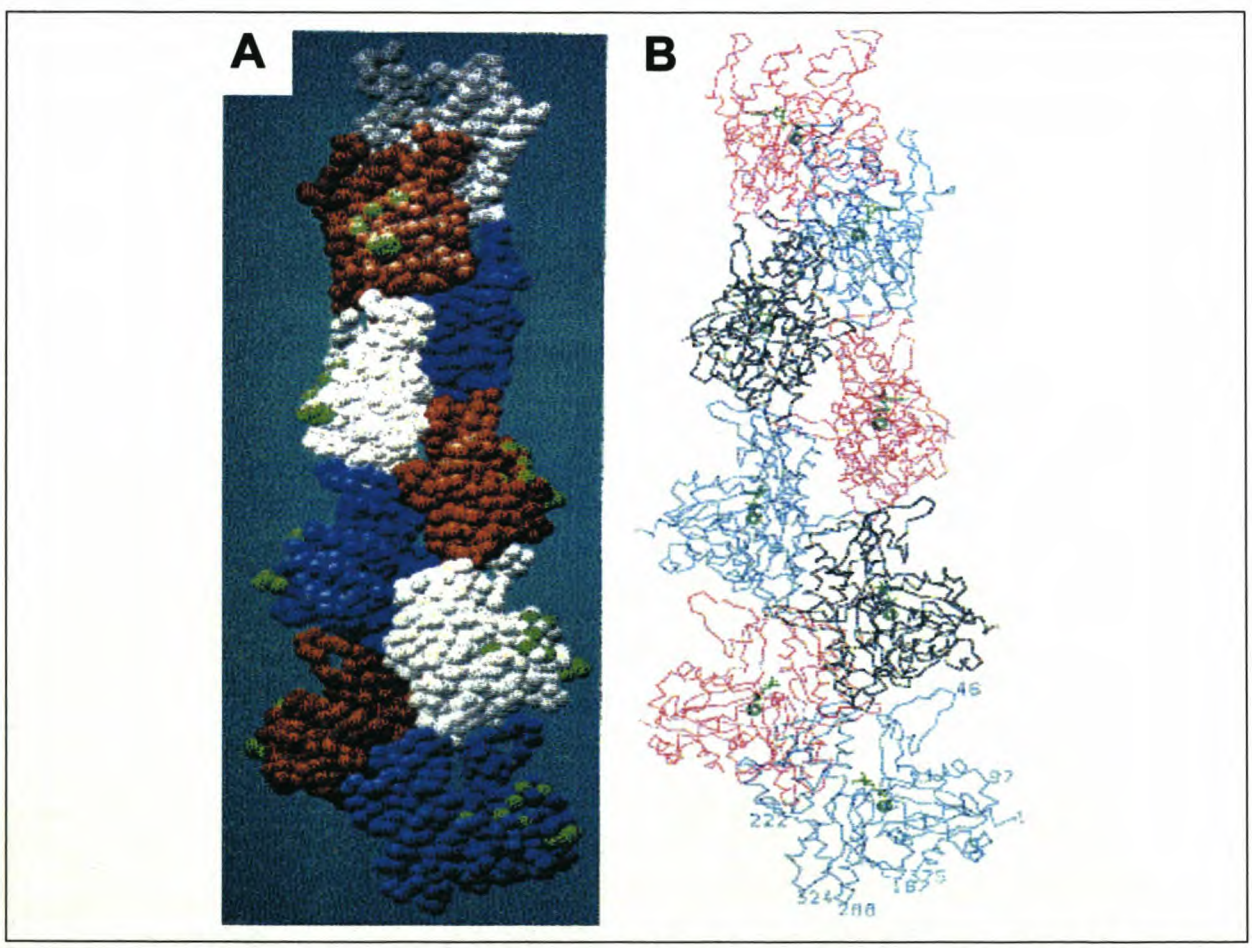


**Figure 2.4** (A) A space filling model representing eight subunits of an actin filament. Amino acid residues involved in the interaction between myosin and the filament are shown in green. (B) Filamentous actin represented by a stereo model. ADP and calcium are shown in green (Holmes *et al.*, 1990).

### 2.2.1.3 CYTOLOGICAL OBSERVATIONS OF ACTIN CONTAINING STRUCTURES IN YEAST

Specialised proteins mediate the ultra-structural arrangement of actin filaments into bundles or cables from pre-existing actin filaments. The cytoplasmic actin cables, also known as microfilaments (5-7 nm in diameter), form an integral part of the cytoskeleton. Together with microtubules (25 nm in diameter), septins and intermediate filaments (approximately 10 nm in diameter), these polymeric molecules constitute the basic protein structures of the cytoskeleton. In comparison to tubulin and microfilaments (reviewed by Winsor and Schiebel, 1997), very little information is available on the yeast intermediate filaments (Botstein *et al.*, 1998).

Apart from the organisation of F-actin into cables, polymeric actin is also incorporated into subcortical actin patches that are observed throughout the yeast cell cycle. Although actin cables and subcortical actin dots are structurally distinguishable, they seem to function in concert to facilitate polarised cell wall growth (Mulholland *et al.*, 1994; Amberg *et al.*, 1998).

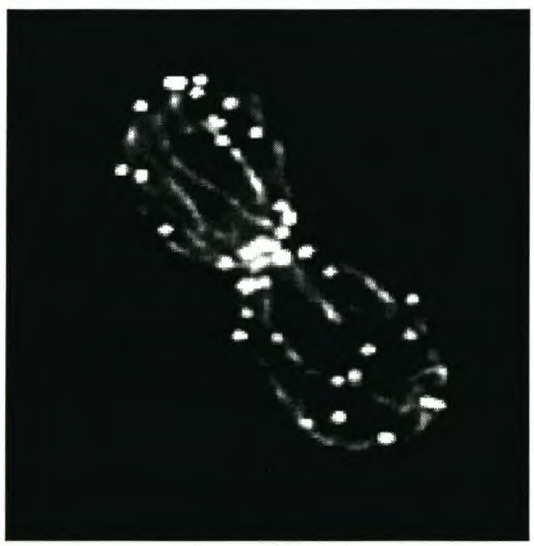

**Figure 2.5** Image of a rhodamine-phalloidin stained actin cytoskeleton that emphasises the interaction between cortical patches and actin cables (Amberg, 1998).

Actin rearrangement during the yeast cell cycle drew considerable attention in the past when the first immunofluorescence microscopy images of actin and tubulin distribution appeared (Adams and Pringle, 1984; Kilmartin and Adams, 1984). More advanced techniques currently in use have led to remarkable revelations, as well as to the elimination of misconceptions concerning the structure and distribution of cytoplasmic actin cables and cortical dots (Mulholland *et al.*, 1994; Amberg, 1998). One observation made by Amberg (1998) was that the cytoplasmic cables are restricted to locations underneath the cell cortex and not throughout the whole cell interior. They associate with actin patches in a lateral fashion or at the respective cable ends (**Figure 2.5**). This result suggested that actin patches function as membrane-anchoring structures from which filament formation is initiated, or,

alternatively, participate in the spatial distribution of the cables by connecting the latter to specific areas of the plasma membrane.

Actin cables and cortical patches are asymmetrically redistributed as the cell progresses through its cycle (**Figure 2.6**). Prior to bud formation, actin patches and cables are evenly distributed in the cell with the latter being randomly orientated. Shortly before the onset of bud formation the cortical patches tend to accumulate in the form of a ring at the incipient bud site, while the actin cables start to assemble in an orientated fashion, facing the preselected bud site. The newly formed bud emerges through the ring as it increases in size. In contrast to previous claims (Kilmartin and Adams, 1984), the patches do not shift to polarised areas during the isotropic growth phase of the bud; instead they are uniformly dispersed throughout the developing bud (Amberg 1998). Bud growth coincides with the elongation of cables extending along the long axis from the mother cell into the daughter cell. Shortly before cytokinesis the patches accumulate at the mother-bud neck from both sides, where they facilitate the formation of a septum, probably by directing secretory vesicles to this area. With the completion of cell separation, the respective actin structures are redistributed again until the next round of bud formation is initiated during the $G_1$ phase.

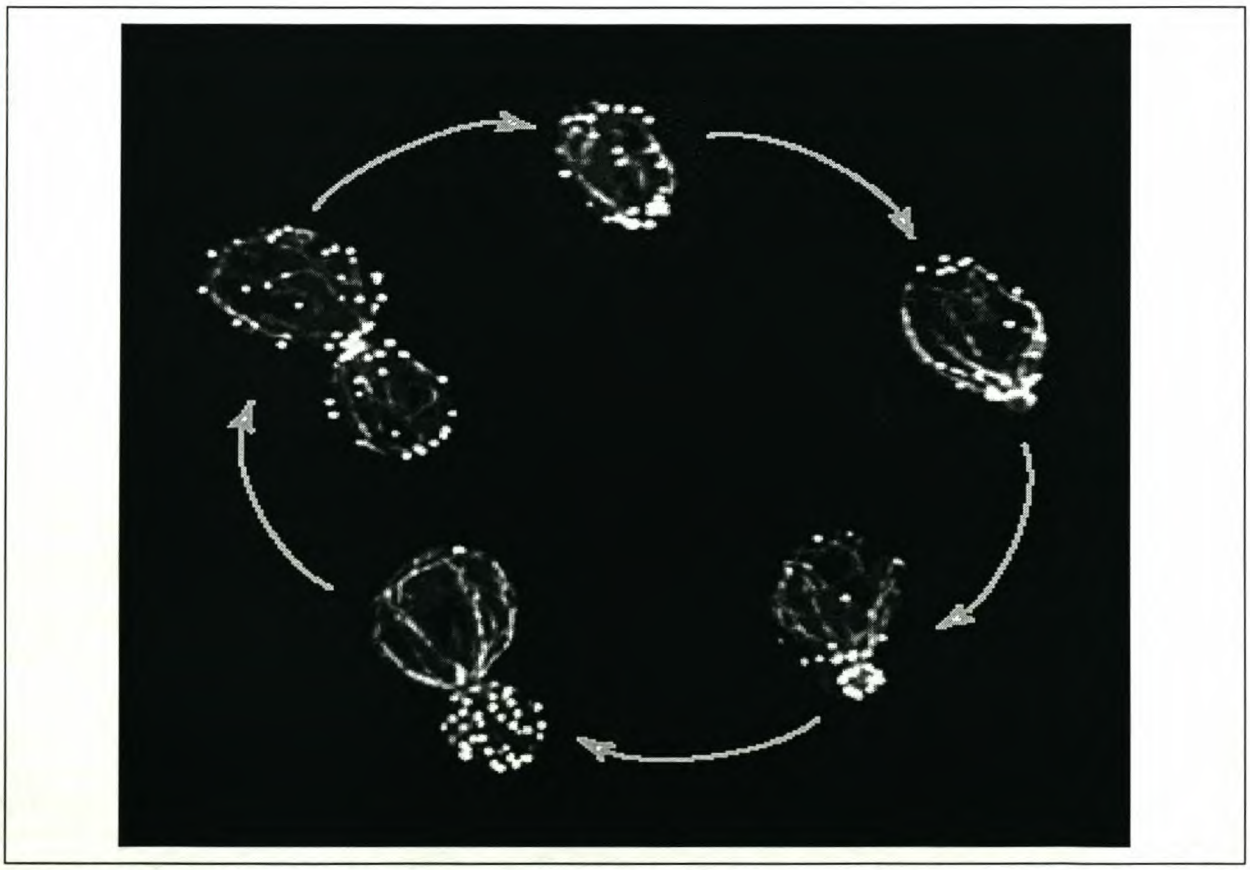

**Figure 2.6** Representation of the yeast cell cycle. The actin cytoskeletons of diploid cells at different cell cycle stages were stained with rhodamine-phalloidin and imaged (Amberg, 1998).

The structural assessment of the yeast actin cytoskeleton (Mulholland *et al.*, 1994), by means of immunoelectron microscopy, indicated that actin cortical patches

are attached to specific areas of the cell periphery *via* finger-like protrusions emanating from the plasma membrane (**Figure 2.7**). A cortical dot, therefore seems to consist of a membrane invagination around which filamentous actin is wrapped and organised in addition to proteins associated with these dense patch-like cortical structures. An interesting hypothesis was put forward by the authors relating to this observation. In short, the actin patch represents sites of active cell growth. Since a constant turgor pressure is maintained by the cell wall as a result of an osmotic gradient, incorporation of new cell wall material, concurring with covalent bond breakage, can be problematic or even detrimental for the cell. This problem is circumvented by the formation of plasma membrane projections, facing the cell interior that can serve as septums onto which new material is added during polarised growth.

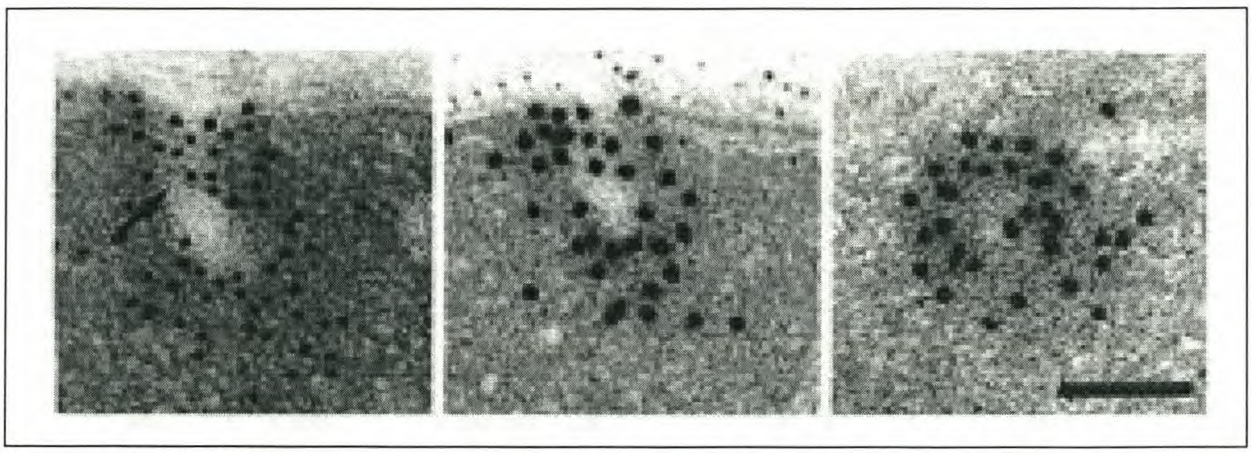

**Figure 2.7** Electron microscopy images of immunogold-labeled actin organised around a membrane invagination forming a cortical patch. The images demonstrated that actin is associated with the plasma membrane in cortical patches *via* membrane protrusions (Mulholland *et al.*, 1994).

## 2.2.2 ACTIN PROCESSING

The actin cytoskeleton is a very dynamic structure, and the biological functions it fulfils are dependent on its ability to change and adjust to the requirements set internally by the different events during the cell cycle, e.g. bud-emergence, organelle segregation, cytokinesis, and extra-cellular signals which demand specific morphological adaptations. It is therefore not surprising that actin is constantly incorporated into growing filaments or disassembled into single units during the different stages of cell growth. Motility and morphological change are the result of the continuous alteration of actin filament length at specific internal sites. In yeast, morphological changes and the construction of sub-cellular structures such as the cortical actin patches, as observed during the initial stages of budding, are equally tightly linked to the dynamic features of the actin cytoskeleton. The morphological appearance and the underlying activities associated with actin structures are the consequences of numerous processing events. The processing of actin is regulated

on several levels, ranging from monomer folding to the rapid spatial distribution of microfilaments in response to signals.

Several processing events are required for the formation of a mature microfilament. The overall process can be subdivided into individual steps that include folding, polymerisation and cross-linking.

### 2.2.2.1 FOLDING

Molecular chaperones are known to assist in the folding of various proteins. The assembly and folding of actin are also subject to this chaperone activity. Extract analysis of pulse-labelled cells, by anion-exchange and size-exclusion chromatography, resulted in the observation that newly synthesised actin enters a large molecular mass complex, TCP1 (for t-complex polypeptide 1) (Sternlicht *et al.*, 1993). The TCP1 complex is an oligomeric particle of approximately 900 kDa, present in the cytosol. It is comprised of five or six polypeptides. Since previous reports suggested a role for the TCP1 complex in the folding of newly translated cytoskeletal proteins in the cytosol of eukaryotes, size-exclusion fractions were immunoblotted with an antibody that recognises one of the polypeptides in the protein complex (Gao *et al.*, 1992; Yaffe *et al.*, 1992). The large molecular mass complexes co-eluted with this polypeptide and actin. These findings strongly suggest a cytosolic pathway for actin folding which requires the activity of the TCP1 complex *in vivo*. In order to avoid any confusion with published mouse gene symbols, Stoldt *et al.* (1996) recommended the use of CCT (chaperonin containing TCP1) as a substitute for TCP1 complex.

Studies on the eight subunits of the *S. cerevisiae* Cct complex in conditional mutants revealed that yeast requires the chaperonin complex to assure the proper assembly of microtubules and actin *in vivo*. Of the eight genes encoding the *S. cerevisiae* chaperonin complex, *CCT1-CCT8*, five were shown to be essential (reviewed by Stoldt *et al.*, 1996). Upon exposure to restrictive conditions, conditional mutants of *CCT1*, *CCT2*, *CCT3* and *CCT4* exhibited an aberrant cytoskeleton resulting from defects in tubulin and actin assembly. Allelic variants of *CCT1* confirmed these results. The various *CCT1*-mutants were stained with rhodamine phalloidin, an agent specific for filamentous F-actin. Fine disrupted actin filaments were observed for the *cct1-1* mutant, in addition to a loss of asymmetrical actin distribution, which is a characteristic of wild-type cells. The phenotype of *CCT1*-mutants seems to be allele specific, since variants thereof exhibit different phenotypes. While the effect of the *cct1-1* mutant appears to be the most severe, *cct1-2* exerts a much lesser effect on actin filaments and their distribution. Moreover, the *cct1-3* allele conferred the formation of intense actin cables in buds instead of the normal patches that are usually observed. Other members of the chaperonin complex were also shown to alter cytoskeletal organisation, suggesting that actin filament formation and its distribution are linked to the activity of the Cct chaperonin complex in *S. cerevisiae*.

To date, the specific role of the Cct complex in filament formation is still not fully understood. It was, however, shown to facilitate the folding of actin and tubulin *in vitro*, as well as to interact with newly synthesised actin and tubulin monomers *in vivo* in higher eukaryotes (Yaffe *et al.*, 1992; Gao *et al.*, 1992; Sternlicht *et al.*, 1993). Monomers are able to associate spontaneously *in vitro*, and it therefore seems likely that the complex assists in preparing the actin monomers for the polymerisation event by keeping it in an assembly-competent state, rather than by mediating filament elongation.

### 2.2.2.2 POLYMERISATION

The *in vitro* assembly of monomers into polymers is a strictly regulated process that is dependent on the concentration of unpolymerised actin. *In vivo*, more than 100 actin-binding proteins, categorised in at least 30 different protein families, have been identified as role players in polymerisation (Sohn and Goldschmidt-Clermont, 1994). It is therefore not surprising that polymerisation *in vivo* can be controlled in such a way that the formation of filaments is targeted to specific areas in the cell, where remodelling of the actin cytoskeleton is required. Translocation of actin to a specific area in the cytosol is the result of filament elongation at a specific end, occurring in parallel with the depolymerisation of the filament at the opposing end. These dynamic features of polymeric actin enable the cell to alter its cytoskeleton rapidly in response to internal or external signals.

Mechanistically, the polymerisation process can be divided into four reversible steps: (i) monomer activation (salt-binding and conformational changes in monomers); (ii) nucleation (formation of stable oligomers prior to elongation); (iii) polymer elongation (extension of filaments at both growing ends) and (iv) annealing (binding of polymers at their respective ends) (Pollard and Cooper, 1986).

#### 2.2.2.2.1 Monomer activation

Actin monomers are activated prior to their incorporation into a growing filament. The association between actin and divalent cations represents the rate-limiting step in the initiation of the polymerisation process (Pollard and Cooper, 1986). The first indications that salt plays a role in the conformational changes of actin, before the onset of nucleus formation, were the alterations observed in UV absorption (Rich and Estes, 1976) and trypsin sensitivity (Rouayrenc and Travers, 1981). Furthermore, the fluorescence of actin labelled with *N*-iodoacetyl-*N'*-(5-sulfo-1-napthyl)ethylenediamine (IAEDANS) is sensitive to the binding of $Mg^{2+}$, due to the slow conformational change induced by the $Mg^{2+}$ (Frieden *et al.*, 1980). Initially the bond between actin and $Mg^{2+}$ is weak, but subsequent isomerisation of actin results in a tight interaction. When ADP occupies the nucleotide site, association with $Mg^{2+}$ does not result in a conformational change required for the activation of the monomer (Frieden and Patane, 1985). However, under similar conditions ATP-bound actin undergoes a conformational change resulting in its subsequent activation. Actin's ability to

associate with both ADP and ATP, in addition to divalent cations, is indicative of a heterogeneous pool of actin species in the cell. Accumulation of ADP-actin is detrimental for the cell, since its polymerisation rate is significantly slower than that of ATP-actin. It is therefore critical that the ADP-bound G-actin should be converted back to its ATP-bound state, a process mediated by the monomer-sequestering protein profilin (Mockrin and Korn, 1980; Goldschmidt-Clermont *et al.*, 1992; Weber *et al.*, 1999).

The polymerisation of $Ca^{2+}$-bound actin in the presence of $Mg^{2+}$ is characterised by a rate-limiting step that is similar to the rate of $Mg^{2+}$-induced conformational change of monomeric actin. When $Ca^{2+}$-bound actin is polymerised in the presence of $Ca^{2+}$, the rate limiting step is absent, therefore the rate limiting step in the polymerisation process is due to binding of $Mg^{2+}$ to the high-affinity, divalent ion binding site (Pollard and Cooper 1986). Both $Ca^{2+}$ and $Mg^{2+}$ compete for the same high-affinity site, situated in the cleft of the monomer. Association with either of the two divalent cations results in different conformational states of G-actin, implying that it plays different roles in the polymerisation process (Frieden *et al.*, 1980). G-actin contains at least five additional binding sites with lower affinities for divalent cations (Martonosi *et al.*, 1964), four of which should be ion-bound before F-actin formation will begin (Barany *et al.*, 1962).

#### 2.2.2.2.2 Nucleation

In order to create a filament *de novo*, the cell has to form a nucleus, i.e. an oligomer that can serve as an initiation point for active filament elongation. The formation of a nucleus or an oligomer is the rate-limiting step in spontaneous polymerisation of actin monomers (Cooper *et al.*, 1983; Frieden, 1983), since the dissociation constant of actin dimer formation is indicative of rapid product dissociation rather than its formation. In defining a nucleus, the most important aspect is the length of a stable oligomer. Prior to 1986, no suitable assays existed to assess the respective reaction properties of dimer and trimer formation. Consequently, based on the experimental data available, general consensus was reached on the likelihood of an actin trimer, rather than a dimer, serving as the actin nucleus in the formation of a new polymer. The stability of the trimeric complex is more likely to allow the incorporation of an additional monomer (Korn, 1982; Pollard and Cooper, 1986). More recently, studies on the *Acanthamoeba* Arp2/3 complex, a stable assembly of two actin related proteins, encoded by *ARP2* and *ARP3*, and complexed with five additional protein subunits, suggested that the actin dimer is the more likely candidate for a filament nucleus (Mullins *et al.*, 1998). The equilibrium binding constants for kinetic models of actin nucleation by the Arp2/3 complex favoured the association of actin dimers with the complex, rather than monomers or trimers. The association rates of both monomeric and trimeric actin seemed too high and were therefore considered as implausible.

Mullins and co-workers (1998) showed that the Arp2/3 complex caps the pointed ends of filaments and promotes the formation of polymers that exhibit a length increase exclusively at their barbed ends. The complex conferred an inducive effect on the rate of spontaneous polymerisation, without affecting monomer addition or dissociation at the barbed ends of filaments. Based on this observation, it was suggested that the accelerated polymerisation rate is attributable to an increase in the rate of nucleation. The gene products of *ARP2* and *ARP3* are believed to form an actin dimer-like structure that might serve as a more suitable template to initiate polymerisation, since it is more stable than an actual actin dimer (Kelleher *et al.*, 1995). Alternatively, the Arp2/3 complex might facilitate nucleation by stabilising small actin oligomers or intermediate fragments to allow the incorporation of a new monomeric subunit in the short actin filament.

It is noteworthy that the natural or induced shearing of filaments leads to an elevated concentration of ends that can serve as nucleation sites. Accordingly, actin filament containing solutions that were treated with a sonication procedure in the presence of a chelating agent, for the extraction of both $Ca^{2+}$- and $Mg^{2+}$-ions, resulted in the induction of actin nucleation (Maruyama, 1981). Although progress has been made in deciphering the various aspects of the nucleation step, gaps exist in our knowledge regarding the mechanism of nucleus formation and the regulatory elements involved. New models, e.g. the proposed role of Arp2/3 serving as a template for nucleation (Mullins *et al.*, 1998), are therefore still being developed.

#### 2.2.2.2.3 Polymer elongation

Cells are equipped with special mechanisms to avoid the spontaneous polymerisation of G-actin into polymers in the presence of large amounts of free actin monomers. This, however, does not apply for G-actin in test tube conditions, where most of the monomers are rapidly consumed into filaments until it reaches the critical G-actin concentration of about 0.1 μM (Sohn and Goldschmidt-Clermont, 1994). G-actin reaches its critical concentration when the rates of polymerisation and depolymerisation are equal at steady state, *in vitro*. When the actin concentration is rapidly induced or reduced, the process that will cause an opposing effect on the alteration in actin levels will accelerate. In other words, a change in actin concentration results in the acceleration of actin polymerisation or depolymerisation, depending on the initial change in concentration levels. This process continues until the critical actin concentration is re-established. Unpolymerised actin *in vivo* is maintained at concentration levels that are significantly higher than the critical concentration. This enables the cells to execute a rapid response when actin polymerisation is required during cellular processes, e.g. movement or protrusion formation. Specialised actin-binding proteins provide the cell with the necessary mechanism for such a response. These proteins assist in maintaining high levels of unpolymerised actin by binding G-actin, blocking the ends of filaments (actin filament capping) and through the depolymerisation of filaments. The capping and

depolymerisation of filaments are facilitated by capping- and severing proteins, respectively. The specific roles of these actin-binding proteins, in addition to other relevant proteins, will be discussed in more detail later in this review.

Filament elongation is not restricted to a specific end of the polymer, but differences in growth speed at the two different ends do exist. As mentioned, the nomenclature distinguishes between faster growing ends, termed plus or barbed ends, and slow growing ends, referred to as the minus or pointed ends (Pollard and Cooper, 1986). The binding of monomers at the opposing ends should differ with regard to their reaction properties, due to the conformational differences of the two end-forming monomeric subunits. Additional work by the Pollard group (1986) provided useful rate constants for the reactions of ADP- and ATP-actin with both the barbed and pointed ends of filaments. The data revealed that the two ends act differently in solutions containing monomeric-actin units (Pollard, 1986).

Profilin (Carlson *et al.*, 1976; Sohn and Goldschmidt-Clermont, 1994) and thymosinβ4 (Safer *et al.*, 1991), are examples of monomer-binding proteins that regulate the availability of free actin, required for the elongation of filaments. Thymosinβ4 is present in cells of higher eukaryotic organisms, but not in *S. cerevisiae*, while profilin is present in all cells (Weber *et al.*, 1999). By sequestering actin monomers these proteins render the binding sites of the free actin units inaccessible for actin-actin interaction. The sequestration of monomeric actin enables motile cells to maintain high levels of unpolymerised G-actin which can be rapidly made available for elongation by means of monomer desequestration, an event elicited by unidentified signals (Pantaloni and Cartier, 1993). In platelets, thymosinβ4 is more abundant than profilin and it also exhibits a higher affinity for actin. Interestingly, profilin is able to desequester monomers bound to thymosinβ4 in the presence of excess ATP, thereby lowering the critical concentration of actin to promote filament elongation. A fascinating role for profilin, functioning as a pump for polymerisation, was proposed by Pantaloni and Cartier (1993). In their model, G-actin bound to profilin can be added onto the barbed ends of filaments. The incorporation of the profilin-ATP-monomer is accompanied by the hydrolysis of the nucleotide, which decreases the affinity of profilin for the terminal subunits and thus results in its dissociation from the polymer. Thymosinβ4 is the major sequestering protein in mammalian cells and its association with monomers leads to a built-up of large pools of unpolymerised G-actin. Upon signal activation, profilin desequester the monomers from thymosinβ4 in order to allow its addition onto the barbed end of a growing filament (Pantaloni and Cartier, 1993). Filament elongation can be turned on or off at spatially defined areas within the cell by means of alternating activities of sequestering and capping proteins. While profilin decreases the critical concentration of G-actin at steady-state conditions, capping proteins cause an increase in the critical concentration. If the critical-state is reduced, the desequestration of monomers is favoured. Conversely, an increase leads to sequestration and, hence, the depolymerisation of filaments.

In its ATP-bound form, the monomer is incorporated at the filament's barbed end to become the next subunit in the growing polymer. When ADP-actin instead of ATP-actin is added onto the growing polymer, it gives rise to a more flexible filament (Janmey *et al.*, 1990). The less rigid appearance of ADP-actin filaments, however, is in contrast with findings from Pollard and co-workers (1992). The authors claim that the elasticity and viscosity of filaments assembled from either ATP-actin or ADP-actin are similar. Electron microscopy images of the filaments after negative staining, did not reveal any apparent morphological differences between the two structures (Pollard *et al.*, 1992). ATP-actin incorporation is accompanied by the hydrolysis of monomer-associated ATP to ADP and inorganic phosphate. During depolymerisation the same actin unit will be released from the F-actin molecule as ADP-actin.

The actin polymer consists of a heterogeneous array of nucleotide bound actin subunits, with ATP-actin at both ends, followed by a few monomers containing ADP-$P_i$-actin and finally, forming the bulk of the filament, ADP-actin. The release of the inorganic phosphate from the hydrolysed ATP-actin molecule is relatively slow. As a result, the $P_i$ moiety stays attach to the ADP-actin subunit after its cleavage from the latter. Hence, the ADP-$P_i$-actin is considered as an intermediate monomer species which can affect the binding rate of new ATP-actin monomers, as described in a model proposed by Pollard (1986), where the rate of monomer association or dissociation is strictly dependent on the nucleotide composition of the neighbouring molecules (Pollard, 1986). Under steady-state conditions the rate of monomer addition at one end is the same as its dissociation at the other end. Consequently, the length of the filament remains static, a process generally refer to as actin translocation or treadmilling (Korn, 1982).The rate of filament elongation at the end of a polymer determines the length of the ATP-actin cap. Depending on the monomer binding rates at the various ends, a filament will elongate, decrease in length or treadmills (Sohn and Goldschmidt-Clermont, 1994).

Data obtained recently by Mullins and co-workers provided some fascinating new insights on the topic of filament elongation. The authors proposed a nucleation model for Arp2/3 mediated assembly of filaments at the protruding edge of motile cells. The model is based on electron micrographs of quick frozen, deep-etched samples, containing F-actin and Arp2/3 complex purified from *Acanthamoeba* (Mullins *et al.*, 1998). As discussed previously under the nucleation section, the Arp2/3 complex promotes nuclei formation. Prior to its activity in the formation of actin nuclei, the Arp2/3 complex must be liberated from an unknown sequestering factor and targeted to the plasma membrane where its activation takes place. The activation process is induced by yet unidentified factors or signals (**Figure 2.8**). The micrographs of Mullins *et al.* (1998), showed that Arp2/3 binds to the sides of filamentous actin. It was also observed at the Y-junction, the point where two filaments are connected at an angle of 70° relative to the barbed ends facing the cell membrane. In addition they showed that Arp2/3 caps the pointed ends of filaments, a characteristic that is only shared with tropomodulin, a tropomyosin-binding protein that was originally isolated

from red blood cells and shown to associate with pointed ends (Weber *et al.*, 1994). The binding properties of Arp2/3 and tropomodulin differ, since the latter requires the association of tropomyosin to fully block the pointed ends of F-actin. The unique attributes of Arp2/3, i.e. the prevention of depolymerisation by capping the pointed ends, the promotion of nucleus formation and the ability to associate with the sides of filaments, enables the complex to attach short actin oligomers to pre-existing filaments. Since the pointed ends are fixed to the side of a filament, growth can only occur in an unipolar fashion at the barbed end (**Figure 2.8**). Subsequent depolymerisation occurs at the cytoplasmic end of the leading edge, the frontal area of the growing filament network, to liberate subunits that can be reapplied for elongation at the barbed ends.

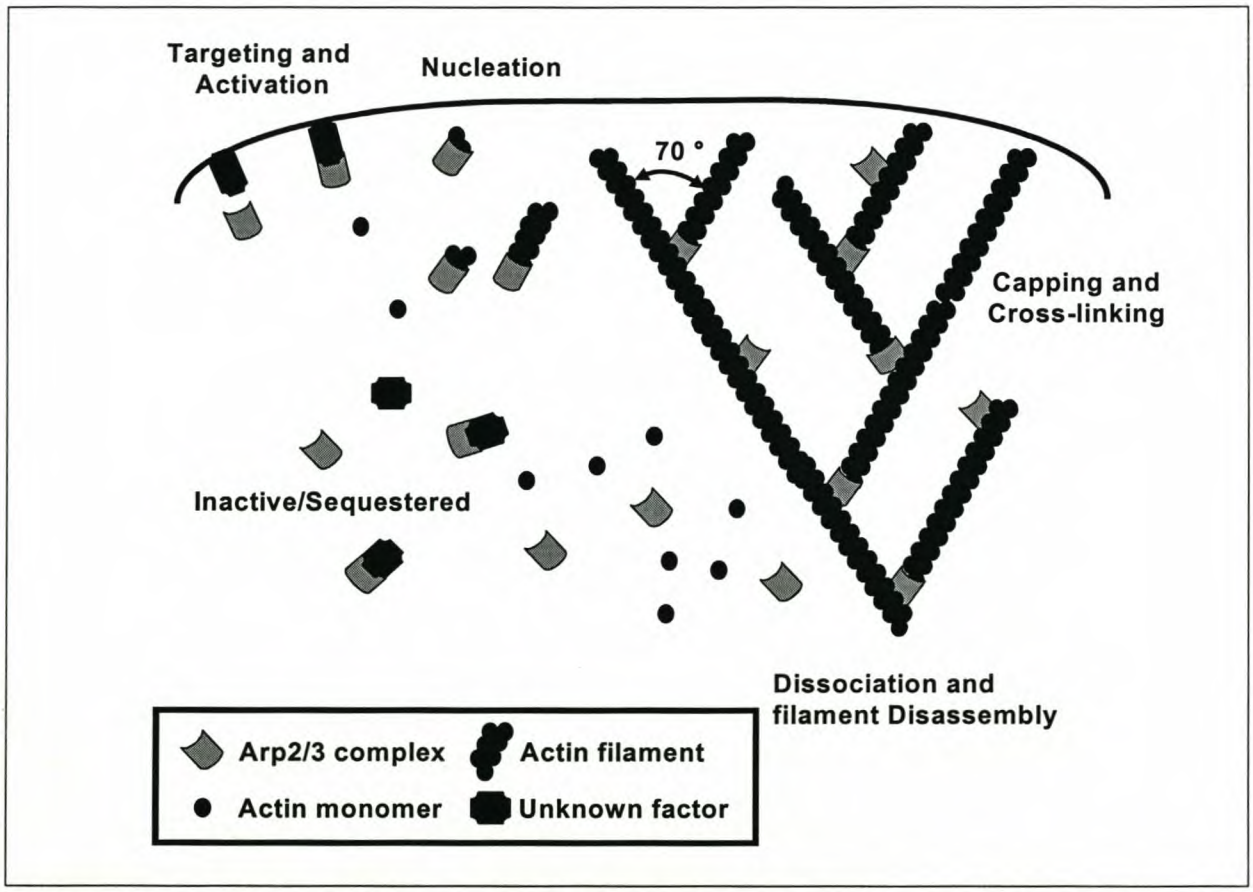


**Figure 2.8** Nucleation model integrating Arp2/3 in actin filament turnover at the leading edge of a motile cell. Arp2/3 complexes are present in the cytoplasm in inactive states when sequestered by unknown factors. External signals or factors activate complexes at the plasma membrane, and subsequently the nucleation of actin monomers is initiated in close proximity to activation sites. Short actin filaments are linked at an angle of 70° to longer filaments. Capping activity of Arp2/3 complexes at crosslinking sites results in filament growth at the barbed ends directly underneath the plasma membrane. Consequently cellular protrusions are formed. Depolymerisation of the filament network occurs at the pointed ends facing the cell interior. Liberated actin units and Arp2/3 complexes are available to be reincorporated in the network (Adapted from Mullins *et al.*, 1998).

Observations made by Svitkina and Borisy (1999) are in accordance with the model presented by the Mullins group (1998). They investigated the characteristics of filament elongation at the leading edge of lamellipodia in *Xenopus laevis* keratocytes

and fibroblasts. Of special interest for this particular study was the effect of the actin depolymerising factor (ADF), cofilin, on filament processing. Cofilin was found to be localised at the ends of elongating actin filament networks that face the interior of keratocyte cells. The authors termed the extensively branched organisation of actin filaments the dendritic brush. This observation fitted a model in which cofilin is responsible for the dissociation of monomers at the pointed termini of filaments, situated deeper in the cytoplasm. Somewhat contradicting, however, is the presence of cofilin at the fast growing ends of the filament network in fibroblasts. It therefore seemed that additional factors, as well as a regulatory step, are required for the depolymerisation of actin in the dendritic brush (Svitkina and Borisy, 1999).

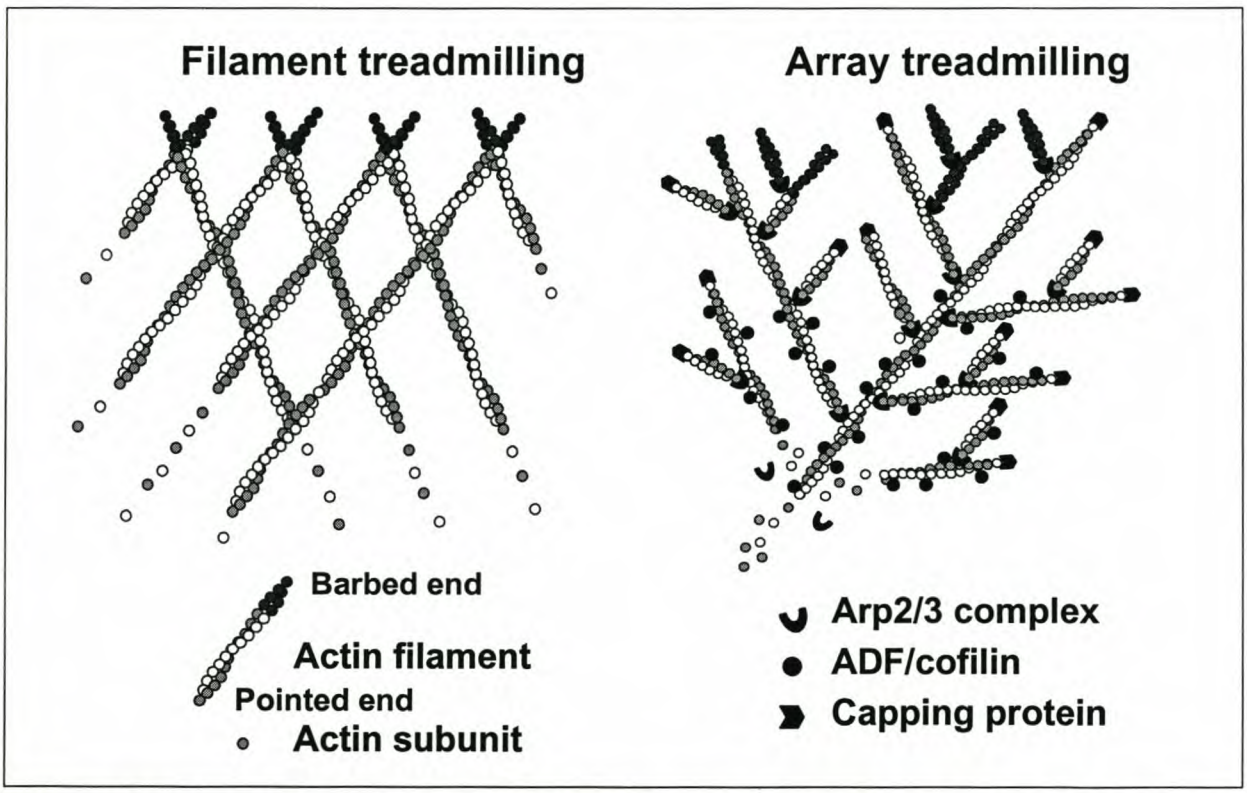


**Figure 2.9** Competing models for actin filament turnover in lamellipodia. Filament treadmilling model illustrating the simultaneous assembly and disassembly of individual filaments in dendritic brush, as opposed to Array treadmilling model in which filaments are portrayed as being interconnected to form a stable network. Actin filament disassembly is prevented by capping proteins associated with the barbed ends, while cofilin, located in the cytoplasm, promotes disassembly of unprotected pointed ends of the filament network. Capping proteins increase filament growth at the few uncapped barbed ends. Arp2/3 complexes link shorter filaments to network (Adapted from Svitkina and Borisy, 1999).

Based on the nucleation model of Mullins and co-workers (1998), Svitkina and Borisy (1999) conceptualised a new model that integrates the role of cofilin in the dynamics of the dendritic brush. The model entails that Arp2/3 promote nucleation and filament elongation by adding actin fragments, growing at their barbed ends exclusively, onto pre-existing filaments (Mullins *et al.*, 1998). Additionally, cofilin is predicted to facilitate depolymerisation at the pointed ends of the filament network, after desequestration of the Arp2/3 complex from these termini. This mechanism

implies that the whole filament network treadmills, termed array treadmilling, instead of individual filaments at the leading edge (**Figure 2.9**).

Capping proteins form another set of $Ca^{2+}$-independent polymerisation regulators that are present in all eukaryotic organisms (Vandekerckhove and Vancompernolle, 1992). Their function entails the binding of the fast growing ends of actin filaments, thereby blocking the addition of new actin monomers to the filament. This process contributes to the polymerisation of uncapped actin polymers, since it results in the acceleration of the rate of monomer addition to the free barbed ends of uncapped F-actin (Carlier and Pantaloni, 1997). *S. cerevisiae* also possesses a homologous gene representative of the capping protein family that binds the barbed ends of filaments. The mere association of a protein to the end of an actin polymer already suggests that it influences the polymerisation process. Accordingly, the gene products of *CAP1* and *CAP2* (Amatruda *et al.*, 1990, 1992) form a heterodimeric protein complex, that blocks polymer elongation of the filament to which it is bound, as well as monomer dissociation when it is associated with the barbed terminus.

