The molecular characterisation of Mss11p, a transcriptional activator of the *Saccharomyces cerevisiae* *MUC1* and *STA1-3* genes

by

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DECLARATION

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

SUMMARY

Upon nutrient limitation, normal cells of the budding yeast, *Saccharomyces cerevisiae*, undergo a transition from ovoid cells that bud in an axial (haploid) or bipolar (diploid) fashion to elongated cells that bud in a unipolar fashion. The daughter cells stay attached to the mother cells, resulting in chains of cells referred to as pseudohyphae. These filaments can grow invasively into the growth substrate (haploid), or away from the colony (diploid), and are hypothesised to be an adaptation of yeast cells that enables them to search for nutrient-rich substrates. This filamentous growth response to nutrient limitation was shown to be dependent on the expression of, amongst others, the *MUC1* gene.

MUC1 (also known as *FLO11*) encodes a large, cell wall-associated, GPI-anchored threonine/serine-rich protein that bears structural resemblance to mammalian mucins and to the yeast flocculins. Deletion and overexpression studies demonstrated that it is critical for pseudohyphal differentiation and invasive growth, and that overexpression of the gene also results in strongly flocculating yeast strains. The upstream regulatory region of *MUC1* comprises the largest yeast promoter identified to date and areas as far as 2.4 kb upstream of the translational start site have been shown to confer regulation on *MUC1* expression. The large promoter region is not unique to *MUC1*, however, since it is almost identical to that of the functionally unrelated *STA2* gene. The *STA2* gene, as well as the identical *STA1* and *STA3* genes, encodes extracellular glucoamylase isozymes that enable the yeast cell to utilise starch as a carbon source. Glucoamylases liberate glucose residues from the non-reducing end of the starch molecule, thereby making it accessible to yeast cells.

The high identity between the promoters of *MUC1* and *STA1-3* suggests that the two genes are co-regulated. In addition, several transcription factors that regulate the transcriptional levels of both *MUC1* and *STA2* have been identified and include Msn1p and the previously uncharacterised Mss11p. Overexpression of either Msn1p or Mss11p results in elevated levels of *MUC1* and *STA2* transcription and a dramatic increase in flocculation, invasive growth, pseudohyphal differentiation and the ability to utilise starch, suggesting that the two genes are indeed co-regulated. The main objective of this study was to characterise Mss11p and its role in the co-regulation of *MUC1* and *STA2* (as a representative member of the *STA* gene family).

A detailed expression analysis, using Northern blots and *lacZ* reporter gene expression studies in different media, confirmed that these genes are indeed co-regulated to a large extent. *MUC1* and *STA2* are also regulated by the same transcriptional regulators, which include not only Msn1p and Mss11p, but also Ste12p, the transcription factor of the mating pheromone/filamentous growth signalling cascade, and Flo8p, a transcriptional activator of the flocculation genes. Overexpression of the genes encoding these factors results in elevated expression levels of both *MUC1* and *STA2* in most nutritional conditions and enhances the

filamentous growth phenotypes of the strain, as well as the ability to degrade starch. On the other hand, the deletion thereof results in severe reductions in the transcription levels of *MUC1* and *STA2*, with equally severe reductions in filamentous growth and the ability to hydrolyse starch. These expression studies also showed that the repressive effect of *STA10*, a previously uncharacterised negative regulator of *STA2*, is actually a phenotype conferred by a *FLO8* mutation in some laboratory strains of *S. cerevisiae*.

The upstream regulatory regions of *MUC1* and *STA2* are the largest promoters in the yeast genome. By sequencing the upstream areas of *STA2* and *STA3* and comparing them to the sequence of *MUC1*, it was shown that these upstream areas are 99.7% identical over more than 3 900 base pairs (bp) upstream of the translational start. With the exception of a few minor substitutions, the only significant difference between the *MUC1* and *STA2* promoters is the presence of a 20-bp and a 64-bp sequence found in the *MUC1* promoter, but not in the promoters of any of the *STA1-3* genes.

Through a promoter-deletion analysis, it was shown that Mss11p, Msn1p and Flo8p exert their control over the transcription of *MUC1* and *STA2* from an 90-bp sequence located at -1 160 to -1 070 in the *STA2* and -1 210 to -1 130 in the *MUC1* promoters. This sequence also mediates the effect of carbon catabolite repression on the transcription of *STA2* and *MUC1*.

Despite the similarities in the expression patterns of *MUC1* and *STA2*, some discrepancies also exist. The most significant difference is that, in wild-type cells and under all nutritional conditions tested, *MUC1* transcription is reduced significantly if compared to the transcription levels of *STA2*. This was attributed to the presence of the 20- and 64-bp sequences, that are present in the promoter region of *MUC1*, but absent from that of *STA2*.

To place the transcriptional regulators of *MUC1* and *STA2* in the context of known signal transduction pathways, an epistasis analysis was conducted between *MSN1*, *MSS11* and components of the mating pheromone/filamentous response MAP kinase cascade and cAMP-PKA pathway that were shown to be required for the filamentous growth response. This analysis revealed that Msn1p functions in a third, as yet uncharacterised, signal transduction pathway, also downstream of Ras2p, but independent of the two identified pathways, i.e. the cAMP-PKA and pheromone response/filamentous growth response MAP kinase pathways. However, Mss11p seems to function downstream of all three the identified pathways. This suggests a critical and central role for Mss11p in determining the transcription levels of *MUC1* and *STA2*.

To further characterise Mss11p and its role in the transcriptional regulation of *MUC1* and *STA2*, it was also subjected to a detailed deletion and mutation analysis. Mss11p was shown to harbour two distinct activation domains required for the activation of *MUC1* and *STA2*, but also able to activate a reporter gene expressed from under the *GAL7* promoter. The more prominent of the activation domains of Mss11p was shown to be one of the domains with homology to Flo8p, designated H2. The H2 domain has significant homology to a number of

proteins of unknown function from a range of different organisms. A multi-sequence alignment allowed the identification of conserved amino acids in this domain. Mutations in two of the four conserved amino acid pairs in the H2 domain completely eliminated the activation function of Mss11p.

The poly-glutamine and poly-asparagine domains of Mss11p are not required for its activation function. The deletion of these domains has no impact on the ability of Mss11p to activate *MUC1* or *STA2* or of the Gal4p-Mss11p fusion to activate the *lacZ* reporter gene expressed from under the *GAL7* promoter. Gal4p fusions of either of these domains were also unable to trans-activate the P*GAL7-lacZ* reporter gene. As such, it was concluded that neither of these domains performs a function in the role of Mss11p as a transcriptional activator. We also demonstrated that the putative ATP/GTP-binding domain (P-loop) is not required for the transcriptional activation function of Mss11p.

In an attempt to identify other target genes of Mss11p, the use of micro-arrays was employed to assess the impact of the overexpression and deletion of *MSS11* on the total yeast transcriptome. These results showed that *MUC1* and *STA2* are the only two genes in the ISP15 genetic background that are significantly (more than 15-fold) enhanced by the overexpression of *MSS11*. Mss11p therefore seems to play a very specific or dedicated role in *MUC1* and *STA2* transcription. This analysis also identified several genes (*DBP2*, *ROM2*, *YPL080C*, *YGR053C*, *YNL179C*, *YGR066C*) that are repressed by overexpression of *MSS11* and activated when *MSS11* is deleted.

To integrate all the results, three possible models for the activation of *MUC1* and *STA2* transcription by Mss11p are proposed: (i) Mss11p performs the role of a transcriptional mediator, possibly in a protein complex, to convey information from upstream regulatory elements to the transcription machinery assembled at the core promoters of *MUC1* and *STA2*; (ii) Mss11p plays a more direct role in transcriptional activation, possibly as a transcription factor itself; and (iii) Mss11p facilitates transcription of the *MUC1* and *STA2* promoters as part of a larger complex that removes or releases the chromatin barrier over the *MUC1* and *STA2* promoters in response to specific nutritional signals.

OPSOMMING

Wanneer voedingstowwe beperkend raak, ondergaan selle van die botselvormende gis, *Saccharomyces cerevisiae*, 'n transformasie vanaf ronde selle, wat in 'n aksiale (haploïede) of bipolêre (diploïede) patroon bot, tot verlengde selle, wat slegs op een punt bot. Die dogterselle bly aan die moederselle geheg, sodat kettings van selle, wat as pseudohifes bekend staan, gevorm word. Hierdie filamente kan 'n groeisubstraat binnedring (haploïede) of vanaf die kolonie weggroei (diploïede), en is moontlik 'n aanpassing van die gisselle wat hulle in staat stel om na meer voedingstofryke substrate te groei. Die vermoë om filamente in respons tot voedingstoftekorte te vorm, is onderhewig aan die uitdrukking van, onder meer, die *MUC1*-geen.

MUC1 (ook bekend as *FLO11*) kodeer vir 'n selwand-geassosieerde treonien/serien-ryke proteïen met 'n GPI-anker wat strukturele verwantskappe met die mukiene van soogdiere en die flokkuliene van giste toon. Delesie- en ooruitdrukkingstudies het bewys dat dit krities is vir die ontwikkeling van pseudohifes en penetrerende groei, terwyl die ooruitdrukking daarvan ook tot sterk flokkulerende gisrasse lei. Die stroom-op regulatoriese area van *MUC1* vorm die grootste promotor wat tot dusver in gis geïdentifiseer is, en daar is bewys dat areas so ver as 2.4 kb stroom-op van die translasie-inisiëringsetel die regulering van *MUC1* beïnvloed. Hierdie groot promotor is egter nie uniek tot *MUC1* nie, aangesien 'n amper identiese promotor die regulering van die funksioneel onverwante *STA2*-geen beheer. Die *STA2*-geen, asook die identiese *STA1*- en *STA3*-gene, kodeer vir ekstrasellulêre glukoamilase isosieme wat die gis in staat stel om stysel as koolstofbron te benut. Dit bevry glukosemolekules vanaf die nie-reduserende punt van die styselmolekuul en stel dit sodoende aan gisselle beskikbaar.

Die hoë vlak van eendersheid tussen dié twee promotors veronderstel dat die twee gene op soortgelyke wyse gereguleer word. Verskeie transkripsiefaktore wat die transkripsievlakke van beide *MUC1* en *STA2* beheer, is ook geïdentifiseer. Dit sluit Msn1p en die tot dusver ongekarakteriseerde Mss11p in. Ooruitdrukking van Msn1p of Mss11p lei tot verhoogde vlakke van *MUC1* en *STA2* se transkripsie en 'n dramatiese toename in flokkulasie, asook die vermoë om penetrerend te groei, pseudohifes te vorm en stysel te benut. Dit bevestig dat die twee gene wel tot 'n groot mate op dieselfde wyse gereguleer word. Die hoofdoel van hierdie studie was om Mss11p en die rol daarvan in die regulering van *MUC1* en *STA2* te karakteriseer.

Gedetailleerde uitdrukkingsanalises met behulp van die Northern-kladtegniek en *lacZ*-verklikkergeeneksperimente in verskillende media het bevestig dat die gene wel tot 'n groot mate op dieselfde wyse gereguleer word. Transkripsie van *MUC1* en *STA2* word ook deur dieselfde transkripsionele reguleerders beheer, wat nie net Msn1p en Mss11p insluit nie, maar ook Ste12p, die transkripsiefaktor van die paringsferomoon/filamentagtige groei seintransduksiekaskade, en Flo8p, 'n transkripsionele aktiveerder van die flokkulasiegene.