In addition to the above mentioned, an extremely wide range of proteins exert an effect on the polymerisation process *via* the binding of actin in both its monomeric and filamentous forms. The topic is reviewed extensively in several papers (Korn, 1982; Pollard and Cooper, 1986; Hartwig and Kwiatkowski, 1991; Stossel *et al.*, 1985; Carlier and Pantaloni, 1997; Weber, 1999). The yeast homologues of these proteins, however, will be discussed briefly in the section on actin binding proteins in yeast (2.2.3).

#### 2.2.2.2.4 Annealing

Actin annealing refers to end-end association of different polymers as part of the polymerisation process. This process was thought to exist for actin *in vivo*, but conclusive evidence was not obtained. For historical reasons, the contradictory evidence that was obtained during earlier studies is included. In favour of fragment annealing is the rapid rate of filament elongation observed for sonicated actin samples, when viewed by electron microscopy (Nakaoka and Kasai, 1969). The rapid increase in filament length was attributed to the formation of high amounts of actin fragments, resulting from sonication, that could re-associate by binding end-to-end to form new filaments. Even more convincing were the formation of longer filaments from a mixture of short bare actin fragments and fragments decorated with myosin heads. The products consist of both decorated and undecorated segments, indicating that the incorporation of monomers is not exclusive to the polymerisation event (Kondo and Ishiwata, 1976). Unfortunately, the assessment of filament formation by means of electron microscopy does not provide any quantitative data on the tempo of fragment annealing, hence an elaborate model for the annealing process is still not conclusive.

To cast more doubt on the matter, evidence exists that argues against the rapid elongation of filaments *via* the association of preformed actin polymers. Quantitative

analysis of the amount of ADP-actin filaments generated after cessation of sonication, does not agree with the predicted values. Instead of a rate proportional to the square of the initial filament concentrations, indicative of annealing, the initial rate of filament decrease was directly proportional to the initial filament concentration (Carlier *et al.*, 1984).

#### 2.2.2.3 CROSS-LINKING

Actin cross-linking proteins mediate the side to side association of filaments, by serving as a link between the individual polymers, to give rise to organised filamentous structures. In order to fulfil this specialised function, the linking protein must contain either two spatially separated binding sites for actin, or it must consist of several polypeptides with single actin-binding sites (Korn, 1982). Both forms are known to exist. The function of these proteins is not restricted to actin-actin interactions exclusively, since actin filaments are connected to microtubules and cellular structures, such as membranes and cell junctions, as well (Dubreuil, 1991). Under *in vitro* conditions several cross-linking proteins are able to promote the formation of gels or lateral bundles of F-actin which resembles similar structures in cells (Stossel *et al.*, 1985).

Relatively well characterised cross-linking proteins include $\alpha$-actinin, the spectrin superfamily, ABP-120, ABP-280, dystrophin, fimbrin, filamin and villin. Dubreuil (1991) stated that many of these proteins are linked with regard to their origin during evolution due to a common, homologous amino acid sequence segment. It is believed that this specific conserved sequence forms the general actin-binding domain. The rest of the protein sequence is composed of repetitive and non-repetitive segments that were shuffled during evolution, to give rise to functional equivalent, but structurally different, products (Drubeuil, 1991).

Fimbrin is an example of a cross-linking protein that is present in both mammalian and yeast cells. It binds to the sides of F-actin filaments in an eight to one ratio in favour of actin. Upon specific conditions this interaction leads to the formation of actin bundles (Namba *et al.*, 1992). The *S. cerevisiae* homologue of mammalian fimbrin, encoded by *SAC6* (Drubin *et al.*, 1988; Adams *et al.*, 1989), exerts a stabilising effect on actin filaments of both cytoplasmic actin cables and cortical dots (Adams *et al.*, 1991).

### 2.2.3 ACTIN-BINDING PROTEINS IN YEAST

Apart from the already mentioned actin binding proteins, several screening techniques (for recent review see Ayscough and Drubin, 1996), resulted in the identification of numerous proteins that are able to complement the defects conferred by actin mutants in yeast. Henceforth, the focus of this literature study is shifted to yeast related actin binding proteins and the effects these proteins exert on the

regulation, structure and distribution of actin, within the framework of the yeast actin cytoskeleton.

### 2.2.3.1 MONOMER-BINDING PROTEINS

Unlike mammalian cells, *S. cerevisiae* only possesses a single monomer sequestering protein, profilin. Mammalian cells contain the major G-actin binding protein, thymosinβ4, which is responsible for a pool of unpolymerised actin, in addition to profilin. No homologue for thymosinβ4 exists in *S. cerevisiae*, and profilin seems to be sufficient to sequester the low levels of monomeric actin as established by Karpova and co-workers (1995).

Profilin fulfils a variety of functions in the cell. It binds actin monomers, promotes the exchange of ADP for ATP when coupled to G-actin, and increases polymerisation by targeting monomers to the barbed ends of filaments (see section on filament elongation). Deletion of *PFY1* results in a slow growth phenotype, large and round cell morphology, and defects in budding (Gerst *et al.*, 1991). It is also suggested to be involved in inositol phospholipid metabolism (Vojtek *et al.*, 1991). The defects of a profilin mutant are similar to those observed for yeast carrying C-terminal deleted or null mutant alleles of *CAP/SRV2*, adenylyl cyclase associated protein. Overexpression of profilin in these mutants restores the wild-type phenotypes (Vojtek *et al.*, 1991). Based on these results, it seems that profilin might act in the RAS-CAP pathway, or in an alternative pathway that interacts with the former, thereby creating a link between CAP function and actin regulation (Goldschmidt-Clermont and Janmey, 1991). Recent data revealed that CAP is able to sequester monomeric actin as well, but the mechanism through which its dual functions, i.e. actin regulation and cAMP regulation, are co-ordinated is still unclear (Freeman *et al.*, 1995).

### 2.2.3.2 BARBED-END CAPPING PROTEINS

*CAP1* and *CAP2* (Amatruda *et al.*, 1990, 1992), encode the *S. cerevisiae* homologue of a barbed-end capping protein. The two genes contain the genetic sequence for a non-homologous α- and β-subunit, respectively, that interact to form a heterodimeric actin binding protein able to bind actin filaments *in vitro*. An interesting feature of the capping-protein subunits is the fact that disruption of one gene leads to a rapid decrease in the levels of the other gene product. It therefore seems that the two proteins stabilise each other. Furthermore, a single disruption of either gene results in similar phenotypes observed for the double mutant. These mutants are characterised by a slow growth phenotype, a mixture of cells with varying cell size, loss of actin cables and depolarisation of cortical actin patches. Since the function of the capping protein is not essential in yeast, screens were conducted to search for genes that are co-lethal in a *cap2* genetic background. Subsequently, *SLC1* and *SLC2* (synthetically lethal with Cap) were identified and characterised (Karpova *et al.*, 1993). Deletions thereof result in an increase in temperature and osmotic sensitivity, as well as

alterations in the normal cytoskeleton distribution, aberrant actin cables and depolarisation of cortical patches.

In the absence of capping protein, actin cables are unstable, and an increase in G-actin and a decrease in F-actin are observed. Karpova *et al.* (1995) suggested that the *in vivo* function of capping proteins in yeast is to stabilise the functional form of actin, i.e. filamentous actin. Deletions of the genes encoding for capping protein in combination with the fimbrin gene are lethal, an effect conferred by the lost of functional actin filaments (Karpova *et al.*, 1995).

### 2.2.3.3 SEVERING PROTEINS

Moon *et al.* (1993) identified the *S. cerevisiae* homologue of cofilin/ADF (actin depolymerising factor) and have subsequently shown that the gene product of *COF1* exhibits similar interactions with actin as its mammalian counterpart. Loss of function mutations are detrimental to yeast cells. Functionally, cofilin assists in the cycle of rapid filament assembly and disassembly, by promoting the depolymerisation of actin filaments (Lappalainen and Drubin, 1997). In accordance with its role in filament turnover, evidence now exist of a role for cofilin in endocytosis, a process that requires rapid filament turnover of F-actin in the cortical actin patches. Furthermore, cofilin is localised at the actin cortical patches, but it does not interact with F-actin of the cytoplasmic actin cables (Moon *et al.*, 1993).

Apart from its ability to associate with filamentous actin, cofilin is also very capable of interacting with monomeric actin. It shows a bias towards the ADP-monomeric subunits within actin filaments, an attribute that facilitates the severing process, since binding of cofilin to these subunits probably causes a curvature of the filament that exposes more favourable binding surfaces which can then be exploited (Moon and Drubin, 1995).

### 2.2.3.4 CROSS-LINKING PROTEINS

Drubin *et al.* (1988) identified the yeast homologue of the cross-linking protein fimbrin as an actin binding protein by means of affinity chromatography. In addition, the fimbrin gene, *SAC6*, was cloned during a genetic screen for suppressors of actin mutants (Adams *et al.*, 1989). The relationship between fimbrin and actin was further investigated by the application of different genetic approaches that include suppressor analysis (Honts *et al.*, 1994), synthetic lethality (Holtzman *et al.*, 1994), two-hybrid analysis (Amberg *et al.*, 1995) and intragenic complementation (Botstein and co-workers, unpublished results). For a recent review on the rationale behind these various genetic approaches, see Ayscough and Drubin (1996). Suppressor analysis resulted in the identification of eight different *act1* mutations, all affected in the same actin domain, which compromise the binding of fimbrin to the actin molecule. Studies on α-actinin, an actin cross-linking protein originally identified in skeletal muscle, pinpointed the actin-fimbrin interface to exactly the same actin domain (Mimura and Asano, 1987). Special allocated areas on the surface of the

actin molecule therefore facilitate the cross-linking of filaments during the formation of actin cables.

Deletion of several genes is co-lethal in a *sac6* genetic background. In particular, *sac6*-mutant alleles result in co-lethality with capping protein mutants. Karpova *et al.* (1995) ascribed the co-lethality phenotype to a loss of functional actin filaments *in vivo*. This result implies that both capping-protein and fimbrin act as filament-stabilising factors in the yeast cell. Synthetically lethal phenotypes are also observed for a *sac6* deletion in combination with an *abp1* deletion, encoding gene for an actin binding protein that localises to cortical actin patches (Drubin *et al.*, 1988). The role of fimbrin as an actin bundling protein is not restricted to filament stabilisation, since the deletion of *SAC6* was shown to confer a decrease in α-pheromone uptake at 24°C, implying that the gene product is involved in endocytosis (Kübler and Riezman, 1993).

### 2.2.3.5 LATERAL BINDING PROTEINS

Tropomyosin is an example of a protein that interacts with the sides of actin filaments, but it differs from the cross-linking proteins, since its association with filamentous actin does not influence the formation of actin bundles. In mammalian striated muscle, the actin-tropomyosin interaction contributes to contraction regulation (Pollard and Cooper, 1986). Budding yeast possesses two genes encoding non-redundant forms of tropomyosin. The gene encoding a major form of tropomyosin, *TPM1* (Liu and Bretscher, 1989, 1992), was identified first, and *TPM2*, encoding the minor form, more recently (Drees *et al.*, 1995).

The gene product of *TPM1* was implicated in directed vesicular transport, since disruption thereof leads to an accumulation of vesicles and abnormal chitin distribution, due to a lack of actin cable stability (Liu and Bretscher, 1992). Its effect on cell development, however, is insignificant (Liu and Bretscher, 1989). Disruption of *TPM2* does not cause morphological defects, but overexpression of *TPM2* leads to an alteration in the budding pattern of haploid wild type strains. These strains exhibited bipolar budding patterns instead of the normal axial pattern (Drees *et al.*, 1995). Furthermore, co-transformation of *TPM1* re-establishes the normal haploid budding pattern. Double mutations of *TPM1* and *TPM2* are lethal.

### 2.2.3.6 MYOSINS

Myosins control cellular functions that range from actin filament movement to the transport of cytoplasmic organelles and vesicles. Myosins also play an indispensable role during cell division, where they mediate the transport of organelles from the mother cell into the newly formed bud. These processes are based on the actin cytoskeleton which promotes vesicular transport along cytoplasmic actin cables, a process that is dependent on myosin's ability to associate with filamentous actin. The myosin molecule consists of distinct domains of which the most important is the N-terminal domain, containing the motor motif (myosin head or S1) and an actin-binding

site. The motor domain possesses an ATPase activity required for force generation. The C-terminal domain, on the other hand, is much less conserved between different myosins and it specifies the functions of each type of molecule (Cope *et al.*, 1996; Wright and Jackson, 1996). Myosin regulation is partly mediated by calmodulins, which interact with the regulatory domain that is defined by a consensus sequence (Cope *et al.*, 1996).

*S. cerevisiae* possesses five myosin representatives that can be categorised in three of the thirteen classes that have been described. The gene products of *MYO2* and *MYO4*, are members of unconventional class V myosins. Mutant alleles of *MYO2* exhibit various phenotypes ranging from defects in polarised secretion and morphogenesis, to an inability to form buds (Santos and Snyder, 1997). Moreover, a loss of function mutation in *MYO2* results in cell death (Johnston *et al.*, 1991). Although *MYO4* (Haarer *et al.*, 1994) is homologous to *MYO2*, it does not encode an essential gene product, and it did not appear to be involved in polarised secretion. Recent data suggests that Myo4p plays a role in mating-type-specific switching, since it was found to be localised in developing buds where Myo4p is required for the accumulation of the *HO* gene repressor, Ash1p (Bobola *et al.*, 1996; Jansen *et al.*, 1996). It therefore seems that Myo4p does, in fact, play a role in polarised secretion of proteins to a designated site within growing buds.

The second group of interest, class I classical myosins, are represented by the two *S. cerevisiae* genes *MYO3* (Goodson and Spudich 1995) and *MYO5* (Goodson *et al.*, 1996). Deletion of either of the two genes has no perceptible phenotypic effects, but disruption of both functional alleles leads to cell death of various strains. Less sensitive strains exhibit an array of cellular and physiological defects, which include an aberrant actin cytoskeleton organisation, random bud-site selection, defects in chitin, invertase and cell wall constituent secretion, cell rounding, sensitivity to high osmotic strength and fluid phase endocytosis (Goodson *et al.*, 1996). The results obtained by Goodson and co-workers (1996) suggested that Myo3p and Myo5p fulfil overlapping functions in the cell, that are linked to actin cytoskeleton organisation.

*MYO1* encodes the only additional myosin representative, apart from those mentioned, in *S. cerevisiae* (Watts *et al.*, 1987). The protein is related to the heavy chain conventional type II myosins. Based on the observation that *MYO1* mutants form chains of cells, its function *in vivo* was suggested to be the facilitation of normal cell separation. Further functional analysis revealed that Myo1p is required for the maintenance of a cell type specific budding pattern, as well as normal chitin and cell wall constituent deposition (Rodriguez and Paterson, 1990).

### 2.2.4 ACTIN RELATED PROTEINS (ARPS)

ARPs form part of a superfamily of actin proteins which differ in function, but share amino acid homology with actin, more specifically within the domain constituting the actin cleft of classical actin proteins. Depending on the degree of sequence

homology, the proteins are categorised into ARP classes, with Arp1p exhibiting the highest homology to actin (Poch and Winsor, 1997). *S. cerevisiae* possesses three actin related proteins in addition to several more distantly related ARPs.

Arp1p is part of a multi-protein complex that plays a role in the activation of dynein, a process required for the promotion of membrane vesicles along microtubules. Deletion of the encoding gene, *ARP1/ACT5/ACT3*, results in misdirected orientation of the mitotic spindle and impaired nuclear migration (Muhua *et al.*, 1994). The second yeast ARP, encoded by *ARP2/ACT2*, is found at sites of active membrane growth, associated with actin cortical patches (Moreau *et al.*, 1996). A specific temperature sensitive *ARP2* allele exhibits various aberrant phenotypes, that include random budding and sensitivity to osmolarity changes, among others. The third ARP in this category is the gene product of *ACT4*, which was recently renamed as *ARP3* to fit the proposed nomenclature of Poch and Winsor (1997). *Arp3* mutants are nonviable and cells are characterised by arresting growth at various stages of the cell cycle (Huang *et al.*, 1996).

Of the five distantly related ARPs identified for *S. cerevisiae*, only one was analysed to date. *ARP4* was shown to encode an essential yeast protein (Harata *et al.*, 1994), that is localised in the cell nucleus. Although these proteins received their respective names based on the sequence homology with actin, *in vivo* function should not necessarily be considered as actin associated.

## 2.3 THE ROLE OF ACTIN IN MORPHOGENESIS

In general, the cytoskeleton can be considered as the intracellular framework of both prokaryotic and eukaryotic cells. It is actively involved in the organisation of the cytoplasm, in force generation required for vesicle transport and cell motility and in the maintenance of cell wall integrity (Botstein *et al.*, 1998). Apart from these common features, the yeast actin cytoskeleton specifically, has also been implicated in secretory vesicle transport and endocytosis (Kübler and Riezman, 1993; Peñalver *et al.*, 1997), nuclear and cell division (Palmer *et al.*, 1992), organelle movement and positioning (Drubin *et al.*, 1993), as well as bud-site selection and bud formation (Drubin *et al.*, 1993). It is interesting to note that in animal cells, the actin network apparently promotes the transport of cellular components over short distances, while the tubulin network is used by the cell to mediate long-range transport (Huang *et al.*, 1999), implying that the functions of both actin and tubulin overlap. The functional interaction between actin cables and tubulin in yeast, however, remains to be resolved.

Actin cables play a vital part in the establishment of polarised growth in yeast, since the actin bundles serve as rails to which transport molecules, in association with secretory vesicles, can bind, to facilitate the intracellular movement of the vesicle cargo to designated areas at the cell periphery. The internalisation step of endocytosis is also dependent on a functional actin cytoskeleton. When exposed to

pheromones, a yeast cell undergoes a morphological change, i.e. the formation of a cellular protrusion in the direction of the mating partner. Binding of α-factor to the product of the *STE2*-gene initiates receptor-mediated endocytosis (Jenness and Spatrick, 1986; Zanolari *et al.*, 1992). Importantly, the internalisation of the α-pheromone receptor does not require the function of the pheromone-response signal transduction pathway (Zanolari *et al.*, 1992). In addition, filamentous growth and the formation of pseudohyphae are also characterised by actin-based morphogenetic changes (Cali *et al.*, 1998). In this case, environmental nutritional cues serve as the signals initialising the development of elongated cells that remain attached to the mother cells, forming branch-like cellular structures (Gimeno *et al.*, 1992). Tolerance to osmolarity fluctuations is also attributable to actin function. Taken together, these data indicate that the yeast actin cytoskeleton provides the underlying structure for a variety of cellular processes.

The overview in the previous sections on actin structure (i.e. monomeric, polymeric and higher levels of structure organisation) and the proteins associated with actin is the basis for the subsequent analysis of the role played by the actin cytoskeleton in these processes. In this regard, the *in vivo* functions of the actin cytoskeleton in various aspects of polarised growth and directed secretion during the establishment of morphological alterations, including bud formation, mating projection formation and pseudohyphal development, are briefly discussed in the following sections.

### 2.3.1 THE ROLE OF THE ACTIN CYTOSKELETON IN SECRETION

The directed transport of secretory vesicles to designated areas of active cell growth under the cell cortex is a prerequisite for the occurrence of polarised growth. An intracellular network of actin cables and cortical patches orientates secretion vesicles to these areas (Kilmartin and Adams, 1984; Mulholland *et al.*, 1994; Amberg 1998). Evidence supporting a role for the actin cytoskeleton in directed transport includes the observations that sites exhibiting cell wall growth are always accompanied by underlying cortical actin patches, and mutant actin alleles confer secretion defects and impaired chitin distribution (Novick and Botstein, 1985; Mulholland *et al.*, 1994). The most direct association is probably the phenotypes observed for various *ACT1* mutants. Several *ACT1* mutations indeed confer secretion defects (Novick and Botstein, 1985).

The direct link between the actin cytoskeleton and secretion was further confirmed when it was showed that mutations in actin-binding proteins like Sac1p were able to suppress mutations in secretion (*sec*-mutants). Several mutations in other genes result in similar defects in polarised secretion. *SAC1* encodes for an actin-binding protein required for the proper localisation of actin patches and chitin (Novick *et al.*, 1989). Mutant alleles were identified that were able to suppress mutations in *SEC14*, which encodes a phosphatidyl inositol transfer protein, as well

as the defects caused by mutations of *SEC9* and *SEC6,* involved in late or post-Golgi steps of the secretion pathway. Furthermore, an additional actin-suppressing *SAC1* mutant allele was shown to be co-lethal in combination with disruptions of either *SEC13*, or *SEC20*, two genes encoding products involved in early steps of the secretory pathway (Cleves *et al.*, 1989). These genetic studies indicate that the actin cytoskeleton is involved in the early stages of the secretion pathway (Cleves *et al.*, 1989).

In order to promote the transport of vesicles to discrete sites at the cell surface, the actin cytoskeleton must be able to associate with the vesicles. Myo2p was suggested to link vesicles destined for the plasma membrane to actin cables (**Figure 2.10**), since expression of temperature sensitive *myo2*-mutant alleles caused vesicle accumulation in the cytoplasm, as well as the accumulation of large unbudded cells (Johnston *et al.*, 1991). It is tempting to assume that this model is a realistic representation of vesicle transport, since Myo2p is located at sites of active growth, i.e. at the bud- and mating-projection tips and at the mother-bud neck. However, both Myo2p and its regulator, calmodulin, did not co-localise with cortical patches and Myo2p was therefore proposed to act as a cortical scaffold that targets and organises actin filaments at sites where cell wall constituents are actively deposited (Govindan and Novick, 1995; Santos and Snyder, 1997).

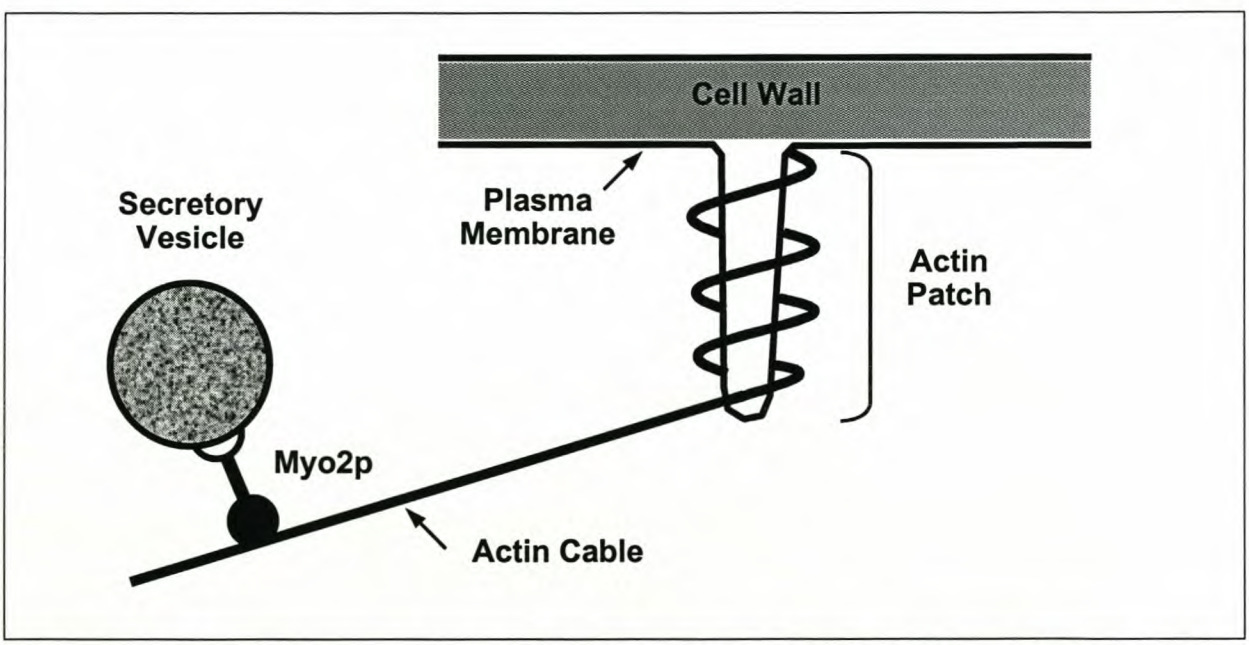


**Figure 2.10** Cartoon of a possible role for Myo2p in polarised secretion. Myo2p acts as a linking-protein between secretory vesicles and actin cables that are attached to cortical patches *via* plasma membrane protrusions (Adapted from Madden and Snyder, 1998).

Secretory vesicles can be divided into distinct classes, depending on the cargo they transport. For example, *sec4* mutants accumulate two different types of 100 nm vesicles (Harsay and Bretscher, 1995). The cargo of the one type consists of both endoglucanase and the major plasma membrane ATPase, while the contents of the second type of vesicle was found to comprise invertase and acid phosphatase. The

actin cytoskeleton is believed to mediate the translocation of at least one class of secretory vesicle, since both *act1* and *myo2* mutants accumulated vesicles that are not required for the deposition of cell wall components at sites of active cell growth. Furthermore, strains deleted for *MYO2* exhibit normal invertase secretion (Govindan *et al.*, 1995; Johnston *et al.*, 1991), while an *act1* mutated strain is able to discriminate between vesicles containing Sec4p, regarded as a factor involved in the secretion of numerous types of proteins, and Ypt1p, a small GTPase implicated in vesicular transport. Vesicles containing Ypt1p tend to accumulate in strains with a specific actin mutant allele (Mulholland *et al.*, 1997). The actin cytoskeleton therefore seems to play a role in the targeting of cargo-specific vesicles to areas within the cell, implying and emphasising that various processes required for morphogenetic events, are at least partially mediated through functional actin cables and subcortical dots.

### 2.3.2 ACTIN-DEPENDENT MORPHOGENESIS

Theoretically, the actin cytoskeleton can be expected to mediate all polarised processes in a mechanistically similar fashion, whether it participates in the establishment of a new bud during vegetative growth, the formation of a mating projection along a pheromone gradient or in establishing an elongated cell shape. The signals initiating these various cellular events and the pathways *via* which the regulatory information is transmitted will differ. This review will therefore mainly focus on two well characterised processes, bud formation and pseudohyphal development, to highlight the involvement of the actin cytoskeleton in morphogenesis. Significant differences between these cellular events and mating-projection formation will also be highlighted.

#### 2.3.2.1 BUD-SITE SELECTION AND BUD FORMATION

Bud formation is the result of the collaboration between numerous factors, each exerting a specific effect on the various underlying processes. Since haploid and diploid strains exhibit different budding patterns, different regulatory elements are involved. The actin cytoskeleton plays a critical role in the selection, as well as the formation of a new bud. Depending on actin polarity, bud formation will occur according to an axial, unipolar, bipolar or random pattern (**Figure 2.11**). The axial budding pattern is characteristic of haploid cells, as well as of diploid cells homozygous for the mating loci, i.e. strains *MAT*$\alpha$/$\alpha$ or *MAT*a/a. Diploid cells, containing both the *MAT*a and *MAT*$\alpha$ alleles, bud in a bipolar fashion. Interestingly, the bipolar budding seems to be the default pattern in haploid cells, since disruption of genes required for axial budding result in the formation of new buds at sites opposite to the previous bud-scar (Chant and Pringle, 1995). Haploid cells are equipped with a regulatory pathway that is able to override the activity of the default regulators responsible for bipolar budding. The unipolar budding pattern is

characteristic of cells growing in pseudohyphae, whereas random budding occurs in mutant cells affected in genes involved in bud-site selection.

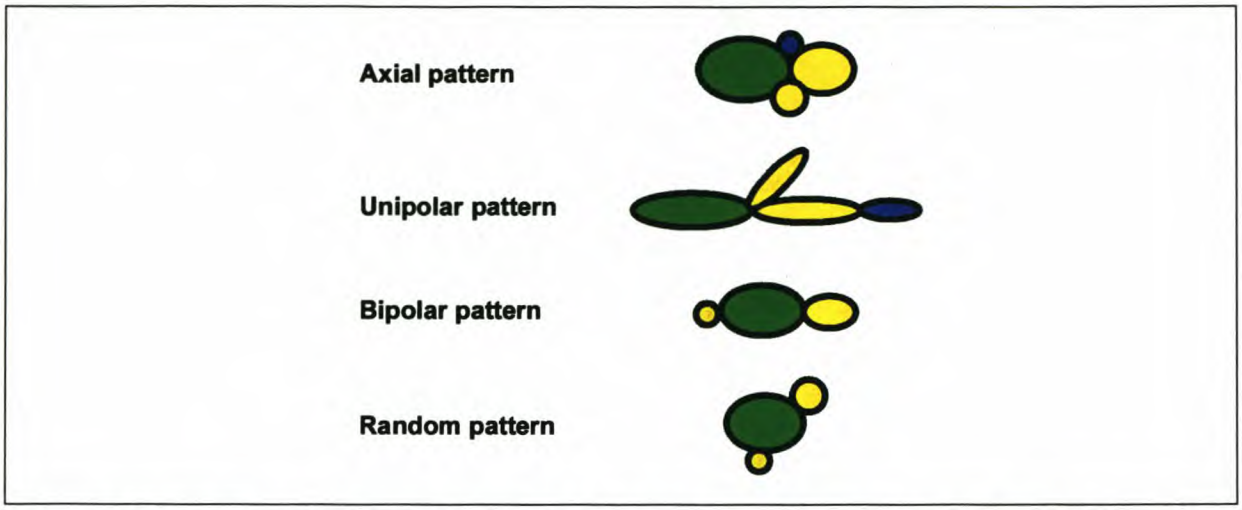


**Figure 2.11** Different budding patterns exhibited by *S. cereviciae* cells. Green, yellow and blue cells represent mother cells, daughter cells and third generation cells, respectively.

The budding pattern is not only the consequence of actin polarity, but various regulatory proteins, polarity establishment factors and targeting proteins, act in concert to govern the formation of a bud at a predetermined location on the surface of the mother cell. Several determinants of bud-site selection have been identified, which include cortical molecules serving as landmarks or tags for the targeting of additional proteins to the incipient bud-site, GTPase modules and other protein complexes involved in bud formation. These factors might interact with the actin cytoskeleton, to mediate actin orientation to a specific subcortical site. Conversely, actin patches might aid in the localisation of tagging proteins to the right locations at the cell periphery. Yang *et al.* (1997) suggested two possible mechanisms for the role of actin in this event. The first has actin responsible for positioning the cortical tags during the initial stages of bud emergence, by retaining their positions at the septal area of the previous budding cycle. The second mechanism suggests that the actin cytoskeleton might mediate the positioning of the cortical tags during bud emergence. In addition, the actin cytoskeleton probably participates in the transport of new components to these landmark proteins. Although some experimental data exist that support the above mentioned hypotheses, compelling evidence to substantiate these speculations still has to be obtained.

Actin, *per se*, is an essential component of bud-site selection. Various *ACT1* mutants were identified that affected the maintenance of a bipolar budding pattern in diploid cells (Yang *et al.*, 1997). Phenotypic analysis of these mutants revealed that bud formation occurs more randomly with every new generation. More strikingly, the mutations were pinpointed to a specific domain of the *ACT1* gene, suggesting that this region is critical for the role of actin in bipolar bud-site selection. Yang *et al.* (1997) speculated that this particular region is required for the interaction between actin and the proteins involved in the positioning of the bipolar bud-site cue.

#### 2.3.2.1.1 Cortical tags

In haploid cells the tagging proteins are located at a position adjacent to the previous bud scar, following the axial budding pattern. This process is probably mediated by a cytokinesis tag, a remnant from the previous budding cycle, that directs other components to the incipient bud-site (Chant and Herskowitz, 1991; Madden *et al.*, 1992). Potential candidates that function as cytokinesis tags include the four septin proteins (Chant *et al.*, 1995; Flescher *et al.*, 1993), Cdc3p, Cdc10p, Cdc11p and Cdc12p, which were originally identified in screens for temperature-sensitive mutations that controls the cell cycle (Hartwell, 1971) and subsequently termed septins to indicate their role in septation and cell division. Other proteins thought to be part of the tagging complex include Bud3p and Bud4p (Chant and Herskowitz, 1991), two proteins identified in a screen for mutants defective in bud-site selection, as well as Axl2p (Roemer *et al.*, 1996), an integral membrane protein. Recognition of any of these factors by proteins required for bud formation, including actin, would result in polarised secretion to the new predetermined bud-site.

The exact mechanism for bipolar bud-site selection is more obscure than for the selection of adjacent sites during axial budding. Growth or polarity factors are believed to be deposited during the initial stages of bud formation. These factors might serve a purpose in the selection of bud-sites in daughter cells by posing as cortical tags for the selection of sites at the distal end of the bud scar during the next round of budding (Chant and Herskowitz, 1991, Zahner *et al.*, 1996). As for the selection of proximal sites, proteins localised at the neck region during cytokinesis are considered as possible candidates (Chant and Herskowitz, 1991, Zahner *et al.*, 1996). Factors such as actin, actin-binding proteins, Spa2p, Pea2p, and possibly Bud9p, might be responsible for conveying the information required for the formation of cortical tags. Alternatively, these factors might associate with the tags at the site of cell separation (Drubin *et al.*, 1993; Yang *et al.*, 1997; Valtz and Herskowitz, 1996).

#### 2.3.2.1.2 GTPase module

The gene products of *RSR1*, *BUD2* and *BUD5* form the three components of a GTPase module that is involved in the establishment of axial, as well as bipolar budding patterns in *S. cerevisiae*. The module consists of a Ras-related GTPase, Rsr1p, a GTPase-activating protein (GAP), Bud2p, and a guanine-nucleotide exchange factor (GEF), Bud5p (Bender, 1993; Chant *et al.*, 1991). The GTPase module functions in directing important bud growth factors to the cortical tags localised at the bud-site (Chant *et al.*, 1991). Of special interest, is the interaction between the Rsr1p GTPase module and Cdc42p, a Rho-type GTPase (Johnson and Pringle, 1990), Cdc24p, the GEF for Cdc42p (Zheng *et al.*, 1994) and Bem1p (Chenevert *et al.*, 1992). Cdc42, Cdc24 and Bem1p are considered to be polarity establishing factors that are implicated in pseudohyphal differentiation and mating projection formation as well.

The mechanism of interaction between the Rsr1p module and the polarity establishing factors is still only vaguely understood, but the current hypothesis is that the former partakes in the positioning of Cdc42p, Cdc24p and Bem1p at the correct subcortical positions (Park *et al.*, 1996). A simple model for the convergence of the Rsr1p- and Cdc42p-GTPase modules is that the Rsr1p localisation is determined by a cortical marker consisting of actin or septin remnants of the previous budding cycle (Drubin and Nelson, 1996; Park *et al.*, 1993). Bud5p is able to associate with the plasma membrane and consequently activates Rsr1p at these intracellular sites. Cdc24p specifically interacts with activated Rsr1p and is recruited to Rsr1p occupied areas. In turn, Cdc24p will activate Cdc42p at these sites. Since Bem1p is able to interact with both Cdc24p and Rsr1p in addition to actin, it might function as a scaffold by bringing Rsr1p, Cdc24p and actin in close proximity (Drubin and Nelson, 1996; Leeuw *et al.*, 1995; Zheng *et al.*, 1995). The targeting of the polarity establishing factors to the cell periphery, however, occurs independently from the Rsr1p module, since both Cdc24p and Bem1p interacts with the plasma membrane in the absence of Rsr1p (Michelitch and Chant, 1996). These factors will be discussed in section 2.4.1.1.1 of this review, since they play a critical role in the link between signalling events and the remodelling of the actin cytoskeleton.

#### 2.3.2.1.3 Additional factors

Alterations in the bipolar budding pattern are observed in strains harbouring mutated alleles of several actin-binding proteins and actin cytoskeleton regulators. Defects in the yeast homologue of fimbrin, Sac6p, as well as Srv2p, Sla1p, Sla2p, Rvs161p and Rvs167p cause impaired actin cytoskeleton organisation in addition to bud-site selection aberrations (Adams *et al*, 1991; Crouzet *et al.*, 1991; Bauer *et al.*, 1993; Holtzman *et al.*, 1993; Sivadon *et al.*, 1995; Zahner *et al.*, 1996). Bni1p, a member of the formin family of proteins, was shown to interact with actin, Cdc42p, profilin and Bud6p (Evangelista *et al.*, 1997). A *BNI1* deletion results in the abolishment of the normal bipolar budding pattern, characteristic of diploid cells, while haploid cells are unaffected (Zahner *et al.*, 1996). Three components, Bud6p, Pea2p and Spa2p, constituting a putative 12S multiprotein complex involved in morphogenetic events were also shown to affect bipolar bud-site selection (Amberg *et al.*, 1997; Snyder, 1989; Valtz and Herskowitz, 1996). These proteins probably act in concert to promote polarised morphogenesis (Sheu *et al.*, 1998).

### 2.3.2.2 MATING PROJECTION FORMATION

In contrast to bud-site selection, the peripheral site for a mating projection is not established by a predetermined tag. Instead, pheromone receptors, dispersed throughout the entire cell periphery, act as the cortical landmarks for mating projection ( also referred to as a shmoo) formation. Upon activation, actin cables are orientated to the projection tip, while actin patches and secretory vesicles accumulate at this site (Hasek *et al.*, 1987). As would be expected, several pivotal factors

involved in bud-site selection and budding also participate in shmoo formation. Accordingly, Bem1p, Spa2p, Pea2p, Bni1p, Bud6p as well as Rom2p, a GEF for the vegetative growth GTPase, Rho1p (Ozaki *et al.*, 1996), are all localised to the shmoo tip (Amberg *et al.*, 1997; Evangelista *et al*, 1997; Manning *et al.*, 1997; Snyder, 1989; Valtz and Herskowitz, 1996). The reorganisation of the actin cytoskeleton towards the recognition site occurs in a similar fashion to that observed during bud emergence.

A very important aspect of mating projection formation is the ability of cells to adjust the direction of cell polarisation along the gradient of pheromone concentration (Segall, 1993). Due to the absence of targeting molecules at preselected sites of cell growth during the mating pheromone response, distinct regulators of protrusion formation should exist to mediate this unique morphogenetic event. The gene products of *FIG1*, *FIG2* and *FIG4* are exclusively transcribed in response to mating pheromone, but not during normal cell growth (Segall, 1993). When exposed to varying concentrations of pheromone, strains lacking any of these genes exhibit aberrant shmoo morphologies (Madden and Snyder, 1998). In addition, *AFR1* encodes a pheromone-induced protein, required for a functional projection morphology that interacts with septin (Giot and Konopka, 1997). It is tempting to speculate that the functions of these proteins are linked to the actin cytoskeleton.

### 2.3.2.3 PSEUDOHYPHAL DEVELOPMENT

The ability to switch cell morphology from oval to elongated is referred to as dimorphic switching (Gimeno *et al.*, 1992). In order to elucidate the role of the actin cytoskeleton in this process, Cali *et al.* (1998) investigated the effect of various *ACT1* mutants on filamentous growth. One of the results is that actin fulfils multiple and distinct functions in various aspects of dimorphic differentiation, particularly cell elongation, unipolar budding and agar invasion. These functions are largely dependent on the interaction between actin and fimbrin. The authors speculate that the multiple actin functions are mediated by different actin binding proteins, based on the varying effects conferred by a set of 12 charged-to-alanine *ACT1* mutants (Wertman *et al.*, 1992) they investigated. Furthermore, they present data that emphasises the importance of a highly polarised actin cytoskeleton during pseudohyphal growth (**Figure 2.12**).

The mutant alleles caused varying defects in filament formation. Some strains were unable to form filaments, while others made many clumpy and disorganised filaments. Similarly, different invasive growth phenotypes were observed due to the different mutations. The phenotypes varied from reduced invasive growth to complete absence of agar penetration. Cell elongation was also affected. The authors investigated whether bud-site selection is altered during pseudohyphal growth as a result of the different *ACT1* mutant alleles and the unipolar budding pattern was indeed abolished in several of the tested strains. Additionally strains lacking functional fimbrin were also examined. These mutants were defective in filament formation, invasive growth and cell elongation (Cali *et al.*, 1998).

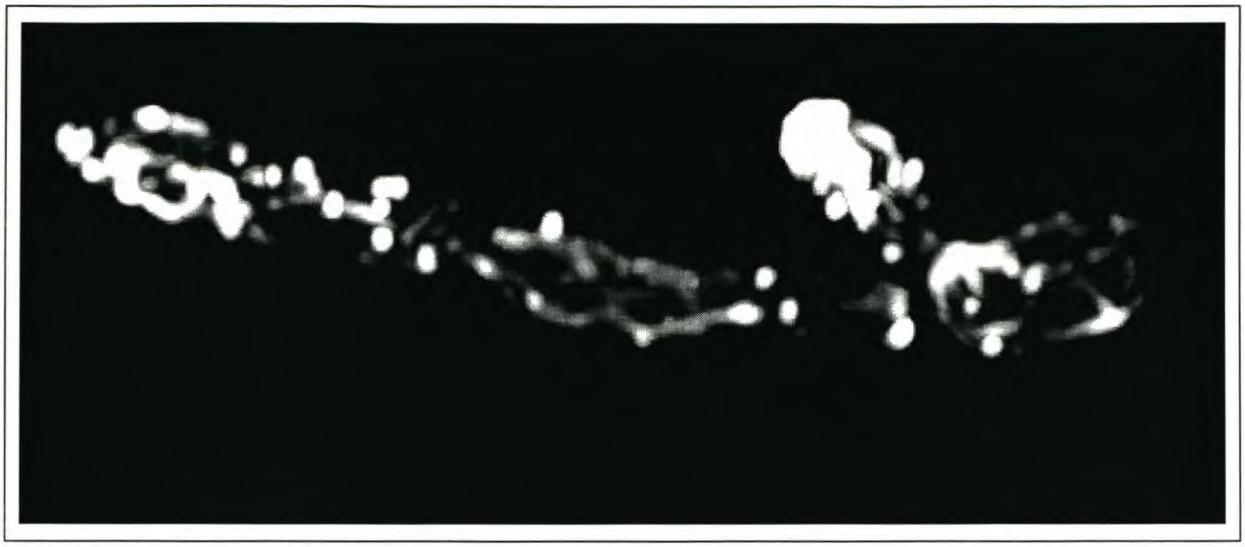

**Figure 2.12** Image of hyper-polarised actin cytoskeleton in elongated cells. Actin was stained with rhodamine-phalloidin (From the Botstein Laboratory Web-site).