Ooruitdrukking van die gene wat vir hierdie faktore kodeer, veroorsaak verhoogde uitdrukkingsvlakke van beide *MUC1* en *STA2* onder die meeste groeitoestande en verbeter die vermoë van die gisras om filamentagtig te groei en om stysel te benut. Andersyds veroorsaak delesies van die gene 'n dramatiese afname in die transkripsievlakke van *MUC1* en *STA2*, met vergelykbare afnames in die vermoë van die gisras om filamentagtig te groei en om stysel te benut. Hierdie uitdrukkingstudies het ook bewys dat die onderdrukkingseffek van *STA10*, 'n tot dusver ongekarakteriseerde, negatiewe reguleerder van *STA2*, aan 'n mutasie in *FLO8* in sekere laboratoriumrasse van *S. cerevisiae* toegeskryf kan word.

Die stroom-op regulatoriese areas van *MUC1* en *STA2* is die grootste promotors in die gis se genoom. Deur die nukleotiedvolgordes van die ver stroom-op areas van *STA2* en *STA3* te bepaal en hulle met dié van *MUC1* te vergelyk, is daar vasgestel dat die stroom-op areas van die gene 99.7% identies is oor meer as 3 900 basispare (bp) stroom-op van die beginsetel van translasie. Met die uitsondering van enkele basispaarverskille, is die enigste noemenswaardige verskil tussen die promotors van *MUC1* en *STA2* die teenwoordigheid van 'n 20 bp- en 'n 64 bp-fragment wat in die *MUC1*-promotor aangetref word, maar nie in die promotors van die *STA1-3* gene nie.

Deur 'n promotordelesie-analise kon daar bewys word dat Mss11p, Msn1p en Flo8p beheer uitoefen oor die transkripsie van *MUC1* en *STA2* vanaf 'n 90-bp-fragment, wat by posisie -1 160 tot -1 070 in die *STA2*-promotor en posisie -1 210 tot -1 130 in die *MUC1*-promotor aangetref word. Koolstofkatabolietonderdrukking van *MUC1* en *STA2* se transkripsie geskied ook deur middel van hierdie fragment.

Ten spyte van die ooreenkomste in die uitdrukkingspatrone van *MUC1* en *STA2*, kom daar tog ook verskille voor. Die mees opvallende verskil is dat, in wilde-tipe selle en onder alle toestande tot dusver getoets, die transkripsievlakke van *MUC1* aansienlik laer is as dié van *STA2*. Dit word toegeskryf aan die teenwoordigheid van die 20 bp- en 64 bp-fragmente, wat in die promotor van *MUC1* teenwoordig is, maar in die promotor van *STA2* afwesig is.

Om die transkripsionele reguleerders van *MUC1* en *STA2* in die konteks van bekende seintransduksieweë te plaas, is 'n epistase-analise gedoen tussen *MSN1*, *MSS11* en komponente van die paringsferomoon/filamentagtige groei MAP-kinasekaskade en die cAMP-PKA-weg wat uitgewys het dat dit 'n rol in die filamentagtige groeirespons speel. Hierdie analise het onthul dat Msn1p in 'n derde, tot dusver onbeskryfde, seintransduksieweg funksioneer, wat ook stroom-af van Ras2p is, maar wat onafhanklik funksioneer van die twee bekende weë, die cAMP-PKA-weg en die paringsferomoon/filamentagtige groei MAP-kinasekaskade. Mss11p blyk egter stroom-af van al drie dié weë te funksioneer. Dit wys dat Mss11p 'n kritiese en sentrale rol in die bepaling van *MUC1* en *STA2* se transkripsievlakke speel.

Om Mss11p en die rol daarvan in die regulering van *MUC1* en *STA2* se transkripsie verder te karakteriseer, is dit aan 'n volledige delesie- en mutasie-analise onderwerp. Dit het gewys dat

Mss11p twee verskillende aktiveringsdomeine bevat wat vir die transkripsionele aktivering van *STA2* en *MUC1* benodig word, maar wat ook 'n verklikkergeen kon aktiveer wat onder die *GAL7*-promotor uitgedruk word. Die prominentste van die twee aktiveringsdomeine van Mss11p is een van die domeine wat homologie toon met 'n soortgelyke domein van Flo8p, die sogenaamde H2-domein. Die H2-domein toon homologie met 'n verskeidenheid van organismes se proteïene, waarvan die funksie onbekend is. 'n Vergelyking van al die relevante aminosuurvolgordes uit dié proteïene het gehelp om 'n aantal gekonserveerde aminosure te identifiseer. Mutasies van twee van die vier gekonserveerde aminosuurpare het die vermoë van Mss11p om transkripsie te aktiveer, heeltemal geëlimineer.

Die poliglutamien- en poliasparagiendomeine van Mss11p word nie vir die aktiveringsfunksie benodig nie. Die delesie van die domeine het geen impak gehad op die vermoë van Mss11p om die transkripsie van *MUC1* en *STA2* te aktiveer nie, of op die vermoë van die Gal4p-Mss11p fusie om die *lacZ*-verklikkergeen onder regulering van die *GAL7*-promotor te aktiveer nie. Gal4p-fusies met enige van die domeine was ook nie in staat om die P*GAL7-lacZ*-verklikkergeen te aktiveer nie. Daar kan dus afgelei word dat nie een van die twee domeine 'n funksie in die rol van Mss11p as transkripsionele aktiveerder het nie. Soortgelyke eksperimente het bewys dat die moontlike ATP/GTP-bindingsdomein (P-lus) nie vir die transkripsionele aktiveringsfunksie van Mss11p benodig word nie.

In 'n poging om ander teikengene van Mss11p te identifiseer, is mikro-ekspressieroosters gebruik om die impak van die ooruitdrukking en delesie van *MSS11* op die totale transkriptoom van die gis te bepaal. Dié resultate het gewys dat *MUC1* en *STA2* die enigste twee gene in die ISP15 genetiese agtergrond is waarvan transkripsie noemenswaardig (meer as 15-voudig) deur die ooruitdrukking van *MSS11* verhoog word. Dit wil dus voorkom asof Mss11p 'n baie spesifieke rol in die transkripsie van *MUC1* en *STA2* speel. Hierdie analise het ook verskeie gene (*DBP2*, *ROM2*, *YPL080C*, *YGR053C*, *YNL179C*, *YGR066C*) geïdentifiseer wat deur die ooruitdrukking van *MSS11* onderdruk word en deur die delesie van *MSS11* geaktiveer word.

Ten einde al die resultate te integreer, word drie moontlike modelle vir die aktivering van *MUC1*- en *STA2*-transkripsie deur Mss11p voorgestel: (i) Mss11p vervul die rol van 'n transkripsionele tussenganger, moontlik as deel van 'n proteïenkompleks, om die inligting van die stroom-op regulatoriese elemente aan die transkripsiemasjinerie wat oor die kernpromotor van *MUC1* en *STA2* gebind is, oor te dra; (ii) Mss11p speel 'n meer direkte rol in transkripsionele aktivering, moontlik as 'n transkripsiefaktor self; en (iii) Mss11p maak die transkripsie van *MUC1* en *STA2* moontlik as deel van 'n groter kompleks wat die chromatienblokkade oor die promotors van *STA2* en *MUC1* in respons tot spesifieke seine verslap of verwyder.

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BIOGRAPHICAL SKETCH

Marco Gagiano was born in Port Elizabeth, South Africa, on 16 November 1971. He matriculated at Swartland High School, Malmesbury, in 1989 and enrolled for a BSc Degree at Stellenbosch University in 1990. He obtained a BSc Degree in Biochemistry, Genetics and Microbiology in 1993, a BScHons in Microbiology in 1994 and an MSc (*cum laude*) in Microbiology in 1998.

PREFACE

With the exception of the first and last chapters (Chapter 1 and Chapter 6), this dissertation is a collection of manuscripts that were published or were submitted for publication in different journals. For the sake of stylistic continuity, all text and figures were formatted according to the same style to avoid the confusion brought about by the different formatting requirements of the specified journals.

Chapter 2. "The sensing of nutritional status and the relationship to filamentous growth in *Saccharomyces cerevisiae*" was submitted as a review paper to *FEMS Yeast Research*.

Chapter 3. "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*" was published in *Molecular Microbiology*.

Chapter 4. "Divergent regulation of the evolutionary closely related promoters of the *Saccharomyces cerevisiae STA2* and *MUC1* genes" was published in *Journal of Bacteriology*.

Chapter 5. "The functional dissection of Mss11p, a transcription factor regulating pseudohyphal differentiation, invasive growth and starch metabolism in *Saccharomyces cerevisiae*" was submitted to *Molecular Microbiology*.

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CHAPTER 6 - CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Chapter 1

General introduction and project aims

1. Introduction

Non-motile organisms such as the yeast, *Saccharomyces cerevisiae*, do not have the privilege of specialised organs or organelles that would allow them the movement to more optimal environments or the movement away from adverse conditions. However, some non-motile organisms have adapted in order to compensate for the lack of motility. In response to nutrient starvation, yeast cells undergo a transition from ovoid cells that bud in axial (haploid) or bipolar (diploid) fashion, to elongated cells that bud in a unipolar fashion (Gimeno et al., 1992; Kron et al., 1994). The daughter cells stay attached to the mother cells, which result in chains of cells that are referred to as pseudohyphae. These chains of cells can grow invasively into the growth substrate and away from the colony (Gimeno et al., 1992; Kron et al., 1994). This adaptation occurs most typically in response to nutrient (e.g. nitrogen) limitation, and is therefore hypothesised to enable yeast to grow towards more optimal growth substrates (Kron, 1997).

Pseudohyphal differentiation and invasive growth require the ability to integrate perceived extracellular signals, such as nitrogen and carbon starvation, into cellular processes that include, among others, the reorientation of the cell's polarity and the changes in budding pattern mentioned above (reviewed in Kron, 1997; Madhani and Fink 1998; Borges-Walmsley and Walmsley, 2000; Pan et al., 2000; Gancedo 2001). These adaptations will ultimately lead to the change from the yeast-form to the filamentous form. Using biochemical and genetic approaches, filamentous growth and the underlying cellular processes were dissected by a number of research groups in recent years. One of the components most frequently identified as being critical for the filamentous growth phenotype, was shown to be the *MUC1* (also known as *FLO11*) gene (Lambrechts et al., 1996a; Lo and Dranginis, 1996, 1998).

MUC1 encodes a large, cell wall-associated, GPI-anchored threonine/serine-rich protein that bears structural resemblance to mammalian mucins and yeast flocculins (Lambrechts et al., 1996a; Lo and Dranginis, 1996, 1998). Deletion of the *MUC1* locus almost completely eliminates the yeast cell's ability to form pseudohyphae or grow invasively, whereas overexpression of the gene results in severe filamentation and flocculation phenotypes (Lambrechts et al., 1996a; Lo and Dranginis, 1996,1998). *MUC1* was recently shown to be a member of a larger family of genes which encode products that contribute to the filamentous growth phenotypes to various extents (Guo et al., 2000). However, considering the impact on filamentous growth phenotypes upon overexpression or deletion of *MUC1*, the gene encodes the protein with the most significant role.

A large and complex regulatory network governs transcription levels of *MUC1*. Its promoter region seems to be the point of convergence for a number of signalling cascades that transmit

specific extracellular signals (Mösch et al., 1999; Rupp et al., 1999). *MUC1* also contains one of the largest promoter regions in the *S. cerevisiae* genome, and areas through which transcriptional regulation is conferred, were identified more than 2400 bp upstream of the open reading frame (Rupp et al., 1999). The large promoter region of *MUC1* is, however, not a unique feature in yeast and it is essentially identical to that of the functionally unrelated *STA1-3* genes.

The *STA1-3* genes of *S. cerevisiae* encode extracellular glucoamylases that enable yeast cells to grow on starch as the sole carbon source (reviewed in Pretorius et al., 1991; Vivier et al., 1997). These glucoamylases catalyse the hydrolysis of the starch molecule through removal of glucose units from the non-reducing end of the starch molecule, thereby making it accessible to the yeast cell. The *STA1-3* genes are, in evolutionary terms, recent acquisitions of *S. cerevisiae* and are hypothesised to have evolved through recombination events between the promoter of *MUC1* and the open reading frame of *SGA1*, which encodes a sporulation-specific, intracellular glucoamylase (Yamashita et al., 1985). The genes have been studied for a number of years and a large pool of knowledge therefore exists on their transcriptional regulation and on factors that mediate their regulation. However, most of the conditions and factors that were shown to determine *STA1-3* transcription levels have not been confirmed as having similar effects on *MUC1* transcription and *vice versa*. This also applies to the analyses of the promoter regions of *MUC1* and *STA1-3*.

Since the promoters of *MUC1* and *STA1-3* are 99.7% identical, the genes are assumed to be co-regulated to a large extent. The promoters of *STA1* (Shima et al., 1989), *STA2* (Lambrechts et al., 1994) as well as *MUC1* (Rupp et al., 1999) have been analysed to some extent and regions required for the transcriptional activation and repression of the genes have been identified. For the *STA1* and *STA2* promoters, these regions are large, but none of the identified regions was shown to be regulated by any specific transcription factor. The more detailed analysis of the *MUC1* promoter revealed a number of smaller regions required for the transcriptional regulation via the Ste12p and Flo8p transcriptional activators (Rupp et al., 1999). However, a very large gap still exists between the mechanistic events occurring at promoter level of these genes and the signal transduction pathways that transmit specific regulatory signals to the genes. The following section will give an overview of what was known of the regulation of *STA2* and *MUC1* prior to the onset of this work in order to place the specific aims in context.

2. Negative regulation of *STA2* and *MUC1*

Transcription of *STA1-3* was described to be repressed in the presence of rapidly fermentable carbon sources such as glucose or sucrose, in diploid strains of *S. cerevisiae* and in strains containing an undefined repressor, *STA10* (Polaina and Wiggs, 1983; Yamashita and Fukui, 1983, 1985; Pretorius et al., 1986; Dranginis, 1989; Inui et al., 1989). This repressor is only present in laboratory strains of *S. cerevisiae*, and renders them unable to grow on starch as a carbon source, due to extremely reduced transcription levels of the *STA1-3*-encoded glucoamylases. However, feral *S. cerevisiae* strains can express the *STA1-3* genes at sufficiently high levels to support growth on starch as sole carbon source, and are therefore reported not to harbour the *STA10* repressor. The *STA10* repressive effect was attributed to the presence of a number of genes, *IST1* and *IST2* (Park and Mattoon, 1987), *INH1* (Yamashita and Fukui, 1984), *SGL1* (Patel et al., 1990) and *SNS1* and *MSS1* (Ahn et al., 1995), but the relationship between the *STA10* repressive effect and any of these genes was never confirmed or reproduced.

In addition to the elusive *STA10*, several other factors have also been identified as negative regulators of *STA1-3* transcription. *HXK2* and *HAP2* were shown to participate in two genetically separable pathways that mediate the repressive effect of rapidly fermentable carbon sources, such as glucose, on *STA2* (Kartasheva et al., 1996). *HXK2* plays a central role in glucose metabolism and is involved in the repression of a large number of genes in the presence of rapidly fermentable carbon sources, not just via the Snf1p-main glucose repression pathway (Vincent et al., 2001), but also via the direct interaction with promoter elements (Hererro et al., 1998). The mechanism through which it represses *STA2* transcription is unknown, but it does not involve the *MIG1*-encoded repressor (Kartasheva et al., 1996).

The mechanism through which *HAP2* regulates transcription of *STA2* is also not very clear (Kartasheva et al., 1996), since *HAP2* was shown to participate in an activation complex required for the transcriptional activation of, among others, the mitochondrial genes (reviewed in Zitomer and Lowry, 1992).

Another factor with a thoroughly characterised repressive effect is Nrg1p. Nrg1p was shown to be a more general repressor that inhibits the transcription of *STA1* by binding to two upstream *cis*-elements in the *STA1* promoter. Nrg1p does not act as a repressor itself, but analogous to the mechanisms employed by the Mig1p repressor, recruits the Tup1p-Ssn6p global transcriptional complex to the promoters of the *STA1* gene (Park et al., 1999), as well as other glucose-repressed genes e.g. *SUC2* and *GAL1* (Zhou and Winston, 2001).

SUD1 was shown to encode a chromatin-associated factor that assists in mediating a repressive effect on not just *STA1*, but on the expression of a number of genes previously

observed to be negatively regulated by repressive chromatin structures (Yamashita, 1993). The relationship between Sud1p and the Tup1-Ssn6p complex, which also repress genes by altering chromatin structure (Gavin et al., 2000; Watson et al., 2000), is unclear at this stage. It does, however, strongly suggest that *STA2* is negatively regulated through condensed chromatin structures in its upstream regulatory regions.

Before this work was initiated, the negative regulation of *MUC1* had not been investigated and, as such, no negative regulators had been identified. Since then, some negative regulators have been identified, but these were all components of specific signal transduction pathways that regulate filamentous growth (e.g. Sfl1p, Tup1p and Ssn6p), and will be discussed in detail in the relevant sections of the next chapter.

3. Positive regulation of *STA2* and *MUC1*

Further suggestive evidence for the potential role of chromatin condensation in the expression of the *STA* genes was obtained when it was shown that most of the genes encoding components of the global Swi-Snf complex are required for *STA1-3* activation. These include *SNF1*, *SNF2*, *SNF5*, *SWI1*, *SWI3* and *SIN3* (Inui et al., 1989; Okimoto et al., 1989, 1991; Yoshimoto and Yamashita, 1991; Yoshimoto et al., 1992; Kuchin et al., 1993), all of which encode factors that associate in a complex to relieve the repressive effect of chromatin on transcription (Kruger et al., 1995; Wilson et al., 1996).

The transcription factor of the mating pheromone cascade, Ste12p, and the transcription factor of the flocculation genes, Flo8p, were also identified as positive regulators of *MUC1* (Lo and Dranginis, 1998; Robertson and Fink, 1998). These factors were shown to regulate the filamentous growth phenotype in response to two distinct signal transduction pathways, the mating pheromone response pathway and the cAMP-PKA pathway, through transcriptional activation of *MUC1*. The effect of these factors and signalling cascades on *STA2* transcription and starch metabolism was never assessed.

A screen for multiple copy suppressors of the repressive *STA10* effect identified *MSS10/MSN1* as a transcriptional regulator of *STA2* (Lambrechts et al., 1996b). *MSN1* was previously identified as a multiple-copy suppressor of *snf1* mutations (Estruch and Carlson, 1990) and of a deficiency of yeast strains to grow on iron-limiting media (Eide and Guarente, 1992). It was later also shown to increase transcription levels of *MUC1* when present on a multiple copy plasmid, and also to regulate, via *MUC1* and *STA2*, filamentous growth and starch metabolism (Lambrechts et al., 1996a). *MSS11* was identified in the same screen and shown to positively regulate starch metabolism by significantly increasing the transcription levels of *STA2* (Webber et al., 1997). Of all the positively regulating factors discussed, Mss11p

was shown to confer the strongest effect on the transcription of *STA2* and *MUC1*. Mss11p also seems to be the most specific factor and was, up to the identification of its role in starch metabolism, unknown. It exhibits no homology to any of the characterised proteins from yeast or other organisms and has no recognisable features that could suggest any specific role in regulating transcription. A number of questions therefore arose around Mss11p and its role in regulating *MUC1* and *STA2* transcription. These questions were partially addressed during the course of the work presented in this thesis.

4. Specific aims

To summarise then, the specific aims of this study were the following:

i. to identify regulatory elements in the upstream areas of the *MUC1/FLO11* and *STA2* genes through which Mss11p, Msn1p and Flo8p exert transcriptional control;
ii. to determine the relationship between *STA10* and the transcription factors encoded by *MSN1*, *FLO8* and *MSS11*;
iii. to assess the extent of the co-regulation between the *MUC1/FLO11* and *STA2* genes with respect to the transcription factors, Mss11p, Msn1p and Flo8p, as well as nutritional conditions;
iv. to place Mss11p in the context of known signal transduction pathways, specifically the MAP kinase and cAMP-PKA cascades;
v. to establish whether Mss11p is a transcriptional activator, i.e. whether it directly or indirectly stimulates the transcription of *MUC1/FLO11* and *STA2* and,
vi. to identify functional domains such as activation domains within Mss11p.

The conclusions to these questions are presented in the published (Chapters 3 and 4) and submitted (Chapter 5) papers. As background to the work, the literature review (Chapter 2) discusses nutritional sensing and signalling in *Saccharomyces cerevisiae*, specifically as it relates to the expression of *MUC1* and ultimately, filamentous growth.

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Chapter 2

Literature Review*

The sensing and signalling of nutritional status and the relationship to filamentous growth in the yeast *Saccharomyces cerevisiae*

* A modified version of this chapter has been submitted for publication as a review paper in *FEMS Yeast Research*.

1. The sensing of nutritional status and the relationship to filamentous growth in *Saccharomyces cerevisiae*

It is essential for all micro-organisms to be able to sense the availability of nutrients in their surroundings and to respond rapidly to changes in this nutritional status. It would, for example, be detrimental to the organisms' survival to engage in energetically expensive cellular processes or to attempt proliferation when nutrients are limiting or absent. Therefore, the cell must be able to perceive how much of a required nutrient is available in its immediate surroundings (sensing), transmit this information to the nucleus (signalling) and switch specific sets of genes on or off (transcription) to initiate cellular programmes that will allow the cell to deal with specific conditions. In the yeast *Saccharomyces cerevisiae*, cellular programmes such as pseudohyphal growth (reviewed in Kron, 1997; Banuett, 1998; Madhani and Fink, 1998) or sporulation (reviewed in Mitchell, 1994) are well-characterised responses to nutritional signals that allow yeast cells to survive changes in their environments.

Pseudohyphal differentiation and the related phenotype, invasive growth, are hypothesised to be adaptations that allow *S. cerevisiae* to grow towards nutrient-rich and therefore more optimal growth substrates (reviewed in Kron, 1997; Madhani and Fink 1998; Borges-Walmsley and Walmsley, 2000; Pan et al., 2000; Bauer and Pretorius, 2001; Gancedo 2001). Although both these phenotypes have been described by several research groups in haploid as well as diploid yeast strains, some differences between the two cell types have been observed. Haploid yeast cells can grow invasively on nitrogen-rich media, whereas diploids cannot (Mösch et al., 1999). Also, haploid invasive growth does not require the *BUD* genes, whose encoded products regulate the budding patterns of haploid and diploid cells (Mösch et al., 1999). Essentially, all other factors, mutants or genes identified in playing a role in the one phenotype were also shown to play a role in the other. For the purpose of this literature review, therefore, the term filamentous growth will be used to describe both haploid invasive and diploid pseudohyphal growth, unless otherwise stated.