Consistent with previous results (Kron *et al.*, 1994), Cali and co-workers (1998) observed that the polarity of cortical actin patches is much more prominent in pseudohyphal cells, as opposed to wild-type cells. Actin cables were also more conspicuous in the case of the former. The mutant strains, on the other hand, exhibited impaired actin cables that included thin, disorganised or absent structures. Interestingly, the *ACT1* alleles that caused a defect in actin patch polarisation were found to affect cell elongation more severely than the other *ACT1* mutant alleles tested.

Filamentous growth was also shown to be dependent on other actin-associated factors, besides the already mentioned Sac6p (fimbrin). Genetic screens to identify factors involved in this phenomena have led to the identification of Tpm1p (Tropomyosin), Srv2p (cyclase associated protein) and Bni1p, a member of the formin family of proteins (Mösch and Fink, 1997). Furthermore, Yang *et al.* (1997) demonstrated that Sla2p, a protein with homology to a focal adhesion protein, (Holtzman *et al.*, 1993), is required for pseudohyphal development, since a strain bearing a *SLA2* deleted allele was unable to undergo the morphological change to elongated cells.

The relationship between the actin cytoskeleton and components of the yeast secretory apparatus could also be important for filamentous growth. The exact mechanism of agar invasion is still not fully understood. Gimeno *et al.* (1992) suggested that the secretion of hydrolytic enzymes might facilitate the process. Enzyme activity could lead to agar degradation, thereby permitting cell growth into the underlying substrate. Glucoamylases encoded by the *S. cerevisiae STA1-3*-genes are potential candidates, since they are required for the degradation of extracellular starch molecules and *STA*-gene transcription is activated by elements regulating invasive growth (Pretorius *et al.*, 1986; Pretorius *et al.*, 1991; Lambrechts *et al.*, 1996a and 1996b; Webber *et al.*, 1997; Gagiano *et al.*, 1999a and 1999b).

Starch is present in great abundance in nature and it therefore seems plausible to suggest that polarised secretion of the glucoamylases to sites of cell-surface contact could enable cells to degrade starch, thereby creating a foothold for invasive growth to occur. This process eventually enables the elongated pseudohyphal cells to use alternative carbon sources. More recently Madhani *et al.* (1999) identified the *PGU1* gene, which encodes endopolygalacturonase, as being regulated by the filamentous growth MAPK signalling pathway as well. *PGU1* encodes a secreted endopolygalacturonase, involved in the hydrolysis of the plant-specific polysaccharide pectin that is present in the cell walls of plants and is considered as a structural barrier for invasion. Although it is not confirmed, it is still tempting to suggest that the actin cytoskeleton might direct the secretion of Pgu1p and the glucoamylases to the cell-substrate interface.

## 2.4. REGULATION OF ACTIN IN RESPONSE TO EXTERNAL AND INTERNAL SIGNALS

Several external stimuli elicit cellular responses that will ultimately lead to changes in cell morphology. In nature, yeast cells are constantly exposed to changes in environmental conditions, some of which have detrimental implications. Complex communication networks are responsible for the perception of the external signal and transmit the information to the interior of the cell.

Environmental factors monitored by the cell include the availability of essential nutrients, changes in osmotic conditions and the presence of pheromones. In order to perceive changes in these parameters the cell is equipped with signal-specific sensing mechanisms. Receptor molecules localised in the cell membrane perceive the information or signal. Once a specific signal is perceived, it will be transmitted by means of sequential modifications of participating elements to the specific targets required for implementing specific cellular responses. Depending on the nature of the signal, gene transcription will commence or be discontinued, while enzyme activities will be modulated, until the requirements set by the information detained in the specific signal are met.

In addition to external cues, internal signals must be initiated to allow the interconnection of the cell cycle with the different events resulting from the external signal. One of the most affected elements is the actin cytoskeleton. The importance of the connection between the cell cycle and actin regulators is clearly demonstrated by the fact that a specific phase in the cell cycle, designated START, must be reached, before shmoo formation or a following round of budding will commence. At this stage the status of the signal must be evaluated in terms of its strength, in the case of exposure to pheromones, and whether it is sufficient enough for a response that will involve, among others, the remodelling of the actin cytoskeleton.

In order to avoid confusion between the multitude of signal inputs, a signal specific response is executed by the application of signal specific transmitters,

although some factors were shown to have partially redundant or dual functions in the sense that they participate in more than one signalling event. Furthermore, a strong line of evidence suggests the existence of cross-communication between signal transduction pathways, thus making the signalling process even more complex in comparison with a single, linear pathway with no additional inputs other than that received by the signal-initiating protein.

### 2.4.1 LINKING SIGNALLING EVENTS TO ACTIN REMODELLING

The establishment and maintenance of cell polarity are governed by a complex network of signal transduction pathways in yeast and higher eukaryotes. Activation of these pathways is a complex process that involves protein-protein interaction as well as the activity of GTPases, among others.

#### 2.4.1.1 FUNCTION AND REGULATION OF RHO-LIKE GTPASES

To date, five *S. cerevisiae* GTPases have been identified that are required for proper actin cytoskeleton organisation. These GTPases include the polarity establishment protein Cdc42p, and several rho-like proteins, Rho1p, Rho2p, Rho3p and Rho4p (Adams *et al.*, 1990; Johnson and Pringle, 1990; Madaule *et al.*, 1987; Matsui and Toh-e, 1992). The different GTPases are distinguishable based on their association with different up- and downstream signalling elements. Due to their association with the regulatory apparatus of different signalling pathways, different exogenous and endogenous signals stimulate their individual activities *via* GTPase-specific regulators. GTPases associate with the nucleotide guanosine-tri-phosphate, which is required for their activation. Moreover, they all contain prenylation sequences, which interact with lipid moieties to facilitate the anchoring of G-proteins to the plasma membrane (Cid *et al.*, 1995).

The factors regulating these GTPases differ with regard to their complex-association properties and amino acid constitution. However, specific domains within the regulators are homologous (reviewed by Johnson, 1999). Guanine nucleotide exchange factors (GEFs) mediate the activation of GTPases by promoting the exchange of GDP (inactive GTPase state) and GTP molecules (active state). GEF activity is required to disrupt $Mg^{2+}$ and GDP binding-sites in the GTPase prior to nucleotide exchange. The conformational changes brought about by this event, result in GDP dissociation. As a consequence, the G-protein contains an unoccupied binding-site that is available for occupation by a new GTP molecule. GTPase activating proteins (GAPs) stimulate GTPase activity through the hydrolysis of the G-protein-associated GTP to GDP and inorganic phosphate ($P_i$). GTP hydrolysis results in the inactivation of the protein. The protein therefore is switched on and off by the alternating activities of GEFs and GAPs. Finally, guanine nucleotide dissociation inhibitors (GDIs) serve as additional regulators of GTPases. GDIs prevent the dissociation of guanine nucleotides from the G-protein, presumably by causing

conformational changes within the G-protein, leading to a fixed association of the latter with either GTP or GDP.

#### 2.4.1.1.1 The role of Cdc42p and associated proteins in morphological processes

The best characterised *S. cerevisiae* GTPase is encoded by the essential gene, *CDC42* (reviewed by Johnson, 1999). The gene encoding for this rho-like GTPase was originally isolated in a screen for mutations that cause growth arrest and render cells unable to bud at elevated temperatures (Adams *et al.*, 1990). Apart from the formation of multi-nucleate cells, another striking feature of this particular mutant was the misdirection of chitin deposition, suggesting a role for Cdc42p in actin polarisation. Genes encoding regulators of Cdc42p were also identified during independent genetic screens (Sloat *et al.*, 1981; Adams *et al.*, 1990; Bender and Pringle, 1989). These genes include *CDC24, CDC43* and *BEM3*, encoding the GEF and geranylgeranyl transferase for Cdc42p, and an SH3-domain containing protein, respectively. Cells deleted for the mentioned genes exhibit randomised actin cytoskeletons, implying that their individual functions are required for proper actin polarisation. Cdc42p, Cdc24p and Bem1p are factors involved in polarised cell growth events, which include bud-emergence, mating projection formation as well as pseudohyphal differentiation (Chant and Herskowitz, 1991; Mösch *et al.*, 1996; Simon *et al.*, 1995; Zhao *et al.*, 1995). However, different targeting factors are required to direct the polarity establishment proteins to sites of polarised growth during these cellular processes.

The role of the Rsr1p GTPase module during budding has been explained in section 2.3.2.1.2. Cdc42p and its two associated proteins Cdc24p and Bemp1 serve as polarity establishment factors at the incipient bud-site. The polarisation of actin to this area requires the collaboration between these factors and additional proteins involved in bud emergence (**Figure 2.13**). The Rsr1p/Bud1p GTPase module is located at the bud-site through a process that involves the GEF, Bud5p. The interaction between the Cdc42p GEF, Cdc24p, and Rsr1p results in the activation of Cdc42p, thereby ensuring that cell growth occurs at the correct spatially tagged region of the cell membrane (Park *et al.*, 1993). Cdc42p function is required for the organisation of cytoskeletal structures at Rsr1p-labeled sites. In its GTP-bound state, Cdc42p interacts with Ste20p or Cla4p, two proteins required for both the localisation of cell growth to the septin ring, and proper cytokinesis (Cvrckova *et al.*, 1995). Cdc42p also physically interact with Bni1p, which in turn, interacts with the actin monomer sequestering protein, profilin. The interaction between profilin and actin allows the polymerisation of actin at the new bud-site (Evangelista *et al.*, 1997). It is important to note that Bni1p is required for actin polarisation during shmoo formation, hence it is only suggested that it could play a role during vegetative growth. Alternatively, Bnr1p (Imamura *et al.*, 1997) might fulfil a similar purpose, but only during bud emergence, and is therefore a Bni1p functional analogue in this particular

event. In addition, Bni1p was found to interact with Rho1p, Rho3p and Rho4p as well (Evangelista *et al.*, 1997; Kohno *et al.*, 1996). The significance of the relationship between Bni1p and Rho-GTPases in this regard, is, however, still not clear. Narumiya (1996) proposed a role for Bni1p as a common mediator through which Rho-type GTPases induce actin polymerisation.

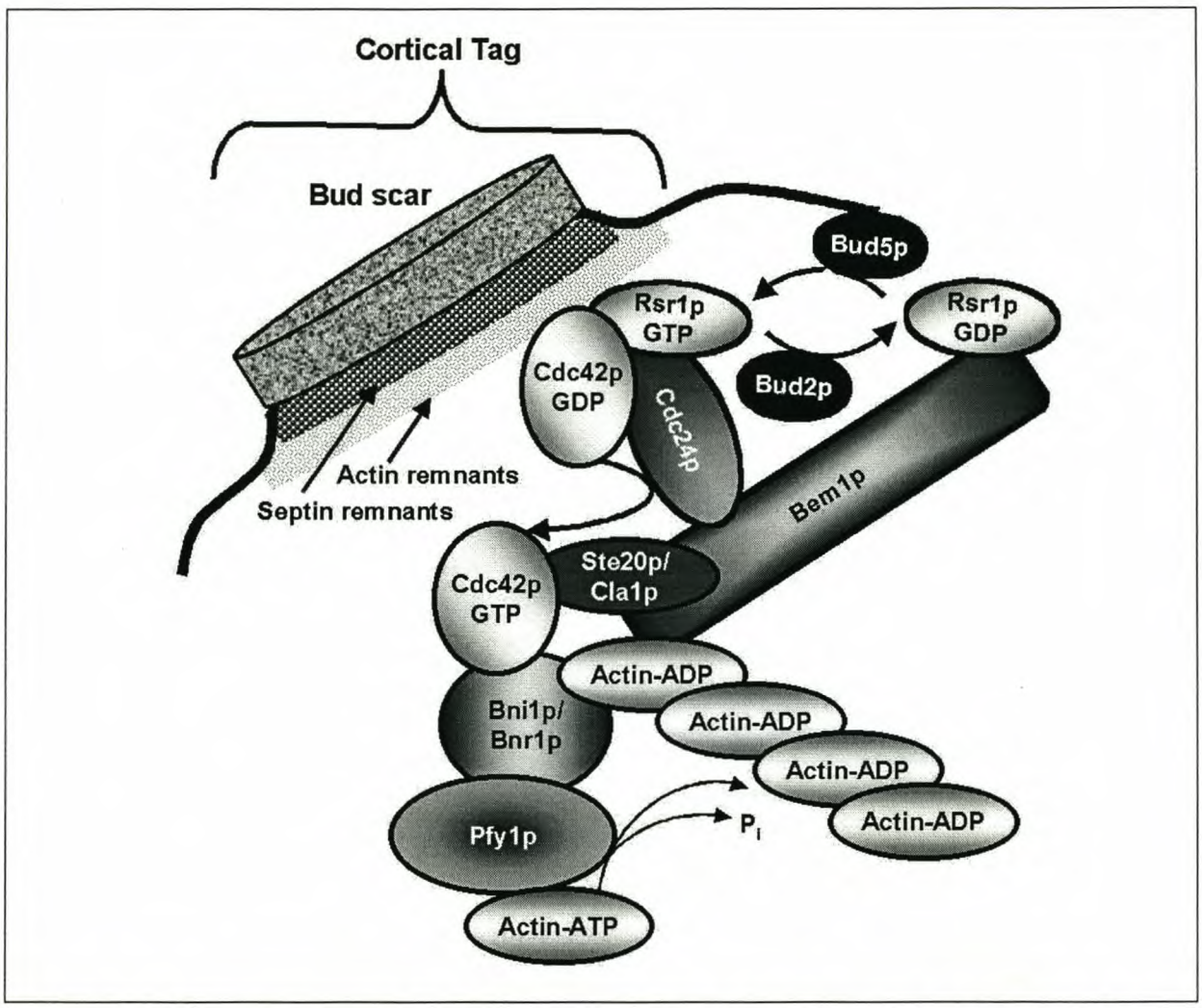


**Figure 2.13** A model demonstrating the interactions between the polarity establishment factors, Cdc42p, Cdc24p and Bem1p, and the Rsr1p-GTPase module at the incipient bud-site prior to axial budding. Remnants of the previous budding cycle serve as tags for the targeting of the Rsr1p GTPase module components to adjacent areas. Interaction between the Cdc42p GEF, Cdc24p, and Rsr1p takes place at the Rsr1p-labelled site. Additional factors required for actin assembly, Bni1p and profilin, and factors involved in directing growth to the septin rings, Ste20p or Cla4p, are included (Adapted from Cabib *et al.*, 1998).

Different cellular events are activated when haploid cells are exposed to mating pheromone. One process entails the activation of the pheromone response MAPK pathway, while the other involves recruitment and activation of the polarity establishment factors. The two events are coupled, since the components regulating actin polarisation, as well as the factors participating in signal transmission through the MAPK pathway, interact. A simplified model for the pheromone response pathway is presented in **Figure 2.14**. Binding of pheromones to G-protein coupled

transmembrane receptors, result in the dissociation of the α-subunit from the βγ-subunits of a heterotrimeric G-protein complex, thereby activating the signalling pathway. An additional factor implicated in the activation step includes Sst2p, a possible GAP for the α-subunit of the heterotrimeric complex (Leberer *et al.*, 1997a). The βγ-complex directly binds to and activates Ste20p (Leeuw *et al.*, 1998), which in turn associates with the MAPK module, comprising Ste11p, Ste7p and Fus3p. This provides a direct physical link not with the receptor, but with the heterotrimeric G-protein. Ste5p acts as a scaffold protein, since it interacts with several components of the MAPK module. The pheromone-initiated signal is conveyed through the MAPK cascade by means of sequential phosphorylation steps. Ste12p and Far1p are two downstream factors that are subsequently activated. Their functions involve the transcriptional activation of several factors required for mating and pheromone induced G1-arrest of the cell cycle, respectively (Dolan *et al.*, 1989; Errede and Ammerer, 1989; Chang and Herskowitz, 1992).

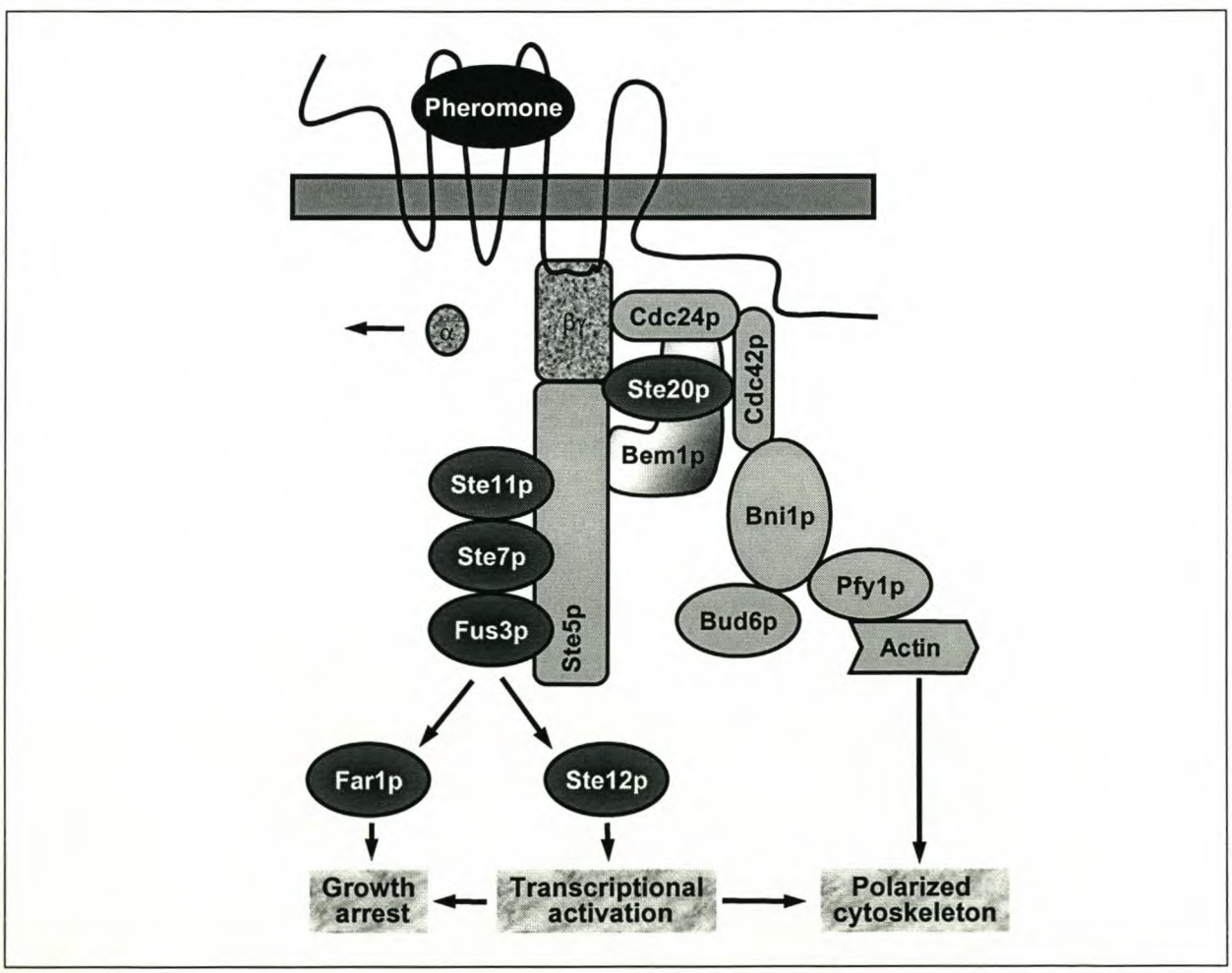


**Figure 2.14** Interaction between components of the pheromone-response pathway and actin cytoskeleton regulators. The polarity establishment factors serve as a link between these components at sites defined by activated pheromone receptors (Adapted from Madden and Snyder, 1998).

Bem1p provides the physical link between one of the components of the pheromone response pathway, Ste20p, and actin (Leeuw *et al.*, 1995). Bem1p was

found to co-immunoprecipitate with Ste20p, Ste5p and actin, implying that these factors function as an actin-associated complex *in vivo*. Association between these proteins was confirmed by two-hybrid analysis. Deletion of *BEM1* abrogates the interaction between Ste20p and actin, Ste20p is therefore speculated to act as the link between regulators of cell polarity and constituents of the pheromone-induced signal transduction pathway (**Figure 2.14**) (Leeuw *et al.*, 1995).

Cdc24p and Far1p were also recently identified as role players in the establishment of the correct orientation of mating projections in response to pheromone (Butty *et al.*, 1998, Nern and Arkowitz, 1998). More specifically, Far1p acts as an adaptor that interacts with the βγ-subunits of the heterotrimeric G-protein, as well as with the members of the polarity establishment factors, Cdc42p, Cdc24p and Bem1p. Furthermore Far1p shuttles from the nucleus to the cytoplasm in response to pheromone-induced activation of the heterotrimeric G-protein. The authors propose a model to integrate Far1p function in the overall process of shmoo-formation. During vegetative growth, Far1p is localised in the nucleus, while the polarity establishment factors organise the actin cytoskeleton for budding purposes. Upon activation of the pheromone-induced MAPK pathway, Far1p is relocated to the cytoplasm, where it is subsequently recruited to the βγ-subunits that mark the site of signal perception. As a consequence, the polarity establishment factors are targeted to the same site by means of association with Far1p. The process finally results in linking the actin cytoskeleton to this particular site, probably *via* Bem1p.

Cdc42p is also a component of the cellular machinery that transmits nutritional information to the nucleus prior to pseudohyphal differentiation. Since Cdc42p act together with additional polarity establishment factors to promote actin polymerisation at sites of active cell growth, specific targeting factors should exist for the pseudohyphal growth response as well. Receptors located in the plasma membrane, perceive the nutritional signals and might therefore serve as landmarks for the recruitment of Cdc42p and associated factors, e.g. the heterotrimeric G-protein α-subunit Gpa2p, to specific areas under the cell cortex. Although the interaction between Ste20p and Cdc42p is required for the activation of the filamentous growth signalling pathway, it is not required for the activation of the pheromone-induced pathway (Leberer *et al.*, 1997a, 1997b). Based on the association of Cdc42p with factors directly downstream of the pheromone receptors and the fact that several similar factors are required for pseudohyphal development and shmoo-formation, it is very possible that similar processes are involved in the recruitment of Cdc42p to sites of polarised growth (**Figure 1.1** and **Figure 2.14**).

#### 2.4.1.1.2 Rho1p and Rho2p

Rho-proteins have been shown to be involved in cytoskeleton organisation in many organisms (for review see Tanaka and Takai, 1998; Arellano *et al.*, 1999; Michiels and Collard, 1999). *RHO1* encodes an essential protein that interacts with (β1-3) glucan synthase and Pkc1p, two proteins involved in cell wall biosynthesis (Qadota *et*

*al.*, 1996; Nonaka *et al.*, 1995). Pkc1p exert a regulatory effect on the transcription of factors involved in cell wall synthesis, *via* a MAPK module comprised of Bck1p, Mkk1p, Mkk2p and Mpk1 (Levin *et al.*,1994). The fact that Rho1p is able to associate with Bni1p suggests that it might be involved in the organisation of the actin cytoskeleton (Imamura *et al.*, 1997; Kohno *et al.*, 1996). In accordance with such a role, Rho1p also localises to sites of growth and co-localises with cortical actin dots (Yamochi *et al.*, 1994). Rho1p seems to link the signal transduction pathway regulating cell wall synthesis with factors involved in the remodelling of the actin cytoskeleton. *RHO2* encodes a non-essential homologue of Rho1p (Madaule *et al.*, 1987). Overexpression of *RHO2* suppresses a dominant negative *RHO1* mutation, suggesting that these proteins fulfil partially overlapping functions *in vivo* (Madaule *et al.*, 1987; Ozaki *et al.*, 1996).

The GTPase activities of Rho1p and Rho2p are regulated by various factors. Rho1p-specific regulators include the three GAPs, Bem2p, Sac7p and Bag7p (Peterson *et al.*, 1994; Schmidt and Hall, 1998). Respective GEFs for Rho1p and Rho2p are encoded by the two homologous genes, *ROM1* and *ROM2* (Ozaki *et al.*,1996). Cells are nonviable in the absence of both these genes. Rom1p activity is subject to two independent signalling pathways. The essential phosphatidylinositol kinase homologue, Tor2p, controls the actin cytoskeleton through mechanisms involving Rho1p, Rho2p, Rom2p and Sac7p (Schmidt *et al.*, 1997). Apart from Tor2p-mediated activation, Rom2p is also activated by defects in the cell wall, suggesting that Rho1p could respond to cell wall damage *via* Rom2p (Bickle *et al.*, 1998).

#### 2.4.1.1.3 Rho3p and Rho4p

Two additional Rho-like GTPases were identified for *S. cerevisiae* (Matsui and Toh-e, 1992). Individually, Rho3p and Rho4p are not essential, but deletion of *RHO3* causes a severe slow growth phenotype. Overexpression of *RHO4* in a strain lacking *RHO3*, restores the normal growth phenotype. Strains carrying null-mutations of both these genes are nonviable, implying that these genes encode partially redundant proteins. The lethality of a double mutant can be rescued if the cells are suspended in osmotic stabilisers, e.g. sorbitol. Double mutants exhibit randomised actin distribution, delocalised chitin deposition and multiple nuclei. Interestingly, overexpression of either Cdc42p or Bem1p, restores the growth phenotype of a *rho3*-mutant strain, while overexpression of *RHO3* and *RHO4* compensate for the growth defects conferred by a *CDC42* mutation (Matsui and Toh-e, 1992). Finally, overexpression of *SEC4* also suppresses the growth defect of a strain lacking *RHO3* (Imai *et al*, 1996). All in all, it seems that Rho3p and Rho4p serve a purpose in directing the actin cytoskeleton and secretory apparatus to subcellular locations where active cell growth takes place. Bem4p and Bni1p might contribute in this process, by serving as targeting factors, since two-hybrid analysis revealed interaction between these proteins and Rho3p and Rho4p (Evangelista *et al.*, 1997; Mack *et al.*, 1997).

### 2.4.1.2 ADDITIONAL FACTORS

Three factors that are implicated in polarised morphogenesis were recently shown to interact with components of different signal transduction pathways (Sheu *et al.*, 1998). Spa2p plays an important role in several cellular processes, which include bud formation, mating projection formation and pseudohyphal development (Chenevert *et al.*, 1994; Mösch and Fink, 1997). Two-hybrid analysis revealed that Spa2p interacts with Bud6p/Aip3p and Pea2p, two proteins required for bud-site selection in diploid cells (Valtz and Herskowitz, 1996; Zahner *et al.*, 1996). Bud6p associates with actin and strains lacking a functional *BUD6* gene are characterised by large round cells and are defective in cytokinesis (Amberg *et al.*, 1997, Zahner *et al.*, 1996) Deletion of Pea2p compromises projection formation and pseudohyphal differentiation (Chenevert *et al.*, 1994; Mösch and Fink, 1997). Several lines of evidence suggest that these proteins function together to establish polarised morphogenesis. Firstly, they localise to similar growth sites (Valtz and Herskowitz, 1996; Zahner *et al.*, 1996) and secondly, null-mutations of these genes cause similar phenotypes. Furthermore, Spa2p localisation is dependent on Pea2p function and in the absence of Spa2p, Pea2p is unstable (Valtz and Herskowitz, 1996). Finally, velocity sedimentation experiments indicated that a large proportion of Spa2p, Pea2p and Bud6p co-sediment and peak at approximately 12S. Taken together, it appears that these proteins constitute a 12S multi-protein complex that is able to bind actin *via* Bud6p (Sheu *et al.*, 1998).

In addition to the interaction among these proteins, two-hybrid analysis also revealed that both Spa2p and Bud6p interact with Ste11p, which is a MAPK kinase kinase for three signalling pathways, i.e. the pheromone-response, filamentous growth and osmolarity pathways. Spa2p also associates with three different MAPK kinases: (i) Ste7p of the mating and filamentous growth pathways, (ii) Mkk1p and (iii) Mkk2p of the cell wall integrity pathway. Previous reports stated that Bud6p interacts with Bni1p, which in turn associates with profilin and actin (Evangelista *et al.*, 1997; Imamura *et al.*, 1997). For this reason Sheu *et al.* (1998) suggested that the mentioned proteins form a large multiprotein complex important for the promotion of polarised morphogenesis. The complex constituents function in concert to regulate actin cytoskeleton organisation in response to various signals (**Figure 2.15**).

## 2.4.2 CO-ORDINATION OF THE CELL CYCLE WITH MORPHOGENETIC EVENTS

The initiating factor for actin polarisation during the cell cycle has eluded researchers. Experimental data point to the cell cycle regulator, Cdc28p, as a potential candidate (Lew and Reed, 1993, 1995). Progression through the yeast cell cycle is governed by complexes consisting of the Cdc28p kinase and various cyclin proteins. Morphogenetic events ensue when the cell cycle reaches the commitment point, designated START, in the G1-phase. Morphogenetic events have to be tightly controlled and co-ordinated with the progression through the cell cycle.

Lew and Reed (1993) demonstrated that the polarisation of the actin cytoskeleton requires the activation of Cdc28p by the G1 cyclins, encoded by *CLN1-3*. Interestingly, the Cdc28p/Cyclin complex is also implicated in depolarisation of the actin cytoskeleton at the bud tip (designated the apical/isotropic switch), since a lack of activity leads to hyperpolarised secretion and consequently, elongated cells (Lew and Reed, 1995). Upon the inactivation of Cdc28p during the M-phase, the cortical actin cytoskeleton is redistributed to the mother-bud junction area. In addition, mitotic Clb cyclin mediated inactivation of Cdc28p is a prerequisite for actin cytoskeleton depolarisation at bud tips (Ghiara *et al.*, 1991). Unfortunately, the downstream elements that are probably regulated by the Cdc28p's kinase activity have not been identified.

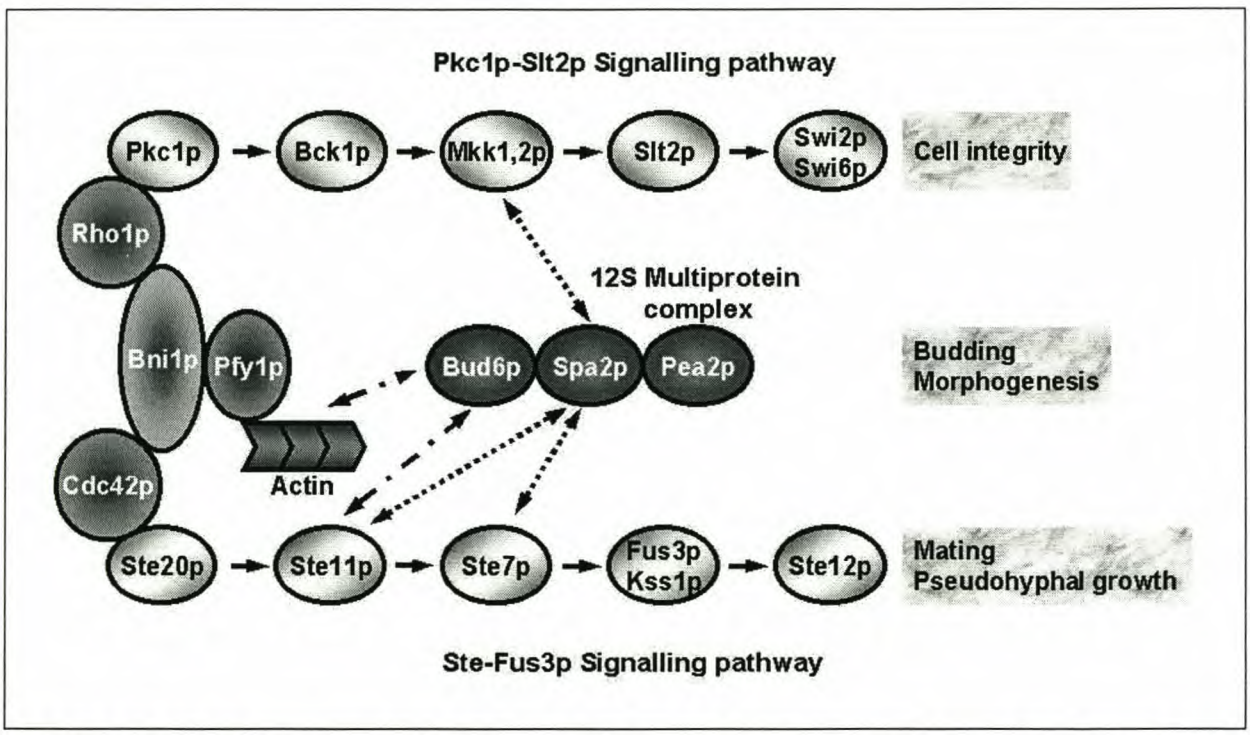


**Figure 2.15** The proposed model illustrating the interactions between constituents of the 12S multiprotein complex, Spa2p, Pea2p and Bud6p, and factors of different signal transduction pathways required for the transmission of signals by means of sequential phosphorylation steps that results in pseudohyphal differentiation, bud formation and cell maintenance, respectively. Bni1p, profilin and actin are general factors involved in the establishment of the three cellular processes (Adapted from Sheu *et al.*, 1998).

Recently Edgington and co-workers (1999) obtained evidence showing that filamentous growth is subject to the activity of a protein kinase pathway that includes Cdc28p. *ELM1* was isolated during a screen for factors that confer cell elongation irrespective of the normal nutritional signal (Blacketer *et al.*, 1993). Null mutation of the *ELM1* gene, which encodes a serine/threonine protein kinase (Koehler and Myers, 1997), results in constitutive filamentous growth on non-specific growth media independent of cell-surface contact and ploidy (Blacketer *et al.*, 1993). Based on the attributes of an *ELM1* deletion, the authors suggested a role for Elm1p as a downstream regulator of morphologic differentiation. Subsequent work by the same

group led to the identification of *ELM7* (Blacketer *et al.*, 1995). The gene was found to be allelic to a *CDC28* mutant in which the cysteine was replaced with a tyrosine 127 residue, termed *cdc28-127*. This particular *CDC28* mutant allele constitutively activates four cellular events which are part of filamentous growth characteristics; i.e. mother and daughter cells budding simultaneously, elongated cellular morphology, unipolar budding and the ability of cells to penetrate the growth substrate. Moreover, the bud-site selection defect conferred by a deletion of *BUD2* (see section on bud-site selection), was found to be epistatic to *cdc28-127*, suggesting that Cdc28p function is required for the selection of proper bud-sites during filamentous growth. In addition, elements that exert a regulatory effect on Cdc28p through the phosphorylation of tyrosine19 were found to affect filamentous growth as well (Edgington *et al.*, 1999). Mutation of *SEL2/HSL1* (suppressor of *elm1*) restores the normal yeast cell morphology in a strain lacking *ELM1*, while Swe1p, an inhibitor of Cdc28p, in turn, was found to be epistatic to Hsl1p.

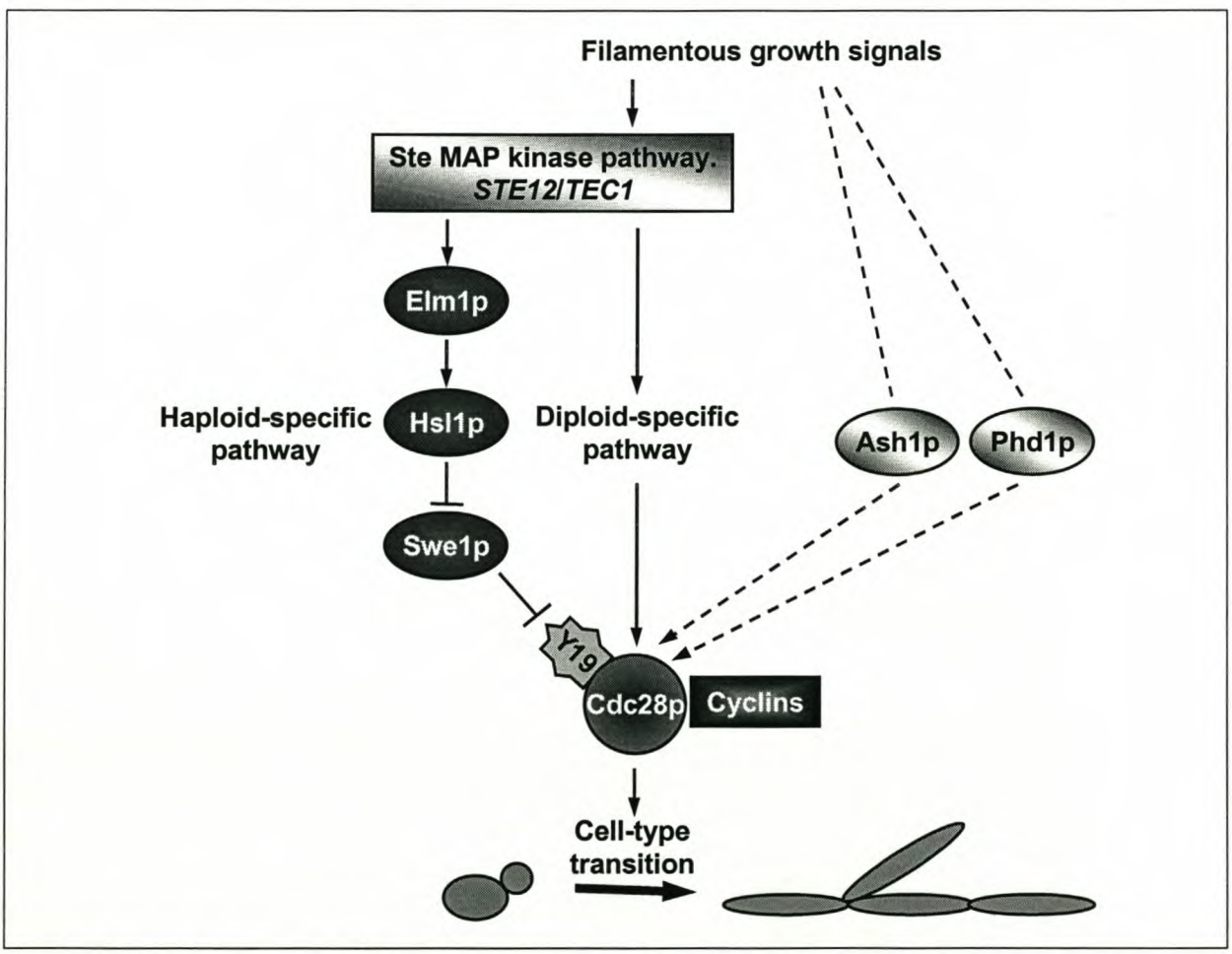


**Figure 2.16** Summary of the elements involved in the modulation of Cdc28p-cyclin complex activity. The Elm1p-Hsl1p-Swe1p module is probably regulated by the Ste MAP kinase signal transduction pathway to establish haploid-specific, but not diploid-specific morphological changes. Additional factors that might exert their effects on the Cdc28p-cyclin complexes include Ash1p and Phd1p. The transition between normal cell shape and elongated cell morphologies is mediated by the Cdc28p-cyclin complexes (Adapted from Edgington *et al.*, 1999).

Edgington *et al.* (1999) proposed a model that summarises the functional relationships between the mentioned factors (**Figure 2.16**). Elm1p serve as the upstream regulator of the Hsl1-Swe1p pathway, which in turn is believed to be regulated by regulators of invasive growth, e.g. the filamentous growth MAP kinase pathway, *PHD1* or *ASH1* (Gimeno and Fink., 1994; Chandarlapaty and Errede, 1998). Hsl1p functions downstream of Elm1p, where its activity results in the inhibition of Swe1p. The latter phosphorylates the tyrosine19 of Cdc28p, thereby inactivating the Cdc28p/Clb G2 kinase activity. As discussed previously, modulation of Cdc28p by the Hsl1p-Swe1p pathway and various cyclins, confers regulatory effects on the actin cytoskeleton. The transition between the yeast form and the filamentous form is most probably mediated by Cdc28p in complex with $G_1$ or $G_2$ cyclins. Cdc28p-cyclin complexes therefore seem to co-ordinate the cell cycle and morphological changes by exerting their effects on actin remodelling and distribution during the various stages of morphogenetic processes. Another link between polarised growth and the cell cycle has recently been suggested by Wu *et al.* (1998). The authors show that Ste20p is a target of Cln2-Cdk-dependent phosphorylation. This phosphorylation step occurs at the early stages of bud emergence while Ste20p is recruited to the site of bud-emergence. These data clearly indicates that morphological processes are tightly regulated and co-ordinated by the cell cycle.

## 2.5 CONCLUSION

For decades researchers focussed on the role of the acto-myosin complex in muscle contraction and cell motility, where actin was first characterised. The identification of the *S. cerevisiae* actin gene caused great excitement in the yeast community, since actin activity in this organism is not related to muscle contraction or cell motility. It therefore provided researchers with the opportunity to investigate non-motile functions of actin. Actin in all cells is a major component of the cytoskeleton, the cellular structure that determines cell morphology. Actin remodelling and distribution therefore contributes directly to morphological changes. This is well established for numerous types of cells, which include *S. cerevisiae*, *Dictyostelium discoideum* and lamellipodia, among others. Information regarding the regulation of actin and cytoskeleton remodelling in response to internal or external signals is lacking. Numerous factors have been identified that participate in actin regulation, but their exact modes of action in a multiprotein environment is still under investigation. This literature review focussed on general properties of actin and also on the advances made in research since the discovery of the *S. cerevisiae* actin homologue. The identity of all the aspects involved in actin remodelling and its effects on morphogenesis are clearly far from being understood. Considering the importance of cytoskeleton remodelling on many aspects of cell physiology, including the ability of cancer cells to invade tissues (Michiels and Collard, 1999), this research topic will continue to receive a high priority in the future.