The switch from the round or ovoid cell shapes that are usually associated with *S. cerevisiae*, to the filamentous invasive and pseudohyphal forms in response to specific signals, became topical research subjects for a number of reasons. This dimorphic transition is an important virulence trait of many human pathogens, including *Candida* (Lo et al., 1997a) and *Cryptococcus* spp. (D'Souza and Heitman, 2001), and insight into the mechanisms and regulation of the phenotype, gained from research on the genetically more tractable and easier manipulated *S. cerevisiae*, can be applied to combat the incidence and severity of infections caused by these organisms. The underlying mechanisms of the dimorphic switch in *S. cerevisiae*, specifically the reorientation and polarisation of the actin cytoskeleton in

response to specific signals, are similar to the transition of cancer cells in mammalian tumours to an invasive or metastatic form associated with the spread of the disease throughout the host body. This invasiveness occurs, amongst others, in response to extracellular signals that elicit a cAMP signal (Stanhill et al., 1999). Since some of the core components of the cytoskeleton (e.g. actin and profilin), factors that establish cell polarity (e.g. Cdc42p) and some of the signalling components (e.g. Ras2p, Ste20p) are conserved between organisms ranging in complexity from yeast to mammalian cells (Gibbs et al., 1987; Powers et al., 1989; Munemitsu et al., 1990; Brown et al., 1996; Winsor and Schiebel, 1997), studies on the regulation of cytoskeletal changes by signals during filamentous growth in *S. cerevisiae* could generate some knowledge on the mechanisms of metastases in human cancers. Also, studies on the integration of multiple signals and the inter-networking of signalling cascades which result in the expression of specific genes in a simple organism such as *S. cerevisiae* generate a wealth of knowledge on the fundamental aspects and mechanisms of signalling in higher eukaryotes.

In *S. cerevisiae*, the switch from the round or ovoid cell form to the filamentous form correlates with the nutritional status of the environment and, subsequently, several nutrient responsive cascades have been shown to regulate this dimorphic switch. The cAMP-PKA pathway was shown to be a critical regulatory cascade for establishing the filamentous growth phenotype in response to nutritional signals (Ward et al., 1995; Kübler et al., 1997; Lorenz and Heitman, 1997; Robertson and Fink, 1998; Mösch et al., 1999; Pan and Heitman, 1999; Rupp et al., 1999; Lorenz et al., 2000; Tamaki et al., 2000). The involvement of another well-characterised nutrient-responsive signalling cascade, the rapamycin-sensitive Tor pathway, on filamentous growth has not been reported to date, but some evidence exists that suggests that it could also play a role in regulating the phenotype (Bertram et al., 2000; Shamji et al., 2000; Kuruvilla et al., 2001; Valenzuela et al., 2001). The core components of the mating pheromone-responsive MAPK cascade were also shown to regulate these phenotypes in response to nutritional signals (Liu et al., 1993; Kron et al., 1994; Roberts and Fink, 1994; Mösch et al., 1996; Cook et al., 1997; Madhani and Fink, 1997, 1998; Madhani et al., 1997; Mösch and Fink, 1997; Bardwell et al., 1998a, b; Rupp et al., 1999). However, this regulatory cascade was, until the discovery of its role in filamentous growth, not considered to be a nutrient-responsive pathway. In addition to the components of the relatively well-characterised pathways mentioned above, several other factors were shown to regulate or contribute to pseudohyphal differentiation and invasive growth in response to nutrient starvation conditions. These include Phd1p (Gimeno and Fink, 1994), Ash1p (Chandarlapaty and Errede, 1998), Elm1p (Blacketer et al., 1993; Garret, 1997; Koehler and Myers, 1997), Msn1p (Gagiano et al., 1999a, b) and Mss11p (Gagiano et al., 1999a, b), but these factors

have either not been placed in the context of known signal transduction pathways, have not been characterised sufficiently or seem to function through alternative pathways.

Filamentous growth is a complex phenotype that requires the integration of not just nutritional signals, but also several other environmental signals into the co-ordinated expression of a large number of genes involved in diverse cellular processes such as the cell cycle, budding, flocculation, cell wall maintenance, etc. (reviewed in Cid et al., 1995; Gancedo, 2001). Most of these cellular processes have been studied extensively and the impact of environmental cues, such as nutritional signals, on them are characterised reasonably well. The impact of nutritional signals on the genes specifically required for the adhesion of yeast cells to substrates or each other is less clear. This group of genes consists of *FLO1*, *FLO5*, *FLO9*, *FLO10*, *MUC1/FLO11*, *FIG2* and *AGA1* (Guo et al., 2000). The ability to adhere to surfaces or other cells is a critical requirement for invasive growth, pseudohyphal differentiation, mating and flocculation (Guo et al., 2000). Of these genes, only *FLO1*, *FLO5* and *MUC1* have been studied to some extent. As a consequence, a large gap between the responses of yeast cells to environmental conditions (e.g. stress responses and nutritional conditions) and the regulation of these genes exists in the literature. This is caused, in part, by the fact that most laboratories prefer to work with *S. cerevisiae* strains from the S288c or W303 genetic backgrounds that do not exhibit flocculation or adhesion phenotypes (Kron, 1997). Consequently, connections between conditions that would act as input signals and the expression of the adhesin-encoding genes were never made.

The expression of some of the genes required for the filamentous growth response, specifically *MUC1*, is also co-regulated with the expression of genes required for the utilisation of the polysaccharides, starch and pectin. The signalling cascades that regulate filamentous growth were demonstrated to regulate two genes that encode polysaccharide-degrading enzymes. The *STA2* gene codes for an extracellular glucoamylase that enables yeast cells to utilise starch as a carbon source (reviewed in Pretorius et al., 1991; Vivier et al., 1997). The pheromone-responsive MAPK cascade (Gagiano et al., 1999b) as well as the cAMP-PKA pathway (Gagiano et al., 1999a) was shown to regulate *STA2* expression in a similar manner than the expression of *MUC1* under the same conditions. The *PGU1* (also known as *PGL1*) gene encodes an endopolygalacturonase that enables yeast cells to depolymerise pectin (Gognies et al., 1999, 2001). *PGU1* is regulated by the mating pheromone responsive MAPK cascade in a similar manner than *MUC1* under the same conditions (Madhani et al., 1999). Therefore, the filamentous growth response in yeast seems to be co-regulated, at least to some extent, with the polysaccharide metabolism.

This literature review focuses on nutritional sensing and signalling as they relate to filamentous growth. The *MUC1* gene is used as a representative member of the *S. cerevisiae*

adhesin/flocculin family, although there are differences in the regulation of the different members of this gene family due to specific requirements for the encoded proteins under specific conditions (Guo et al., 2000). Some of the gaps in the literature are tentatively filled by using analogies to published work on the co-regulated *STA2* glucoamylase gene, and others by reasoning and speculation. Therefore, to give an updated review of nutritional signalling in *S. cerevisiae* and its relation to filamentous growth, this chapter will discuss the mechanisms through which *S. cerevisiae* senses nutrients in its environment, specifically carbon and nitrogen sources, and how it transmits these signals to the nucleus to regulate the transcription of *MUC1*, as a representative factor, to establish the pseudohyphal and invasive growth phenotypes.

1.1 The sensing of carbon sources

Glucose is the most abundant monosaccharide in nature and the preferred carbon and energy source for most organisms, including yeast (reviewed in Carlson, 1998, 1999; Kruckeberg et al., 1998; Johnston, 1999). The pathway for glucose catabolism in *S. cerevisiae* is glycolysis, but a variety of other sugars can also be utilised as sources of carbon and energy through this pathway (reviewed in Barnett, 1976; Johnston and Carlson, 1992) (Fig. 1). Fructose, like glucose, is readily phosphorylated and enters glycolysis directly, whereas galactose and mannose are first converted to glucose-6-P and fructose-6-P, respectively, before entering glycolysis. Di-, tri- and oligosaccharides have to be hydrolysed into monosaccharides that can enter the glycolytic pathway. Sucrose is therefore cleaved into glucose and fructose by invertase, maltose into glucose by maltase (α-D-glucosidase) and melibiose into galactose by melibiase (α-D-galactosidase). Poly- or trisaccharides consisting of one or more than one type of sugar are hydrolysed into monosaccharides by a combination of enzymes. Raffinose, for example, is cleaved into the monosaccharides, galactose, glucose and fructose, through the combined efforts of melibiase and invertase. The polysaccharide, starch, however, requires glucoamylase to be hydrolysed to glucose (reviewed in Vivier et al., 1997). The enzymatic breakdown of the di-, tri- and oligosaccharides can occur intracellularly if transporters for the sugars exist, e.g. maltose which is transported by a maltose permease, otherwise it occurs extracellularly and the liberated monosaccharides are transported into the cell before entering glycolysis, e.g. starch (Barnett, 1976; Johnston and Carlson, 1992; Vivier et al., 1997).

The mechanisms by which yeast cells detect the presence of carbon sources in their environment have been investigated only in recent years (reviewed in Johnston, 1999; Kruckeberg et al., 1998; Rolland et al., 2001). Most of this work focused on the sensing of

glucose concentrations, although a substantial amount of data on the utilisation of other carbon sources, such as maltose and galactose, also became available. Glucose, however, seems to be sensed by the most complex and extensive array of mechanisms. The reason for this might be that yeast cells are exposed to a wide range of glucose concentrations and therefore need specialised sensing and transport mechanisms to make optimal use of this (Boles and Hollenberg, 1997; Kruckeberg et al., 1998). *S. cerevisiae* therefore developed highly specialised mechanisms that allow it to rapidly perceive and communicate the levels of glucose in the environment to the regulatory machinery of the cell. This, in turn, results in the fast and exclusive utilisation of all the glucose in the environment and the conversion thereof to ethanol, which gives a selective advantage to the ethanol-tolerant yeast cells (Kruckeberg et al., 1998; Rolland et al., 2001). The regulation of the transport of carbon sources such as glucose, maltose and galactose, the regulation of the metabolism of alternative carbon sources and the levels of expression of genes encoding components of the glycolytic pathway, all allow *S. cerevisiae* to respond effectively to fluctuations in glucose concentrations and are all examples of mechanisms directly regulated by the sensing of glucose (reviewed in Johnston and Carlson, 1992; Carlson, 1998, 1999; Kruckeberg et al., 1998; Johnston, 1999).

The major mechanisms by which yeast cells can sense carbon sources are either through the specific association of the molecules with specific proteins, or through the monitoring of metabolic derivatives of glucose (Johnston, 1999; Rolland et al., 2001). Examples of both such mechanisms exist in *S. cerevisiae*. The G-protein-coupled receptor, Gpr1p, and the two glucose transporter homologues, Rgt2p and Snf3p, were proposed to be glucose-binding proteins that relay the concentration of glucose to the regulatory machinery as a nutritional signal. Although a large amount of evidence suggests that this is highly likely, the physical binding of glucose to these proteins has not been demonstrated to date. Both maltose and galactose, however, bind to specific inducer proteins that orchestrate the rapid utilisation of these carbon sources, while at the same time mediating the repression of genes required for the utilisation of lesser-preferred carbon sources (Trumbly, 1992; Gancedo, 1998; Carlson, 1999). In response to glucose, the glucose-phosphorylating enzymes, glucokinase (Glk1p), hexokinase 1 (Hxk1p) and hexokinase 2 (Hxk2p), were also shown to perform a sensing or monitoring function (Johnston, 1999; Rolland et al., 2001) and to regulate a large number of genes in response to the presence of glucose.

The Hxt glucose transporters, to which both Rgt2p and Snf3p are highly homologous, can transport other monosaccharides that are structurally similar to glucose, e.g. fructose and mannose, albeit with lesser efficiency (Barnett, 1976; Boles and Hollenberg, 1997). Since the glucose-binding domains of Rgt2p and Snf3p, are proposed to correspond to the modified or

mutated transport domain (Özcan and Johnston, 1999), it is possible that these glucose sensors are able to bind some of the monosaccharides that are structurally similar to glucose, and relay the presence and concentration of these carbon sources to the cell. Most of these carbon sources are, however, also converted to glucose or fructose before entry into glycolysis (Fig. 1.), and it is therefore possible that such carbon sources are not sensed in their original state, but that the cell senses them in terms of the glucose or fructose molecules they are converted into. The actual sensing would then occur through an intracellular glucose-sensing mechanism made up by, for example, the hexokinases and/or glucokinase. A similar scenario might apply to the di-, tri-, oligo- and polysaccharides for which transporters do not exist. The hydrolysis of these molecules generates the constituting monosaccharides extracellularly, and these are then transported into the cell by a transporter protein (Fig. 1). These monosaccharides can then enter glycolysis as either glucose or fructose. The possibility, however, also exists that other unidentified mechanisms are in place for the specific sensing of these carbon sources.

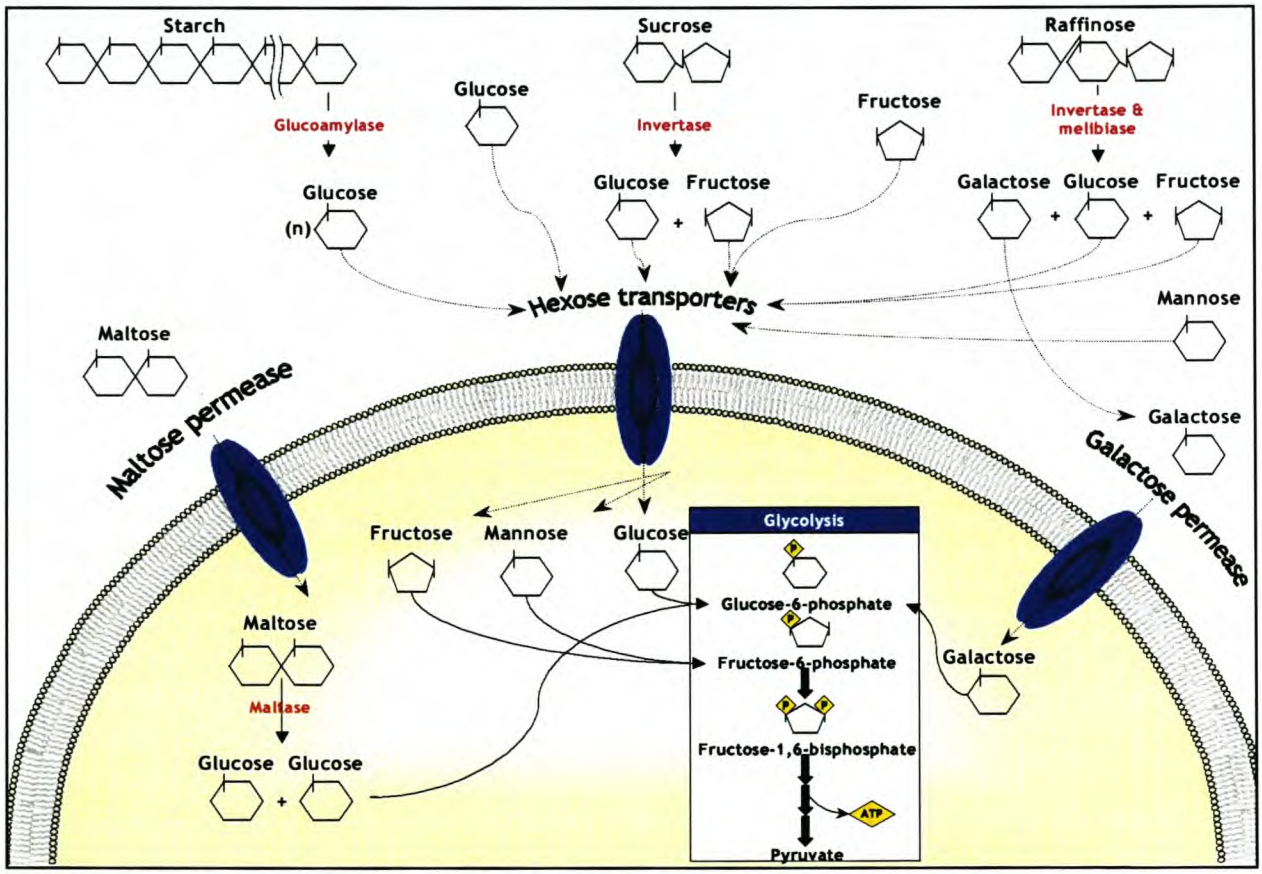

Figure 1. A diagram illustrating the different steps required for the uptake and utilisation of carbon sources by *S. cerevisiae*. See text for details.

The sensing of carbon sources other than glucose, maltose and galactose has not received much attention to date. As a consequence, some components involved in the sensing of

carbon sources and the subsequent signal generation processes remain to be identified. These gaps make it difficult to connect the sensing of the different carbon sources to specific signalling processes at this stage. Furthermore, not all of the sensing mechanisms have been shown to impact on the pseudohyphal differentiation and invasive growth phenotypes. Nevertheless, the next section will attempt to give a complete overview of the specific carbon source sensing mechanisms that have been identified in *S. cerevisiae* to date, with some reference to the signal components that were shown to respond to these sensing mechanisms. The specific transmission of the signals generated by these sensing mechanisms, however, will be discussed as a separate topic in the next section (section 2).

1.1.1 Sensing of carbon sources through a G-protein-coupled receptor (GPCR)

Heterotrimeric G-proteins are important regulators of cell growth and development in eukaryotic cells. These protein complexes are primarily responsible for the generation of intracellular signals in response to extracellular cues such as hormones, neurotransmitters, pheromones, light and odorants in higher eukaryotic systems (reviewed in Neer, 1995). The signals are subsequently passed on to intracellular effectors such as adenylate cyclase, phospholipases or protein kinases. The G-protein activity is regulated through a guanidine nucleotide exchange cycle wherein the ligand-bound or active receptor stimulates the exchange of GDP for GTP on the α-subunit of the complex. This association of the α-subunit with GTP stimulates its dissociation from the $\beta\gamma$-dimer (Neer, 1995). Either the free α-subunit or the $\beta\gamma$-dimer then regulates the downstream effectors. The α-subunit has an intrinsic GTPase activity, which hydrolyses the GTP to GDP. The hydrolysis of GTP promotes the re-association of the heterotrimeric G-protein complex and terminates the signalling event (Neer, 1995).

The molecular mechanisms and functioning of G-protein-coupled signalling have remained evolutionarily conserved to the extent where G-protein-coupled receptors from higher eukaryotes can be functionally expressed in yeast. The human β-andrenergic receptor and $G_s\alpha$-subunit, for example, were functionally expressed in *S. cerevisiae* and were shown to result in the ligand-dependent activation of the mating pheromone-responsive pathway (King et al., 1990). The molecular structure of the components also remained evolutionarily conserved and were used to identify G-protein α-subunits in several fungal systems, including *Ustilago maydis*, *Cryptococcus neoformans*, *Cryptonectria parasitica*, and most significantly, *S. cerevisiae* (Yun et al., 1997, 1998; Xue et al., 1998; Kraakman et al., 1999; Lorenz et al., 2000; Tamaki et al., 2000).

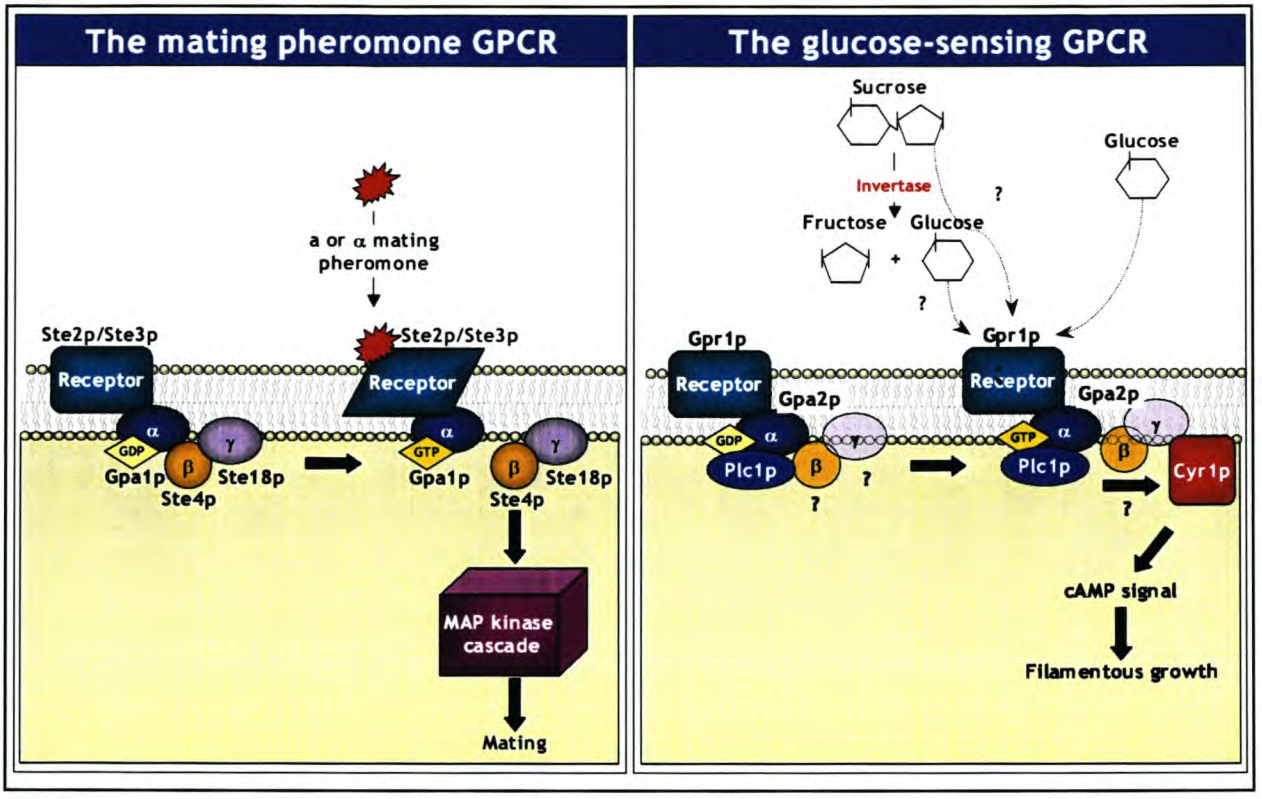

Figure 2. A diagram illustrating the structure and key features of the G-protein-coupled receptors of *S. cerevisiae*. Unknown aspects and components of the Gpr1-Gpa2p GPCR, implicated in filamentous growth, are indicated with question marks (see text for details).

Only three GPCR systems have been identified in *S. cerevisiae* to date. Of these, two are required for regulating the pheromone-responsive MAP kinase cascade during mating in haploid cells and consist of the mating pheromone receptors Ste2p and Ste3p (the α- and a-factor receptors, respectively) and the associated G-protein subunits, Gpa1p (α-subunit), Ste4p (β-subunit) and Ste18p (γ-subunit) (reviewed in Sprague and Thorner, 1992) (Fig. 2). These GPCR systems transmit mating signals to the conserved MAP kinase cascade through interaction with a large multimeric complex. The third GPCR system, of which only the receptor, Gpr1p, and the α-subunit, Gpa2p, have been identified until now, was shown to regulate cAMP levels in response to specific nutritional conditions (Kübler et al., 1997; Yun et al., 1997, 1998; Xue et al., 1998; Kraakman et al., 1999; Lorenz et al., 2000; Tamaki et al., 2000). This Gpr1p GPCR system was shown to be involved in the sensing of both carbon and nitrogen sources and transmits the presence, absence or limitation of these nutrients via cAMP signals to regulate, amongst others, the transcription of genes involved in pseudohyphal and invasive growth (Ansari et al., 1999; Lorenz et al., 2000; Tamaki et al., 2000). Although the other components of the GPCR have not been identified, several elements residing downstream thereof were shown to be required for the signalling of the carbon or nitrogen source. These include the cAMP-dependent kinases, Tpk1p, Tpk2p and Tpk3p, and the transcription factors, Sfl1p and Flo8p (Pan et al., 2000). The genes specifically required for

pseudohyphal differentiation and invasive growth, such as those encoding the cell surface flocculins, Flo1p, Flo5p, Flo9p, Flo10p and Muc1p, are probably all regulated via this GPCR-cAMP pathway (Lorenz and Heitman, 1997; Gagiano et al., 1999a; Rupp et al., 1999; Guo et al., 2000; Lorenz et al., 2000).