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# CHAPTER 3

# Research Results

## Genetic analysis of factors involved in invasive growth and starch metabolism

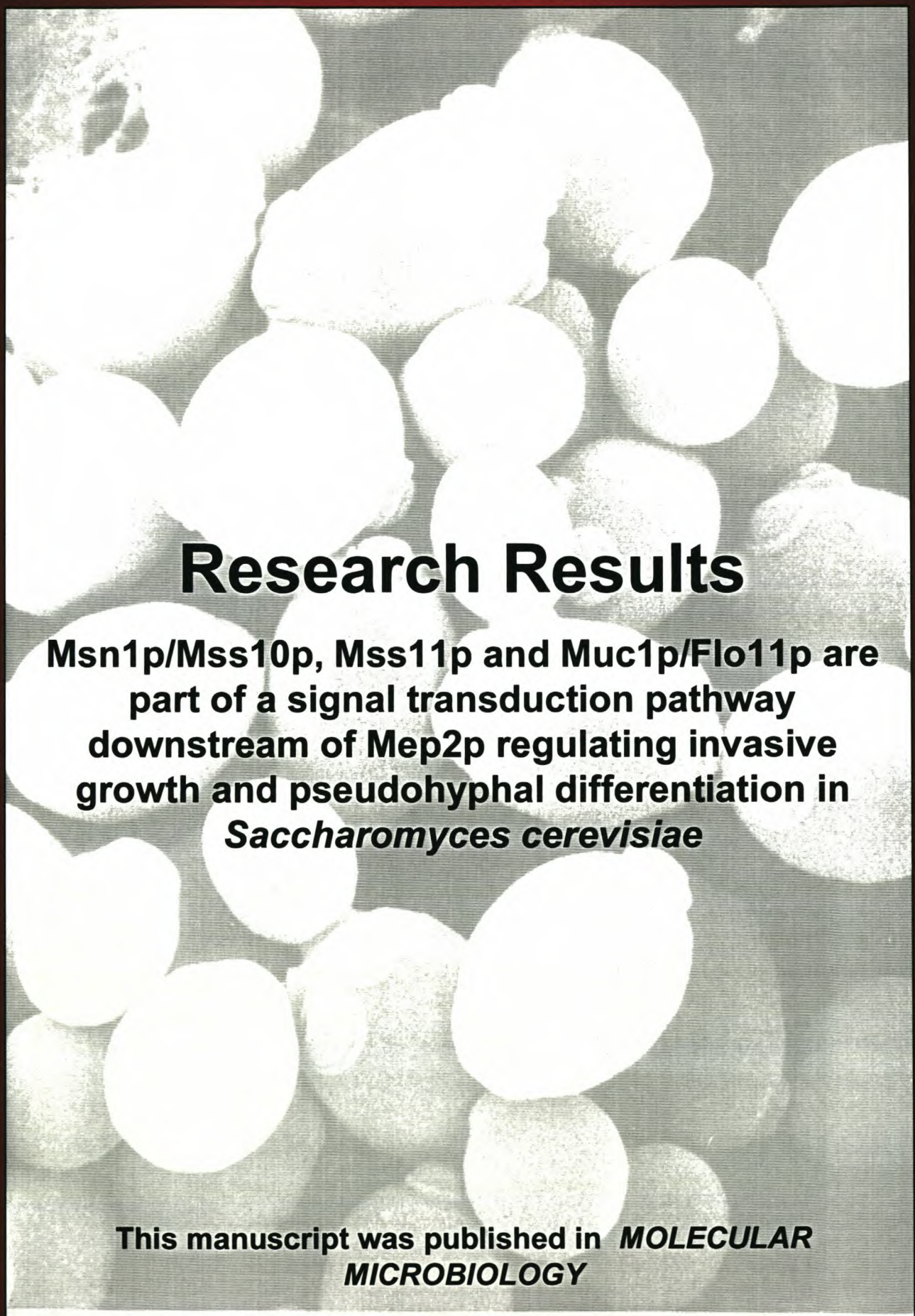
# 3. RESEARCH RESULTS

## 3.1 INTRODUCTION

The data generated during this M.Sc. study contributed to two scientific publications in international peer-reviewed journals (Gagiano *et al.* 1999a and b). In the first publication (Gagiano *et al.* 1999a), the author is the co-first author, and in the second (1999b) a co-author. The first paper therefore forms the first chapter of this section, whereas the second is attached in the appendix. Both papers are presented in the style of the journal in which they were published. The second chapter of this section briefly summarises the specific contributions by the author to these publications. Finally, the third chapter in this section lists additional, unpublished results that will be part of future publications.

This M.Sc. study was conducted as one of several projects investigating genetic, biochemical and physiological aspects of pseudohyphal differentiation, invasive growth and starch metabolism in *Saccharomyces cerevisiae*. The specific objective was to characterise the genetic relationships between several genes that are involved in these phenomena. In the process, a wide range of mutant-strains and plasmids were constructed that serve as tools for the analysis of genetic interactions or epistatic relationships, and will provide the genetic backgrounds for the investigation of (i) protein-protein interactions, (ii) protein-DNA interactions, and (iii) protein localisation experiments. Several of the factors will be investigated with regard to all these aspects as parts of a Ph.D. study that is a continuation of this M.Sc. project.

The two articles should be read before the section describing the unpublished results, because much of the background information and the methodologies used are explained in these publications. This information is therefore not repeated in the unpublished result section.



# Research Results

## Msn1p/Mss10p, Mss11p and Muc1p/Flo11p are part of a signal transduction pathway downstream of Mep2p regulating invasive growth and pseudohyphal differentiation in *Saccharomyces cerevisiae*

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# 3.2 RESEARCH RESULTS

## Msn1p/Mss10p, Mss11p and Muc1p/Flo11p are part of a signal transduction pathway downstream of Mep2p regulating invasive growth and pseudohyphal differentiation in *Saccharomyces cerevisiae*

***Marco Gagiano, Dewald van Dyk, Florian F. Bauer, Marius G. Lambrechts and Isak S. Pretorius***

*Institute for Wine Biotechnology and Department of Microbiology, University of Stellenbosch, Stellenbosch, ZA-7600, South Africa*

### 3.2.1 SUMMARY

In *Saccharomyces cerevisiae*, a network of signal transduction pathways governs the switch from yeast-type growth to pseudohyphal and invasive growth that occurs in response to nutrient limitation. Important elements of this network have been identified, including nutrient signal-receptors, GTP-binding proteins, components of the pheromone-dependent MAP kinase cascade and several transcription factors. However, the structural and functional mapping of these pathways is far from being complete. Here we present data regarding three genes, *MSN1/MSS10, MSS11* and *MUC1/FLO11*, which form an essential part of the signal transduction network establishing invasive growth. Both *MSN1* and *MSS11* are involved in the co-regulation of starch degradation and invasive growth. Msn1p and Mss11p act downstream of Mep2p, Ras2p, and regulate transcription of both *STA2* and *MUC1*. We show that *MUC1* mediates the effect of Msn1p and Mss11p on invasive growth. In addition, our results suggest that the activity of Msn1p is independent of the invasive growth MAP kinase cascade, but that Mss11p is required for activation of pseudohyphal and invasive growth by Ste12p. We also show that starch metabolism in *S. cerevisiae* is subject to regulation by components of the MAP kinase cascade.

### 3.2.2 INTRODUCTION

Pseudohyphal differentiation and invasive growth of diploid and haploid cells of the yeast *Saccharomyces cerevisiae* have been described as a cellular adaptation to growth on substrates containing either limiting amounts of - or inefficiently utilised - nutrients (Gimeno *et al.*, 1992; Gimeno and Fink, 1994; Lambrechts *et al.*, 1996a; Roberts and Fink, 1994). Research on the processes responsible for this cellular differentiation has focused on signal transduction mechanisms that transmit information regarding the nutritional status of the substrate and initiate the molecular, morphological and physiological changes observed during the switch from yeast-type

unicellular growth to pseudohyphal and invasive growth. These studies revealed a complex network of interacting signal transduction pathways of both inhibitory and activating nature and contributed vastly to our knowledge on signal transduction in eukaryotic organisms, as well as to our understanding of cellular differentiation processes. The two phenomena, pseudohyphal differentiation and invasive growth, are closely related and seem to be regulated by the same signal transduction mechanisms. However, they can be genetically separated and could correspond to different implementations of similar developmental pathways (Mösch and Fink, 1997).

One of the most outstanding aspects of signal transduction to emerge from recent data has been the modular nature of the pathways involved (reviewed in Elion, 1995; Herskowitz, 1995; Levin and Errede, 1995; Madhani and Fink, 1998). Modules include small and heterotrimeric G-proteins, MAP kinase cascades, second messengers and transcription factors, with some of these elements playing important roles in several signal transduction events.

Recent data suggest that the mating-specific MAP kinase cascade comprised by the MEKK, Ste11p, the MEK, Ste7p and the MAPK, Fus3p, has an inhibitory effect on establishing an invasive phenotype in haploids (Cook *et al.*, 1997; Madhani *et al.*, 1997). The same MEKK and MEK activate a second MAPK, Kss1p, which induces invasive growth when phosphorylated. The absence of this cascade, however, does not eliminate an appropriate regulation of pseudohyphal differentiation, indicating that MAPK-independent pathways play a major part in the process. Elements identified as being involved in MAPK-independent regulation include the small G-protein, Ras2p (Kübler *et al.*, 1997; Lorenz and Heitman, 1997), the $\alpha$-subunit of a heterotrimeric G-protein, Gpa2p (Kübler *et al.*, 1997; Lorenz and Heitman, 1997), Whi2p, a regulator of cell proliferation under starvation conditions (Radcliffe *et al.*, 1997) and Ash1p, a negative regulator of *HO* expression in daughter cells (Chandarlapaty and Errede, 1998). In addition, the ammonium specific receptor Mep2p has been shown to signal via MAP kinase independent pathways (Lorenz and Heitman, 1997).

Several regulators of transcription, acting downstream of the elements described above, have furthermore been identified. They include proteins such as Ste12p (Liu *et al.*, 1993), which, together with Tec1p (Gavrias *et al.*, 1996), acts downstream of the MAP kinase cascade to activate specific genes involved in the process. Other genes directly or indirectly responsible for the transcriptional regulation of genes involved in the invasive growth response are Phd1p (Gimeno and Fink, 1994) and Flo8p (Liu *et al.*, 1996).

Finally, another set of proteins such as Cdc42p, a Rho-like small G-protein and Ste20p, a MEKKK, form complexes with proteins that have been shown to transmit the spatial information necessary for the modulation of the cytoskeleton and for polarised growth (Evangelista *et al.*, 1997; Leberer *et al.*, 1992, 1997; Leeuw *et al.*, 1995, 1998; Mösch *et al.*, 1996).

However, whereas the MAP kinase dependent signal transduction process is relatively well mapped, little data are available regarding the MAP kinase

independent processes. In particular, a very limited amount of information is available about downstream elements responding to Ras2p and Gpa2p.

In this paper, we present data characterising the role of three previously identified genes, *MSN1/MSS10*, *MSS11*, and *MUC1/FLO11*, in the establishment of the invasive and pseudohyphal growth phenotypes. Two of these genes, *MSN1* and *MUC1*, have previously been shown to be important for invasive and pseudohyphal growth. *MSN1* has first been identified as a multicopy suppressor of *snf1* mutants (*MSN1*) (Estruch and Carlson, 1990). Other authors cloned the same gene as *FUP1*, an enhancer of iron-limited growth of *S. cerevisiae* (Eide and Guarente, 1992), as *PHD2*, a multicopy inducer of pseudohyphal growth (Gimeno and Fink, 1994) and, in our laboratory, as *MSS10*, a multicopy suppressor of the repression exerted by *STA10* on the *STA1-3* glucoamylase-encoding genes, involved in starch metabolism of *S. cerevisiae* (Lambrechts *et al.*, 1994, 1996b). *MSN1* has been suggested to encode a transcriptional activator since multiple copies of the gene seem to enhance the transcription of several genes, most of which are involved in nutrient utilisation. In addition, *MSN1* has been shown to activate reporter gene expression if fused to the LexA DNA-binding domain (Estruch and Carlson, 1990).

*MSS11*, like *MSN1*, was identified as a suppressor of the *STA10* dependent phenotype and was shown to induce *STA1-3* encoded glucoamylase expression when present on a 2μ plasmid (Webber *et al.*, 1997). The protein displays homologies with a number of transcriptional activators and suppressors, such as *S. cerevisiae* Snf5p, Ssn6p/Cyc8p and *Drosophila* NTF-1, and in particular with Flo8p, a protein activating genes involved in flocculation (Kobayashi *et al.*, 1996). The third gene investigated here, *MUC1*, cloned in our laboratory and later isolated as *FLO11* (Lo and Dranginis, 1996), encodes a cell wall-bound protein with homologies to mammalian membrane-bound mucins and to dominant yeast flocculation genes. *MUC1* was shown to be necessary for both invasive growth and filamentation to occur and to induce invasive growth when overexpressed (Lambrechts *et al.*, 1996a). Lo and Dranginis (1998) recently confirmed these data and presented additional evidence showing that *MUC1* was regulated by Ste12p and induced in response to nitrogen limitation in diploid cells. Here we show that Mss11p is an essential factor in the establishment of the invasive and pseudohyphal growth responses. We further show that the two genes *MSN1* and *MSS11* define a critical part of the signal transduction pathway regulating these adaptive responses, and that this regulation occurs in part through the transcriptional regulation of *MUC1*. Through genetic analysis, we show that both Msn1p and Mss11p act in a linear pathway downstream of Mep2p. Both genes show complex epistatic interactions with other elements of the signal transduction cascade. Our data show that Mss11p, as Msn1p, regulates the transcription of *MUC1*. We also show that starch metabolism in *S. cerevisiae* is regulated by components of the MAP kinase cascade that regulates the invasive growth response.

## 3.2.3 RESULTS

### 3.2.3.1 *MSS11* is involved in the regulation of pseudohyphal development and invasive growth

*MSS11* has initially been cloned as a gene that, when present on a 2μ plasmid, enhances starch utilisation by *S. cerevisiae* strains containing the *STA1-3* glucoamylase genes (Webber *et al.,* 1997). In these strains, we observed that the presence of *MSS11* on a multiple copy plasmid leads, in addition to more effective starch degradation (Webber *et al.,* 1997) and flocculation phenotypes (unpublished results), to strong invasive growth (Fig. 1A, B), including filaments of elongated cells (pseudohyphae) in a haploid background (Fig. 1C). In fact, strains bearing multiple copies of *MSS11* grow invasively directly after plating and at the beginning of growth, including in rich YPD medium. This phenotype was verified in several haploid and diploid laboratory strains, including the Σ1278 and S288C genetic backgrounds, as well as on different growth media. *MSS11* induced invasive growth in all genetic backgrounds and in all growth conditions tested (data not shown). Invasive growth by strains containing *MSS11* on a 2μ plasmid was directly correlated to colony growth and was clearly visible after only 24 hours. Control strains, containing only the plasmid without *MSS11*, were unable to grow invasively in media containing glucose and showed invasive growth only in media with a limited nitrogen source (SLAD) and media containing starch (SCS) or glycerol/ethanol (SCGE) as carbon sources after prolonged incubation periods. Multiple copies of *MSS11* therefore seem to induce the genes necessary for invasive growth on a permanent and signal-independent basis.

To ascertain whether *MSS11* played an important role in the invasive growth process, the gene was disrupted in several genetic backgrounds. The Δ*mss11* strains were unable to grow invasively (Fig. 1B), even after prolonged incubation periods under all conditions tested. *MSS11* therefore seems to encode an important component in the ability of yeast cells to grow invasively. Disruption of the gene, however, did not affect the general growth of the strains in liquid and solid media in any of the growth media tested (with the exception of starch containing media), the mating ability of the strain, osmosensitivity or heat shock resistance (data not shown), indicating that *MSS11* is specifically required for some cellular differentiation processes, but does not affect the general yeast physiology.

### 3.2.3.2 Epistatic relationship between Msn1p, Mss11p and Muc1p

To assess whether the two genes *MSN1* and *MSS11* act in the same pathway, we established their epistatic relationship. Several ISP15 strains, in which either *MSN1* or *MSS11* or both were deleted, were used for this study. These strains were transformed with 2μ plasmids bearing *MSN1*, *MSS11* or the vector without any insert as a negative control. The strains were subsequently spotted onto different media to assess the extent of the invasive growth phenotypes. The invasive growth phenotypes of these strains on nitrogen limited SLAD medium are shown in Fig. 2A.

If compared to wild-type ISP15 transformed with the vector alone, it can be seen that multiple copies of both *MSN1* and *MSS11* lead to more pronounced invasive growth phenotypes, whereas the deletion of *MSN1* or *MSS11* or both, lead to strongly reduced or absent invasive growth phenotypes.

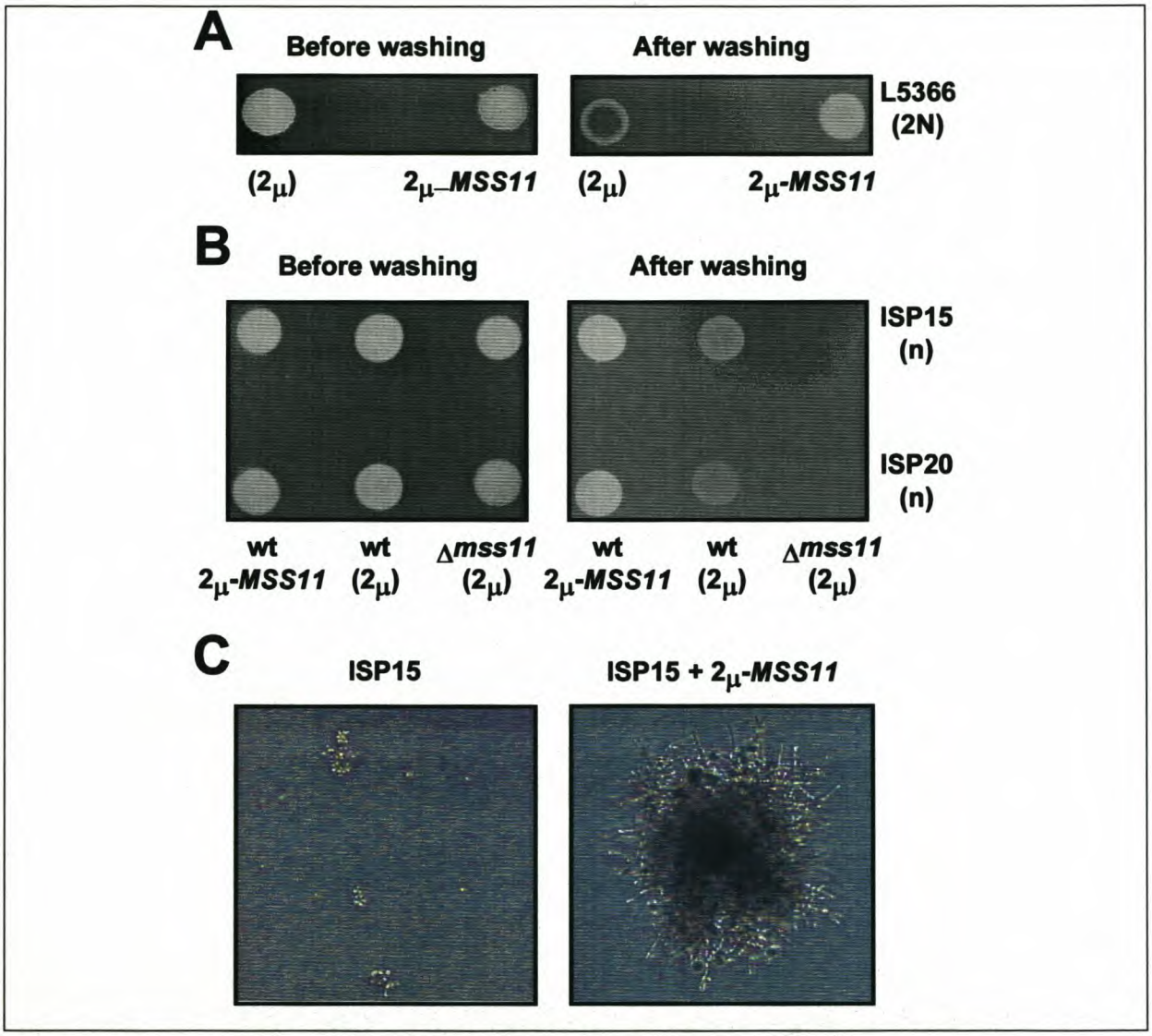


**Fig. 1.** Role of Mss11p in invasive and filamentous growth. A. The effect of multiple copies of *MSS11* on the invasive growth of a Σ1278 diploid strain, L5366, transformed with plasmid YEpLac195-MSS11. The control consists of L5366 transformed with YEpLac195 without insert. B. The effect of multiple copies and of deletion of *MSS11* in haploid strains ISP15 and ISP20, transformed with YEpLac112-MSS11. The control shows strains ISP15 and ISP20 transformed with YEpLac112 without insert. C. Filament formation induced by *MSS11* in the haploid strain, ISP15. Photos show colonies of ISP15 transformed with YEpLac112 and YEpLac112-MSS11, respectively, photographed beneath the agar surface of SLAD medium 3 days after plating. At this stage, the wild-type strain transformed with YEpLac112 as control, showed very limited invasive growth.

Multiple copies of *MSN1* are unable to overcome the effect of an *MSS11* disruption, since no invasive growth was observed in the corresponding strain. This result suggests that the function of Msn1p depends on Mss11p, or that Msn1p functions upstream of Mss11p in a linear signal transduction pathway. Multiple copies of *MSS11,* however, are able to overcome the effect of a deletion in *MSN1* very

efficiently, resulting in very strong invasive growth. We therefore propose that Msn1p is situated upstream of Mss11p in a signal transduction pathway resulting in invasive growth. Interestingly, the invasive phenotype of strains carrying multiple copies of *MSS11* is significantly stronger in strains with disrupted *MSN1* loci than in the wild-type strains.

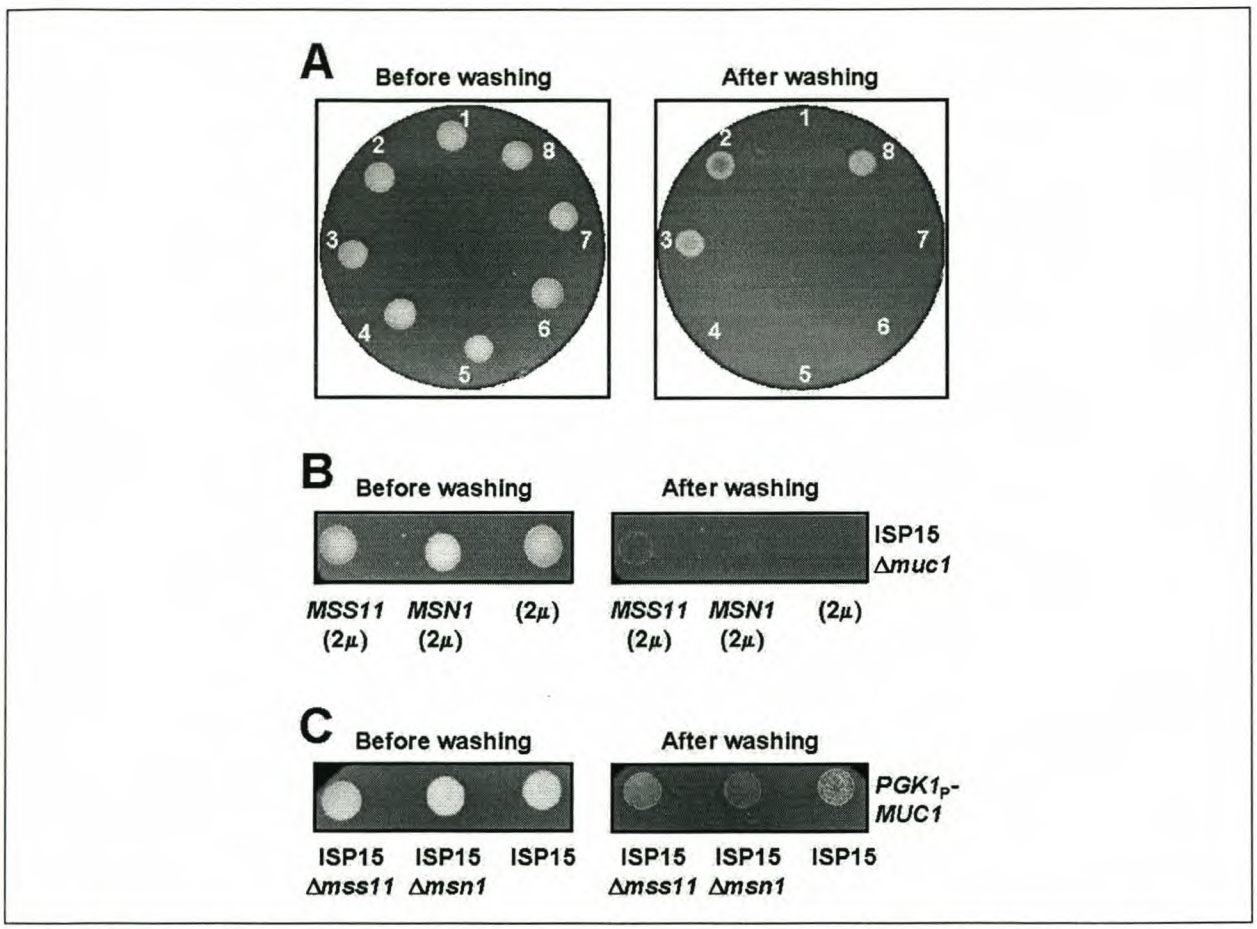


**Fig. 2.** Epistasis analysis of *MSN1*, *MSS11* and *MUC1*. Photographs show strains spotted on SLAD medium as described in Experimental Procedures. Surface growth was washed off after incubating the plates for 3 days at 30°C. A. Relation between *MSN1* and *MSS11*. The strains spotted are: (1) ISP15 transformed with YEpLac112; (2) ISP15 with YEpLac112-MSN1; (3) ISP15 with YEpLac112-MSS11; (4) ISP15Δ*msn1* with YEpLac112; (5) ISP15Δ*mss11* with YEpLac112; (6) ISP15Δ*msn1*Δ*mss11* with YEpLac112; (7) ISP15Δ*mss11* with YEpLac112-MSN1 and (8) ISP15Δ*msn1* with YEpLac112-MSS11. B. Effect of multiple copies of *MSN1* and *MSS11* on a *MUC1* deletion strain. ISP15Δ*muc1* was transformed with YEpLac112-MSS11, YEpLac112-MSN1 and, as control, YepLac112 without any insert. Strains were spotted onto SLAD (limited nitrogen) plates and surface growth washed off after 6 days. Only multiple copies of *MSS11* allow for a partial restoration of invasive growth, which is less efficient than in the wild-type strain (see Fig. 1). C. Effect of *MUC1* overexpression on invasive growth in strains with deleted *MSN1* and *MSS11* loci. Strains ISP15, ISP15Δ*msn1* and ISP15Δ*mss11* were transformed with the *MUC1* overexpression plasmid, YEpLac112-PGK1$_p$-MUC1 and incubated on SLAD medium for 4 days.

To assess the relation of Msn1p, Mss11p and Muc1p, further epistasis studies were carried out using strains with either deletions of *MUC1* (Fig. 2B) or carrying a plasmid with the *MUC1* gene fused to the constitutive *PGK1* promoter (Fig. 2C). Multiple copies of *MSN1* were unable to overcome the effect of a deletion in *MUC1*

since no invasive growth could be observed in this case, even after prolonged periods of incubation (Fig. 2B). However, strains carrying multiple copies of *MSS11* were able to grow invasively, but with reduced efficiency. In the opposite situation (Fig. 2C), strains with disrupted *MSN1* or *MSS11* loci were able to grow invasively when *MUC1* was expressed under the control of the *PGK1* promoter. This suggests that both Msn1p and Mss11p act above Muc1p in a linear signal transduction pathway that establishes the invasive growth phenotype. It is also evident that Mss11p does not function through Muc1p alone, since multiple copies of *MSS11* were still able to induce invasive growth in strains with deleted *MUC1* loci.

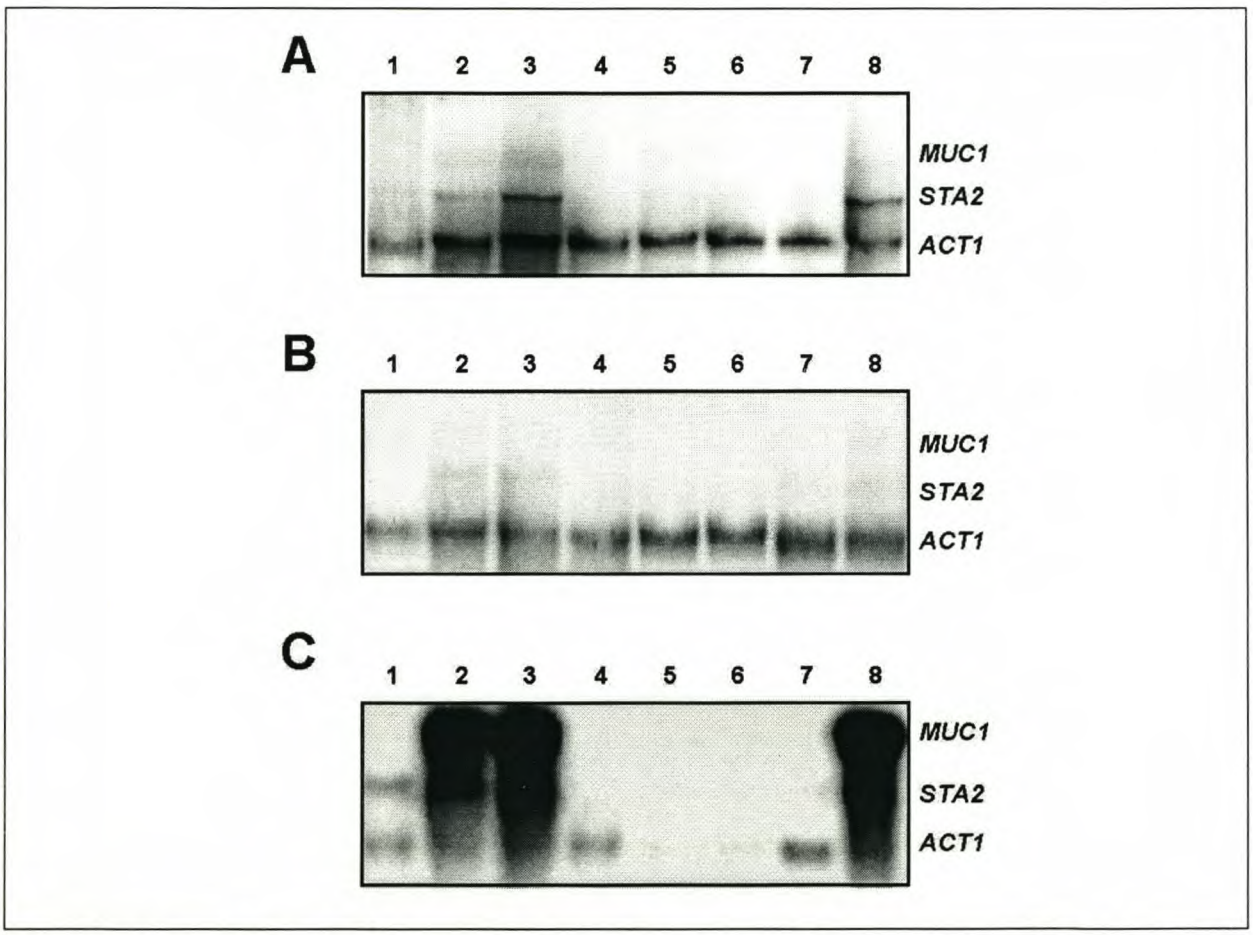


**Fig. 3.** Northern blot analysis of the different ISP15 strains on different carbon sources. Each lane contains 10 μg of total RNA from the different strains in the following order: (1) ISP15 transformed with YEpLac112; (2) ISP15 with YEpLac112-MSN1; (3) ISP15 with YEpLac112-MSS11; (4) ISP15Δ*msn1* with YEpLac112; (5) ISP15Δ*mss11* with YEpLac112; (6) ISP15Δ*msn1*Δ*mss11* with YEpLac112; (7) ISP15Δ*mss11* with YEpLac112-MSN1 and (8) ISP15Δ*msn1* with YEpLac112-MSS11. A. Northern blot analysis of *STA2* and *MUC1* expression in liquid SLAD medium. B. Northern blot analysis of *STA2* and *MUC1* expression in liquid SCD medium. C. Northern blot analysis of *STA2* and *MUC1* expression in liquid SCS medium.

### 3.2.3.3 Mss11p, like Msn1p, enhances transcription of *MUC1*

Fig. 3 presents the effect of deleted or multiple copies of *MSN1* and *MSS11* on the transcription levels of *MUC1* and *STA2* in different strains and growth media. If compared to transcript levels of wild-type ISP15 (lane 1) grown on nitrogen limited

media (SLAD) (Fig. 3A), SCD (Fig. 3B) or media containing starch as carbon source (SCS) (Fig. 3C), multiple copies of either *MSN1* or *MSS11* lead to enhanced levels of both *STA2* and *MUC1* mRNA. In SLAD and SCD media, which both contain glucose as carbon source, transcript levels of *MUC1* as well as *STA2* are significantly reduced in all strains when compared to media with starch or glycerol/ethanol (data not shown) as carbon sources. Since the promoter areas of *MUC1* and *STA2* are to a large extent homologous (Lambrechts *et al.*, 1996a,b) and since the transcription of *STA2* was shown to be subject to glucose repression (Pretorius *et al.*, 1986b), this phenomenon is probably the result of glucose repression on the transcription of *STA2* and *MUC1*. Strains in which *MSN1*, *MSS11* or both *MSN1* and *MSS11* were deleted, showed a dramatic reduction in transcript levels of both *MUC1* and *STA2* mRNA, irrespective of the carbon source used. These results, considered together with the increased mRNA levels in strains with multiple copies of *MSN1* and *MSS11*, suggest that *MSS11*, like *MSN1*, mediates the transcriptional activation of *MUC1*.

The results of the invasive growth epistasis analysis were also confirmed by the mRNA levels of *STA2* and *MUC1*. The presence of multiple copies of *MSN1* in a strain with a deleted *MSS11* locus, did result in very low levels of *STA2* or *MUC1* mRNA. In the reverse situation, however, multiple copies of *MSS11* in a strain with a disrupted *MSN1* locus resulted in very high mRNA levels of *STA2* and *MUC1*. This again confirmed that Mss11p functions downstream of Msn1p in establishing the transcriptional state of *MUC1* and *STA2* and correlates with the stronger invasive growth observed in strains carrying multiple copies of *MSS11* in a Δ*msn1* background.

### 3.2.3.4 Msn1p and Mss11p function downstream of Ras2p

To verify whether Msn1p and Mss11p act in a Ras2p-dependent pathway, we transformed ISP15Δ*msn1* and ISP15Δ*mss11* with either the hyperactivated *RAS2* allele, $RAS2^{val19}$, or with the wild-type *RAS2* allele as a control. The effect on invasive growth can be seen on nitrogen limited SLAD medium (Fig. 4A). Whereas a hyperactivated Ras2p results in an increased invasive growth response in a wild-type strain, it is unable to do so in the strain with a disrupted *MSS11* locus, even after prolonged periods of incubation on all media tested. However, the figure shows that the $RAS2^{val19}$ allele is able to weakly induce invasive growth in the Δ*msn1* strain.

These results were confirmed on media containing starch as carbon source. In all cases the strength of the invasive growth response correlated to the efficiency of starch degradation (data not shown).

### 3.2.3.5 Both Msn1p and Mss11p act downstream of Mep2p

The *MEP2* gene encodes one of several ammonium permeases and was shown to be responsible for ammonium dependent signalling (Lorenz and Heitman, 1997). This signal is, at least in part, transmitted by Ras2p. We therefore transformed the *MSN1* and *MSS11* multiple copy plasmids into strains with a *MEP2* deletion and into the

isogenic wild-type strain. When spotted onto media with glycerol/ethanol as carbon source, the Δ*mep2* and wild-type strains showed similar invasive behaviour, which in both cases was strongly amplified by the presence of *MSN1* or *MSS11* on 2μ plasmids (data not shown). On SLAD medium (Fig. 4B) where the nitrogen source, ammonium, is limiting, the wild-type strain again showed increased invasive behaviour when *MSN1* and *MSS11* were present on 2μ plasmids. The Δ*mep2* strain transformed with a control plasmid alone did not show any invasive growth on this medium, confirming the results obtained by Lorenz and Heitman (1997). The same strain transformed with either *MSN1* or *MSS11* on 2μ plasmids, regained the ability to invade, with *MSS11* being more efficient than *MSN1*. The efficiency of invasion in these strains never reached the level of the untransformed wild-type strain. These data suggest that Msn1p and Mss11p act in a pathway downstream of the Mep2p permease.

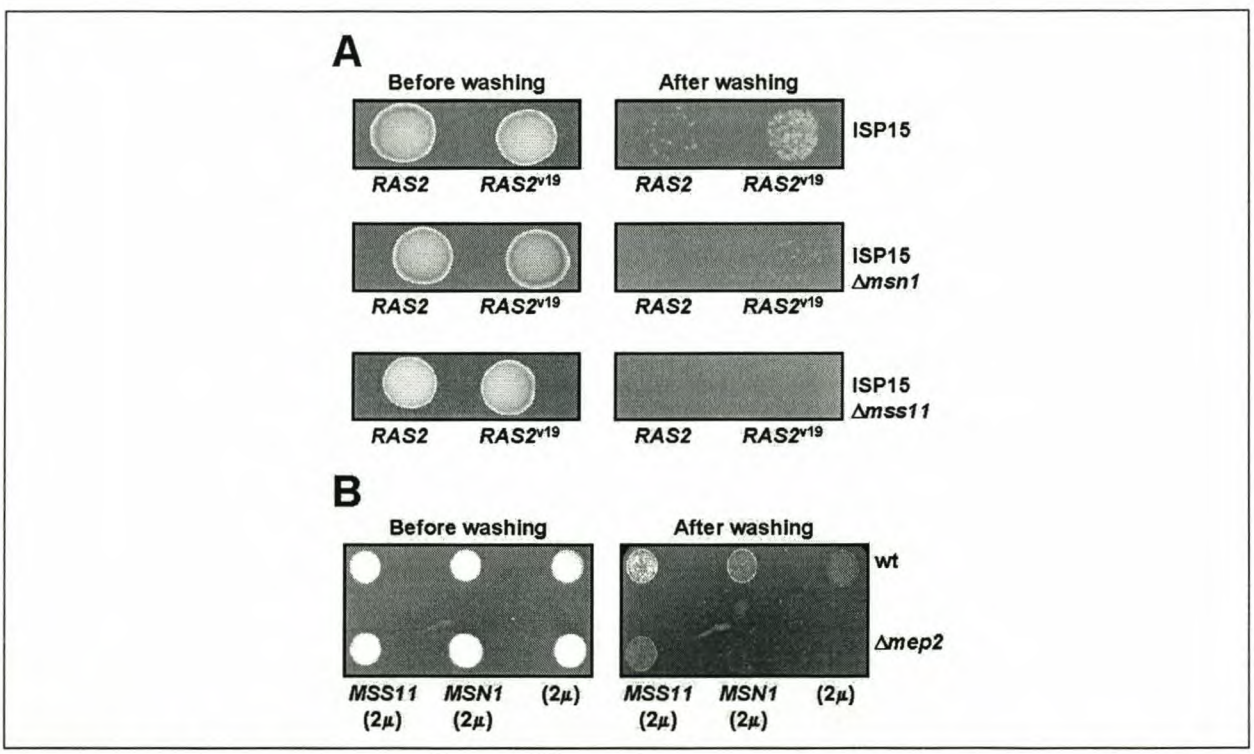


**Fig. 4.** Msn1p and Mss11p act downstream of Mep2p and Ras2p in a pathway resulting in invasive growth. A. The effect of the hyperactivated *RAS2* allele, *RAS2*$^{val19}$ on the ability to grow invasively in a strain with a disrupted *MSS11* locus. ISP15 and ISP15Δ*mss11* were transformed with YCpLac22-RAS2$^{val19}$, and, as control, YCpLac22-RAS2 bearing the wild-type RAS2 allele. Strains were spotted on nitrogen-limited SLAD plates and surface growth was washed off after 3 days. B. The effect of multiple copies of *MSN1* and *MSS11* in a Δ*mep2* strain on SLAD medium. The wild-type Σ1278 strain, 23344c, and 31021c, an isogenic strain with a disrupted *MEP2* locus, were transformed with YEpLac195-MSS11, YEpLac195-MSN1 and YEpLac195 as control. Surface growth was washed off after 3 days.

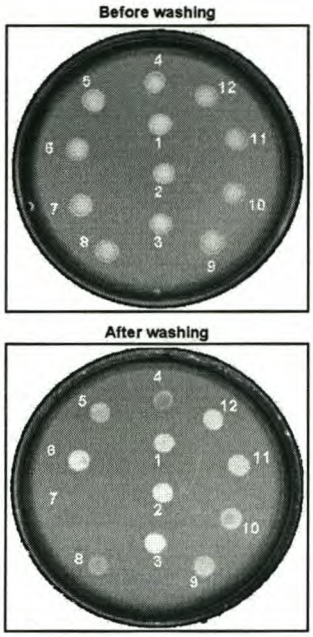


**Fig. 5.** Effect of multiple copies of *MSN1* and *MSS11* in wild-type, Δ*ste20*, Δ*ste11 and* Δ*ste7* strains on SLAD medium. The strains were transformed with YEpLac195-MSN1, YEpLac-MSS11 and YEpLac195 as control and spotted onto the plates in the following order: (1) L5366h (wt) transformed with YEpLac195; (2) L5366h (wt) with YEpLac195-MSN1; (3) L5366h (wt) transformed with YEpLac195-MSS11; (4) L5624h (Δ*ste20*) with YEpLac195; (5) L5624h (Δ*ste20*) with YEpLac195-MSN1; (6) L5624h (Δ*ste20*) with YEpLac195-MSS11; (7) L5625h (Δ*ste11*) with YEpLac195; (8) L5625h (Δ*ste11*) with YEpLac195-MSN1; (9) L5625h (Δ*ste11*) with YEpLac195-MSS11; (10) L5626h (Δ*ste7*) with YEpLac195; (11) L5626h (Δ*ste7*) with YEpLac195-MSN1 and (12) L5626h (Δ*ste7*) with YEpLac195-MSS11. Surface growth was washed off after 3 days. Multiple copies of *MSN1* and *MSS11* re-establish invasive growth in the Δ*ste20*, Δ*ste11* and Δ*ste7* strains.

### 3.2.3.6 Msn1p and Mss11p act independently or downstream of Ste20p, Ste11p and Ste7p MAP kinase cascade

To determine the epistatic relationship between the kinases Ste20p, Ste11p and Ste7p, and Msn1p and Mss11p, 2μ plasmids bearing *MSN1* or *MSS11* were transformed into Σ1278 strains in which *STE20, STE11* or *STE7* were deleted, and, as control, the isogenic wild-type strain. Results are shown in Fig. 5. The wild-type strain carrying only the vector was able to form pseudohyphae and grow invasively into the agar after short (48 hours) incubation on nitrogen limited SLAD medium. The presence of multiple copies of *MSN1* and *MSS11*, as expected, resulted in significantly improved invasive growth and strong pseudohyphae formation, similar to the results obtained with the ISP15 strain (Fig. 1). Strains with disrupted *STE20*, *STE11* or *STE7* loci transformed with the vector alone showed significantly reduced invasive growth when compared to the wild-type. This reduction was most prominent in the case of Δ*ste11*, and least pronounced in Δ*ste7*, with Δ*ste20* showing an intermediate phenotype. This confirms data obtained previously showing that both *STE11* and *STE20* are required for additional functions outside of the

pheromone/invasive growth MAP kinase cascade (Leberer *et al.*, 1997; Posas and Saito, 1997). Multiple copies of *MSN1* and *MSS11* re-established the invasive growth phenotype in all the strains to close to - or above - wild-type level. In every case, multiple copies of *MSS11* proved more efficient in overcoming the invasive growth defect than multiple copies of *MSN1*.