1.1.1.1 The G-protein-coupled receptor, Gpr1p

GPR1 was identified by different research groups in two-hybrid screens for proteins that interact with the putative G-protein α-subunit, Gpa2p (Yun et al., 1997; Xue et al., 1998; Kraakman et al., 1999). The only gene identified in these screens encoded a product with some resemblance to eukaryotic G-protein-coupled receptors, and was therefore designated *GPR1* (G-protein-coupled Receptor). *GPR1* encodes a 961 aa protein with the characteristic seven transmembrane helices of G-protein-coupled receptors, a large intracellular loop (aa 273-622) between transmembrane helices five and six, a long C-terminal tail (aa 679-961) and an asparagine-rich domain (aa 471-587) (Yun et al., 1997; Xue et al., 1998; Kraakman et al., 1999). In addition to these structures, it also contains five phosphorylation sites for the cAMP-dependent kinases (Yun et al., 1997). Several amino acids located in the transmembrane domains of Gpr1p are conserved within the G-protein-coupled receptor superfamily (Xue et al., 1998; Kraakman et al., 1999) and are hypothesised to maintain the structure of the receptor in the membrane and allow it to bind to the G-protein (Xue et al., 1998). The last ~120 C-terminal aa of Gpr1p were shown to associate with the G-protein α-subunit, Gpa2p in the two-hybrid system (Yun et al., 1997; Xue et al., 1998; Kraakman et al., 1999). Genetic evidence suggests that this association is required for the transmission of the cAMP signal that is generated in response to changes in the nutritional status (Xue et al., 1998).

1.1.1.2 The G-protein α-subunit, Gpa2p

GPA2 was originally cloned based on sequence homology to a G-protein α-subunit isolated from rat brain tissue. *GPA1*, the only other yeast gene encoding a G-protein α-subunit, was cloned via the same strategy (Nakafuku et al., 1987, 1988). *GPA1* was shown to be required for the mating pheromone response (Dietzel and Kurjan, 1987), whereas a possible physiological role for *GPA2* was suggested only recently, when it was shown to regulate the filamentous and invasive growth phenotypes via cAMP levels in diploid *S. cerevisiae* cells (Lorenz and Heitman, 1997). The expression of *GPA2* from a 2μ-based multiple copy plasmid, resulted in two-fold increased cAMP levels (Nakafuku et al., 1988; Papasavvas et al., 1992), a phenotype similar to that observed with the overexpression of *RAS2* (Toda et al., 1985). It was also shown that *GPA2* overexpression could suppress the growth defect of a temperature-

sensitive *ras2* mutant (Nakafuku et al., 1988), but that the deletion of both *RAS2* (but not *RAS1*) and *GPA2* resulted in a severe growth defect (Kübler et al., 1997; Lorenz and Heitman, 1997; Xue et al., 1998). This phenotype could only be rescued by the deletion of the gene encoding a phosphodiesterase, *PDE1* (Xue et al., 1998). Further epistasis experiments with deletions and hyperactive alleles of *RAS2* and *GPA2* showed that they have a partially redundant function, but that they act in separate pathways to regulate cAMP levels (Xue et al., 1998). The deletion of *GPA2* has no effect on mating, sporulation, or growth (Lorenz and Heitman, 1997; Yun et al., 1997), but eliminates glucose-induced cAMP signalling completely (Colombo et al., 1998). The fission yeast homologue of *GPA2* has also been cloned and the overexpression and deletion thereof in *Schizosaccharomyces pombe* were shown to have similar phenotypes to those observed for *S. cerevisiae* (Isshiki et al., 1992).

Gpa2p has a highly conserved GXGXXG motif that is characteristic of all G-proteins, including Ras2p (Lorenz and Heitman, 1997). The integrity of this motif is coupled to the intrinsic GTPase activity of these proteins and any mutations in these domains impair the function of the G-protein (Graziano and Gilman, 1989; Masters et al., 1989). One specific mutation, the substitution of the second glycine in the motif for valine to generate a GXVXXG motif, reduces the GTPase activity of the protein almost 100-fold and renders it active by promoting the GTP-bound form (Graziano and Gilman, 1989; Masters et al., 1989). This constitutively active form of Gpa2p also results in increased levels of cAMP, similar to the overexpression thereof, and results in increased pseudohyphal differentiation and invasive growth in some strains of *S. cerevisiae* (Lorenz and Heitman, 1997). The mechanism through which Gpa2p stimulates the adenylate cyclase encoded by *CYR1* to increase the levels of cAMP in the cell is also unknown at this stage. Analogous to well-characterised mammalian and fungal GPCR systems, this could occur through the G-protein β- and γ-subunits.

1.1.1.3 Other components of the Gpr1p-Gpa2p GPCR

The β- and γ-subunits of the Gpr1p-Gpa2p G-protein-coupled receptor system remain to be identified. Attempts to identify and clone such subunits have been unsuccessful. Deletion of eight genes with sequence homology to identified fungal β-subunits (*S. pombe* Gpb1p and *S. cerevisiae* Ste4p) did not reveal a role for any of these proteins in regulating cAMP levels or pseudohyphal differentiation (Lorenz and Heitman, 1997). The deletion of three potential GPCR γ-subunits, identified through homology searches to known γ-subunits, also did not reveal any function in cAMP signalling or pseudohyphal differentiation (Lorenz and Heitman, 1997). If such subunits exist, they will have to be identified through alternative means, since

the sequence homology to known fungal β- and γ-subunits seems to be too low to allow for their identification.

Besides the receptor, Gpr1p, the only other protein that has been shown to interact with Gpa2p is the phospolipase C, Plc1p (Ansari et al., 1999). Phospholipase C was shown to bind to the C-terminal domain of Gpr1p through co-immunoprecipitation and two-hybrid analysis (Ansari et al., 1999). This interaction between Gpr1p and Plc1p is dependent on an interaction between Gpa2p and Gpr1p and is also required for pseudohyphal differentiation (Ansari et al., 1999). The enzymatic function of Plc1p is the hydrolysis of phosphatidylinositol 4,5 bisphosphate to produce diacylglycerol and inositol 1,4,5-triphosphate (Ansari et al., 1999). Both these products were shown to be important second messengers in animal cells, where their activity is usually stimulated by G-protein-coupled hormone receptors (Wilcox et al., 1998; Lennartz, 1999). Plc1p is not essential for viability at 25°C, but the deletion of *PLC1* results in multi-budded, enlarged cells that are unable to complete cytokinesis at 35°C. It also renders the cell sensitive to osmotic stress, nitrogen starvation and unable to utilise fermentable carbon sources or to sporulate (Ansari et al., 1999). These phenotypes suggest that an important G-protein-associated signalling function is coupled to Plc1p. However, the exact function of the Plc1p-Gpr1p-Gpa2p association is unknown at this stage.

1.1.1.4 The Gpr1p GPCR-generated signal and transmission

The exact mechanisms through which ligands bind to the Gpr1p-Gpa2p GPCR to stimulate signals are unknown. Besides having a role in the sensing of nitrogen sources, Gpr1p and Gpa2p were shown to be required for the sensing of glucose (Kraakman et al., 1999; Lorenz et al., 2000; Rolland et al., 2000) and sucrose (Lorenz et al., 2000; Rolland et al., 2000). As with the sensing of nitrogen sources, the function of Gpr1p in the sensing of carbon sources was shown to control pseudohyphal differentiation (Lorenz et al., 2000). The actual binding of these molecules to Gpr1p has not been demonstrated to date. Gpr1p was also shown not to respond to fructose, mannose, galactose, xylose or any glucose analogues, such as 2-deoxyglucose or 6-deoxyglucose (Lorenz et al., 2000; Rolland et al., 2000), and therefore seems specific for the sensing of glucose and sucrose as far as carbon sources are concerned. However, the turnover of glucose and fructose from the hydrolysis of sucrose by secreted invertase is quite rapid and the possibility therefore exists that the GPCR system might actually be specific to glucose. The reported sensing of sucrose via the GPCR could therefore be artefactual.

Overexpression and deletion experiments with both Gpr1p and Gpa2p established a direct connection between the intracellular cAMP levels and the Gpr1p-Gpa2p GPCR system and suggested with reasonable certainty that the GPCR relays the nutritional signal to the cell via

a cAMP signal. The exact mechanism through which the GPCR stimulates adenylate cyclase to increase the cAMP levels is unknown, which is largely due to the fact that two critical components of the GPCR remain to be identified. In characterised GPCR systems from other organisms, the β- and γ-subunits are responsible for contacting adenylate cyclase directly to stimulate an increase in cAMP levels. It is also possible that the Gpr1p-Gpa2p GPCR system does not have β- and γ-subunits. The finding that Gpr1p and Gpa2p associate with the *PLC1*-encoded phospholipase C (Ansari et al., 1999) also opens up other signalling possibilities. It is clear that Plc1p plays an important physiological role in the yeast cell and that this is probably associated with its enzymatic activity, rather than having a structural role in the receptor complex. This implies that the products, diacylglycerol and inositol 1,4,5-triphosphate, could act as second messengers and perform a signalling function in *S. cerevisiae*, similar to the situation in mammalian cells where these molecules act as second messengers in response to G-protein stimulation upon ligands binding to the receptor (reviewed in Wilcox et al., 1998; Lennartz, 1999).

It is unclear at this stage how the carbon source signal generated by the GPCR system relates to the transcriptional activity of *MUC1* to result in pseudohyphal differentiation and invasive growth. Several reports have shown that the transcription levels of *MUC1* correlate well with the invasive growth and pseudohyphal phenotypes. The transcription of *MUC1* is, however, repressed in the presence of abundant rapidly fermentable carbon sources, such as glucose (Gagiano et al., 1999a, b; Rupp et al., 1999), and activated when glucose concentrations are low (Gagiano et al., 1999a; Rupp et al., 1999; Cullen and Sprague, 2001) or in the presence of poor carbon sources such as starch (Lambrechts et al., 1996). This would suggest that a carbon source starvation signal, rather than an abundance of carbon source, results in the expression of *MUC1*. However, there are reports that suggest the contrary, i.e. that *MUC1* and the associated phenotypes are activated by an abundance of carbon source (Lorenz et al., 2000). Although the authors failed to separate the nitrogen-sensing function of the GPCR system from the carbon source-sensing function (they used plates with limiting concentrations of nitrogen but high concentrations of carbon source), it is nevertheless clear that *MUC1* transcription can be induced in the presence of increased levels of cAMP and that it is regulated by the GPCR (see section 2.1. for cAMP signalling).

1.1.2 Sensing of carbon sources through transporter homologues

A second potential glucose-binding receptor system in *S. cerevisiae* was shown to consist of hexose transporter homologues, Rgt2p and Snf3p (reviewed in Kruckeberg et al., 1998; Özcan and Johnston, 1999; Rolland et al., 2001). These proteins have some homology and structural similarity to the hexose transporter family, but are unable to perform any transport function.

Genetic experiments suggested a role for these proteins in the sensing of glucose, although the physical binding of glucose to these proteins has yet to be demonstrated. The exact nature of the generated signal is at this stage also unknown, since cAMP signalling was eliminated as a possible candidate mechanism (Rolland et al., 2001). Rgt2p and Snf3p, however, were shown to be required for a regulatory network that controls the expression levels of glucose-repressed genes, e.g. those that encode products required for the utilisation of alternative carbon sources such as maltose, as well as glucose-induced genes, e.g. the glycolytic genes and hexose transporters, in the presence of glucose. The structure as well as the function of these proposed receptors are discussed in the next section. The signal transduction pathways and the regulation of transcription stimulated by these receptors are discussed in section 2.4.

1.1.2.1 The hexose transporter homologues, Rgt2p and Snf3p

The hexose transporter family of *S. cerevisiae* consists of 20 proteins encoded by the *HXT1-HXT17*, *RGT2*, *SNF3* and *GAL2* genes (reviewed in Boles and Hollenberg, 1997; Özcan and Johnston, 1999). The encoded products are highly conserved with 50-100% identity on amino acid level. Snf3p and Rgt2p are exceptions to this, since they only have 26 and 30% identity, respectively, to the other members of the family, but 60% identity to each other. The major characteristics of the hexose transporters are 12 transmembrane domains and a C-terminal extension of ~50 aa. Rgt2p and Snf3p, however, possess much larger C-terminal domains consisting of 218 and 341 aa, respectively (Özcan and Johnston, 1995, 1996, 1999).

Of the entire family of hexose transporters, only Hxt1p to Hxt7p were shown to be functional hexose transporters (Boles and Hollenberg, 1997; Özcan and Johnston, 1999). None of these transporters is essential and only the deletion of all seven of the encoding genes renders yeast cells unable to grow on glucose as a sole carbon source. The introduction of any one of the *HXT1-7* genes into such an *hxt1-7* mutant restores its ability to grow on glucose to various degrees, which illustrates the extent of functional redundancy between these transporters. Gal2p was initially identified as a galactose permease, but it is structurally similar to the hexose transporters and was also shown to transport glucose. Multiple copies of *GAL2* are therefore able to restore the growth defect of an *hxt1-7* mutant on glucose media. No hexose transport function has been observed for Hxt8p-Hxt17p, Snf3p or Rgt2p. The genes encoding Hxt8p-Hxt17p, however, are expressed at very low levels under most conditions and this is proposed as a possible reason why no transport function can be observed (Diderich et al., 1999).

The hexose transporters of *S. cerevisiae* import the hexoses, glucose, fructose and mannose, into the cell by means of passive, energy-independent facilitated diffusion along a

gradient (Özcan and Johnston, 1999). None of the hexose transporters of *S. cerevisiae* is specific for the import of mannose or fructose, whereas a transporter specific for fructose was identified in the closely related yeast, *S. carlsbergensis* (Gonçalves et al., 2000). The hexose transport proteins vary in their affinities for the hexoses as well as the transport rates of the different hexoses. The affinity correlates with their transcription patterns, e.g. transcription of *HXT1*, which encodes a low affinity transporter, is induced only by high levels of glucose (> 1%), and transcription of *HXT2*, *HXT6* and *HXT7*, which encodes high affinity transporters, is induced only in low glucose concentrations. *HXT1* and *HXT3* are therefore used for growth in high glucose concentrations, whereas *HXT2*, *HXT4*, *HXT6* and *HXT7* are used for growth in low glucose concentrations. Snf3p and Rgt2p do not transport glucose and were shown to be the glucose sensors that are required for sensing glucose in either high (Rgt2p) or low (Snf3p) glucose concentrations (Özcan and Johnston, 1996, 1999; Özcan et al., 1996, 1998).

The expression levels of the hexose transporters are governed by the signals generated by these two glucose sensors and are ultimately determined by the transcription factor Rgt1p (Özcan and Johnston, 1999). Rgt1p is a DNA-binding protein that acts as a permanent repressor of the *HXT* and other glucose-repressed genes. The repressor function of Rgt1p is regulated by Grr1p, which associates with the SCF ubiquitin-conjugating complex and probably regulates Rgt1p function through an ubiquitin-directed process (Özcan and Johnston, 1995; Li and Johnston, 1997). The expression levels of *RGT2* and *SNF3* also reflect on the roles of their encoded products as sensors of high and low glucose concentrations. *RGT2* is constitutively expressed (Özcan et al., 1996), whereas *SNF3* is repressed in the presence of high levels of glucose (Neigeborn et al., 1986).

The deletion of *SNF3* renders yeast cells unable to grow on raffinose as carbon source, since the ability to ferment raffinose is dependent on invertase expression that is impaired in a *snf3* strain (Marshall-Carlson et al., 1990; Özcan et al., 1996, 1998; Schmidt et al., 1999). The deletion of *RGT2*, on the other hand, does not result in growth defects on any media and displays wild-type levels of derepression for *SUC2* (Özcan et al., 1996, 1998; Schmidt et al., 1999), although minor defects in the glucose repression of *SUC2* in *rgt2* strains have been observed (Schmidt et al., 1999). Deletion of both *RGT2* and *SNF3* generates a strain with a slow-growth phenotype on media with glucose as carbon source (Schmidt et al., 1999). The *snf3 rgt2* strain is also unable to grow in the presence of the drug, antimycin, which inhibits mitochondrial function. However, such a strain can grow with wild-type rates on non-fermentable carbon sources, thereby indicating an inability to grow fermentatively (Schmidt et al., 1999).

As discussed, Rgt2p and Snf3p are structurally similar to the hexose transporters, with the only major exceptions being some sequence divergence and the large, extended C-terminal cytoplasmic tails (Özcan and Johnston, 1999). The 12 transmembrane domains of Rgt2p and Snf3p are proposed to form glucose-binding pockets that, because of the sequence divergence between the glucose transporters, are unable to facilitate the import of glucose into the cell, but that are still able to bind glucose (Özcan and Johnston, 1999). The C-terminal tails were identified as the domains required for the generation of intracellular glucose signals when glucose is bound to the extracellular parts of Rgt2p and Snf3p, since they were shown to be required for glucose induction of *HXT* expression (Özcan et al., 1998; Özcan and Johnston, 1999). Overexpression of the Snf3p C-terminal domain alone is sufficient to suppress the growth defect of *snf3* mutants in low glucose concentrations (Coons et al., 1997; Vagnoli et al., 1998) and the attachment of the C-terminal tail to Hxt1p or Hxt2p also complements the glucose induction defects of *snf3 rgt2* mutants (Özcan et al., 1998). The only significant homology between the sequences of the Rgt2p and Snf3p C-terminal tails resides in a stretch of 25 aa, where 16 out of the 25 aa are identical. Snf3p contains two of these 25 aa stretches, whereas Rgt2p contains only one. These stretches are required for the generation of the glucose induction signal, since deletion thereof, while leaving the remainder of the C-terminal tail intact, eliminates the glucose induction signal (Coons et al., 1997; Özcan et al., 1998). The exact mechanism through which these 25 aa stretches generate a glucose-induction signal has not been shown to date.

1.1.2.2 Proteins interacting with Snf3p and Rgt2p

Two proteins, Std1p and Mth1p, which interact with the cytoplasmic tails of Snf3p and Rgt2p, have recently been identified (Schmidt et al., 1999). *STD1* has also been cloned as a multicopy suppressor of *TBPΔ57*, a dominant negative mutation of the TATA binding protein (TBP) (Ganster et al., 1993). It was also cloned as *MSN3*, for being able to partially suppress a *snf4* mutation (Hubbard et al., 1994). It was later shown to interact with both TBP and Snf1p (Hubbard et al., 1994; Tillman et al., 1995).

Mth1p was isolated based on homology to Std1p, since the two proteins are 61% identical (Hubbard et al., 1994). A dominant negative mutation of *MTH1* was identified as a mutation in *HTR1*, which severely impairs yeast cells for glucose uptake (Schulte et al., 2000). Mutant forms of *MTH1* were also identified as being allelic to the mutant genes *DGT1-1* and *BPC1-1*, which impair glucose transport and catabolite repression in yeast (Lafuente et al., 2000).

The deletion of either *STD1* or *MTH1* has no apparent effect on cell growth or on invertase (*SUC2*) expression, whereas a double deletion results in a four-fold reduction in invertase expression. This would suggest that the two encoded products are redundant to some extent

(Hubbard et al., 1994). Subsequent work, however, showed distinct functions for the two proteins in receiving and transmitting the Rgt2p- and Snf3p -mediated signals (Schmidt et al., 1999; Lafuente et al., 2000; Schulte et al., 2000). Mutations in *MTH1*, but not *STD1*, are sufficient to restore the growth defects of *snf3* strains on raffinose media (Schmidt et al., 1999). Mutations in *MTH1* are also able to suppress the fermentation defects of *rgt2 snf3* strains, whereas mutations in *STD1* have no effect on this phenotype (Schmidt et al., 1999).

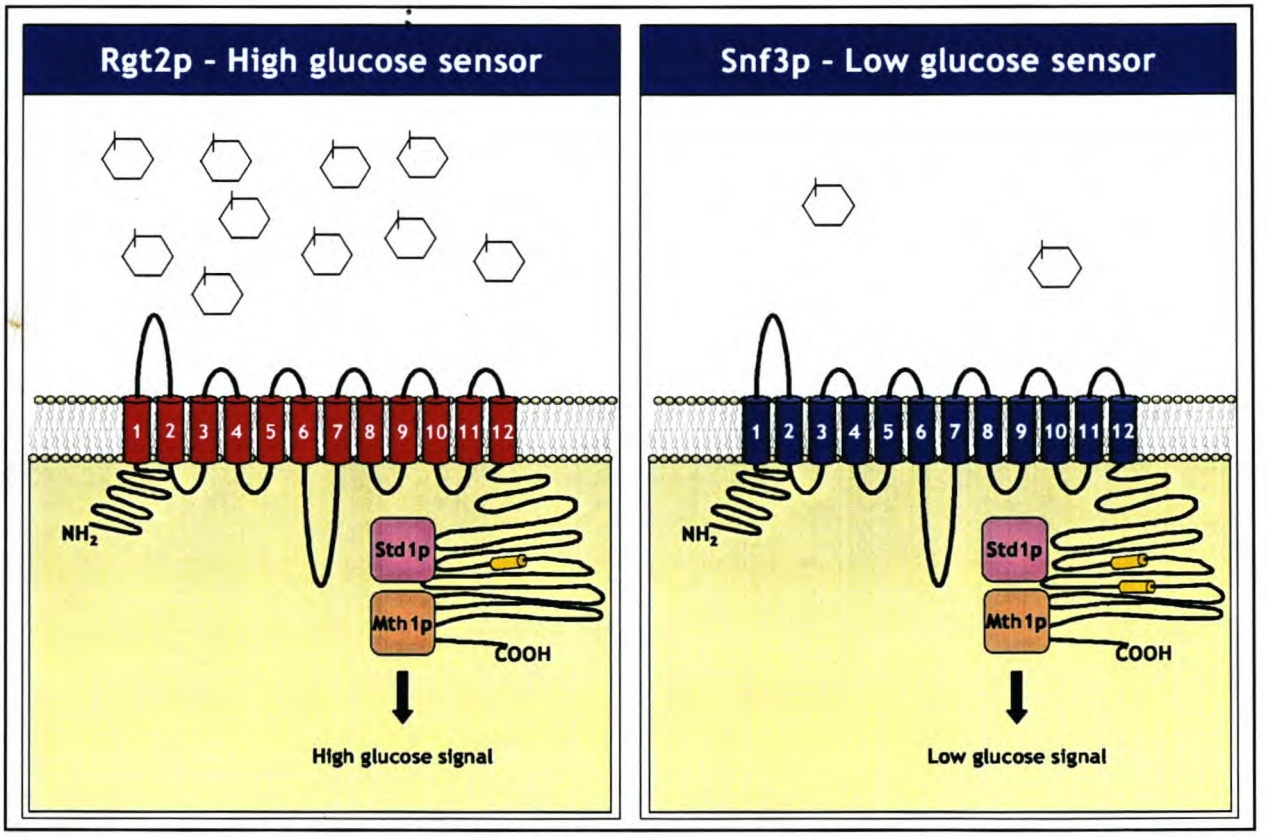

Figure 3. A diagrammatic representation depicting the structures of the glucose sensors, Rgt2p and Snf3p. Mth1p and Std1p were demonstrated to associate with the C-terminal domain of the proteins and relay the signals in different concentrations of glucose (Schmidt et al., 1999; Lafuente et al., 2000). The 12 transmembrane helices of Rgt2p and Snf3p are represented by the red and blue cylinders, respectively. The yellow cylinders illustrate the relative positions of the conserved 25 aa sequences of the proteins (see text for details).