The results indicate that both Msn1p and Mss11p either act downstream of the MAP kinase cascade or in a pathway functioning in parallel to this cascade.

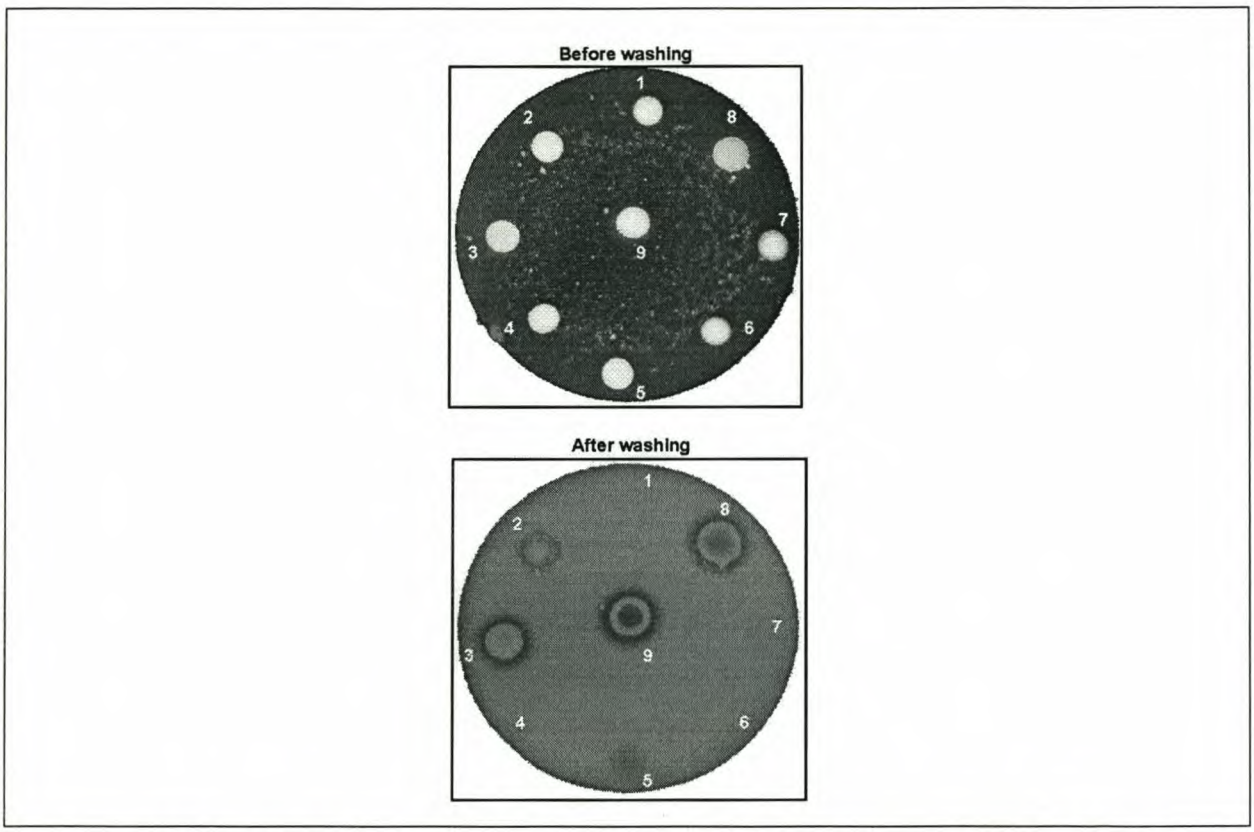


**Fig. 6.** Effect of multiple copies of *MSN1* and *MSS11* in a Δ*ste12* background. Strains were spotted onto SCS plates and incubated for 6 days. The following strains were spotted: (1) ISP15Δ*ste12* transformed with YEpLac112; (2) ISP15Δ*ste12* with YEpLac112-MSN1; (3) ISP15Δ*ste12* with YEpLac112-MSS11; (4) ISP15Δ*msn1* with YEpLac112; (5) ISP15Δ*msn1* with YEpLac112-STE12; (6) ISP15Δ*mss11* with YEpLac112; (7) ISP15Δ*mss11* with YEpLac112-STE12 (8) ISP15 with YEpLac112-STE12 and (9) ISP15 with YEpLac112. After washing off surface growth, invasive growth can be seen at the periphery of the wild-type strain (9) and below the entire colonies of (2), (3) and (8). The halos around the colonies are indicative of starch utilisation. ISP15Δ*msn1* transformed with YEpLac112-STE12 (5) shows increased starch utilisation. This correlates with increased invasive growth observed for the same strain on all other test media (data not shown).

### 3.2.3.7 Msn1p induces invasive growth independent of Ste12p whereas Mss11p functions downstream of, or in conjunction with, both Ste12p and Msn1p

A putative binding site for Ste12p was identified in the promoter of *MUC1*, suggesting that it could be the final step in the activation of a gene required for the pseudohyphal or invasive growth response (Lo and Dranginis, 1998). We therefore had to establish

whether Msn1p and Mss11p function through Ste12p or independent thereof in activating transcription of *MUC1*. *MSN1* and *MSS11* present on 2μ plasmids, were transformed into strains with a disrupted *STE12* locus to assess the effect thereof on invasive growth on media containing starch as carbon source (SCS) (Fig. 6) or nitrogen limited SLAD media (data not shown). Whereas the Δ*ste12* strain transformed with the vector alone is unable to invade the substrate, the strains transformed with either 2μ-*MSN1* or 2μ-*MSS11* regained the ability to invade the agar efficiently, indicating that both Msn1p and Mss11p function either downstream of Ste12p or independent thereof in the signalling pathway resulting in invasive growth. In the reverse experiment, a 2μ plasmid bearing *STE12* was used to transform ISP15 strains with a deletion of either *MSN1* or *MSS11*. The results (Fig. 6) indicate that multiple copies of *STE12* result in invasive growth in both the wild-type and the Δ*msn1* strain, but not in a Δ*mss11* strain. This suggests that Msn1p functions independently of Ste12p in establishing the invasive growth phenotype, whereas Mss11p either acts downstream or in combination, but not independently of Ste12p.

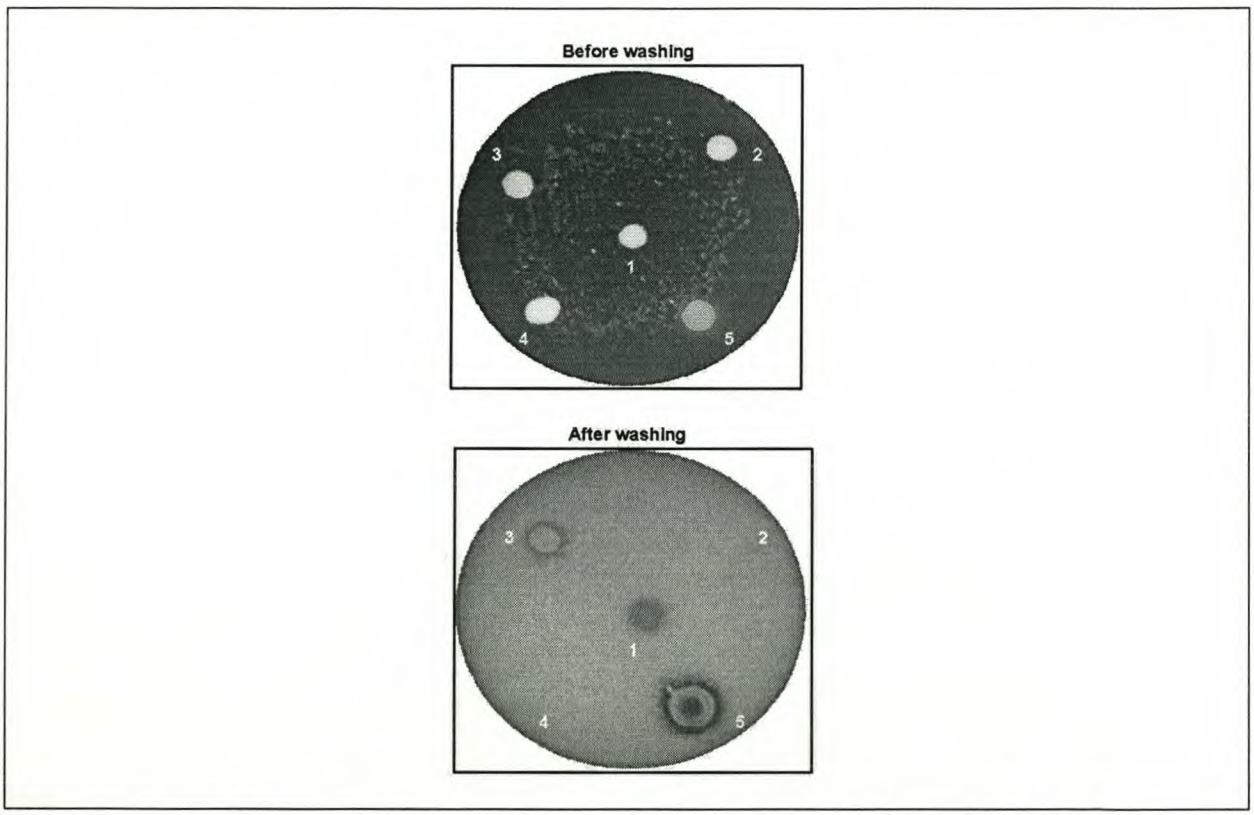


**Fig. 7.** The MAP kinase cascade activates glucoamylase activity. The following strains were spotted onto SCS medium: (1) ISP15 transformed with YEpLac112; (2) ISP15Δste7 with YEpLac112; (3) ISP15 with YEpLac112-STE7; (4) ISP15Δste12 with YEpLac112 and (5) ISP15 with YEpLac112-STE12. Surface growth was washed off after incubating for 3 days. The strains with disrupted *STE7* (2) and *STE12* (4) loci were unable to degrade starch, even after prolonged incubation periods. Strains with multiple copies of *STE7* (3) and *STE12* (5) degraded starch more efficiently with *STE12* being the most efficient.

### 3.2.3.8 Starch metabolism is regulated by the MAP kinase cascade

Some strains of *S. cerevisiae* carry any one (or more) of three genes, *STA1*, *STA2* or *STA3*, which encode extracellular glucoamylases (reviewed by Vivier *et al.*, 1997). Once secreted, glucoamylases hydrolyse starch molecules by liberating glucose molecules from the non-reducing end of the molecule, thereby making it available to the yeast cell. This enables the yeast cell to grow on starch as the sole carbon source. *MUC1* and the *STA1-3* genes have highly homologous promoter areas. Since *MUC1* was shown to be an important role-player in pseudohyphal differentiation and invasive growth, both processes under regulation of the mating pheromone/invasive growth MAP kinase cascade, the question arose whether starch metabolism is also regulated by the same cascade. In addition, a putative Ste12p binding site was identified in the upstream region of *MUC1* (Lo and Dranginis, 1998) and the same sequence is present in the promoter of *STA2* as well.

The effect of deletions in two of the MAP kinase modules, *STE7* and *STE12*, as well as the presence of multiple copies thereof, on a yeast strain's ability to degrade starch can be seen in Fig. 7. The sizes of the halo's around these colonies are indicative of the ability of these strains to degrade starch and indicate that multiple copies of both *STE7* and *STE12* result in enhanced starch utilisation. Deletion of either *STE7* or *STE12* results in a severe decrease in this phenotype, which can be overcome by multiple copies of either *MSN1* or *MSS11* (Fig. 6 and Fig. 7).

### 3.2.3.9 Effect of growth phase on invasive growth

*S. cerevisiae* L5366 was inoculated into liquid SCD medium and grown to an optical density at 600 nm ($OD_{600}$) of 1.0. From this culture, four precultures were inoculated and grown to optical densities of 0.6, 1.2, 2.0 and 3.0, respectively. From these cultures equal amounts of cells ($1.5 \times 10^5$) were taken, the volumes adjusted to 20 µl and dropped onto nitrogen limited SLAD plates. Plates were incubated for 4 days after which the plates were investigated for invasive growth. Fig. 8 clearly shows the effect that the growth phase of the precultures had on the ability of the yeast cells to grow invasively into the agar. Cells taken at later growth phases ($OD_{600}$ of 3.0) started growing invasively at a much earlier stage than those taken from the mid log cultures ($OD_{600}$ of 0.6, 1.2 and 2.0). This was repeated with strains ISP15 and L5366-h1 and the observations confirmed (data not shown). The invasive phenotype did not increase in a linear manner with corresponding increases in $OD_{600}$. Indeed, phenotypes were similar for cells taken at an $OD_{600}$ of 0.6, 1.2 and 2.0, but not for cells taken at an $OD_{600}$ of 3, suggesting a sudden switch in cell physiology occurring between mid and late log phase. The effect of multiple copies of *MSN1* or *MSS11* was, however, always clearly visible, and even strains spotted at $OD_{600}$ of 3 showed a marked increase in invasion when transformed with those plasmids (data not shown).

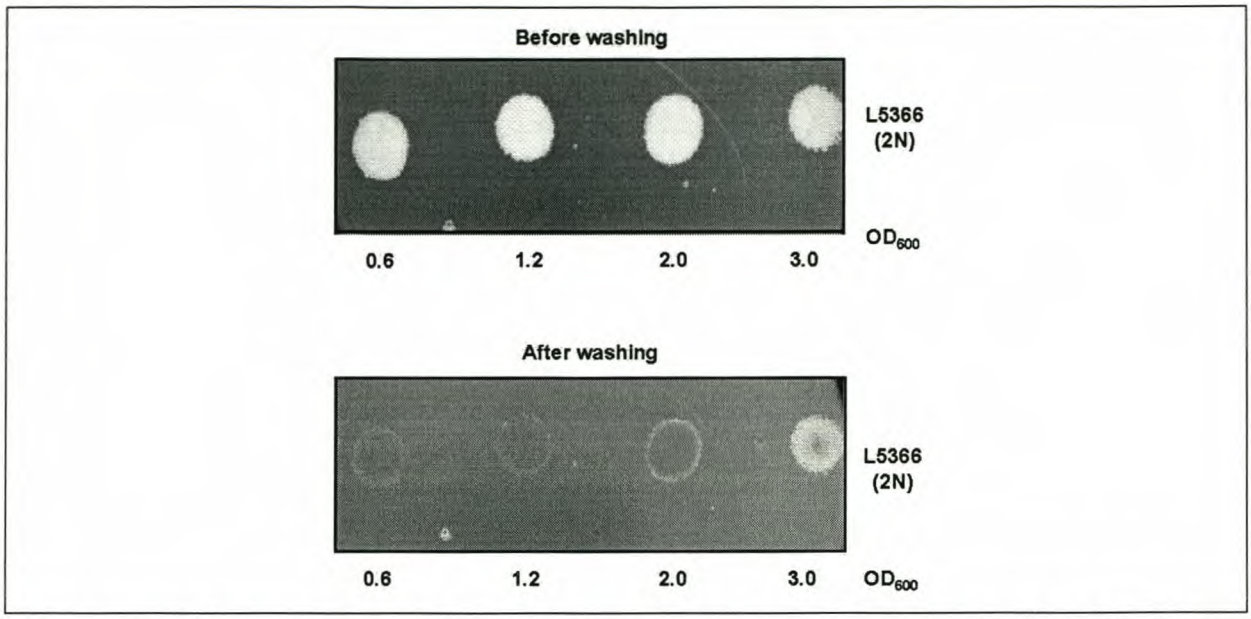


**Fig. 8.** Effect of growth phase on invasive growth. The same number of cells ($1.5 \times 10^5$ cells/ml) of the diploid Σ1278 strain, L5366, was spotted onto SLAD medium from cultures grown in rich media (YPD) to different growth phases. Surface growth was washed off after 3 days. Similar results were obtained with all other strains tested.

## 3.2.4 DISCUSSION

In this paper, we present data positioning three genes, *MSN1, MSS11* and *MUC1*, in a signal transduction pathway downstream of *MEP2* and *RAS2*. As expected for a network of signal transduction cascades, the epistasis analysis does reveal complex interactions between the different components. Our genetic data clearly suggest that Msn1p, Mss11p and Muc1p act in this hierarchical order to activate invasive growth in yeast cells. Msn1p has been suggested to act as a transcriptional activator. The position of Mss11p downstream of Msn1p could suggest that it is either itself a target of Msn1p-mediated activation, or that an interaction between the two proteins is required to allow Msn1p to exert its effects. This second hypothesis is more plausible for several reasons. First, multiple copies of *MSS11* are more efficient in inducing invasive growth in a strain deleted for *MSN1* than in a wild-type strain, and it is therefore unlikely that Msn1p is required to activate *MSS11*. Secondly, these same data suggest that a genomic copy of *MSN1* somehow attenuates the effect of *MSS11* overexpression. This would suggest a more direct interaction between Msn1p and Mss11p.

The expression of *MUC1* from a strong, constitutive promoter increases the invasiveness of yeast cells significantly. The Northern blots clearly demonstrate the strong induction of *MUC1* by multiple copies of *MSN1* and *MSS11*. The increased invasiveness of these cells is therefore, at least in part, due to the transcriptional activation of *MUC1*. This is further confirmed by a very strong reduction of invasive growth in Δ*muc1* strains. *MUC1*, however, is not the only target gene of Mss11p, since deletion thereof still allows Mss11p to re-establish invasive growth in a *MUC1*

deletion strain, although at a significantly reduced level. The Northern blot data correlate well with the observed phenotypes described above. Indeed, the effect of *MSS11* overexpression on *MUC1* and *STA1-3* transcription is significantly stronger in strains with disrupted *MSN1* loci than in a wild-type strain. In all cases, the level of *MUC1* transcription reflects the strength of the invasive growth observed.

Our data suggest that Msn1p and Mss11p act downstream or in parallel with the MAP kinase cascade. Both genes overcome deletions in *STE7, STE11* and *STE20*. Multiple copies of both *MSN1* and *MSS11* are, however, less efficient in overcoming the invasive growth defect of a Δ*ste20* and a Δ*ste11* strain than of a Δ*ste7* strain. This is in accordance with previous reports indicating that Ste20p has functions independent of the MAP kinase cascade in establishing the invasive growth phenotype (Leberer *et al.*, 1992), and that Ste11p functions independently in several other signal transduction events.

Our data suggest that Msn1p acts independently of Ste12p in a parallel pathway, since overexpression of any of the two proteins partially overcomes the deletion of the other. Mss11p, however, functions downstream of, or in conjunction with, Ste12p. Indeed, multiple copies of *MSS11* still result in increased invasive growth in strains with deletions of *STE12*, whereas the overexpression of *STE12* is unable to overcome the effects of a deletion of the *MSS11* locus. In all cross-complementation experiments involving *MSN1* (disruptions in *MSN1* complemented by multiple copies of *STE12* or disruptions in genes encoding MAP kinase cascade elements or *STE12* complemented by multiple copies of *MSN1*), the invasive phenotypes observed were reduced compared to those induced by multiple copies of *MSN1* or multiple copies of *STE12* in a wild-type background. This reduction of the ability to invade again suggests that the MAP kinase cascade and Msn1p act in parallel pathways with additive effects on invasiveness. Mss11p seems to be situated at the confluence of two signalling pathways, one depending on the invasive growth MAP kinase cascade, the other signalling via Msn1p.

The *RAS2* gene has already been shown to act via at least two different signal transduction pathways, one of which is MAPK-dependent (Lorenz and Heitman, 1998; Mösch *et al.*, 1996). Disruption of *MSS11* completely eliminates invasive growth in strains carrying a plasmid encoding the hyperactivated form of Ras2p, $Ras2^{val19}p$. This clearly places Mss11p downstream of the Ras2p signal. Our data furthermore suggest that the transmission of the signal via Msn1p is under the control of the *RAS2*-dependent, but MAP kinase-independent pathway, since the *RAS2*-dependent signal is partially blocked by a deletion of *MSN1*. We are currently investigating the relation of Msn1p and Mss11p with Ash1p, a DNA-binding protein that was shown to act as an activator of pseudohyphal growth and has similar epistatic relations with *RAS2* and the pheromone-associated MAP kinase cascade. Interestingly, the *MUC1* gene is activated by both Ras2p-dependent pathways, since we observe a strong induction of the *STA1-3* and *MUC1* genes in a strain carrying *STE12* on a 2μ plasmid. *MUC1* could therefore be the first common target of the two

*RAS2*-dependent signal transduction pathways. This suggests that Muc1p plays a role in different events requiring cell-cell or cell-substrate adhesion, both during mating and pseudohyphal differentiation.

Our data suggest that Msn1p and Mss11p act downstream of the ammonium specific permease Mep2p which specifically signals ammonium limitation. The ability to invade the agar of Δ*mep2* strains carrying multiple copies of *MSN1* or *MSS11* is restored, but at a significantly weaker level than in any other of the investigated genetic backgrounds. This reinforces the idea that Msn1p and Mss11p are situated downstream of Mep2p. Indeed, of all the strains used, the Δ*mep2* strain is the only one where the nutritional signal itself is absent. All other mutants used for the epistasis analysis are affected in one of several parallel signal transduction pathways. In those mutants, the signal itself will still be perceived and transmitted via non-affected parallel pathways, if perhaps with reduced efficiency. The fact that multiple copies of *MSN1* and *MSS11* are able to re-establish invasive growth in a *MEP2* deletion strain at very reduced levels indicates that their activity is partly dependent on the presence of the signal itself. This suggests that these proteins do not only amplify the signal simply through stoichiometrical effects, as might be suggested by the effects of the overexpression, but that some type of signal-dependent modification has to take place in order for them to function efficiently. This signal is specifically Mep2 dependent in ammonium limited conditions. The data obtained from our epistasis analysis suggest a model that is summarised in Fig. 9.

The effect of multiple copies of *MSN1* and *MSS11* or the deletion of genomic copies thereof on transcription of *MUC1* and *STA1-3* suggests that both genes encode either transcriptional activators or proteins that directly affect transcription factors. Both genes were shown to induce the transcription of *MUC1* as well as the *STA1-3* genes and in all cases deletions or overexpression had similar effects on invasive growth and starch utilisation. This co-regulation of invasive growth and starch metabolism was also confirmed through the deletion or overexpression of genes encoding components of the invasive growth/pheromone response MAP kinase cascade. The ability to degrade starch through activation of the *STA1-3* genes is therefore an excellent reporter system for invasive growth in strains bearing these genes.

The exact role of *MSS11* is not yet understood. The data presented here suggest that Mss11p could be specifically required in the establishment of the invasive and pseudohyphal growth phenotypes in response to a signal emanating from Ras2p. Data presented elsewhere (Gagiano *et al.*, submitted), show that the effect of *MSS11* overexpression on *MUC1* transcription can be pinpointed to a specific area within the *MUC1* promoter. In addition, the sequence homologies of Mss11p with Flo8p and other transcription factors strongly suggest that Mss11p itself could be a transcription factor. The presence of a ATP- or GTP binding-loop within the protein sequence gives an indication on the possible regulation of this factor. We are currently

investigating whether Mss11p is binding ATP or GTP and which proteins might be directly involved in this regulation. In addition, we are establishing the interactions of this protein and of Msn1p, with some of the other transcription factors involved in pseudohyphal differentiation like Phd1p, Ste12p and Ash1p. We suggest that Mss11p mediates transcriptional activation specifically of genes required for pseudohyphal and invasive response.

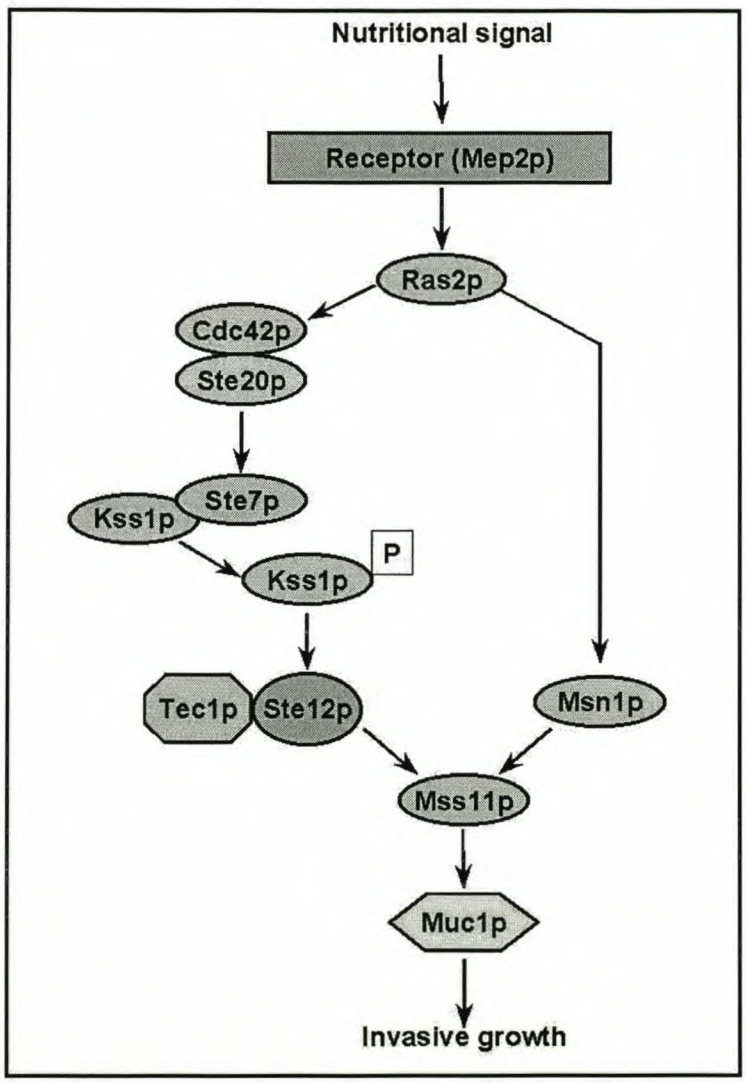


**Fig. 9.** Proposed model for the positions of Msn1p, Mss11p and Muc1p in the signal transduction pathways resulting in invasive growth.

The role for *MUC1* in mediating invasive growth is unclear. Overexpression results in increased invasive growth phenotypes whereas deletion thereof diminishes strongly the invasive growth phenotype. Based on the structure of Muc1p, which resembles the mammalian mucins (Lambrechts *et al.*, 1996a) and yeast flocculins (Lo and Dranginis, 1998), an adhesion function can be suggested for Muc1p. Whether this involves only cell-cell adhesion or cell-substrate adhesion remains to be

verified. Adhesion to a specific substrate was shown to be a prerequisite for invasion by *Candida albicans*, since elimination of the ability to adhere to a substrate also eliminated the ability to invade that substrate (Gale *et al.*, 1998).

Several of our results point towards a more complex picture of nutrient-dependent signal transduction leading to invasive and pseudohyphal growth than the model developed here and elsewhere, which is based on the combined action of several linear signalling pathways resulting in pseudohyphal differentiation. First, phenotypes are in general more complex than the simplified description of increases or reductions in invasive or pseudohyphal growth might suggest. These observations are consistent and reproducible, but they hide a multitude of aspects that characterise specifically each of the mutants used in the epistasis analysis. For example, invasiveness might be a generalised feature of a colony or only occur in some areas below it, pseudohyphae might be formed by cells of different morphological appearance in different genetic backgrounds, as well as many other aspects not analysed in detail during this study.

Secondly, an important factor for the efficiency of the invasiveness of all the strains proved to be the growth phase (not cell concentration) at which strains were spotted from the liquid preculture onto the test plate. When cells of the same strain were sampled at different growth phases and spotted at adjusted cell densities, they would show markedly different invasion efficiencies, a higher $OD_{600}$ resulting in a more invasive phenotype. This behaviour could be accounted for by a difference in transcription patterns between early, mid and late log phase cells. The latter might have induced genes in response to limited nutrients, including some of the genes responsible for invasion, prior to being spotted onto the plates. However, more interestingly, the change in $OD_{600}$ did not only result in a difference in invasive efficiency, but reproducible differences were observed with regard to the behaviour of different strains. The results of epistasis analysis could indeed be different according to the growth phase of the cells used for plating. This might explain some of the differences seen between papers published in the past by different groups. However, the effect of multiple copies of *MSN1* and *MSS11* were not affected by the growth phase of the culture.

Further considerations concern the genetic background of the strains used in epistasis analysis. In this work, the effects of mutations and overexpression were verified in several strains with different genetic backgrounds. This includes the strain that has been used as the reference strain for most pseudohyphal research work, Σ1278, FY23 (S288C) and yeast strains constructed in our laboratory i.e. ISP15 and ISP20. In general, results obtained in one of the strains were always reproducible in all the others. However, during epistasis analysis, clear differences in the intensity of responses in the different strains where observed. For example, the increase in invasiveness after transformation with multiple copy plasmids containing *MSN1* or *MSS11* was significant in all strains investigated. However, the relative strength of the invasion varied. In some strains (ISP15, ISP20) the efficiency of invasion was

increased similarly by multiple copy plasmids carrying either *MSN1* or *MSS11*, whereas in other strains (Σ1278, FY23), *MSS11* was significantly more efficient than *MSN1*.

Finally, our results were always verified for several types of either nitrogen or carbon limitation. Again, we found that, as a rule, a result obtained on one medium could be reproduced on another. However, as for the different genetic backgrounds, significant differences in the relative strength of the invasive response emerged. Some of the mutants responded stronger in one medium rather than in another. We are conducting experiments to verify whether this specificity can be linked to specific genes.

## 3.2.5 EXPERIMENTAL PROCEDURES

### 3.2.5.1 Yeast strains and culture conditions

Yeast strains used in these experiments are listed in Table 1. All strains were grown at 30°C in standard yeast media, prepared according to Sherman *et al.* (1991). Standard protocols were employed in the transformation of yeast strains (Ausubel *et al.,* 1994). Selective media contained 0.67% yeast nitrogen base, the specific amino acids required by each strain, as well as 2% glucose for SCD, 2% starch for SCS or 3% glycerol and 2% ethanol for SCGE. Agar was added to a final concentration of 2% for all plates. SLAD media, which contains 50 μM of ammonium sulphate as sole nitrogen source, were prepared as described by Lorenz and Heitman (1997).

### 3.2.5.2 Yeast strain construction

*S. cerevisiae* strains, ISP15 and ISP20, both exhibiting the abilities to utilise starch as a carbon source, form pseudohyphae and grow invasively into the agar, were used for strain constructions. Yeast strains of the Σ1278 genetic background for which the pseudohyphal and invasive phenotypes are well established, were used as control strains. FY23, a standard S288C laboratory strain (Winston *et al.,* 1995) which cannot form pseudohyphae or grow invasively due to a naturally occurring mutation in the *FLO8* gene, was transformed with the wild-type *FLO8* gene on centromeric plasmids, YCpLac22-FLO8 or pF415-1, and also used as a control strain for the pseudohyphal and invasive growth phenotypes. To create a wild-type haploid Σ1278 strain, L5366 was sporulated and 15 tetrads analysed. A single haploid strain, L5366-h1, was selected and used for these experiments.

An existing Δ*mss11*::*LEU2* disruption cassette (Webber *et al.,* 1997) was used to disrupt the *MSS11* open reading frame (ORF) in strains ISP20 and FY23 by means of homologous recombination and integration (Ausubel *et al.,* 1994). Disruptions were verified by the polymerase chain reaction (PCR) and Southern blots. The Δ*ste7*::*LEU2* and Δ*ste12*::*URA3* disruption cassettes constructed for this work, pΔste7 and pΔste12, were used to disrupt the *STE7* and *STE12* loci in strains FY23, ISP15 and ISP20. *STE7* and *STE12* disruptions were verified by Southern blot

analysis and the inability of successfully disrupted strains to mate with strains of opposing mating type (data not shown).

**Table 1.** *S. cerevisiae* strains used in this study.

| Strain | Relevant genotype | Source or reference |
|---|---|---|
| ISP15 | *MATa his3 leu2 thr1 trp1 ura3 STA2* | Lambrechts *et al.*, 1996a |
| ISP15Δ*muc1* | *MATa his3 leu2 thr1 trp1 STA2 Δmuc1::URA3* | Lambrechts *et al.*, 1996a |
| ISP15Δ*msn1* | *MATa his3 leu2 thr1 trp1 STA2 Δmsn1::URA3* | Lambrechts *et al.*, 1996b |
| ISP15Δ*mss11* | *MATa his3 thr1 trp1 ura3 STA2 Δmss11::LEU2* | Webber *et al.*, 1997 |
| ISP15Δ*msn1*Δ*mss11* | *MATa his3 thr1 trp1 STA2 Δmsn1::URA3 Δmss11::LEU2* | Webber *et al.*, 1997 |
| ISP15Δ*ste7* | *MATa his3 leu2 thr1 trp1 STA2 Δste7::LEU2* | This study |
| ISP15Δ*ste12* | *MATa his3 leu2 thr1 trp1 STA2 Δste12::URA3* | This study |
| ISP20 | *MATa leu2 thr1 trp1 ura3 STA2* | This laboratory |
| ISP20Δ*mss11* | *MATa thr1 trp1 ura3 STA2 Δmss11::LEU2* | This study |
| ISP20Δ*ste7* | *MATa thr1 trp1 ura3 STA2 Δste7::LEU2* | This study |
| ISP20Δ*ste12* | *MATa leu2 thr1 trp1 STA2 Δste12::URA3* | This study |
| FY23 | *MATa leu2 trp1 ura3* | Winston *et al.*, 1995 |
| FY23Δ*mss11* | *MATa trp1 ura3 Δmss11::LEU2* | This study |
| FY23Δ*ste7* | *MATa trp1 ura3 Δste7::LEU2* | This study |
| FY23Δ*ste12* | *MATa leu2 trp1 Δste12::URA3* | This study |
| L5366 | *MATa/MATα ura3/ura3* | Liu *et al.*, 1993 |
| HLY492 | *MATa/MATα ura3/ura3 ste20::TRP1/ste20::TRP1* | Liu *et al.*, 1993 |
| L5366h | *MATa ura3* | Radcliffe *et al.*, 1997 |
| L5624h | *ura3 Δste20* | Radcliffe *et al.*, 1997 |
| L5625h | *ura3 Δste11* | Radcliffe *et al.*, 1997 |
| L5626h | *ura3 Δste7* | Radcliffe *et al.*, 1997 |
| L5366-h1 | *MATα ura3* | This study |
| L5981 | *MATα his3 leu2 ura3 ste20::TRP1* | Mösch *et al.*, 1996 |
| 23344c | *MATα ura3* | Marini *et al.*, 1997 |
| 31021c | *MATα ura3 mep1 mep2* | Marini *et al.*, 1997 |

### 3.2.5.3 Plasmid construction and recombinant DNA methods

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 the enzymatic manipulation of DNA were obtained from Boehringer-Mannheim (Randburg, South Africa) and used according to the specifications of the supplier. *Escherichia coli* DH5α (GIBCO-BRL/Life Technologies) was used as host for the construction and propagation of all plasmids.

All plasmids used in or constructed for this study are listed in Table 2. A 1675 bp *Xho*I-*Sna*BI fragment containing *MSN1* was obtained from the plasmid pMS2A (Lambrechts *et al.*, 1996b) and cloned into the unique *Sal*I and *Sma*I sites of plasmids YEpLac112 and YEpLac195 (Gietz and Sugino, 1988) to generate

YEpLac112-MSN1 and YEpLac195-MSN1. A 3326 bp *Eco*RI fragment containing *MSS11* was derived from the plasmid pMSS11-g (Webber *et al.,* 1997) and cloned into the unique *Eco*RI site of plasmids YEpLac112 and YEpLac195 to generate plasmids YEpLac112-MSS11 and YEpLac195-MSS11. *STE12* was obtained as a 2889 bp *Sac*I-*Nar*I fragment from plasmid YCp12-3 (Pi *et al.*, 1997) and cloned into the unique *Sac*I and *Nar*I sites of plasmid YEpLac112 to generate plasmid YEpLac112-STE12. A 2094 bp *Hin*dIII fragment containing *STE7* was obtained from plasmid STE7-1 (Chaleff and Tatchell, 1985) and cloned into the unique *Hin*dIII site of plasmid YEpLac112 to generate plasmid YEpLac112-STE7. *RAS2* and the mutant allele, *RAS2*$^{val19}$, were obtained as 1637 bp *Stu*I-*Hin*dIII fragments from pRAS2 and pRAS2$^{val19}$ respectively and cloned into the unique *Sma*I and *Hin*dIII sites of plasmid YCpLac22 (Gietz and Sugino, 1988) to generate YCpLac22-RAS2 and YCpLac22-RAS2$^{val19}$. FLO8 was obtained as a 3252 bp *Sph*I-*Eco*RV fragment from plasmid pF415-1 (Kobayashi *et al.*, 1996) and cloned into the unique *Sma*I and *Sph*I sites of plasmid YCpLac22 (Gietz and Sugino, 1988) to generate plasmid YCpLac22-FLO8.

A 1129 bp *Bal*I-*Bln*I fragment was deleted from plasmid YEpLac112-STE7, removing most of the *STE7* ORF. A 1680 bp *Sma*I-*Nhe*I fragment containing the entire *LEU2* gene, obtained from YDp-L (Berben *et al.*, 1991), was subsequently inserted, resulting in plasmid pΔste7. A *STE12* disruption construct was created by deleting a 647 bp *Mlu*I-*Xba*I fragment from plasmid YCp12-3, removing the translational start site (ATG) and a large part of the ORF in the process. A 1175 bp fragment containing the *URA3* gene from plasmid YDp-U (Berben *et al.*, 1991) was inserted to generate pΔste12.

To create a plasmid for overexpressing *MUC1* in the different yeast strains, a 1872 bp *Hin*dIII fragment containing the *PGK1* promoter and terminator was obtained from plasmid pHVX2 (Volschenk *et al.*, 1997) and inserted into the unique *Hin*dIII site of plasmid YEpLac112. A 4101 bp *Eco*RI fragment containing the entire *MUC1* ORF was then obtained from plasmid pADMU (Lambrechts *et al.*, 1996a) and subsequently inserted into the *Eco*RI site between the *PGK1* promoter and terminator, resulting in plasmid YEpLac112-PGK1$_P$-MUC1.

### 3.2.5.4 Invasive growth and pseudohyphal development assays

Yeast strains were transformed with plasmids bearing *MSN1*, *MSS11*, *STE7*, *STE12* and *RAS2*$^{val19}$, as well as all the control plasmids and plated onto selective plates. Three colonies from each transformation were inoculated into SCD and grown to an $OD_{600}$ of 1.0. To assess the ability of these yeast strains to grow invasively into the agar, 10 μl of this liquid culture suspension was dropped onto SLAD, SCS, SCGE and SCD plates. Plates were incubated at 30°C and investigated for invasive growth at intervals of 2 days. Yeast colonies were washed off the surface of the agar by rubbing the surface of the plates with a gloved finger under running water. Cells that invaded the agar cannot be washed off and are clearly seen below the surface of the

agar. Plates were photographed both before and after the washing process. After washing off the cells, each of the colonies were investigated for elongated cells or filaments under the 10X magnification of a light microscope (Nikon Optiphot-2) and photographs of cells below the agar surface taken with a Matrox Intellicam 2 (Matrox Electronics Inc.).

**Table 2.** Plasmids used in this study.

| Plasmid | Relevant genotype | Source or Reference |
|---|---|---|
| YEpLac112 | 2μ *TRP1* | Gietz and Sugino, 1988 |
| YEpLac195 | 2μ *URA3* | Gietz and Sugino, 1988 |
| YCpLac22 | *CEN4 TRP1* | Gietz and Sugino, 1988 |
| YDp-L | *LEU2* | Berben *et al.*, 1991 |
| YDp-U | *URA3* | Berben *et al.*, 1991 |
| pHVX2 | 2μ *LEU2 PGK1*$_P$ *PGK1*$_T$ | Volschenk *et al.*, 1997 |
| YCp12-3 | *CEN4 STE12* | Pi *et al.*, 1997 |
| STE7-1 | 2μ *URA3 STE7* | Chaleff and Tatchell, 1985 |
| pMSS11-g | 2μ *LEU2 MSS11* | Webber *et al.*, 1997 |
| pADMU | 2μ *LEU2 ADH1*$_P$ *MUC1 ADH1*$_T$ | Lambrechts *et al.*, 1996a |
| pMS2A | 2μ *URA3 MSN1* | Lambrechts *et al.*, 1996b |
| pF415-1 | *CEN6 LEU2 FLO8* | Kobayashi *et al.*, 1996 |
| pRAS2 | *CEN4 URA3 RAS2* | M. Vanoni |
| pRAS2$^{val19}$ | *CEN4 URA3 RAS2*$^{val19}$ | M. Vanoni |
| YEpLac112-MSN1 | 2μ *TRP1 MSN1* | This work |
| YEpLac195-MSN1 | 2μ *URA3 MSN1* | This work |
| YEpLac112-MSS11 | 2μ *TRP1 MSS11* | This work |
| YEpLac195-MSS11 | 2μ *URA3 MSS11* | This work |
| YEpLac112-PGK1$_p$-MUC1 | 2μ *TRP1 PGK1*$_P$ *MUC1 PGK*$_T$ | This work |
| YEpLac112-STE7 | 2μ *TRP1 STE7* | This work |
| YEpLac112-STE12 | 2μ *TRP1 STE12* | This work |
| YCpLac22-RAS2 | 2μ *TRP1 RAS2* | This work |
| YCpLac22-RAS2$^{val19}$ | 2μ *TRP1 RAS2*$^{val19}$ | This work |
| YCpLac22-FLO8 | *CEN4 TRP1 FLO8* | This work |
| pMSS11-Δ | Δ*mss11*::*LEU2* | Webber *et al.*, 1997 |
| pΔste7 | Δ*ste7*::*LEU2* | This work |
| pΔste12 | Δ*ste12*::*URA3* | This work |

### 3.2.5.5 Plate assays to determine starch utilisation

The *STA2* gene encodes an extracellular glucoamylase which hydrolyses starch by liberating glucose molecules from the non-reducing end of the starch molecule (Vivier *et al.*, 1997). The presence of the *STA2* gene therefore enables most yeast strains to grow on starch as the sole carbon source. On plates containing starch (SCS), a clear zone is formed around such starch-degrading colonies and the diameter of the zone

is indicative of the amount of glucoamylase secreted (Pretorius *et al.*, 1986a; Yamashita *et al.*, 1985). The expression of *STA2* in the different yeast strains, transformed with the plasmids bearing *MSN1*, *MSS11*, *STE7*, *STE12* and $RAS2^{val19}$, as well as all control plasmids, were therefore determined by the size of the clear zone around each of the colonies on the SCS plates.

### 3.2.5.6 RNA isolation and Northern blot analysis

Using standard protocols (Ausubel *et al.*, 1994), total RNA was isolated from the wild-type ISP15 strain or ISP15 strains of which *MSN1*, *MSS11* or both were deleted. RNA preparations were also obtained from different ISP15 strains transformed with 2μ plasmids bearing copies of either *MSN1* or *MSS11*. Cultures were inoculated from an overnight culture and grown to an $OD_{600}$ of 1.0 in selective SCD, SLAD, SCS and SCGE media. For electrophoresis analysis of the samples, 10 μg of each RNA preparation was subjected to electrophoresis on a formamide gel. The RNA was transferred to MSI Magnacharge membranes and Northern blotting performed according to standard procedures (Ausubel *et al.*, 1994). A 777 bp *Xho*I-*Bst*EII fragment, unique to the *MUC1* ORF, was used to probe for *MUC1* transcripts whereas a *Ball*-*Sal*I fragment from the ORF of *STA2* was used as a probe for *STA2* transcripts. *ACT1* was used as internal control and a 563 bp *Cla*I fragment was used to probe for *ACT1* transcripts. All probes were radioactively labelled with $P^{32}$ dATP using the Prime-It II random primer labelling kit (Stratagene).

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## 3.3 AUTHOR'S CONTRIBUTION TO PUBLISHED ARTICLES

Two genes, *MSS10* and *MSS11*, were identified in our laboratory as multiple copy suppressors of *STA10*, which had been described previously as the repressive effect on the transcription of the *STA1-3* genes in most laboratory strains (Pretorius *et al.* 1986, Lambrechts *et al.* 1996a, Webber *et al.* 1997). When present in multiple copies in strains carrying glucoamylase-encoding genes, both *MSS10* and *MSS11* induce starch degradation. While the regulatory roles of the *MSS10* and *MSS11* gene products were being assessed, a third gene, *MUC1*, was also investigated, since a large portion of the promoter region of this gene exhibits a high degree of nucleotide sequence homology with the promoter of the *STA2* gene. *MUC1*, encoding a protein with sequence homology to mammalian membrane-bound mucins, was subsequently shown to play an important role in invasive growth and pseudohyphal differentiation (Lambrechts *et al.*, 1996b). Overexpression of Mss10p and Mss11p was found to enhance the invasive growth phenotype and to result in pseudohyphal differentiation. Other groups identified numerous additional genes, encoding either elements of signal transduction modules or transcriptional regulators, which are required for this cellular differentiation process, but no link between these genes and those isolated in our laboratory was established. The project therefore focused on establishing the functional relationships between the genes identified in our laboratory and some of the other factors involved in or required for invasive growth.

The first aim of this study was to investigate the effects on invasive/pseudohyphal growth and starch degradation of the overexpression and the deletion of *MSS10*/*MSN1*, *MSS11* and *MUC1*/*FLO11* and to establish the epistatic relationship between these factors themselves as well as their relation with the filamentation MAPK pathway. In this regard, various mutant-strains with different genetic backgrounds were created and are listed in **Table 3.1** (Gagiano *et al*, 1999a). These mutants were subsequently transformed with 2μ-based plasmids carrying copies of *MSN1*, *MSS11* and *MUC1*, respectively. Furthermore, the strains deleted for *MSN1*, *MSS11* and a combination thereof were transformed with hyper-activated $RAS2^{val19}$, as well as with multiple copies of *STE7* and *STE12*. The results obtained are presented in Gagiano *et al.* (1999a). During the course of the investigation, interesting functional relationships were observed between Msn1p, Mss11p and Ste12p (see unpublished results).

Additional genes were incorporated in the genetic analysis, since other factors are expected to contribute to the regulation of *MUC1* transcription. *FLO8*, a transcriptional regulator of flocculation genes, and two genes shown to enhance invasive growth when overexpressed, *PHD1* and *TEC1*, were included. The genetic relationship between *FLO8*, *MSN1* and *MSS11* is discussed in Gagiano *et al.* (1999b), whereas the results obtained during the genetic analysis of *PHD1* and *TEC1* are presented in the section relating the as yet unpublished results. In addition,

several plasmids and strains were constructed for the promoter analysis of *MUC1* and *STA2* and are presented in **Table 3.1** and **Table 3.2** (Gagiano *et al.* 1999b).

**Table 3.1 Published plasmids constructed by the author.**

| Plasmid | Reference |
|---|---|
| YEpLac112-MSS11 | Gagiano *et al.*, 1999a |
| YEpLac112-PGK1$_p$-MUC1 | Gagiano *et al.*, 1999a |
| YEpLac112-STE7 | Gagiano *et al.*, 1999a |
| YEpLac112-STE12 | Gagiano *et al.*, 1999a |
| YCpLac22-FLO8 | Gagiano *et al.*, 1999a |
| YCpLac22-RAS2 | Gagiano *et al.*, 1999a |
| YCpLac22-RAS2$^{val19}$ | Gagiano *et al.*, 1999a |
| pΔste7 | Gagiano *et al.*, 1999a |
| pΔste12 | Gagiano *et al.*, 1999a |
| pΔflo8 | Gagiano *et al.*, 1999b |
| pΔura3::kan | Gagiano *et al.*, 1999b |
| PPMUC1-lacZ | Gagiano *et al.*, 1999b |
| PPMUC1-ΔUAS1-lacZ | Gagiano *et al.*, 1999b |
| PPSTA2-lacZ | Gagiano *et al.*, 1999b |
| PPSTA2-ΔUAS1-lacZ | Gagiano *et al.*, 1999b |

**Table 3.2 Published strains constructed by the author.**

| Strains | Reference |
|---|---|
| ISP15wt | Our laboratory |
| ISP15Δ*ste7* | Gagiano *et al.*, 1999a |
| ISP15Δ*ste12* | Gagiano *et al.*, 1999a |
| ISP15Δ*flo8* | Gagiano *et al.*, 1999b |
| ISP20Δ*mss11* | Gagiano *et al.*, 1999a |
| ISP20Δ*ste7* | Gagiano *et al.*, 1999a |
| ISP20wt | Our laboratory |
| ISP20Δ*ste12* | Gagiano *et al.*, 1999a |
| ISP20Δ*flo8* | Gagiano *et al.*, 1999b |
| FY23wt | Winston *et al.*, 1995 |
| FY23Δ*mss11* | Gagiano *et al.*, 1999a |
| FY23Δ*ste7* | Gagiano *et al.*, 1999a |
| FY23Δ*ste12* | Gagiano *et al.*, 1999a |

## 3.4 UNPUBLISHED RESULTS

The functional relationships between components of various signal transduction pathways regulating invasive growth and starch degradation are still not well understood. The analysis of phenotypic consequences of hyperactive and null mutant alleles, and combinations thereof, provides a very effective tool for the elucidation of these relationships. The following section provides a summary of some of the experiments that have not been included in the two previously presented publications. The results of these experiments will be part of future publications.

### 3.4.1 INTRODUCTION

Several additional factors besides those investigated by Gagiano *et al.* (1999a and 1999b) are involved in the regulation of invasive growth. These factors include Gpa2, a homologue of the α-subunits of heterotrimeric G-proteins which is required for the regulation of c-AMP levels (Lorenz and Heitman, 1997), and the transcription regulators Flo8p, Phd1p and Tec1p (Gimeno *et al.*, 1994; Gavrias *et al.*, 1996). Previous reports suggested that these proteins are components or targets of the signalling pathways that regulate invasive growth as well as pseudohyphal development (Lorenz and Heitman, 1997).

Gagiano *et al.* (1999b) and Rupp *et al.* (1999) presented evidence that both the filamentous growth MAP kinase module and the c-AMP pathway control invasive and filamentous growth at least in part through the transcriptional regulation of *MUC1.* Data presented by Gagiano *et al.* (1999b) showed in addition that the glucoamylase-encoding *STA2* gene, which is required for starch degradation, follows similar regulation patterns, and is indeed regulated by some of the same factors. In a previous paper, the same authors demonstrated that Msn1p and Ste12p function independently of each other and that both factors require Mss11p for the transcriptional activation of *MUC1* and *STA2* (Gagiano *et al.*, 1999a).

This section presents some of the more interesting preliminary data obtained while investigating the functional relationships between the above mentioned factors, including *RAS2, GPA2*, *MSN1*, *MSS11*, *FLO8, PHD1, STE12* and *TEC1.* Single genes and combinations thereof were deleted in various genetic backgrounds, and these strains were transformed with multiple copies or hyperactivated alleles to assess the effects on invasive and filamentous growth as well as on starch degradation.

### 3.4.2 MATERIALS AND METHODS

The growth conditions and media used during this study were identical to those in Gagiano *et al.* (1999a, 1999b). The additional strains described in this study are listed in **Table 3.4.2.2**, whereas the additional plasmids are listed in **Table 3.4.2.1**. The description of the DNA manipulations involved in constructing these plasmids and strains will be made in future publications.

Deletion mutants were made in several genetic backgrounds (ISP15, ISP20, FY23, Σ1278b), since pseudohyphal differentiation, invasive growth and glucoamylase activity are strain-dependent phenotypes, indicating that laboratory strains carry mutations in one or more of the genes involved.

**Table 3.4.2.1. Plasmids used in this study**

| Plasmids | Reference |
|---|---|
| YEpLac112 | Gietz and Sugino, 1988 |
| YEpLac112-FLO8 | Gagiano *et al.*, 1999b |
| YEpLac112-MSN1 | Gagiano *et al.*, 1999a |
| YEpLac112-MSS11 | Gagiano *et al.*, 1999a |
| YEpLac112-STE12 | Gagiano *et al.*, 1999a |
| YEpLac112-PHD1 | This study |
| YEpLac112-TEC1 | This study |
| YCpLac22 | Gietz and Sugino, 1988 |
| YCpLac22-GPA2$^{val132}$ | This study |
| YCpLac22-RAS2$^{val19}$ | Gagiano *et al.*, 1999a |
| pΔflo8 | Gagiano *et al.*, 1999b |
| pΔgpa2 | This study |
| pΔmsn1 | This study |
| pΔmss11 | This study |
| pΔphd1 | This study |
| pΔste7 | Gagiano *et al.*, 1999a |
| pΔste12 | Gagiano *et al.*, 1999a |
| pΔtec1 | This study |

Hyper-activated *GPA2*$^{val132}$ and *RAS2*$^{val19}$ alleles, as well as multiple copies of *FLO8*, *MSN1*, *MSS11, PHD1, STE12*, *TEC1* and of the cloning vector without insert (YL112 for YEpLac112) were transformed into the following mutant-strains: Δ*msn1*, Δ*ste12*, Δ*mss11*, Δ*msn1*Δ*ste12*, Δ*msn1*Δ*mss11*, Δ*mss11*Δ*ste12*, Δ*msn1*Δ*ste12*Δ*mss11*. The transformants were spotted on SCD- (glucose), SCS- (starch), SLAD- (limited ammonium), and SCGE-media (glycerol and ethanol). After six days of incubation at 30°C, the plates were washed off and photographed. In order to induce starch precipitation SCS-plates were placed at 4°C subsequently to the six day incubation period. Transparent zones surrounding the colonies represent glucoamylase activity. Figures for ISP15Δ*msn1*Δ*ste12*Δ*mss11* are omitted, since the strain was unable to grow invasively or to degrade starch, irrespective of the genes introduced.

**Table 3.4.2.2 Strains used in this study.**

| Strains | Reference |
|---|---|
| ISP15wt | Our laboratory |
| ISP15Δ*flo8* | Gagiano *et al.*, 1999b |
| ISP15Δ*msn1* | This study |
| ISP15Δ*mss11* | Webber *et al.*, 1997 |
| ISP15Δ*phd1* | This study |
| ISP15Δ*ste7* | Gagiano *et al.*, 1999a |
| ISP15Δ*ste12* | Gagiano *et al.*, 1999a |
| ISP15Δ*tec1* | This study |
| ISP15Δ*flo8*Δ*mss11* | This study |
| ISP15Δ*flo8*Δ*tec1* | This study |
| ISP15Δ*msn1*Δ*mss11* | This study |
| ISP15Δ*msn1*Δ*ste12* | This study |
| ISP15Δ*msn1*Δ*tec1* | This study |
| ISP15Δ*mss11*Δ*ste12* | This study |
| ISP15Δ*mss11*Δ*tec1* | This study |
| ISP15Δ*ste12*Δ*tec1* | This study |
| ISP15Δ*ste7*Δ*flo8* | This study |
| ISP15Δ*ste7*Δ*msn1* | This study |
| ISP15Δ*ste7*Δ*mss11* | This study |
| ISP15Δ*ste7*Δ*ste12* | This study |
| ISP15Δ*ste7*Δ*tec1* | This study |
| ISP15Δ*flo8*Δ*mss11*Δ*ste12* | This study |
| ISP15Δ*msn1*Δ*mss11*Δ*ste12* | This study |
| ISP15 Δ*mss11*Δ*ste12*Δ*tec1* | This study |
| ISP20wt | Our laboratory |
| ISP20Δ*gpa2* | This study |
| ISP20Δ*msn1* | This study |
| ISP20Δ*msn1*Δ*mss11* | This study |
| ISP20Δ*msn1*Δ*ste7* | This study |
| FY23wt | Winston *et al.*, 1995 |
| FY23Δ*msn1* | This study |
| FY23Δ*msn1*Δ*mss11* | This study |
| FY23Δ*mss11*Δ*ste12* | This study |
| L5366-h1 | Gagiano *et al.*, 1999a |
| L5366-h1Δ*flo8* | This study |
| L5366-h1Δ*msn1* | This study |
| L5366-h1Δ*mss11* | This study |
| L5366-h1Δ*msn1*Δ*ste7* | This study |
| L5366-h1Δ*msn1*Δ*ste12* | This study |
| L5366-h1Δ*mss11*Δ*ste12* | This study |

## 3.4.3 RESULTS

### 3.4.3.1 Msn1p-mediated invasive growth on glucose-containing media requires Gpa2p.

Gpa2p regulates invasive growth and acts as a regulator of the cAMP-dependent signal transduction pathway (Lorenz and Heitman, 1997). Msn1p, on the other hand, encodes a transcriptional regulator which was shown to function independently of the filamentous growth MAP kinase pathway, whereas Mss11p, another transcriptional regulator, is required for both this cascade and for Msn1p-dependent induction of invasive growth and starch degradation (Gagiano *et al.*, 1999a). In order to ascertain whether Msn1p and Mss11p are dependent on Gpa2p activity, the ISP20 wild type and the mutant-strain ISP20Δ*gpa2*, were transformed with 2μ-plasmids bearing either no insert, or the *MSN1* or *MSS11* genes, respectively. The phenotypes of three different transformants were assessed for each of the respective transformations. Invasive growth was monitored after spotting cells from three independent colonies of each transformation on glucose (SCD), starch (SCS), and limited ammonium (SLAD) media.

After six days, the wild-type strain started to invade the agar substrate on all media tested. This invasion was strongly enhanced in wild-type ISP20 transformed with 2μ-*MSN1* and 2μ-*MSS11* (data not shown). ISP20Δ*gpa2* strains transformed with the plasmid alone, on the other hand, showed strongly reduced or no invasion, confirming the results of Lorenz and Heitman (1997) (**Figure 3.1**). Indeed, in this case, weak invasion could only be detected on starch containing media. The 2μ-*MSS11* transformed Δ*gpa2* strain, on the other hand, showed strong invasive growth, similar to wild-type levels, on all media tested, indicating that Mss11p activity is not dependent on Gpa2p. 2μ-*MSN1* was able to restore invasive growth in a Δ*gpa2* background on nitrogen-limited (SLAD) or starch (SCS) containing media, but was unable to do so on SCD media.

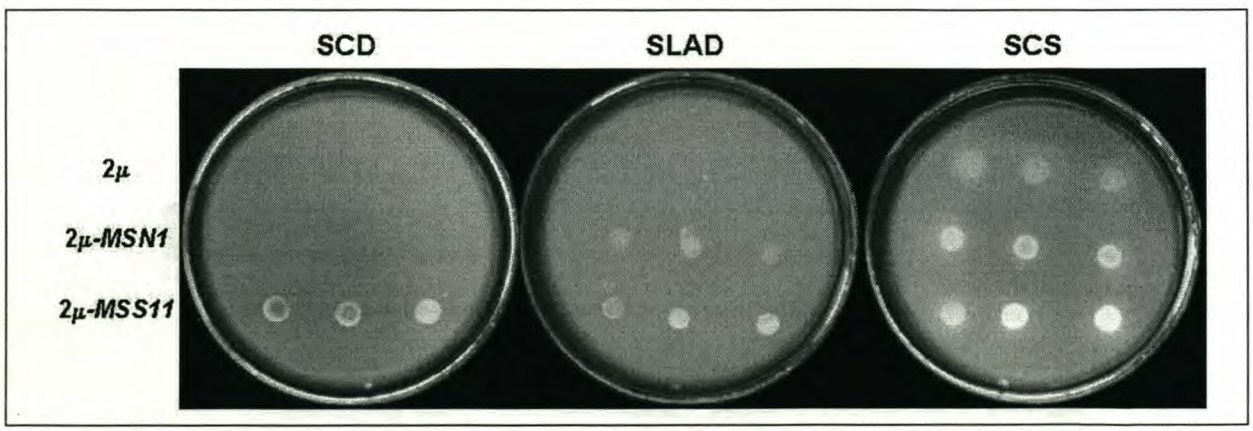


**Figure 3.1** Invasive growth of strains ISP20Δ*gpa2* transformed with either 2μ, 2μ-*MSN1* and 2μ-*MSS11*. Three independent colonies were spotted for each strain. The plates were washed of after 6 days of incubation. Cells that invaded the agar remained on the plates after the wash.

This result indicates that Gpa2p activity is required for Msn1p to induce invasive growth in glucose containing media, but does not affect Msn1p activity in conditions of nitrogen limitation. These results suggest that Mss11p is not regulated by Gpa2p and is able to act independently of this pathway, whereas Msn1p function is dependent on Gpa2p in some conditions.

### 3.4.3.2 Multiple copies of *PHD1* but not *FLO8*, *MSN1* and *TEC1*, partially restore invasive growth in Δ*mss11* and Δ*msn1*Δ*mss11* genetic backgrounds.

None of the multiple copy plasmids or hyperactivated alleles transformed into ISP15Δ*mss11* and ISP15Δ*msn1*Δ*mss11* were able to re-establish invasive growth, except *PHD1* (**Figure 3.2**). This suppression of the Δ*mss11* phenotype was only observed when the transformants were grown on media containing glucose as the sole carbon source (SCD and SLAD), and not on SCS or SCGE media, suggesting that Phd1p function is not strictly dependent on Mss11p in response to glucose signals.

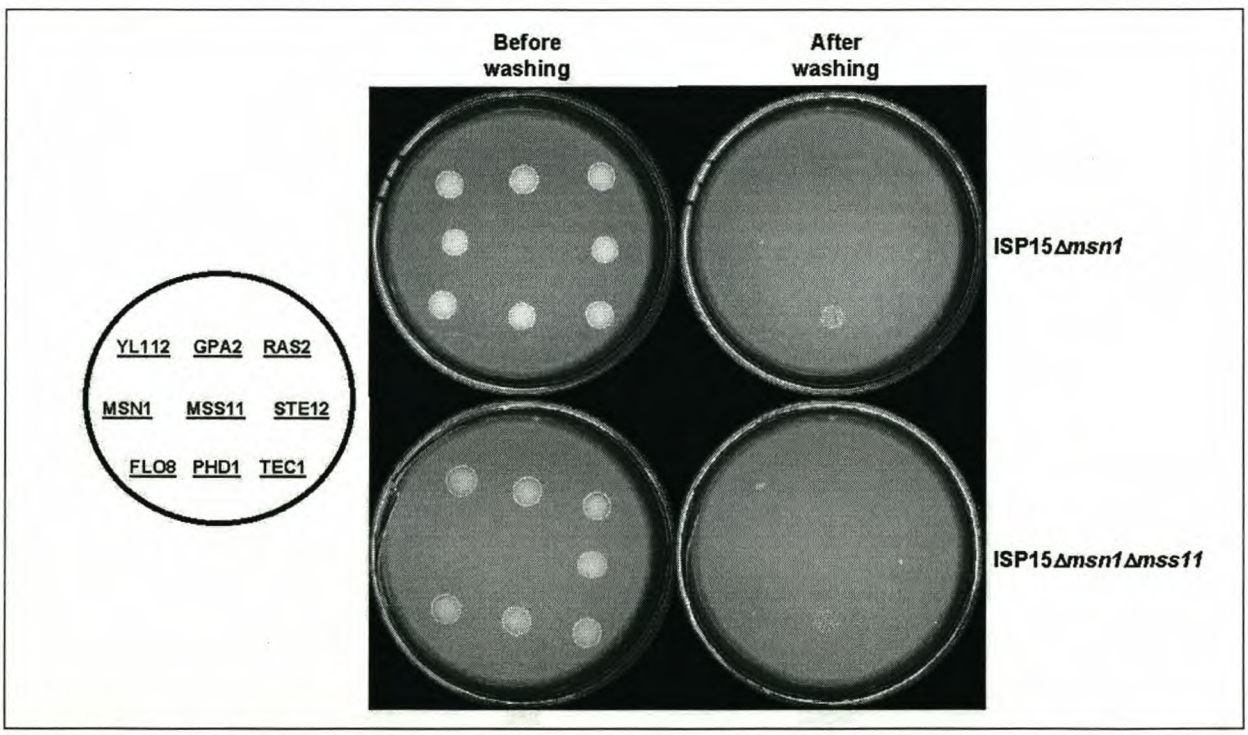


**Figure 3.2** Phd1p partially restores invasive growth in mutants lacking *MSS11* or both *MSS11* and *MSN1* on SLAD plates. This effect is exclusive to media containing glucose as a carbon source (SCD and SLAD).

### 3.4.3.3 The effect of deletions of *MSN1* and *STE12*.

ISP15 strains lacking functional copies of *MSN1*, *STE12*, or of both genes, were transformed with all the other factors investigated. The results are presented in **Figures 3.3** and **3.4**, which represent the same combinations of strains and plasmids spotted on either SLAD or SCS media, respectively. From these plates it can be seen that multiple copies of both *FLO8* and *MSS11* efficiently overcome the inability to

invade or to degrade starch of the three mutant strains. These results are in accordance with the data presented by Rupp *et al.* (1999), suggesting that Flo8p functions independently of the MAP kinase module as a downstream factor of the cAMP-pathway. Furthermore, Gagiano *et al.* (1999a and b) provided evidence that both genes, *MSS11* and *FLO8*, act independently or downstream of Msn1p.

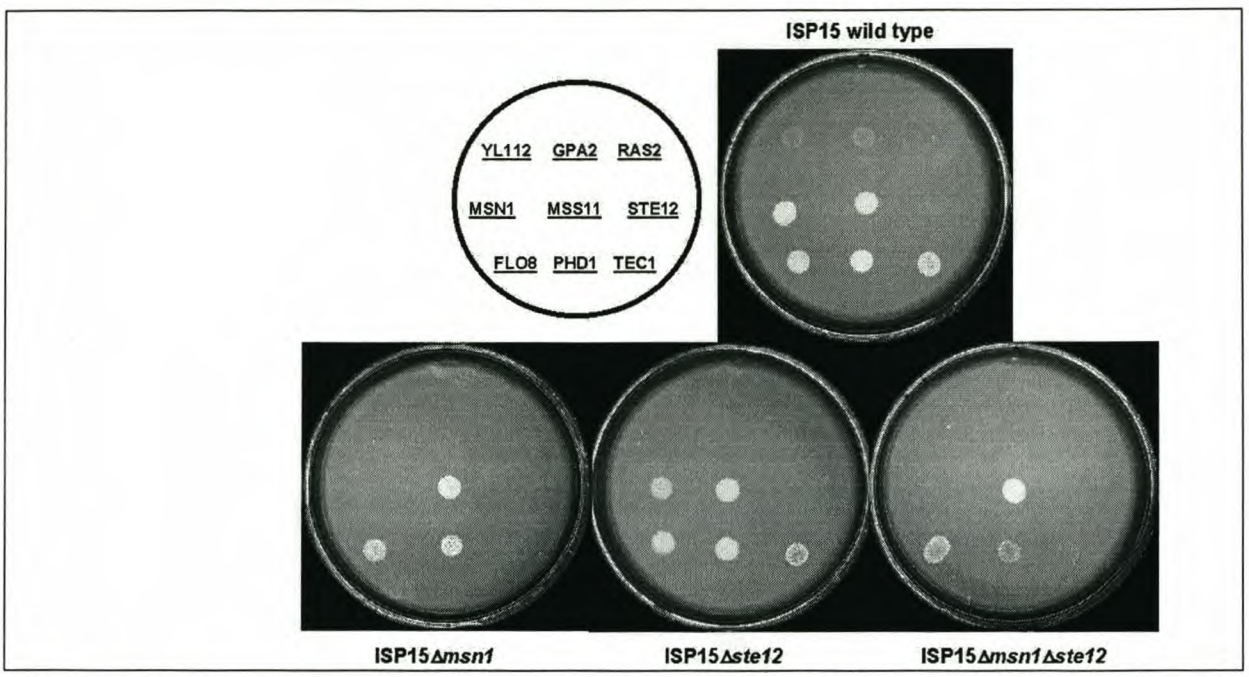


**Figure 3.3** Invasive growth tests of ISP15 wild-type, ISP15Δ*msn1*, ISP15Δ*ste12* and ISP15Δ*msn1*Δ*ste12* strains transformed with multiple copies of *MSS11*, *STE12*, *FLO8*, *PHD1* and *TEC1* or hyperactive alleles of *GPA2* and *RAS2* on SLAD (nitrogen-limited) medium.

Multiple copies of *TEC1* also suppressed the invasive growth defect conferred by a disruption of *STE12* (**Figure 3.3**). In this case, efficient agar penetration was observed for SCD-, SLAD- and SCS-media, but not for SCGE-media (results not shown). However, *TEC1* was unable to efficiently overcome the absence of Msn1p, since it failed to significantly enhance invasive growth in both the *MSN1*-deletion strain and in the double mutant. Interestingly, *TEC1* efficiently induced starch degradation in the Δ*msn1* background, indicating differences in the regulation of the *MUC1* and *STA2* genes. Overexpression of Phd1p, on the other hand, resulted in an efficient suppression of the invasive growth defects of both single mutants on SLAD medium, similar to the phenotypes observed with multiple copies of *MSS11* or *FLO8*. However, *PHD1* was less efficient in overcoming the defect of the double mutant, indicating that it requires the presence of at least one of the two gene products. Interestingly, while strengthening the invasive growth phenotypes of these strains, *PHD1* was unable to increase starch degradation **(Figure 3.4)**. Furthermore, as for multiple copies of *TEC1*, multiple copies of *PHD1* in wild-type and mutant-strains did not result in increased invasive growth when grown on SCGE, suggesting that both Phd1p and Tec1p are not activated when grown on these carbon sources (results not

shown). Tec1p activity therefore seems to depend specifically on Msn1p in the presence of glucose.

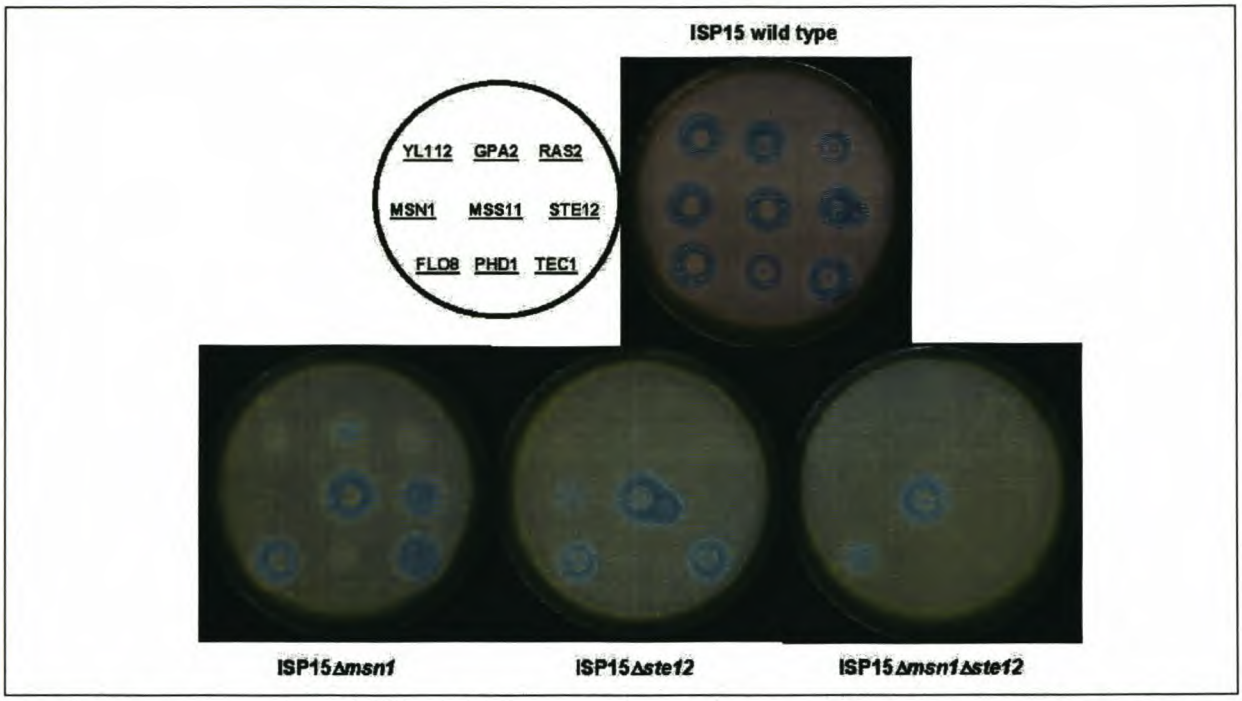


**Figure 3.4** Starch degradation tests of ISP15 wild type, ISP15Δ*msn1*, ISP15Δ*ste12* and ISP15Δ*msn1*Δ*ste12* strains transformed with multiple copies of *MSS11*, *STE12*, *FLO8*, *PHD1* and *TEC1* or hyperactive alleles of *GPA2* and *RAS2* on SCS (starch) medium.

### 3.4.3.4 Differences in the genetic backgrounds of laboratory yeast.

Gagiano *et al.* (1999b) presented evidence that the inability of most laboratory strains to grow invasively, or to degrade starch after transformation with a *STA2* gene containing plasmid, was at least partially due to a mutation in the *FLO8* gene present in several laboratory yeast. We furthermore observed a significant difference in the ability to invade between two other strains used in our laboratory, ISP15 and ISP20, which both carry functional copies of *FLO8*, with ISP15 displaying significantly stronger invasive growth than ISP20.

Interestingly, wild-type ISP15 transformed with single copy plasmids carrying the hyperactivated alleles *GPA2*$^{val132}$, *RAS2*$^{val19}$ or multiple copies of 2μ-*STE12* did not display increased invasive growth or starch degradation (**Figures 3.3** and **3.4**). A possible explanation for the absence of increased invasive growth is that the signalling pathways regulated by these factors are constitutively activated in this specific genetic background. Transformation with these hyperactive alleles will therefore have no significant effect on the wild-type invasive growth phenotype. This is similar to recently published data by Stanhill *et al.* (1999), who show that the strain used in most laboratories to assess invasive and pseudohyphal growth, Σ1278b, might have an overactive Ras2 pathway. Overexpression of *STE12* on the other hand is known to be lethal in some strains (Liu *et al.*, 1993).

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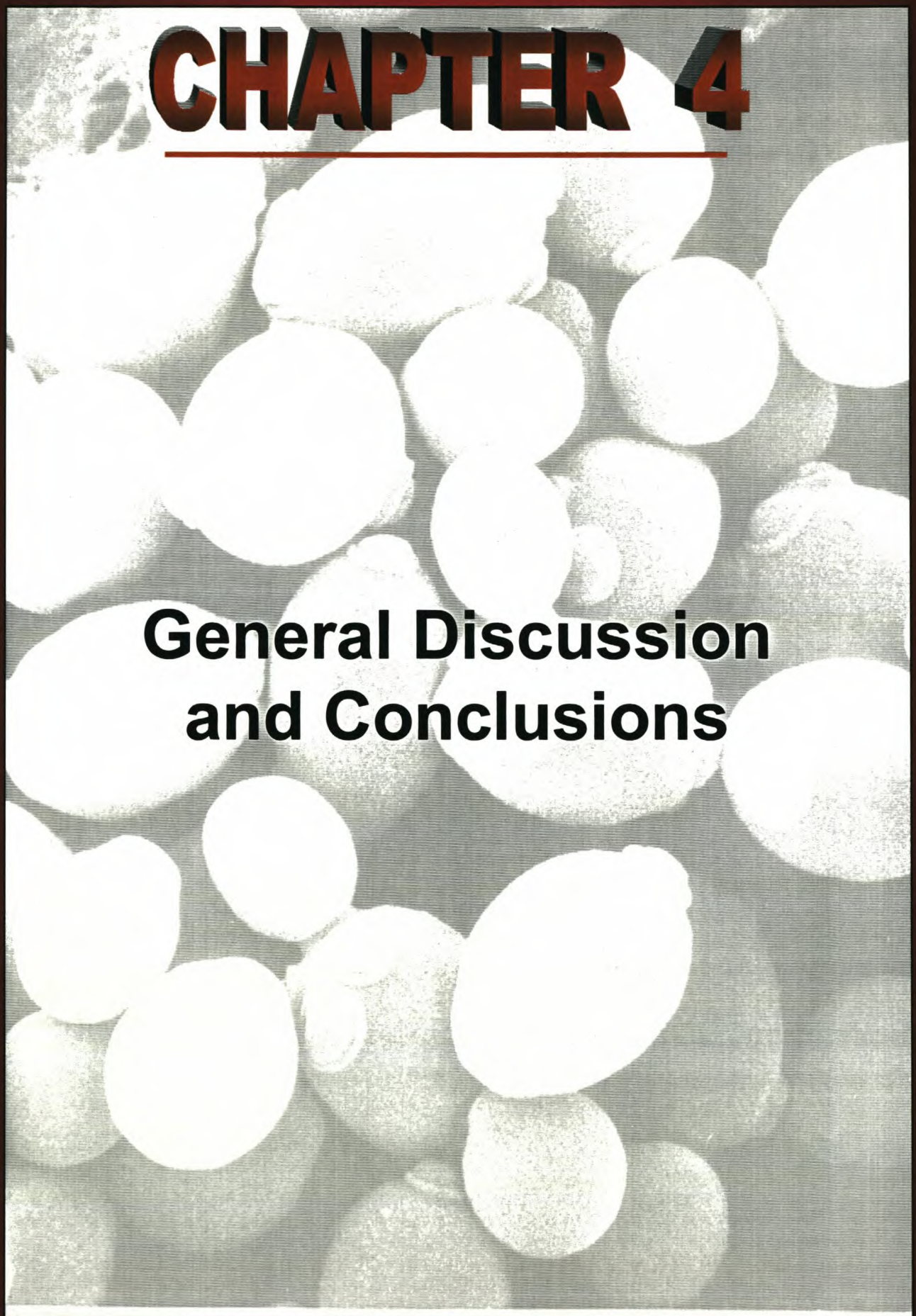

# CHAPTER 4

# General Discussion and Conclusions

# 4. GENERAL DISCUSSION AND CONCLUSION

## 4.1 CONCLUDING REMARKS AND PERSPECTIVES

The actin cytoskeleton plays a critical role in the establishment of polarised growth, a process required for budding, mating projection formation and pseudohyphal differentiation. This cytoskeleton is regulated and distributed according to internal or external information perceived by the cell. Signal transduction pathways transmit the information provided by these signals to polarity establishment factors as well as to transcriptional regulators residing in the nucleus. Signals that result in morphogenetic events include the presence of pheromone of the opposite mating-type and limited amounts of nutrients. Morphologically, these signals result in mating projection formation and cell elongation/pseudohyphal growth, respectively.

Polarity establishment factors are required for the targeting of actin and actin-associated proteins to sites where polarised growth will occur, and the activated transcriptional regulators control the transcription of numerous genes involved in these events, including actin-binding proteins and actin-regulators. The cell cycle is also implicated in morphogenetic events, since the information determining the timing at which polarised growth will occur, is provided by cell cycle regulators during the G1-phase. Morphogenesis is therefore the combined result of activation of signal transduction pathways, cell-cycle reprogramming and actin cytoskeleton redistribution.

The literature review focuses on various aspects of actin-structure, on the roles played by actin in the establishment of polarised growth and on signal transduction elements that are involved in the regulation of the actin cytoskeleton in response to internal and external stimuli. The experimental section of this thesis, on the other hand, investigates several genes which play a role in the regulation of one of those actin dependent processes, pseudohyphal differentiation. Several elements of the filamentous growth signalling pathways, which include Cdc42p, Cdc24p, Ste20p, and CAP, as well as factors that interact with elements of the Ste-MAP kinase pathway, Spa2p, Bud6p and Pea2p, are indeed required for the distribution and regulation of the actin cytoskeleton (Goldschmidt-Clermont and Janmey, 1991; Mösch *et al.*, 1996; Sheu *et al.*, 1998). In addition, preliminary data from genome wide transcription analysis with two mutant strains created during this M.Sc. indicate that some actin cytoskeleton regulators are directly controlled by the factors investigated (unpublished results).

The primary objective of this study was to determine whether the same factors are involved in the regulation of *MUC1* and the *STA1-3* genes. In addition to the previously described roles for *MSN1* and *MSS11* as regulators of the *STA*-genes (Lambrechts *et al.*, 1996a, Webber *et al.*, 1997), data was obtained that confirmed that these factors act on the promoters of *MUC1* and *STA2* (Gagiano *et al.*, 1999a). Apart from the 99% nucleotide sequence homology between the promoters of *MUC1*

and *STA2*, differences in the levels of expression were nevertheless observed. *MUC1* transcription is generally very low in comparison to that of *STA2*. Different regulatory elements, including transcriptional inhibitors and activators, therefore exist, which are able to distinguish between the two promoters. Alternatively, signal-specific factors might be activated in response to distinct nutritional information and are subsequently targeted to general protein complexes that are required for the transcriptional regulation of both genes. The addition of such a signal-specific factor to the general transcription complexes, could alter the function of the protein complex in such a manner that it is able to discriminate between the promoters of *MUC1* and *STA2*.

In order to ascertain the co-regulation between *MUC1* and *STA2*, several genes regulating invasive growth, including *FLO8*, *PHD1*, *STE12* and *TEC1*, were investigated to determine if they affect starch metabolism. Conversely, *MSS10* and *MSS11*, cloned for their ability to induce starch degradation when present in multiple copies, were tested to establish whether they are involved in the regulation of invasive growth. Strains with different genetic backgrounds were used, since some of the genes involved in invasive growth encode for non-functional proteins in several laboratory strains. Multiple copies of *FLO8*, *MSN1*, *MSS11*, *PHD1* and *TEC1* resulted in induced levels of starch degradation and invasive growth in all the genetic backgrounds tested, which exclude mutant-strains (Gagiano *et al.*, 1999a, 1999b, unpublished results). However, in the case of *PHD1* the opposite was observed. While inducing invasive growth upon overexpression, starch degradation was not activated in the same conditions. It appears that Phd1p functions as an activator for elements of the invasive growth pathway, while it inhibits, or is neutral, in starch metabolism. The results emanating from this study are summarised in a model presented in **Figure 4.1**.

The repressive effect on *STA* transcription observed in so-called *STA10*-strains, was established to be due to the absence of *FLO8* and not the result of a repressor (Gagiano *et al.*, 1999b). Furthermore, laboratory strains lacking *FLO8* are unable to grow invasively. Overexpression of *FLO8* in some of these strains does not lead to the re-establishment of invasive growth, suggesting that additional factors are absent in these strains (unpublished results).

An additional objective of this project was the assessment of the functional hierarchy of several factors implicated in invasive growth and starch metabolism. For this purpose, phenotypes were monitored in well-defined conditions. In addition, *MUC1*- and *STA2*-promoters fused to a *lacZ*-reporter plasmid were used to quantify the observed effects. The epistasis analysis and the *lacZ*-expression data suggested that complex relationships exist among the various transcription factors involved in invasive growth and starch degradation (Gagiano *et al.*, 1999a, 1999b, unpublished results).

Results derived from this study show that the activation of transcriptional regulators involved in invasive growth and starch metabolism is dependent on the

nature of the nutritional signals. Different factors are required for the activation of both Tec1p and Phd1p in response to limited amounts of different carbon sources and to limited amounts of ammonium. This suggests parallel pathways, which use different factors for the transmission of nutritional information. The term "pathways" might be misrepresentative of the actual mechanism present in a biological system, since it suggests a linear relationship between several molecules. In fact, it appears that intertwined networks are rather responsible for the processing of stimuli. Moreover, as described previously, protein-complexes control the transcriptional activation of a multitude of genes involved in invasive growth and starch metabolism, but the association or dissociation of a specific factor from these complexes might alter transcriptional specificity significantly. Our data show that Mss11p is a pivotal factor in the transcriptional regulation of *MUC1* and *STA2*, since a deletion of *MSS11* abrogates invasive growth and starch degradation. From all the factors tested to date, only overexpression of *PHD1* was able to weakly suppress the invasive growth defect conferred by a deletion of *MSS11* on glucose containing media.

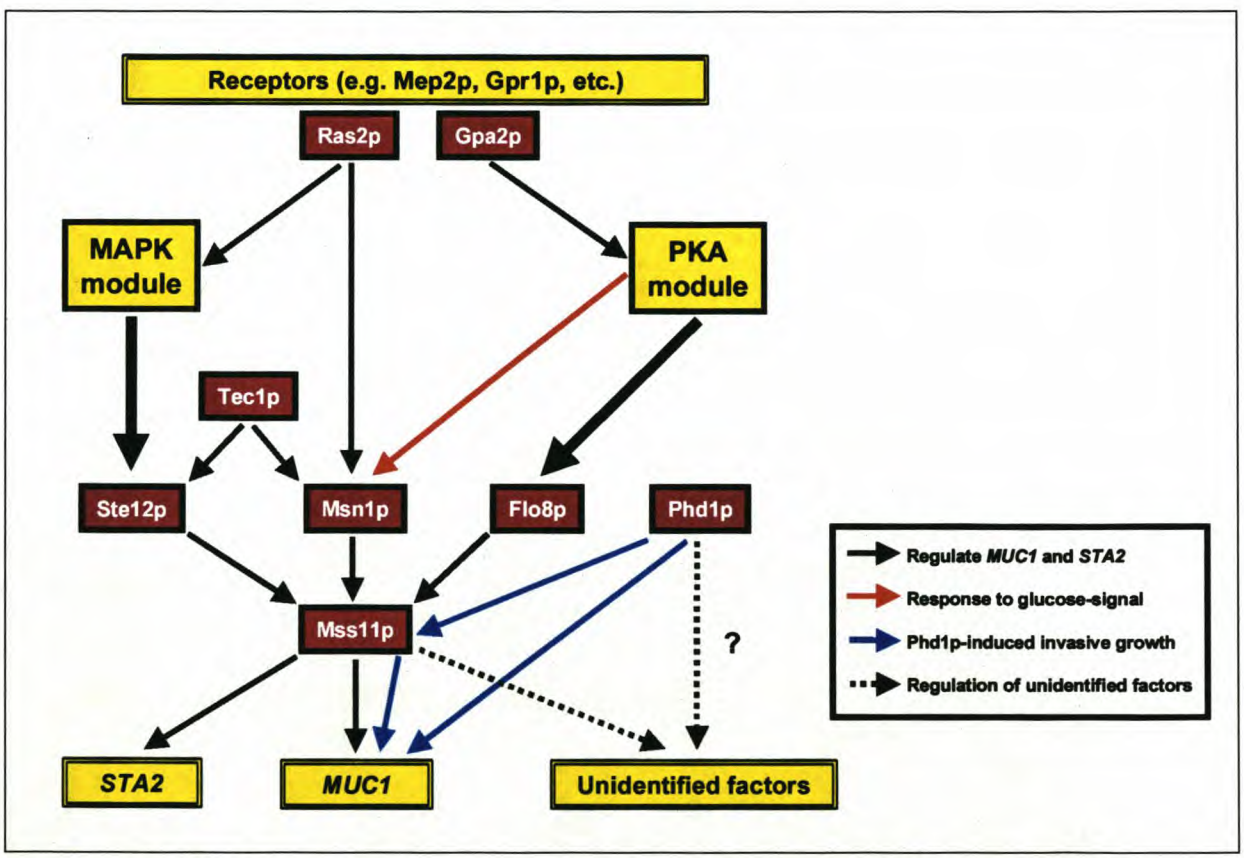


**Figure 4.1** A model illustrating the genetic interactions between the factors investigated.

The genetic analysis of these transcriptional regulators is far from complete. As a matter of fact, a wide range of mutant-strains with different genetic backgrounds were constructed but were not yet characterised or tested due to the great amount of time and effort that are required for a comprehensive analysis of the genetic relationships between various combinations of these factors. These genetic studies form the basis for additional experimental work that entails the identification and characterisation of

specific protein-protein interactions. Furthermore, protein localisation will be investigated in the mutant-strains that were created during this study.

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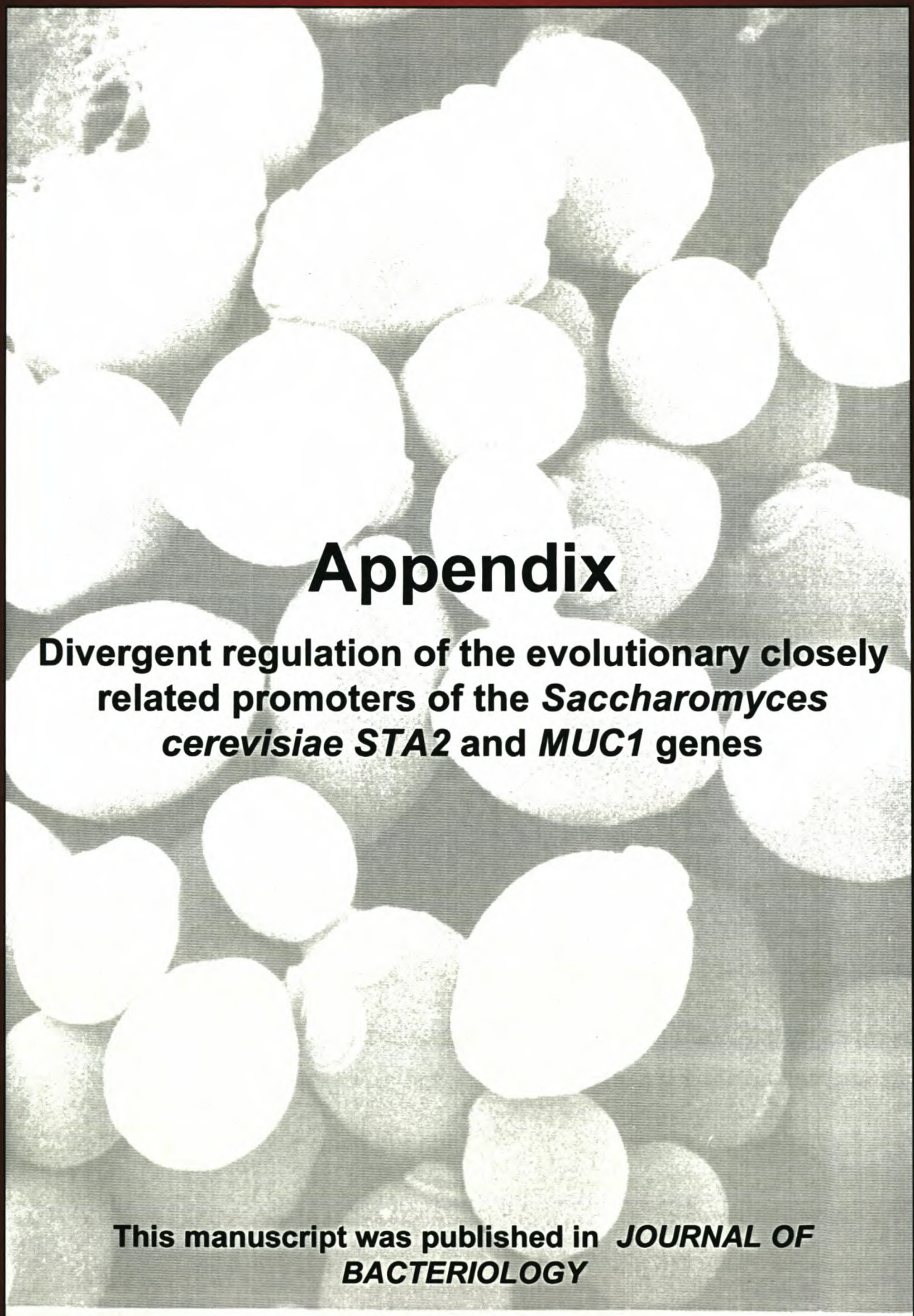

# Appendix

## Divergent regulation of the evolutionary closely related promoters of the *Saccharomyces cerevisiae STA2* and *MUC1* genes

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# APPENDIX

## Divergent regulation of the evolutionary closely related promoters of the *Saccharomyces cerevisiae STA2* and *MUC1* genes

**MARCO GAGIANO[1,2], DEWALD VAN DYK[1,2], FLORIAN F. BAUER[1], MARIUS G. LAMBRECHTS[1] AND ISAK S. PRETORIUS[1,2]**

*Institute for Wine Biotechnology[1] and Department of Microbiology[2], University of Stellenbosch, Stellenbosch, ZA-7600, South Africa*

The 5' upstream regions of the *Saccharomyces cerevisiae* glucoamylase-encoding genes, *STA1-3*, and of the *MUC1/FLO11* gene, which is critical for pseudohyphal development, invasive growth and flocculation, are almost identical and the genes co-regulated to a large extent. Besides representing the largest yeast promoters identified to date, these regions are of particular interest from both a functional and an evolutionary point of view. Transcription of the genes seems indeed dependent on numerous transcription factors which integrate the information of a complex network of signalling pathways, while the very limited sequence differences between them should allow study of promoter evolution on a molecular level. To investigate the transcriptional regulation, we compared the transcription levels conferred by the *STA2* and *MUC1* promoters under various growth conditions. Our data show that transcription of both genes responded similarly to most environmental signals, but also indicated significant divergence in some aspects. We identified distinct areas within the promoters that show specific responses to the activating effect of Flo8p, Msn1p (Mss10p/Fup1p/Phd2p) and Mss11p as well as to carbon catabolite repression. We also identified the *STA10* repressive effect as the absence of Flo8p, a transcriptional activator of flocculation genes in *S. cerevisiae*.

The *STA1*, *STA2* and *STA3* genes encode extracellular glucoamylase isozymes which enable *Saccharomyces cerevisiae* cells to utilise starch as a carbon source (reviewed in 38, 40, 50). The three genes have nearly identical sequences, and are located on chromosomes II (*STA2*), IV (*STA1*) and XIV (*STA3*). All three members of the *STA*-gene family are located in subtelomeric positions, similar to the *FLO* (reviewed in 48), *SUC* (reviewed in 15) and *MAL* (reviewed in 32) gene families, which probably evolved through genomic duplications and chromosomal rearrangements. The 5′ upstream region of *STA1* and *STA2* (the nucleotide sequence of *STA3* has not previously been determined) is almost identical to that of *MUC1* which encodes a large membrane-bound, mucin-like protein that plays an important role in the processes of invasive growth, pseudohyphal development and flocculation (8, 23, 27, 28). The homology extends over more than 3 500 basepairs

(bp) upstream of the ATG start codon and includes the first 60 bp of the open reading frame (ORF) encoding a secretion signal sequence (52). With the exception of a few single nucleotide dissimilarities, the only significant differences between the promoters of *STA2* and *MUC1* are two inserts of 20 bp and 64 bp in the *MUC1* promoter, which are absent from the *STA2* promoter (23). These inserts stretch from nucleotides -1 333 to -1 313 and nucleotides -933 to -869, respectively. This very limited sequence divergence between the *STA* and *MUC1* promoter regions suggests a recent origin of the *STA* genes. The *STA* genes probably evolved through a recombination and sequence duplication process between the promoter and signal sequence of *MUC1* and the ORF of the *SGA1* gene that encodes a sporulation-specific intracellular glucoamylase. *MUC1* and *SGA1* are located on the right and left arms of chromosome IX, respectively (59, 60). Besides the strong sequence conservation between these genes, other arguments in favour of a recent origin of the *STA* genes and of the proposed molecular mechanism are (i) the subtelomeric position of the *STA* genes compared to the more central position of both *MUC1* and *SGA1* (ii) the presence of *STA* genes in only some *S. cerevisiae* strains, compared to the general presence of *MUC1* (4, 59, 60) and *SGA1* (59, 60) in all *S. cerevisiae* strains investigated so far and (iii) the existence of homologous repeated sequences on either side of the proposed junctions (59, 60).

Analyses of the upstream areas of *STA1* (1, 46), *STA2* (22) and *MUC1* (44) demonstrated that elements at distances of up to 2 800 bp from the translation start codon (ATG) are involved in the transcriptional control of the respective genes, therefore representing the largest *S. cerevisiae* promoters identified to date (7, 44). The *STA* and *MUC1* upstream regions are thus of particular interest from both an evolutionary and functional point of view.

The extent of the promoter homology would suggest that genes involved in starch metabolism and pseudohyphal differentiation/invasive growth are co-regulated to a large extent, and experimental data so far have supported this hypothesis. Lambrechts *et al.* (23, 24) and Gagiano *et al.* (8) showed that two transcriptional regulators, Msn1p and Mss11p, strongly induce transcription of both the *STA2* and *MUC1* genes when present on multiple copy plasmids. Conversely, Δ*msn1* or Δ*mss11* strains show strongly reduced transcription of these genes. Furthermore, Lo and Dranginis (28) demonstrated that *MUC1* is regulated by Ste12p, a transcription factor responsible for both pheromone-specific (reviewed in 21), and, in combination with the TEA/ATTS family transcription factor, Tec1p, filamentation-specific gene regulation (9, 30). Gagiano *et al.* (8) presented evidence that the same factor regulates the *STA* genes in a similar way.

Other regulatory factors have so far only been associated with regulation of *MUC1* or *STA1*, *STA2* and *STA3* independently. Recent data suggest that the transcription of *MUC1* might be specifically regulated by a network of signal transduction pathways which controls invasive growth and pseudohyphal differentiation (8, 28, 44). This network combines inputs from at least three interacting

signal transduction modules, including (i) the filamentation-specific MAP kinase cascade (25, 31, 42), (ii) the cAMP and cAMP-dependent kinase (29, 43), and (iii) the cyclin-dependent kinase Cdc28p (6). In addition to the above mentioned result that *MUC1* was subjected to MAPK-dependent regulation by Ste12p/Tec1p, the gene was shown to be regulated by cAMP levels, a regulation that occurs via Flo8p (44), a transcription factor initially identified for its role in flocculation (18). The gene was also shown to be negatively regulated by a suppressor of flocculation, Sfl1p, which interacts specifically with the yeast A kinase, Tpk2p, to repress *MUC1* transcription in the absence of a cAMP signal (43).

Numerous data concerning the regulation of the *STA* genes have been published. Expression of *STA1-3* is negatively regulated at several levels. Transcription is repressed on most readily metabolised carbon sources, including glucose, sucrose, maltose and galactose (5, 17, 20, 41, 47). Carbon catabolite repression was reported to involve two separate pathways, of which one requires *HXK2* and the other *HAP2* (17). It was also reported that repression of *STA2* does not require Mig1p, the common repressor of genes under carbon catabolite control. *MUC1* was also shown to be repressed in media containing glucose as carbon source (8, 27), probably via the same mechanisms as *STA1* and *STA2*. Transcription of *STA1-3* is repressed in most, but not all, diploid strains of *S. cerevisiae* (5, 41). The mechanism through which repression occurs is not defined, since the removal of the putative a1/$\alpha$2 binding sites from the *STA2* promoter does not relieve the repressive effect observed in diploid strains (22). In rich media, *MUC1* is also repressed in diploid strains, but in nitrogen-starvation conditions seems to be more repressed in haploid than diploid strains (28, 44).

Most laboratory strains of *S. cerevisiae* contain an undefined repressor, *STA10*, which reduces transcription of the *STA1-3* genes at least 20-fold (37, 41). It has been reported that the repressive effect of *STA10* results from interaction between two unlinked genes, *IST1* and *IST2* (35), but this was not confirmed. The negative effect of several other genes, i.e. *INH1* (58), *SGL1* (36), *SNS1* and *MSS1* (1) on the transcription of the *STA* genes have also been reported but the relationships between these negatively-acting genes and the repressive effect of *STA10* remains to be determined.

Transcription of *STA1-3* is subject to the repressive effect of chromatin on promoters, since *SUD1*, a component of a global chromatin-associated repressor of promoter activity, was shown to act on the *STA1* promoter (57). Furthermore, transcription of *STA1-3* also requires the presence of components of the SWI-SNF global activation complex (13, 20, 33, 61, 62, 63), which associates with the RNA polymerase holoenzyme at specific promoters and relieves the repressive effect of chromatin on transcription (19, 55).

*Cis*-acting promoter elements in several regions within the *STA1* (1, 46), *STA2* (22) and *MUC1* (44) promoters were shown to be required for transcriptional regulation. Two areas hosting upstream activating sequences (UASs), (UAS1

between nucleotides -1 390 and -1 074 and UAS2 between nucleotides -1 940 and -1 815), as well as three upstream repression sequences (URSs) were identified in the *STA2* promoter (22). URS1 was found to reside in the area between nucleotides -1 390 and -1 074 that also hosts UAS1. URS2 was identified between nucleotides -1 650 and -1 390 and URS3 upstream of position -2 457. Similar regions were defined for the *STA1* promoter (1, 46). A recent, more systematic, analysis of the *MUC1* promoter (44), revealed a vast array of regulatory elements which confer the regulation of several nutritional and cell-type signals on *MUC1* expression levels. In good agreement with the previous studies on the highly homologous *STA1* and *STA2* promoters, four areas required for the activation of *MUC1* and nine areas required for the repression thereof were identified. The transcriptional activator encoded by *FLO8* was found to exert its activating effect through a 200 bp sequence stretching from nucleotides -1 200 to -1 000 in the upstream region of *MUC1* (44).

The 5' upstream areas of *MUC1*, *STA1* and *STA2*, are predicted to contain a single small ORF, YIR020c, of unknown function, situated from nucleotides -1 285 to -882 in the upstream region of *MUC1*. YIR020c lies in an area identified and experimentally defined as a regulatory region for *STA1*, *STA2* and *MUC1*, and other regulatory regions were shown to exist upstream of this ORF (1, 22, 44, 46). Its occurrence therefore does not affect conclusions regarding the transcriptional regulation of *STA1*, *STA2* or *MUC1*, independently of whether this ORF encodes a functional protein or not.

The homologous sequences from nucleotides -1 390 to -1 074 of the *STA2* promoter and from nucleotides -1 479 to -1 136 of the *MUC1* promoter are of particular interest since they (i) have previously been identified as areas hosting an upstream activating sequence as well as an upstream repression sequence (22), (ii) confer increased levels of activity from a far upstream position, and (iii) include one of the two significant differences between the upstream areas of *MUC1* and *STA1-3* (a sequence of 20 bp that is deleted in the *STA2* promoter). The region might therefore contain an evolutionary, significant molecular change explaining differences in the regulation of *STA1-3* and *MUC1*.

In this paper we compare expression levels conferred by the full *MUC1* and *STA2* promoters on reporter gene expression. We furthermore present a detailed analysis of the promoter region from nucleotides -1 390 to -1 074 of *STA2* and the corresponding area of *MUC1*, from nucleotides -1 479 to -1 136. We show that these regions of *MUC1* and *STA2* confer both similar and divergent regulation and contain sequences involved in general repression as well as areas for (i) activation by the transcriptional activators encoded by *MSN1* and *MSS11*, (ii) activation by the transcriptional activator encoded by *FLO8*, (iii) carbon catabolite repression and (iv) diploid repression. Our data indicate that differences in expression levels observed between *MUC1* and *STA2* are largely due to the two deletions of 20 and 64 bp that have occurred in the *STA* promoters. We also show that the repressive effect identified as *STA10* in most laboratory *S. cerevisiae* strains is due to the absence of

the *FLO8*-encoded transcriptional activator. Epistasis analysis furthermore suggests that *FLO8/sta10* requires or is situated upstream of *MSS11*, but acts independently of *MSN1*.

## MATERIALS AND METHODS

### Strains, growth media and genetic methods.

The *S. cerevisiae* strains used in this study, along with the relevant genotypes, are listed in Table 1. Transformation of *S. cerevisiae* cells was carried out by the lithium acetate procedure (3). The one-step gene replacement method (3) was used to disrupt the *FLO8* loci with the *flo8*::*URA3* cassette, p$\Delta$*flo8*, in the genomes of strains ISP15 and ISP20, to generate strains ISP15$\Delta$*flo8* and ISP20$\Delta$*flo8*, respectively. Successful disruptions of the *FLO8* loci in these strains were verified by Southern blot analysis and confirmed by PCR analysis. The *URA3* marker of strains ISP15$\Delta$*flo8* and ISP15$\Delta$*msn1* was regenerated through transformations with the *ura3*::*kan*$^R$ disruption cassette, p$\Delta$*ura3*::*kan*, and selected for on media containing 125 mg/ml kanamycin and 1 mg/ml 5-fluoroorotic acid (5-FOA). *S. cerevisiae* strain FY23 (56) is isogenic to the S288C genetic background and L5366 (25) and L5366h (8) to the $\Sigma$1278b genetic background. Strain JM2508 does not contain any of the *STA1-3* genes and is from the culture collection of the late Dr. Julius Marmur.

Unless specified differently, yeast cells were grown at 30°C in synthetic media containing 0.67% yeast nitrogen base without amino acids (Difco laboratories, Detroit, MI, U.S.A.), supplemented with the required amino acids and 2% glucose for SCD, 3% glycerol and 3% ethanol for SCGE and 2% corn or potato starch (Sigma Chemical, St. Louis, MO, U.S.A.) for SCS. SLAD media, used for induction of invasive growth and pseudohyphae, were prepared as described previously (11). Solid media contained 2% agar (Difco laboratories). SPD medium contained 0.17% yeast nitrogen base without $(NH_4)_2SO_4$ and without amino acids (Difco laboratories), 2% glucose and 0.1% filter-sterilised proline as sole nitrogen source.

*E. coli* strain DH5$\alpha$ (Gibco BRL/Life Technologies, Rockville, MD, U.S.A.) was used for propagation of all plasmids and was grown in Luria-Bertani (LB) broth at 37°C. All *E. coli* transformations and isolation of DNA were done according to Sambrook *et al.* (45).

### Construction of plasmids.

*FLO8* was isolated as a 3 252 bp *Sph*I-*Eco*RV fragment from plasmid pF415-1 (18) and ligated to plasmids YEplac112 and YEplac181 (10), digested with *Sph*I and *Sma*I, to generate plasmids YEplac112-*FLO8* and YEplac181-*FLO8*. YEplac112-*FLO8* was subsequently used to construct p$\Delta$*flo8*, a cassette for disrupting the *FLO8* locus. In order to do this, a 760 bp *Pst*I-*Bgl*II fragment, comprising the translational start codon (ATG) and a large part of the *FLO8* ORF,

was removed and replaced with a 1 084 bp *Nsi*I-*Bam*HI fragment containing the *URA3* marker, isolated from plasmid pJJ242 (16).

**TABLE 1.** Yeast strains used in this study

| Strain | Relevant genotype | Source/reference |
|---|---|---|
| ISP15 | *MATa STA2 his3 thr1 trp1 leu2 ura3* | This laboratory |
| ISP15Δ*flo8* | *MATa STA2 his3 thr1 trp1 leu2 flo8::URA3 ura3::kan*$^R$ | This work |
| ISP15Δ*msn1* | *MATa STA2 his3 thr1 trp1 leu2 msn1::URA3 ura3::kan*$^R$ | This work |
| ISP15Δ*mss11* | *MATa STA2 his3 thr1 trp1 ura3 mss11::LEU2* | 8 |
| ISP20 | *MATa STA2 thr1 trp1 leu2 ura3* | This laboratory |
| ISP20Δ*msn1* | *MATa STA2 thr1 trp1 leu2 msn1::URA3* | 8 |
| ISP20Δ*mss11* | *MATa STA2 thr1 trp1 ura3 mss11::LEU2* | 8 |
| ISP20Δ*flo8* | *MATa STA2 thr1 trp1 leu2 flo8::URA3* | This work |
| JM2508 | *MATa leu2 ura3* | Julius Marmur |
| FY23 | *MATα leu2 ura3 flo8* | 56 |
| L5366 | *MATa/MATα ura3/ura3* | 25 |
| L5366h | *MATa ura3* | 8 |

YCplac33-*STA2* was constructed by inserting a *Xho*I-*Eco*RV fragment from plasmid pSP*STA2* (22) into the unique *Sal*I-*Sma*I sites of YCplac33 (10). A 953 bp *Dra*III-*Xba*I fragment containing the entire UAS1 region and the area downstream thereof was removed from the promoter region of *STA2* of plasmid YCplac33-*STA2* and replaced with the corresponding area from the *MUC1* promoter, a 1 045 bp *Dra*III-*Xba*I fragment isolated from plasmid pMUU (23). This generated YCplac33-P*MUC1*-*STA2*, a plasmid identical to YCplac33-*STA2* with the only difference being the presence of the two *MUC1* promoter inserts of 20 bp and 64 bp.

A 1 675 bp *Xho*I-*Sna*BI fragment containing *MSN1* was obtained from the plasmid pMS2A (24) and cloned into the unique *Sal*I and *Sma*I sites of plasmid YEplac181 (10) to generate YEplac181-*MSN1*. A 3 326 bp *Eco*RI fragment containing *MSS11* was derived from the plasmid pMSS11-g (53) and cloned into the unique *Eco*RI site of plasmid YEplac181 to generate plasmid YEplac181-*MSS11*. A construct for regenerating the *URA3* marker in strains ISP15Δ*flo8* and ISP15Δ*msn1* was made by ligating a 1 586 bp *Eco*RV-*Pvu*II fragment, containing the kanamycin resistance marker from plasmid pUG6, into plasmid pJJ242 of which a 248 bp *Eco*RV-*Stu*I fragment was deleted from *URA3*.

The construction of plasmids with sequentially deleted promoter fragments upstream of *lacZ* is shown in Fig. 1. The sequences of all the primers used for these and other constructions are listed in Table 2. The forward primers contain *Sal*I sites and the reverse primers *Xho*I sites so that, when used in combination during PCR reactions, these primers yield fragments with 5′ *Sal*I and 3′ *Xho*I restriction sites.

Primers FP3, FP11 and FP12 were used together with primer RP10 to amplify PCR fragments M3-10, M11-10 and M12-10 from the *MUC1* promoter, using pMUU (23) as template. The 20 bp insert, present in the *MUC1* promoter but absent from *STA2*, occurs in the area between primers FP12 and FP13. The rest of the *MUC1* UAS1 area is identical to that of *STA2*. Primers FP3, FP11, FP12, FP13, FP14 and FP15 were used together with RP10 to generate PCR fragments S3-10, S11-10, S12-10, 13-10, 14-10 and 15-10, using YCplac33-*STA2* as template. Expand High Fidelity polymerase, obtained from Roche Diagnostics (Randburg, South Africa), was used for all PCR reactions. Primers F-M20 and R-M20 were hybridised to generate fragment M20, the 20 bp *MUC1* promoter insert, and primers F-M64 and R-M64 hybridised to generate fragment M64, the 64 bp *MUC1* promoter insert. These primers were designed to generate *Sal*I and *Xho*I compatible single stranded overhangs after pairwise annealing.

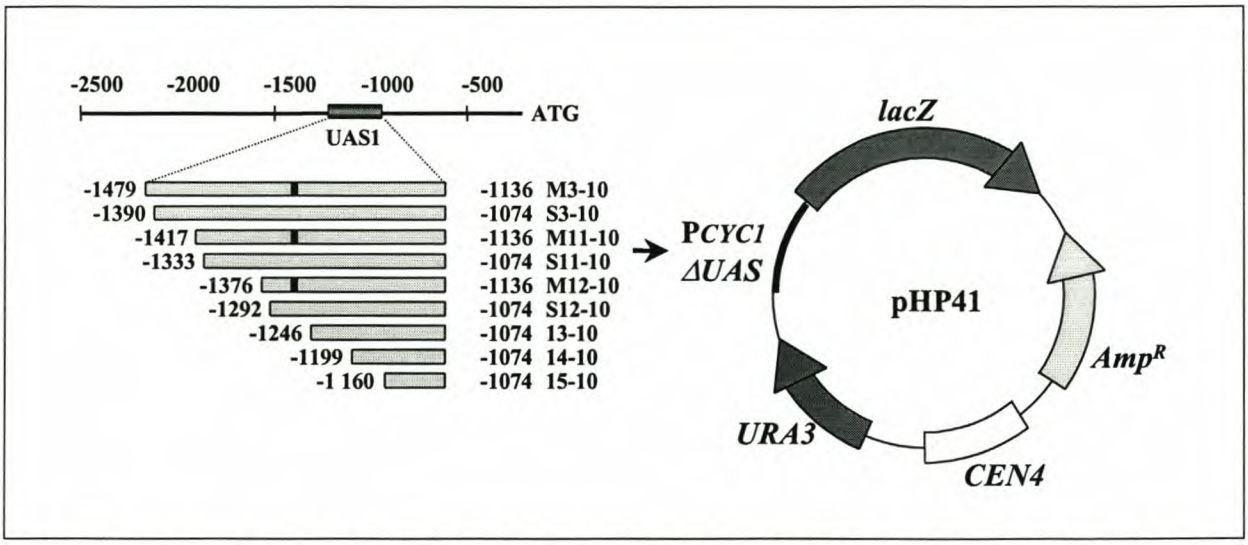


FIG. 1. Construction of a series of plasmids containing sequential deletions of the *STA2* and *MUC1* UAS1, upstream of the *lacZ* reporter gene in plasmid pHP41. The position of UAS1 and UAS2 relative to the translation initiation codon (ATG) of the *STA2* and *MUC1* ORFs are indicated and the positions of the fragments in the respective promoters given. The position of the 20 bp insert of the *MUC1* promoter is indicated by the black square.

Plasmids pHP41 (34), pLG670-Z (12) and pLGΔ312 (54) contain the *CYC1* promoter, fused in-frame to the *lacZ* reporter gene. The *CYC1* promoters present in pHP41 and pLG670-Z were modified in that the UASs were removed to yield low expression levels of *lacZ*, which makes it possible to identify sequences conferring activation. Plasmid pLGΔ312 contains the wild-type UAS which results in high levels of *lacZ* expression, thereby making it possible to identify sequences conferring repression. The *Xho*I site in the linker of pHP41 is not unique, therefore the plasmid was partially digested with *Xho*I, purified and subsequently digested with *Sal*I. Plasmid pLG670-Z was digested with both *Sal*I and *Xho*I and plasmid pLGΔ312 with only *Xho*I. The PCR amplification products was digested with *Sal*I and *Xho*I and subsequently ligated to pHP41, pLG670-Z and pLGΔ312.

**TABLE 2.** List of primers used to generate deletion fragments and *lacZ* fusions of the *STA2* and *MUC1* promoters

| Name | Sequence | Position relative to *STA2* ORF | Position relative to *MUC1* ORF |
|---|---|---|---|
| FP3 | 5′-acgc**gtcgac**aataaaggatccacgggtaa-3′ | -1 395 to -1 376 | -1 478 to -1 459 |
| FP11 | 5′-acgc**gtcgac**ctttgaggaataccggattg-3′ | -1 333 to -1 313 | -1 417 to -1 398 |
| FP12 | 5′-acgc**gtcgac**gtatgttctcacggctgtaa-3′ | -1 292 to -1 273 | -1 376 to -1 357 |
| FP13 | 5′-acgc**gtcgac**attaaactttcgcggcagga-3′ | -1 246 to -1 227 | -1 310 to -1 291 |
| FP14 | 5′-acgc**gtcgac**tcagtttctcggaatgtggc-3′ | -1 199 to -1 180 | -1 263 to -1 244 |
| FP15 | 5′-acgc**gtcgac**ctttgaggaataccggattg-3′ | -1 160 to -1 140 | -1 223 to -1 203 |
| RP10 | 5′-gatc**ctcgag**ataacggccgaaactctttg-3′ | -1 093 to -1 074 | -1 155 to -1 136 |
| RP16 | 5′-gatc**ctcgag**cgtaccagtgaagcctaatt-3′ | -990 to -1 110 | -1 058 to -1 077 |
| F-M20 | 5′-**tcgac**cccaataggaacgccggtaggc-3′ | | -1 313 to -1 333 |
| R-M20 | 5′-**tcgag**cctaccggcgttcctattgggg-3′ | | -1 313 to -1 333 |
| F-M64 | 5′-**tcgac**tccgagcgtttagaaggtgattgtaggcagaaattaactttg cggtaaaagaatgacattctttcc-3′ | | -869 to -933 |
| R-M64 | 5′-**tcgag**gaaagaatgtcattcttttaccgcaaagttaatttctgcctac aatcaccttctaaacgctcggag-3′ | | -869 to -933 |
| P*MUC1*-FX | 5′-gatctctagagaaatgtgggtcatcttttt-3′ | -437 to -463 | -439 to -465 |
| P*MUC1*-RB | 5′-ttaaggatccggtcatagtgtgcgtatatg-3′ | | -1 to -14 |
| P*STA2*-RB | 5′-cgcgggatccggtcatagtgtgcgtatatggatt-3′ | -1 to -18 | |

[a] The *Sal*I sites, present in all the forward primers (F), and the *Xho*I sites, present in all the reverse primers (R*), are indicated with bold text. The *Bam*HI and *Xba*I sites in the primers used for fusing the *MUC1* and *STA2* promoters to the *lacZ* reporter gene are underlined.

To generate plasmids containing the *MUC1* and *STA2* promoters fused in-frame to the *lacZ* reporter gene, a forward primer, P*MUC1*-FX, was used in combination with primers P*MUC1*-RB and P*STA2*-RB to amplify a 472 bp fragment containing the ATG and first 9 bp of the *lacZ* ORF fused to the first 460 bp of the *MUC1* and *STA2* promoters, respectively. The *Bam*HI site in the *lacZ* ORF and the *Xba*I site that occurs around position -460 in both the *MUC1* and *STA2* promoters were used to

clone these fragments into the unique *Bam*HI and *Xba*I sites of plasmid pHP41. The rest of the *MUC1* upstream region was inserted as a 3 257 bp *Avr*II-*Xba*I fragment, isolated from plasmid pMUU, and the rest of the *STA2* promoter as a 3 173 bp *Avr*II-*Xba*I fragment, isolated from pSP*STA2*, into the *Xba*I site of the plasmids with the 460 bp *MUC1* and *STA2* promoters fused to *lacZ*, generating plasmids p*MUC1-lacZ* and p*STA2-lacZ*, respectively. To delete the UAS1 areas from these plasmids, a partial *Bam*HI digestion was done, followed by complete digestion with *Eag*I. The 360 bp *STA2* UAS1 region and 380 bp *MUC1* UAS1 region were removed, the ends filled in using Klenow enzyme and subsequently religated to generate plasmids p*MUC1*ΔUAS1-*lacZ* and p*STA2*ΔUAS1-*lacZ*.

All constructed plasmids were sequenced to verify that no mutations occurred during the PCR amplification of the promoter fragments and that the constructs were in the correct orientation. All the constructs are listed in Table 3. Enzymes for DNA modification and restriction digestions were obtained from Roche Diagnostics (Randburg, South Africa). All DNA manipulations were done according to Sambrook *et al.* (45).

**Sequencing of the *STA2* and *STA3* promoters.**

To sequence the 5′ upstream region of *STA3*, a series of nine primers was synthesised, covering the entire promoter area and first part of the *STA3* ORF. The primers were designed from the available sequences of *STA1* and *STA2*. Plasmid p*STA3*-6-4 (59) was used as template to determine the nucleotide sequence. A 2 779 bp sequence comprising the *STA3* promoter and the first part of the ORF was submitted to the GenBank database and assigned accession number U95022.

The sequence of the *STA2* gene, upstream of position -2 500 was also determined to establish how far the homology between the *STA* genes and *MUC1* extends. For this purpose, a single reverse primer was designed from the *STA2* sequence and plasmid YCplac33-*STA2* was used as template for determining the nucleotide sequence. From the obtained sequence, an additional primer was made and again used with YCplac33-*STA2* as template. A 1462 bp sequence comprising the far upstream region of the *STA2* promoter was submitted to the GenBank database and assigned accession number AF169185.

**β-Galactosidase assays.**

After transformation, at least three colonies of each transformation were grown overnight in 10 ml of selective SCD media. From each overnight culture, 10 ml cultures of SCD, SCGE, SLAD and SPD were inoculated to an optical density (OD) of 0.1 at 600 nm and incubated to grow for 4-5 generations at 30°C to an OD of ~1.0. To obtain post-diauxic shift cultures, SCD cultures were incubated for longer periods until it reached an OD of > 3.0. The effect of osmotic shock on expression levels was determined in 10 ml selective SCD cultures that were grown to an OD of 1.0. Sterile NaCl was added to a final concentration of 0.7 M after which the cultures were

incubated at 30°C for 1h. The effect of heat shock was determined in 10 ml selective SCD cultures, grown to an OD of 1.0 and placed at 42°C for 1h. β-Galactosidase assays were done according to Ausubel *et al.* (3). Error margins were calculated for each set of assays and were usually less than 7.5% and never higher than 15%.

**Invasive growth and pseudohyphal development assays.**

Three colonies from a transformation were inoculated into SCD and grown to an $OD_{600}$ of 1.0. To assess the ability of these yeast cells to grow invasively into the agar, 10 µl of this liquid culture suspension was spotted onto SLAD, SCS, SCGE and SCD agar plates. Plates were incubated at 30°C and investigated for invasive growth at intervals of 2 days. Yeast colonies were washed off the surface of the agar by rubbing the surface of the plates with a gloved finger under running water. Cells that grew invasively into the agar cannot be washed off and are clearly seen below the surface of the agar.

Plates were photographed both before and after the washing process. After washing off the cells, each of the colonies were investigated for elongated cells or filaments under the 10X magnification of a light microscope (Nikon Optiphot-2) and photographs of cells below the agar surface were taken with a Matrox Intellicam 2 (Matrox Electronics Inc.).

**Plate assays to determine starch utilisation.**

The *STA2* gene encodes an extracellular glucoamylase which hydrolyses starch by liberating glucose molecules from the non-reducing end of the starch molecule (50). The presence of the *STA2* gene therefore enables most yeast strains to grow on starch as the sole carbon source. On plates containing starch as carbon source (SCS), a clear zone is formed around such starch-degrading colonies and the size of the colony, as well as the diameter of the zone, are indicative of the amount of glucoamylase secreted (39, 59). The expression of *STA2* in yeast strains was therefore determined by the size of the colonies and the clear zone around each of the colonies on SCS plates.

Yeast cells were grown in a 10 ml SCD culture until it reached an OD of 1.0. Of these cultures, 10 µl were spotted onto the different starch plates. Plates were incubated at 30 °C for 4-6 days, after which it was placed at 4 °C for 2 days to allow for the starch to precipitate. This precipitation of unutilised starch results in a clear zone around the colony where secreted glucoamylase hydrolysed the starch.

**Sequence analysis and homology searches.**

Homology searches in the yeast genome subdivision of GenBank were done with the BLAST software (2). Sequence fragment assembly and individual alignments between the STA genes and MUC1 were done using the OMIGA v1.1 package (Oxford Molecular Ltd, UK).

**TABLE 3.** Plasmids and constructs used in this study

| Plasmid | Relevant genotype | Source/reference |
|---|---|---|
| p*STA3*-6-4 | 2μ *URA3 STA3* | 59 |
| pSP*STA2* | *STA2* | 22 |
| PMS2A | *MSN1* | 24 |
| pMUU | *2μ URA3 MUC1* | 23 |
| pF415-1 | *CEN4 LEU2 FLO8* | 18 |
| pJJ242 | *URA3* | 16 |
| pUG6 | $kan^R$ | J. H. Hegemann |
| YEplac112 | 2μ *TRP1* | 10 |
| YEplac112-*MSN1* | 2μ *TRP1 MSN1* | 8 |
| YEplac112-*MSS11* | 2μ *TRP1 MSS11* | 8 |
| YEplac112-*FLO8* | 2μ *TRP1 FLO8* | This work |
| YEplac181 | 2μ *LEU2* | 10 |
| YEplac181-*MSN1* | 2μ *LEU2 MSN1* | This work |
| YEplac181-*MSS11* | 2μ *LEU2 MSS11* | This work |
| YEplac181-*FLO8* | 2μ *LEU2 FLO8* | This work |
| pΔ*flo8* | Δ*flo8*::*URA3* | This work |
| pΔ*ura3*::*kan* | Δ*ura3*::$kan^R$ | This work |
| YCplac22 | *CEN4 TRP1* | 10 |
| YCplac22-*FLO8* | *CEN4 TRP1 FLO8* | 8 |
| YCplac33 | *CEN4 URA3* | 10 |
| YCplac33-*STA2* | *CEN4 URA3 STA2* | This work |
| YCplac33-P*MUC1-STA2* | 2μ *URA3* P*MUC1-STA2* | This work |
| pHP41 | *CEN4 URA3* P*CYC1*ΔUAS-*lacZ* | 34 |
| pP*MUC1-lacZ* | *CEN4 URA3* P*MUC1-lacZ* | This work |
| pP*MUC1*ΔUAS1-*lacZ* | *CEN4 URA3* P*MUC1*ΔUAS1-*lacZ* | This work |
| pP*STA2-lacZ* | *CEN4 URA3* P*STA2-lacZ* | This work |
| pP*STA2*ΔUAS1-*lacZ* | *CEN4 URA3* P*STA2*ΔUAS1-*lacZ* | This work |
| pHP41 + S3-10 | *CEN4 URA3* P*CYC1*ΔUAS-*lacZ* + S3-10 | This work |
| pHP41 + M3-10 | *CEN4 URA3* P*CYC1*ΔUAS-*lacZ* + M3-10 | This work |
| pHP41 + S11-10 | *CEN4 URA3* P*CYC1*ΔUAS-*lacZ* + S11-10 | This work |
| pHP41 + M11-10 | *CEN4 URA3* P*CYC1*ΔUAS-*lacZ* + M11-10 | This work |
| pHP41 + S12-10 | *CEN4 URA3* P*CYC1*ΔUAS-*lacZ* + S12-10 | This work |
| pHP41 + M12-10 | *CEN4 URA3* P*CYC1*ΔUAS-*lacZ* + M12-10 | This work |
| pHP41 + 13-10 | *CEN4 URA3* P*CYC1*ΔUAS-*lacZ* + 13-10 | This work |
| pHP41 + 14-10 | *CEN4 URA3* P*CYC1*ΔUAS-*lacZ* + 14-10 | This work |
| pHP41 + 15-10 | *CEN4 URA3* P*CYC1*ΔUAS-*lacZ* + 15-10 | This work |
| pHP41 + M20 | *CEN4 URA3* P*CYC1*ΔUAS-*lacZ* + M20 | This work |
| pHP41 + M64 | *CEN4 URA3* P*CYC1*ΔUAS-*lacZ* + M64 | This work |
| pLG670-Z | 2μ *URA3* P*CYC1*ΔUAS-*lacZ* | 12 |
| pLG670-Z + M20 | 2μ *URA3* P*CYC1*ΔUAS-*lacZ* + M20 | This work |
| pLG670-Z + M64 | 2μ *URA3* P*CYC1*ΔUAS-*lacZ* + M64 | This work |
| pLGΔ312 | 2μ *URA3* P*CYC1-lacZ* | 54 |
| pLGΔ312 + M20 | 2μ *URA3* P*CYC1-lacZ* + M20 | This work |
| pLGΔ312 + M64 | 2μ *URA3* P*CYC1-lacZ* + M64 | This work |

## RESULTS

### Similar and divergent regulation of *STA2* and *MUC1*.

To determine the extent of the co-regulation between *MUC1* and *STA2*, we determined the β-galactosidase activity of the *MUC1* and *STA2* promoters fused to the *lacZ* reporter gene with plasmids pP*MUC1-lacZ* and pP*STA2-lacZ*, respectively, in different growth conditions as well as in the presence of multiple copies of the transcriptional activators *FLO8*, *MSN1* and *MSS11*. The results for these assays are given in Tables 4 and 5.

**TABLE 4.** Expression levels from the *MUC1* and *STA2* wild-type promoters fused to the *lacZ* reporter gene on a centromeric plasmid in *S. cerevisiae* strains L5366h (n) and L5366 (2n) from the Σ1278b genetic background

| | SCD (mid-log) $OD_{600}$ of ~1 | | SCD (post-diauxic) $OD_{600}$ of >3 | | SLAD 50μM $(NH_4)_2SO_4$ | | SCGE 3% glycerol 3% ethanol | | SCD osmoshock 0.7 M NaCl | | SCD heat shock 42°C for 1h | | SPD 0.1% proline | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **n** | **2n** | **n** | **2n** | **n** | **2n** | **n** | **2n** | **n** | **2n** | **n** | **2n** | **n** | **2n** |
| pP*STA2-lacZ* | 1.02 | 0.33 | 2.66 | 0.77 | 0.30 | 0.15 | 13.9 | 0.68 | 0.31 | 0.30 | 0.34 | 0.49 | 0.28 | 0.32 |
| pP*MUC1-lacZ* | 0.21 | 0.22 | 0.24 | 0.22 | 0.40 | 0.30 | 0.60 | 0.14 | 0.23 | 0.33 | 0.24 | 0.25 | 0.22 | 0.19 |

The data show that reporter gene expression levels observed for both pP*MUC1-lacZ* and pP*STA2-lacZ* were low in most conditions, similar to those reported for genes transcribed at low levels, e.g. P*HIS3*-lacZ (3). *MUC1* promoter-dependent expression levels were however consistently lower than *STA2* promoter-dependent levels.

The data indicate that in haploid strains, both pP*MUC1-lacZ* and pP*STA2-lacZ* are repressed in rich glucose media, derepressed in glycerol/ethanol media and can be induced by multiple copies of *FLO8*, *MSN1* and *MSS11*. In the haploid Σ1278b strain (Table 4), pP*STA2-lacZ* has 13.6-fold higher expression levels when grown in glycerol/ethanol (SCGE) media than on media containing glucose as carbon source (SCD). In the same strain and the same conditions, expression levels of the pP*MUC1-lacZ* construct increased 3-fold. Interestingly, this increase is nearly completely absent in diploid strains, where pP*STA2-lacZ* expression was only increased 2-fold, and no increase at all was observed for pP*MUC1-lacZ*. A 2.6-fold increase in expression levels of pP*STA2-lacZ* was also seen in the post-diauxic shift SCD cultures, where most of the glucose has been utilised. Again, this post-diauxic shift induction could not be observed for the pP*MUC1-lacZ* construct. These data are in good agreement with previous reports on the transcriptional activity of either *STA2* or *MUC1*, determined by Northern blot analysis. Transcription of *MUC1* was reported to be repressed in rich media (27, 28, 44) or media containing glucose as carbon source (8) whereas *STA1* and *STA2* was reported to be repressed in all media containing readily metabolised carbon sources such as glucose (5, 8, 17, 20, 41, 47).

Despite the high homology between the *STA2* and *MUC1* promoters, p*PMUC1-lacZ* responds differently to some of the growth conditions. It is, in particular, activated in media containing limiting amounts of $(NH_4)_2SO_4$ as nitrogen source (SLAD), where a 2-fold increase in expression levels of p*PMUC1-lacZ* was observed, whereas p*PSTA2-lacZ* was not. Another clear difference can be seen in the response to multiple copies of *FLO8*. Whereas the *STA2* promoter is strongly induced in both glucose and glycerol/ethanol media, the *MUC1* promoter was only activated in media containing glucose. The data show that both promoters do not respond to osmo- (NaCl) or heatshock (42°C) conditions, and are not induced by poor nitrogen sources like proline (SPD).

The effect of the genetic background on the expression levels of the two genes can be observed when comparing the p*PMUC1-lacZ* and p*PSTA2-lacZ* expression levels of the wild-type ISP20 strain in SCD and SCGE media (Table 5), to that of the Σ1278b haploid strain, L5366h, in the same conditions (Table 4). Whereas expression levels in SCGE media was 13.6-fold higher than on SCD media for p*PSTA2-lacZ* in the Σ1278b haploid strain, only a 3.7-fold difference was observed for ISP20. A similar effect was seen for p*PMUC1-lacZ* where expression levels in SCGE media was 2.9-fold higher than in SCD media in the Σ1278b haploid strain, but only 1.5-fold in ISP20. The general tendencies with regard to repression and activation, however, were always the same.

**The *STA10* repressive effect in S288C derived strains is due to a mutation in *FLO8*.**

When compared to feral *S. cerevisiae* strains, most laboratory strains, e.g. S288C, exhibit a 20-fold reduction in *STA1-3* expression (41). This phenomenon was believed to be due to the presence of a repressor, designated *STA10* (37). It was, however, recently reported that most laboratory strains contain a point mutation in *FLO8*, a transcriptional activator of the flocculation genes, which render these strains unable to flocculate, grow invasively or form pseudohyphae (26). Due to the extensive homology between the *STA2* and *MUC1* promoter regions and since *FLO8* was shown to be required for transcription of *MUC1* (44), we investigated whether a genetic relationship between *STA10* and *FLO8* exists.

From Fig. 2a it is evident that, in the S288C genetic background, the *STA10* repressive effect is due to the lack of the *FLO8*-encoded activator and not due to the presence of a repressor. Strain FY23, isogenic to the S288C genetic background (56), was transformed with a centromeric plasmid, YCplac33, bearing *STA2* and the centromeric vector, YCplac22, without any insert. This strain is unable to utilise starch as sole carbon source. The same strain, transformed with centromeric plasmids, YCplac33-*STA2* and YCplac22-*FLO8*, bearing *STA2* and *FLO8*, respectively, was fully able to degrade starch. To verify the requirement of *FLO8* for *STA1-3* expression, *FLO8* was deleted from the genomes of *sta10* strains ISP15 and ISP20. As can be seen in Fig. 2b, the absence of *FLO8* reduced the ability of these

strains to utilise starch, resulting in a phenotype, similar to what was reported for *STA10*.

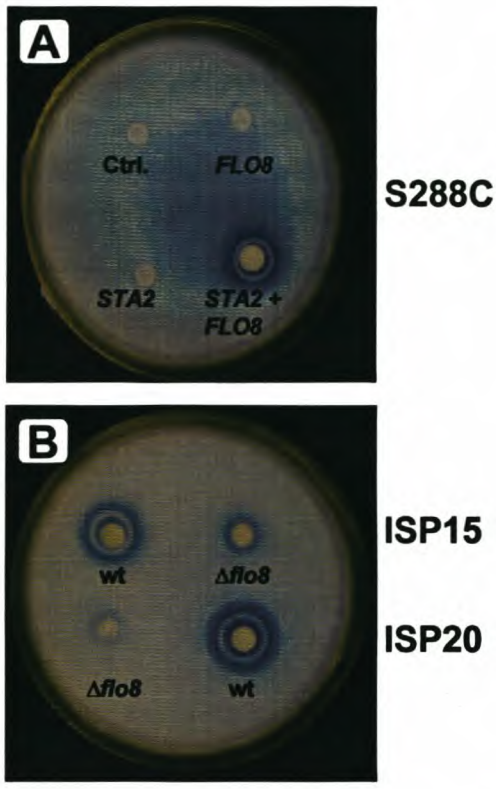


FIG. 2A. The S288C-derived *STA10* strain, FY23, transformed with the centromeric plasmids YCplac22 and YCplac33 (Ctrl.), YCplac33 and YCplac22-*FLO8* (*FLO8*), YCplac22 and YCplac33-*STA2* (*STA2*) and YCplac22-*FLO8* and YCplac33-*STA2* (*STA2* + *FLO8*) on plates containing potato starch as sole carbon source (SCS). Cells that are unable to express *STA2* are unable to grow, whereas cells that do express *STA2* sufficiently, produce extracellular glucoamylase which enable them to grow. The clear zone around the $Sta^+$ colony is due to the hydrolysis of the starch in the media. FIG. 2B. The *sta10* strains ISP15 and ISP20 with wild-type (wt) and disrupted (Δ*flo8*) *FLO8* loci on media containing starch as sole carbon source. The wild-type strains express *STA2* sufficiently to sustain growth on starch, whereas the Δ*flo8* strains show a clear reduction in glucoamylase expression and are therefore unable to grow.

**Flo8p acts independently of Msn1p but upstream of Mss11p.**

*FLO8* is one of several transcriptional regulators required for the transcriptional activation of the *STA1-3* genes and *MUC1*. The epistatic relationships between these transcriptional regulators revealed a complex signal transduction network that converge at the promoter of the *MUC1* (8, 44) and *STA1-3* (8) genes. To establish the epistatic relationship between *FLO8* and other transcriptional regulators required for *MUC1* and *STA1-3* expression, *MSN1* and *MSS11* present on 2μ-plasmids, were transformed into strains with deleted *FLO8* loci, ISP15Δ*flo8* and ISP20Δ*flo8*. A 2μ-plasmid carrying *FLO8* was also transformed into strains with deleted *MSN1* and *MSS11* loci. These strains were spotted onto SLAD (limited nitrogen source) and

SCS (potato starch as carbon source) plates and scored for their ability to grow invasively into the agar and to utilise starch.

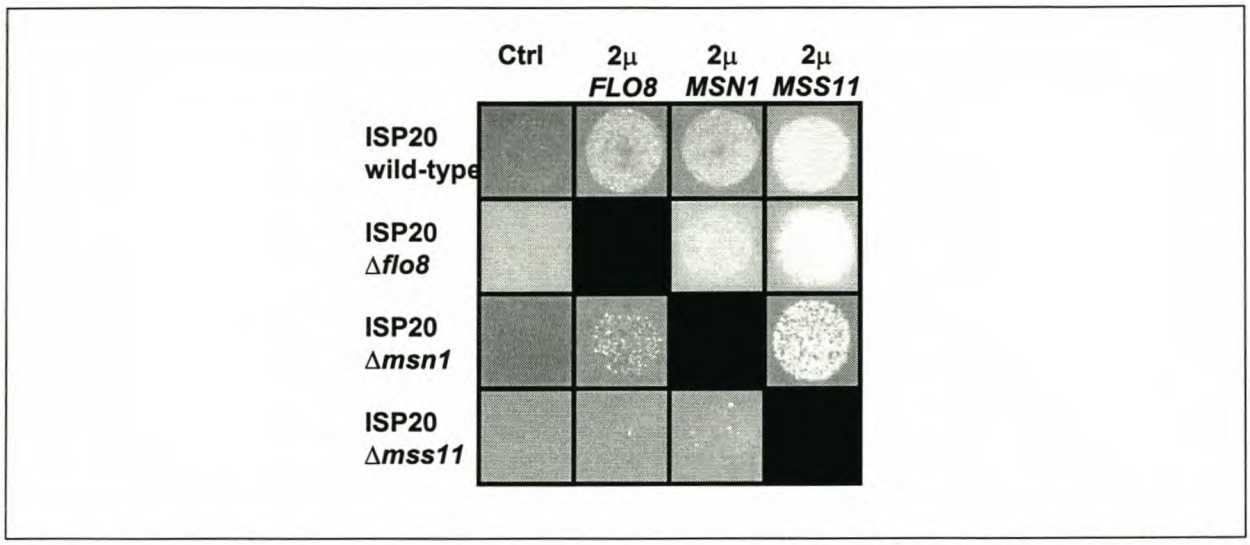


FIG. 3. Determining the epistatic relationships between *MSN1*, *MSS11* and *FLO8* on limited nitrogen (SLAD) media in strain ISP20. Wild-type ISP20, ISP20Δ*flo8*, ISP20Δ*msn1* and ISP20Δ*mss11* were transformed with YEplac112 without insert (Ctrl.), YEplac112-*FLO8* (2μ *FLO8*), YEplac112-*MSN1* (2μ *MSN1*) and YEplac112-*MSS11* (2μ *MSS11*). Cells were grown in SCD media until mid-log phase whereupon 10 μl of the respective cell suspensions were spotted onto limited nitrogen (SLAD) media. Plates were incubated for 6 days after which the growth on top of the agar was washed off. Cells that grew invasively into the agar could not be washed off and were photographed.

The results of the epistasis analysis on limited nitrogen SLAD media can be seen in Fig. 3. The wild-type strain was able to grow invasively into the agar. Multiple copies of *FLO8*, *MSN1* and especially *MSS11*, significantly increased the invasive growth of the strain. Deletions of the *FLO8*, *MSN1* and *MSS11* loci on the other hand, completely eliminated invasive growth. In strains with deleted *FLO8* loci, multiple copies of *MSN1* and *MSS11* were able to restore invasive growth to above wild type levels, with *MSS11* being the more efficient. Similar results were obtained when multiple copies of *FLO8* and *MSS11* were transformed into strains with a deleted *MSN1* loci. However, multiple copies of *MSN1* or *FLO8* were unable to restore invasive growth in a strain with deleted *MSS11* loci. The data indicate that (i) *FLO8* and *MSN1* act independently of each other when relaying the invasive growth signal, and that (ii) Mss11p functions downstream of - or is required for activity by - both Msn1p and Flo8p. Similar results were obtained with strain ISP15 (data not shown). The epistasis analysis was also performed with respect to the ability to utilise starch as a carbon source, and the same conclusion was reached (data not shown).

**Role of UAS1 in determining expression levels of *STA2* and *MUC1*.**

Deletions of the UAS1 area from the promoters of *MUC1* and *STA2* (Table 5), indicated that this area is required for glucose repression and transcriptional activation by *MSS11*, *FLO8* and *MSN1*. The data show that multiple copies of *MSN1* or of *MSS11* were still able to increase expression levels conferred by the *MUC1* and

*STA2* promoters when UAS1 is deleted. This suggests that the corresponding gene products act through regulatory sequences both within and outside of UAS1. Interestingly, the same does not apply for multiple copies of *FLO8*, which are unable to induce reporter gene expression when UAS1 is deleted. Flo8p is therefore completely dependent on sequences within the UAS1 region to assert its effect on *MUC1* and *STA2* transcription.

The data furthermore show that UAS1 plays a significant role in glucose-dependent repression of the two promoters. In media that contain glucose as carbon source (SCD), the pP*STA2*ΔUAS1-*lacZ* and P*MUC1*ΔUAS1-*lacZ* constructs exhibited a 1.8 and 1.7 fold increase, respectively, in expression when compared to the wild-type promoter. The UAS1 region therefore confers some glucose repression on the *STA2* and *MUC1* promoters.

However, both ΔUAS1 promoters no longer showed any significant increases between glucose (SCD) and glycerol-ethanol (SCGE) media, suggesting that glucose-dependent repression has been eliminated. In addition, the ΔUAS1 constructs failed to reach expression levels conferred by the wild-type promoter in derepressed conditions, indicating that sequences required for activation must have been deleted.

**TABLE 5.** Effect of multiple copies of *FLO8*, *MSN1* and *MSS11* on expression levels of *MUC1* and *STA2* wild-type promoters, as well as promoters from which the UAS1 area were deleted, fused to the *lacZ* reporter gene on a centromeric plasmid in *S. cerevisiae* strain ISP20

| **Constructs** | **Wild-type** | | **2μ-*MSS11*** | | **2μ-*FLO8*** | | **2μ-*MSN1*** | |
|---|---|---|---|---|---|---|---|---|
| | **SCD** | **SCGE** | **SCD** | **SCGE** | **SCD** | **SCGE** | **SCD** | **SCGE** |
| pP*STA2-lacZ* | 1.10 | 4.03 | 12.76 | 13.31 | 20.67 | 13.30 | 36.42 | 19.62 |
| pP*STA2*-ΔUAS1-*lacZ* | 2.03 | 2.66 | 19.63 | 6.32 | 0.82 | 1.81 | 7.83 | 34.50 |
| pP*MUC1-lacZ* | 0.33 | 0.51 | 1.41 | 3.15 | 0.66 | 0.36 | 6.33 | 2.88 |
| pP*MUC1*-ΔUAS1-*lacZ* | 0.55 | 0.34 | 1.64 | 1.06 | 0.49 | 0.23 | 1.22 | 3.81 |

Compared to the wild-type strain, multiple copies of *MSS11* resulted in a 4.3-fold increase in expression levels from the native *MUC1* promoter and an 11.6-fold increase from the native *STA2* promoter on SCD media. The effect of multiple copies of *MSS11* in SCGE medium was, however, more pronounced for the native *MUC1* promoter, since a 6.2-fold increase in *lacZ* expression was observed, whereas a 3.3-fold increase in expression was observed for the native *STA2* promoter under the same conditions. Expression levels of *lacZ* under control of the *STA2* promoter was, however, always much higher than those observed for the *MUC1* promoter.

In the presence of multiple copies of *MSS11* on SCGE media, deletion of the UAS1 area from the promoters of *MUC1* and *STA2* still results in increased promoter activity, but at levels which are respectively 2.9- and 2.1-fold lower than those of the wild-type promoter under the same conditions. This indicates that *MSS11* exerts its activating effect in part via this area. In SCD media, however, the opposite happens

since an increase in activity was observed for both the *MUC1* and *STA2* promoters. This again indicates that other areas are required for activation by *MSS11*. However, the elimination of the glucose repression exerted by the UAS1 region allows higher levels of activation by multiple copies of *MSS11*.

Multiple copies of *FLO8* have a more pronounced effect on expression levels of *STA2* than *MUC1*. For the native *STA2* promoter, an 18.8-fold increase in *lacZ* expression was observed in SCD, whereas only a two-fold increase in *lacZ* expression levels was observed for the *MUC1* promoter. In SCGE, multiple copies of *FLO8* were able to significantly activate expression of the *STA2* dependent reporter gene, but not of the *MUC1* promoter dependent reporter gene. Deletion of the UAS1 area from both the *MUC1* or *STA2* promoters resulted in a complete loss of *FLO8* dependent activation.

**TABLE 6.** Identification of *FLO8* responsive regions in the UAS1 area of *STA2* and *MUC1* in strain ISP15 in SCD media

| **Constructs** | ***FLO8* (wild-type)** | **2μ-*FLO8*** | ***Δflo8*** |
|---|---|---|---|
| pHP41 | 0.55 | 0.39 | 0.53 |
| pHP41 + S3-10 | 0.17 | 0.49 | 0.15 |
| pHP41 + M3-10 | 0.14 | 0.27 | 0.12 |
| pHP41 + S11-10 | 0.13 | 0.33 | 0.10 |
| pHP41 + M11-10 | 0.12 | 0.23 | 0.10 |
| pHP41 + S12-10 | 0.13 | 0.23 | 0.04 |
| pHP41 + M12-10 | 0.09 | 0.24 | 0.09 |
| pHP41 + 13-10 | 0.23 | 0.81 | 0.16 |
| pHP41 + 14-10 | 0.16 | 0.21 | 0.06 |
| pHP41 + 15-10 | 0.88 | 1.50 | 0.33 |

Multiple copies of *MSN1*, on the other hand, had a more pronounced effect on expression levels from both promoters in both SCD and SCGE media. In SCD medium, the wild-type *MUC1* and *STA2* promoters yielded 19.2- and 33-fold increases in activity, respectively, in the presence of multiple copies of *MSN1* and a 5.6- and 4.9-fold increase in activity in SCGE medium. Deletion of the UAS1 area from the promoters of *STA2* and *MUC1*, resulted in reductions in expression levels in the presence of multiple copies of *MSN1* in SCD medium. Compared to the levels of activity from the native promoters under the same conditions, a 5.2-fold decrease for the *MUC1* promoter and a 4.7-fold decrease for the *STA2* promoter were observed. In SCGE medium, however, multiple copies of *MSN1* resulted in higher expression levels from the *STA2* and *MUC1* promoters of which the UAS1 region was deleted, than the native promoters. Compared to the wild-type promoters under the same conditions, a 1.3-fold increase for the *MUC1* promoter and a 1.8-fold increase for the *STA2* promoter, both of which the UAS1 area were deleted, were observed.

**Identification of regulatory regions within the UAS1 area.**

Both a previous report (44) and the data presented in Table 5, suggest that *FLO8* confers regulation via a sequence within the UAS1 area. Our data (Table 5) furthermore show that Msn1p and Mss11p act in part via the same region. To better define this area, sequential deletions of UAS1 were generated through PCR amplification, using the promoters of *MUC1* and *STA2* as templates. These fragments were introduced into the UAS-less *CYC1* promoter fused to *lacZ* as reporter gene on the centromeric vector pHP41 (Fig. 1). These constructs, as well as the vector without any insert as control, were transformed into different genetic backgrounds and the levels of β-galactosidase conferred by these fragments determined.

**TABLE 7.** Identification of *MSS11* responsive regions in the UAS1 area of *STA2* and *MUC1* in strain ISP15 in SCGE media

| **Constructs** | ***MSS11* (wild-type)** | **2μ-*MSS11*** | ***mss11*** |
|---|---|---|---|
| pHP41 | 0.47 | 0.42 | 0.53 |
| pHP41 + S3-10 | 0.32 | 0.70 | 0.15 |
| pHP41 + M3-10 | 0.18 | 0.39 | 0.13 |
| pHP41 + S11-10 | 0.23 | 0.48 | 0.13 |
| pHP41 + M11-10 | 0.14 | 0.39 | 0.12 |
| pHP41 + S12-10 | 0.04 | 0.26 | 0.08 |
| pHP41 + M12-10 | 0.08 | 0.26 | 0.09 |
| pHP41 + 13-10 | 0.30 | 0.47 | 0.15 |
| pHP41 + 14-10 | 0.22 | 0.29 | 0.20 |
| pHP41 + 15-10 | 1.04 | 1.33 | 0.64 |

To locate the sequences in UAS1 through which *FLO8*, *MSS11* and *MSN1* confer activity, we transformed the UAS1 sequential deletion constructs and the vector without any insert as control, into strains ISP15, ISP15Δ*flo8*, ISP15Δ*mss11* and ISP15Δ*msn1*. The wild-type strain represent the expression levels conferred by single copies of *FLO8*, *MSS11* and *MSN1*, and the deletion strains the absence of the respective factors. To determine the effect of multiple copies of *FLO8*, *MSS11* and *MSN1* on expression levels, we co-transformed the deletion constructs into the wild-type strain, ISP15, along with YEplac112-*FLO8*, YEplac112-*MSS11* or YEplac112-*MSN1*, i.e. 2μ-plasmids bearing *FLO8*, *MSS11* and *MSN1*, respectively. The expression levels conferred by the deletion constructs in these strains are given in Table 6 (*FLO8*), Table 7 (*MSS11*) and Table 8 (*MSN1*). From the data in these tables, it is clear that the UAS1 area, inserted in the *CYC1* promoter upstream of the *lacZ* reporter gene, conferred largely similar regulation patterns to the full *STA2* and *MUC1* promoters, which is a confirmation of the results obtained with deletions of this area from the native promoters (Table 5). It is repressed in media containing glucose

as carbon source, derepressed in media containing glycerol/ethanol as carbon source and subject to activation by *FLO8*, *MSS11* and *MSN1*.

For the *MUC1* UAS1 region (pHP41 + M3-10), a 1.9-fold increase in expression was observed with *FLO8* present in multiple copies (Table 6). Expression levels of the same construct, however, were only slightly lower in the *flo8* strain. For the *STA2* UAS1 region (pHP41 + S3-10), a similar expression pattern was observed, since only a slight reduction was observed in the *flo8* strain but a 2.9-fold increase when *FLO8* was present in multiple copies. The smallest fragment, 15-10, is still subject to activation by *FLO8*, since multiple copies of *FLO8* resulted in an almost 1.7-fold increase in expression levels for this 80 bp fragment. A deletion of *FLO8* also resulted in a 2.6-fold decrease in expression levels, suggesting that Flo8p acts through a sequence in this fragment to confer activation of *STA2* and *MUC1*.

**TABLE 8.** Identification of *MSN1* responsive regions in the UAS1 area of *STA2* and *MUC1* in strain ISP15 in SCD media

| **Constructs** | ***MSN1* (wild-type)** | **2μ-*MSN1*** | ***msn1*** |
|---|---|---|---|
| pHP41 | 0.55 | 0.77 | 0.69 |
| pHP41 + S3-10 | 0.17 | 0.79 | 0.15 |
| pHP41 + M3-10 | 0.14 | 0.75 | 0.14 |
| pHP41 + S11-10 | 0.13 | 0.60 | 0.15 |
| pHP41 + M11-10 | 0.12 | 0.59 | 0.10 |
| pHP41 + S12-10 | 0.13 | 0.68 | 0.10 |
| pHP41 + M12-10 | 0.09 | 0.56 | 0.11 |
| pHP41 + 13-10 | 0.23 | 0.70 | 0.42 |
| pHP41 + 14-10 | 0.16 | 2.21 | 0.21 |
| pHP41 + 15-10 | 0.88 | 3.61 | 0.23 |

When compared to the wild-type strain, it is evident that expression levels conferred by all the UAS1 deletion fragments were higher in the presence of multiple copies of *MSS11* and lower in the Δ*mss11* background (Table 7). As observed for *FLO8*, the smallest fragment, 15-10, still conferred a 1.3-fold increase in reporter gene expression when *MSS11* was present in multiple copies and a 1.6-fold decrease in expression in a Δ*mss11* strain, suggesting that Mss11p also acts through a sequence in this area to confer activation of *STA2* and *MUC1*.

Expression levels conferred by all fragments were the highest in the presence of multiple copies of *MSN1*. As with *MSS11* and *FLO8*, the smallest fragment, 15-10, still exhibited *MSN1*-dependent behaviour. Multiple copies of *MSN1* resulted in a 4.1-fold increase and the deletion of *MSN1* resulted in a 3.8-fold decrease.

With the data from Tables 6, 7 and 8, it is clear that a strong repressive element was present in all fragments except fragment 15-10. The deletion of the area immediately upstream of 15-10, i.e. the area still present in 14-10 but removed from 15-10, resulted in the biggest increases in expression levels. This would suggest that

a *cis*-acting element, conferring repression on UAS1, is present in the sequence immediately upstream of 15-10. Fragment 15-10 was, however, still susceptible to activation by Flo8p, Mss11p and Msn1p, since strains transformed with multiple copy plasmids bearing *FLO8*, *MSS11* or *MSN1*, resulted in higher expression levels than the wild-type strain. Concomitantly, expression levels for fragment 15-10 were also lower in strains with deleted *FLO8*, *MSS11* and *MSN1* loci.

Fragments M12-10 and S12-10 exhibited low levels of activity in most conditions tested and in all genetic backgrounds investigated, except when *FLO8*, *MSS11* or *MSN1* was present in multiple copies. A Mig1p binding site present in this fragment might explain some of the observed decreases, e.g. as in SCD media. It was shown that Mig1p is not involved in repression of the *STA* genes (17), but at the large distance from the ORF in the native promoter context, the presence of this binding site might not be relevant. However, in the *CYC1* promoter of the reporter plasmid, pHP41, this site is much closer to the open reading frame and might therefore become relevant. In this case, this result would be artefactual.

**Effect of the small *MUC1* promoter inserts on expression levels.**

Unlike *MUC1*, the *STA1-3* genes are not present in the genomes of the S288C-derived laboratory strains that were used in the sequencing of the *S. cerevisiae* genome. Laboratories working on starch metabolism in *S. cerevisiae* therefore contributed the sequences of the *STA1* and *STA2* genes. *STA3* is the only member of the *STA* gene family that had not been sequenced to date. To establish whether the promoter is identical to those of the other members of the family, the 5′ upstream region of *STA3* was sequenced and compared to the available sequences of *STA1* and *STA2*. The sequence proved to be identical to those of the *STA1* and *STA2* promoters, with the exception of some single nucleotide substitutions (data not shown). The sequence was submitted to GenBank and assigned accession number U95022.

Only 2 500 bp of the upstream regions of the *STA2* gene had been sequenced to date. An additional 1 462 bp of the *STA2* promoter, upstream of position -2 500 relative to the *STA2* ORF, was sequenced to see how far the homology between the upstream regions of *MUC1* and *STA2* stretches. The sequence was submitted to GenBank and assigned accession number AF169185. An alignment of this sequence with the upstream sequence of *MUC1* revealed that the homology extends over more than 3.9 kb. The 20 bp and 64 bp sequences found at nucleotides -1 333 to -1 313 and nucleotides -933 to -869 of the *MUC1* promoter are not present in any of the *STA1-3* upstream regions and thorough BLAST homology searches (2) revealed that the sequences thereof do not have significant homology to any other submitted sequence. This suggests the possibility of a unique regulatory role for these inserts in the *MUC1* promoter.

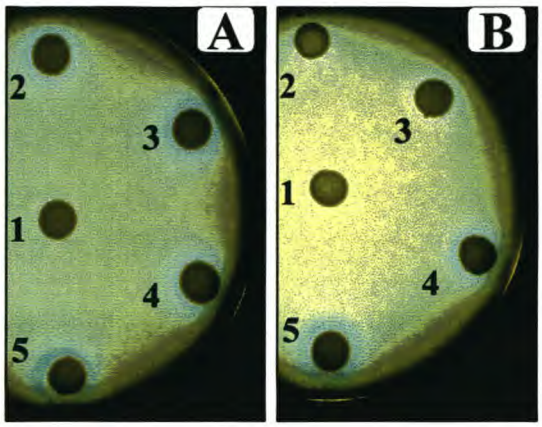


FIG. 4A. SCS plate (containing 2 % corn starch as carbon source) to investigate the starch utilisation of *S. cerevisiae* JM2508 transformed with (1) plasmids YCplac33 and YEplac112 without any inserts; (2) YCplac33-*STA2* and YEplac112; (3) YCplac33-*STA2* and YEplac112-*FLO8*; (4) YCplac33-*STA2* and YEplac112-*MSN1* and (5) YCplac33-*STA2* and YEplac112-*MSS11*. FIG. 4B. SCS plate (containing 2 % corn starch as carbon source) to investigate the starch utilisation of *S. cerevisiae* JM2508 transformed with (1) plasmids YCplac33 and YEplac112 without any inserts; (2) YCplac33-P*MUC1-STA2* and YEplac112; (3) YCplac33-P*MUC1-STA2* and YEplac112-*FLO8*; (4) YCplac33-P*MUC1-STA2* and YEplac112-*MSN1* and (5) YCplac33-P*MUC1-STA2* and YEplac112-*MSS11*.

From the expression levels of the *STA2* and *MUC1* UAS1 deletion constructs, given in Tables 6, 7 and 8, it is evident that the presence of the 20 bp *MUC1* promoter insert in constructs M3-10 and M11-10 resulted in decreases in expression levels. This reduction was reproducible in all strains and most conditions tested (data not shown). The only other differences between the *STA1-3* upstream regions and that of *MUC1* exist around the TATA-boxes. *MUC1* has only one functional TATA-box at position -100 whereas *STA1-3* has two at positions -75 and -100 (51). To investigate whether these inserts are the major factors determining the decreased expression levels observed for the *MUC1* upstream region, and that it is not contributed by any other dissimilarity between the two promoters, e.g. the use of different TATA-boxes, we took advantage of the fact that the *STA1-3* genes could be used as reporter genes in a glucoamylase plate-assay. Plasmids YCplac33-*STA2*, bearing the wild-type *STA2* gene under its native promoter, and YCplac33-P*MUC1-STA2*, which is identical but for the presence of the 20 and 64 bp *MUC1* promoter inserts, were transformed into strain JM2508, which does not contain any of the *STA1-3* genes in its genome. In addition, the different transcriptional activators of *STA2*, i.e. *FLO8*, *MSN1* and *MSS11* present on 2μ plasmids, were co-transformed along with YCplac33-*STA2* and YCplac33-P*MUC1-STA2* into strain JM2508. The different transformants were grown on SCD media until it reached mid-log phase ($OD_{600}$ = 1.0) before 10 μl of these cell suspensions were spotted onto corn starch plates (SCS). Expression levels of the *STA2* gene are reflected in the size of the halo around the different colonies. In Fig. 4a, it is evident that the yeast strain containing only the plasmids YCplac33 and YEplac112, was unable to utilise starch whereas the cells transformed with the wild-type *STA2* gene were able to degrade starch efficiently. The presence of

multiple copies of *FLO8*, *MSN1*, and *MSS11* clearly resulted in increased production of glucoamylase when the *STA2* gene was regulated by its native promoter. Fig. 4b shows the expression levels of the different colonies of JM2508, transformed with a copy of the *STA2* gene, which has the two *MUC1* promoter inserts in its upstream region. The strain without *STA2* was unable to degrade starch, as expected. Glucoamylase production from *STA2* with the *MUC1* promoter inserts in its upstream area, YCplac33-P*MUC1*-*STA2*, was almost undetectable. Only multiple copies of *FLO8*, *MSN1* or *MSS11* were able to overcome this repressive effect, resulting in visually detectable expression levels of *STA2*, albeit at more reduced levels when compared to strains bearing *STA2* under regulation of its native promoter (Fig. 4a). Interestingly, multiple copies of *MSN1* and *MSS11* were able to overcome the repressive effect conferred by the *MUC1* promoter fragments much more efficiently than multiple copies of *FLO8*.

**TABLE 9.** Expression levels of *MUC1* promoter inserts in different plasmids on SCD in strain ISP20

| **Constructs** | **SCD** |
|---|---|
| pHP41 | 1.23 |
| pHP41 + M20 | 0.72 |
| pHP41 + M64 | 0.74 |
| pLG670-Z | 4.6 |
| pLG670-Z + M20 | 57.2 |
| pLG670-Z + M64 | 42.4 |
| pLGΔ312 | 39.4 |
| pLGΔ312 + M20 | 45.6 |
| pLGΔ312 + M64 | 64.4 |

The levels of expression conferred by the 20 bp and 64 bp *MUC1* promoter inserts alone were also determined. The two fragments were cloned into vectors pHP41, pLG670-Z and pLGΔ312 and the effect on the expression levels of *lacZ* determined in different strains and in different growth conditions. Only in the low-copy number plasmid, pHP41, did these fragments confer the expected repressive effect. In the multiple copy vectors, pLG670-Z and pLGΔ312, the fragments seemed to confer activation rather than repression, since, even on repressive SCD media (Table 9), high levels of *lacZ* activity were observed. These high levels of activity were observed in all the conditions tested and at no stage was any specific regulation observed. This data could illustrate the unsuitability of multiple copy plasmids in the functional analysis of promoter fragments. The large number of *cis*-elements created by the use of multiple copy plasmids could titrate out regulatory factors, leaving a percentage of a DNA sequence that would normally be subject to regulation, in an unregulated state, thereby masking the true nature of the fragment.

## DISCUSSION

The *MUC1* and *STA1-3* promoters are of particular interest since they (i) consist of evolutionary closely related sequences allowing the study of promoter evolution on a molecular level, (ii) represent the largest *S. cerevisiae* promoters identified to date, and (iii) might integrate the information transmitted by several separated signal transduction pathways to specifically result in an adaptive cellular differentiation process. Our results confirm previously published data suggesting that the expression of the *MUC1* and the *STA* genes is indeed controlled by the complex interaction of a large number of factors which are regulated by several independent signalling pathways.

Our data regarding the transcriptional activity of the entire promoters reveal several general features. Firstly, P*MUC1*-dependent reporter gene expression is very low in most conditions, and generally well below levels observed for the UAS-less reporter plasmid alone, indicating that the entire promoter has a repressive effect. The *STA2* promoter, on the other hand, is in a less repressed state. Indeed, expression levels of the P*STA2*-dependent reporter gene are consistently higher than for the P*MUC1*-dependent reporter gene.

Secondly, the data show that overall variations in expression levels conferred by the entire promoters in a wild-type strain is much more important for the *STA2* promoter than for the *MUC1* promoter, even if the general regulation patterns are very similar. Since the *STA* genes encode extracellular glucoamylases, and can therefore provide otherwise inaccessible nutrients, high expression levels and strong induction can obviously be advantageous to the cell. *MUC1* expression, on the other hand, has to be more tightly controlled, since overexpression of the gene could result in profound physiological changes. *MUC1* is essential for pseudohyphal differentiation and invasive growth, and both processes can be induced through overexpression of *MUC1* from a heterologous promoter or, to a lesser degree, by multiple copies of the gene (8, 23, 28). From an evolutionary perspective, the changes between the two promoters have therefore allowed the retention of a similar regulation pattern, insuring a co-regulation of pseudohyphal differentiation and invasive growth with starch degradation, while allowing for much stronger production of glucoamylases.

The data show that parameters which affect expression of both genes are (i) the presence or absence of a fermentable carbon source, (ii) the ploidy of the strain, and (iii) the presence or absence of several transcriptional regulators. As stated above, in all these cases we found that changes conferred by the *STA2* promoter are generally more significant than those conferred by the *MUC1* promoter.

The *STA2* gene seems to have retained most specific regulatory elements, but has evolved a less attenuated or less repressed promoter. This could indicate that the sequences which are found in the *MUC1* promoter, and which are deleted in the *STA1-3* promoters, are required for general repression. Our data suggest that this is

indeed the case since (i) the two inserts reduce *STA2* expression strongly when present upstream of this gene and (ii) the 20 bp insert has a repressive effect as the analysis of the UAS1 region clearly demonstrates. Our data in addition show that this repression is specifically linked to the Flo8p transcriptional activator. Multiple copies of *FLO8* indeed result in strong production of glucoamylases when the *STA2* gene is controlled by its own promoter, but fail to do so when the two *MUC1* promoter inserts are present. Multiple copies of *MSN1* and *MSS11* do not result in a similar difference between the two promoters, but efficiently increase *STA2* expression in the presence or absence of the inserts. The repressive effect of these sequences might therefore depend on inhibiting directly or indirectly the Flo8p dependent regulation of *MUC1*.

The sequence does not seem to confer a repressive effect on its own, but its regulatory activity seems context specific. When tested in the pHP41 plasmid, both the M20 and M64 inserts reduce transcription of the reporter gene. However, and surprisingly, both sequences confer activation to a reporter gene when tested in a different reporter plasmid. The strong activation observed in the case of the pLG670-Z plasmid might be due to the creation of a spurious activating sequence, even if this hypothesis is difficult to reconcile with the fact that the two insert sequences do not present any homologies. These results could nevertheless suggest that the two inserts are the target of a DNA-binding protein, whose binding could result in repression in the specific context of the *MUC1* gene promoter.

Our study of the entire promoters confirms that *MUC1* and *STA2* respond similarly to the deletion or the presence of multiple copies of *MSN1* and *MSS11*. However, and as suggested by the effect of the *MUC1* promoter inserts on *STA2* expression, the response of the two genes to multiple copies and deletion of *FLO8* differ. Multiple copies of *FLO8* result in strongly increased expression of the *STA2* gene in media containing either glucose or glycerol/ethanol as carbon source, but induce *MUC1* expression only in media containing glucose as carbon source. Rupp *et al.* (44) showed that Flo8p was required for the cAMP dependent regulation of invasive and pseudohyphal growth. The only physiologically significant variation in intracellular cAMP concentration is observed when glucose is added to cells grown on non-fermentable carbon sources (reviewed in 14, 49), and data suggest that the main role of cAMP could be the sensing of fermentable sugars. Our data could therefore indicate that Flo8p is only required for *MUC1* induction during growth on substrates containing fermentable sugars, as is the case on nitrogen limited SLAD medium, which is the main or only media used for the assessment of filamentation by most authors. Flo8p could interact with other factors to induce filamentation during nitrogen limitation, when glucose levels are still high, but might not be required or act differently in other conditions.

The size of the promoter, coupled to the apparent complexity of the regulatory processes, renders the detailed molecular analysis of the entire promoter a difficult task. For most promoter-studies in yeast published so far, a reasonable correlation between mechanistically (i.e. the binding of a regulatory protein to a specific

sequence) and physiologically relevant data (i.e. the resulting change in transcription levels) can easily be achieved. However, in the case of the *MUC1* and the *STA1-3* genes, data suggesting specific molecular interactions and regulatory events in a specific area of the promoters might not result in expected and corresponding changes in the overall transcriptional activity of the genes. The activating or repressing effect expected after the binding of a transcription factor to a region within the promoter might frequently be masked and covered by other regulatory signals acting through other areas.

Physiologically, the only significant data are those that relate to the activity of the promoter as a whole. However, in order to establish mechanistically relevant data concerning for example *cis*-acting transcription factor binding sites, it is necessary to dissect the promoter by using smaller sequence fragments. For analysis purposes, these fragments are placed in a new, very different sequence context (i.e. plasmid sequences), and data obtained have to be interpreted carefully when considering effects on the native promoters. For this reason, we have focused our investigation on a small section of the *STA2* and *MUC1* promoters that combines several of the interesting features of the entire, intact promoters within a relatively short stretch of DNA. Our data show that this area (i) confers transcriptional regulation from a far upstream (>1000 nt) position in the context of the native promoter, and (ii) regulates reporter gene expression very similarly to the entire promoters when analysed on its own. More specifically, this area of the *MUC1* and *STA2* promoters indeed (i) confers a general repressive effect on reporter gene transcription in most conditions, and (ii) contains sequences responsible for both specific activation and (iii) specific repression. Furthermore, the area contains one of the two significant changes between the *MUC1* and *STA2* promoters.

Our data clearly establish that this additional sequence contributes to the general repression or attenuation of the *MUC1* promoter, giving a clear indication of a molecular rearrangement during promoter evolution. In addition, the sequence is a target of glucose repression. The three transcriptional regulators investigated during this study, Flo8p, Msn1p and Mss11p, all act, at least in part, via UAS1 to activate transcription of *MUC1* and *STA2*.

The deletion analysis pinpoints the sequences within UAS1 which confer these regulations and these short sequences can now be investigated further to establish the binding sites of the factors involved. Flo8p and Mss11p clearly act in the 80 bp region between nucleotides -1 160and -1 070 in the *STA2* promoter and nucleotides -1 210 to -1 130 in the *MUC1* promoter.

We also identify the *STA10* repressive effect as being due to a mutation in the gene encoding the transcriptional regulator Flo8p. Indeed, we clearly demonstrate that a single copy of *FLO8* in a S288C genetic background allows production of a similar amount of glucoamylase to that observed in naturally occurring starch-degrading strains. *FLO8* was shown to be required for invasive growth in S288C-derived strains (26), since transformation of these strains with a single copy

of wild-type *FLO8* restored the ability to invade the growth media. W303, another commonly used laboratory strain contains, in addition to a mutation in *FLO8*, mutations in other activators required for invasive growth and pseudohyphal differentiation (26) and is therefore unable to form pseudohyphae or grow invasively. In this strain, a single copy of *FLO8* was also unable to restore glucoamylase expression from a plasmid-borne *STA2* gene (data not shown), suggesting that the *STA10* phenotype in W303 strains might be due to the requirement of *FLO8* as well as other transcriptional activator(s). We also show that Flo8p requires Mss11p to induce both starch degradation and pseudohyphal differentiation and invasive growth. Since Mss11p is able to overcome mutations in *FLO8*, we suggest that Mss11p is situated downstream of Flo8p in a linear signal transduction cascade. However, Flo8p apparently acts independently of Msn1p, which is probably situated in a parallel pathway.

As expected for such a complex promoter, and as discussed above, some of the data obtained for UAS1 do not correlate properly with those seen for the entire promoter. Most tendencies are, however, conserved, and the data are mechanistically significant. Only a combination of studies on all UAS and URS sequences of the *MUC1* and *STA* promoters will reveal a complete picture of how transcription factors combine to result in either repression or activation.

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