STD1 is expressed constitutively, whereas *MTH1* is expressed only when glucose is depleted (Schmidt et al., 1999). This expression pattern is identical to that of *RGT2* and *SNF3*, which are also expressed constitutively and in low glucose concentrations, respectively. This suggests a role for Std1p in the signalling of high glucose concentrations and for Mth1p in the signalling of low glucose concentrations (Schmidt et al., 1999). Both Mth1p and Std1p interact with the C-terminal cytoplasmic tails of Rgt2p and Snf3p (Schmidt et al., 1999; Lafuente et al., 2000) and these interactions were shown to depend on the extracellular glucose concentrations (Lafuente et al., 2000). Genetic (Schmidt et al., 1999; Schulte et al., 2000) and biochemical (Lafuente et al., 2000) analyses revealed that Mth1p and Std1p act

antagonistically to the glucose sensors to establish different glucose-induced signals. Based on these data and the detailed analysis of different combinations of *rgt2*, *snf3*, *std1* and *mth1* mutations, models were proposed for the role of Std1p and Mth1p in signalling the concentration and availability of glucose (Schmidt et al., 1999; Lafuente et al., 2000; Schulte et al., 2000) (Fig. 3).

Experiments to detect the possibility of stable or permanent physical interactions between Mth1p, Snf3p and Std1p were unable to reveal any form of such a complex (Schmidt et al., 1999). Also, localisation studies with green fluorescent protein fusions revealed differences in subcellular localisation of Mth1p, Snf3p and Std1p. Whereas Snf3p-GFP localised to the cytoplasmic membrane, Mth1p-GFP and Std1p-GFP showed punctuated fluorescence in the nucleus and at the cytoplasmic periphery that was not dependent on glucose concentration (Schmidt et al., 1999). This suggests that the proteins are not components of a stable complex, but that they rather would have transient interactions with each other. It also raises the possibility that Std1p and Mth1p might have more general roles in signalling events and that they could be involved in other signalling events through associations with membrane signalling proteins such as Ssy1p that signals ammonium availability (see section 1.2.2. for Ssy1p) (Schmidt et al., 1999).

1.1.2.3 The Rgt2p and Snf3p-generated signals and transmission

In high glucose concentrations (Fig. 3), Rgt2p senses the availability of glucose, supposedly through physical interaction with glucose. This generates a signal that results in the activation of *HXT1*, which encodes a low affinity hexose transporter, while also inhibiting the activity of Std1p (Özcan et al., 1996, 1998; Schmidt et al., 1999). *MTH1* and the gene encoding the low glucose sensor, *SNF3*, are repressed under these high glucose conditions. Mth1p, however, is still functional under high glucose conditions, since the deletion thereof results in increased expression of *HXT2-4*, which encodes the high affinity glucose transporters and which is normally only expressed in low glucose conditions (Schmidt et al., 1999). In low glucose conditions (Fig. 3), Snf3p instead of Rgt2p interacts with glucose (Özcan et al., 1996, 1998; Schmidt et al., 1999). The transcription of the genes encoding the high affinity hexose transporters, *HXT2-4*, increases while Snf3p also inhibits the activity of Mth1p. In these conditions, Std1p acts upstream of the Snf1p kinase complex, which is required for the derepression of the genes encoding high affinity hexose transporters, *HXT2*, *HXT3* and *HXT4*, as well as *SUC2*. The activated Snf1p complex is also involved in the repression of *HXT1*. Std1p also negatively regulates Snf3p-mediated signalling. Since Mth1p and Std1p showed interactions with the C-terminal tail of Snf3p, this could be through direct competition for the same domain (Schmidt et al., 1999). In the absence of glucose, neither

Snf3p nor Rgt2p is able to generate a signal. Either Mth1p or Std1p is sufficient for the repression of the low affinity transporter gene, *HXT1*, and Mth1p plays an important role in the repression of the high affinity transporter genes, *HXT2*, *HXT3* and *HXT4* (Schulte and Ciriacy, 1995; Schmidt et al., 1999). The exact mechanism through which Mth1p and Std1p communicate the signals generated by Rgt2p and Snf3p, respectively, to the downstream components required for the activation or repression of genes under glucose control remains to be identified.

Two important questions concerning the sensing and transmission of the glucose-induction signal by Rgt2p and Snf3p and the associated proteins, Mth1p and Std1p, therefore remain. The first is the nature of the signal itself. It does not seem to be intracellular concentrations of glucose or metabolic products thereof, since glucose transport or metabolism is not required for the generation of the glucose-induction signal (Özcan et al., 1998). It is clear that glucose elicits the signal, probably via interaction with the glucose-binding pockets of Rgt2p and Snf3p that result in conformational changes at the cytoplasmic C-terminal tail, although no physical evidence exists that supports this link. The interaction of the Snf3p and Rgt2p cytoplasmic tails with other proteins besides Mth1p and Std1p, or the interaction of Mth1p and Std1p with other proteins, and subsequent conformational changes upon glucose binding to the receptors, could elicit a physical signal. Alternatively, the production of a secondary messenger such as cAMP might be stimulated by the binding of glucose to these receptors. However, recent work excluded cAMP as the signalling molecule for the *RGT2* and *SNF3*-encoded sensors (Rolland et al., 2000, 2001).

The second remaining question is whether Rgt2p and Snf3p signal the availability of other carbon sources, and whether this relates to morphological and physiological adaptations of the yeast cell. Most of the carbon sources are converted to monosaccharides that are readily (although with variation in affinity) transported by the hexose transporters into the cell. The 12 transmembrane domains that are common features of the hexose transporters as well as the glucose sensors constitute the glucose-transporting domains in the transporters and glucose-binding domain in the sensors. This allows one to speculate that these mutated transporter domains of Rgt2p and Snf3p could bind the monosaccharides that are transported by the hexose transporters. This would imply that Rgt2p and Snf3p could act as sensors for most of the carbon sources transported by the hexose transporters, i.e. glucose, fructose and mannose, as well as all the di-, tri- and polysaccharides that are hydrolysed to liberate the different monosaccharides transported by the hexose transporters.

Most of the studies on the Rgt2p and Snf3p glucose-induced signals exploited the well-characterised invertase expression levels and the ability of yeast cells to ferment sucrose or raffinose as a reporter system. Yeast cells tend to grow in invasive and filamentous forms on

carbon sources that are difficult to utilise such as raffinose or starch, but not on carbon sources that are readily utilised such as galactose or fructose (Lambrechts et al., 1996; Lorenz et al., 2000). Yeast cells also tend to grow invasively or in filamentous form upon glucose starvation (Gagiano et al., 1999a, b; Cullen and Sprague, 2001). One can speculate that the low amounts of monosaccharides produced by the extracellular hydrolysis of complex carbon sources, such as raffinose or starch, constitute a carbon source starvation signal. If this is indeed the case, Snf3p could be required for the generation of a carbon source-induced starvation signal that results in morphological adaptations such as pseudohyphal and invasive growth.

1.1.3 Sensing of carbon sources through hexose kinases

The sensing mechanisms that are based on the interaction of extracellular glucose with membrane-associated proteins such as Rgt2p, Snf3p and Gpr1p, are relatively new discoveries. The regulatory effect of glucose metabolism on gene regulation, however, is well established and has been investigated since the early advent of yeast genetics. Glucose metabolism provides an intricate and complex internal mechanism for the sensing of carbon sources. The majority of genes encoding the enzymes involved in glycolysis are expressed constitutively. In the presence of glucose, however, these genes can be induced to the extent where glycolytic enzymes can make up 30-60% of the total soluble protein in the cell (Fraenkel, 1982; Heinisch et al., 1991). By using a number of different glycolytic mutant strains, the induction of several glycolytic enzymes was shown to require the metabolism of glucose up to specific points in glycolysis (Boles and Hollenberg, 1997). This would suggest that several specific metabolic intermediates could act as inducers of the glycolytic genes.

The most prominent metabolic mechanism for glucose sensing involves hexokinases, the enzymes required for the phosphorylation of hexoses prior to entry into glycolysis. As described earlier, genes required for gluconeogenesis and respiration as well as for the utilisation of alternative carbon sources such as sucrose or maltose, are severely repressed in the presence of glucose or other rapidly fermentable carbon sources (Trumbly, 1992; Gancedo, 1998; Carlson, 1999). This regulation was shown to require the phosphorylation of the available monosaccharide (glucose, fructose or mannose), but not the further metabolism thereof. In *S. cerevisiae*, monosaccharides are phosphorylated by two hexokinase isozymes, which are encoded by *HXK1* and *HXK2*. A third hexose kinase, glucokinase, is encoded by *GLK1* and only phosphorylates mannose and glucose. The hexose kinases are not redundant and are expressed differentially (Herrero et al., 1995). The yeast cell also has different requirements for each in establishing the carbon catabolite repressed state (De Winde et al., 1996; Sanz et al., 1996). All three of the kinases are required for short-term glucose repression, whereas

only Hxk2p is required for sustained repression (De Winde et al., 1996). Furthermore, a specific role has been identified for Hxk2p in establishing the repressed state of a large number of genes in the presence of glucose or fructose (Trumbly 1992; Gancedo, 1998; Carlson, 1999). Hxk1p can also mediate repression when grown on glucose or fructose as carbon sources, but since it is regulated negatively by Hxk2p, Hxk2p plays the dominant role in mediating glucose repression (Herrero et al., 1995; De Winde et al., 1996).

The characterisation of this Hxk2p-mediated sensing mechanism has proven to be as difficult, if not more so, than the mechanisms discussed in previous sections (see sections 1.1.1 and 1.1.2). The main reason for this is the multiple roles of Hxk2p. As with the two previous mechanisms, it also controls a regulatory cascade that ensures that the preferred carbon sources are used rapidly and optimally when available, but it was also shown to play a more direct role by acting at the DNA level (Herrero et al., 1998). In addition to this, it can also contribute through metabolic regulatory mechanisms via its catalytic function, a function that it probably shares with the other hexose kinase, Hxk1p, and the glucokinase, Glk1p (reviewed in Gancedo, 1998; Rolland et al., 2001).

HXK2 was shown to be required for the repression of the *STA2* gene, which encodes a glucoamylase required for the utilisation of starch (Kartasheva et al., 1996). *STA2* and *MUC1*, which is required for filamentous growth and pseudohyphal differentiation, are co-regulated to a large extent and their promoters present a high degree of homology (Gagiano et al., 1999a, b). It is therefore highly likely that Hxk2p is also required for the negative regulation of *MUC1*, and consequently pseudohyphal differentiation and invasive growth, in the presence of rapidly fermentable carbon sources. Since the expression levels of *MUC1* were shown to be reduced in the presence of rapidly fermentable carbon sources such as glucose, sucrose and galactose, but not in poor carbon sources such as starch, or non-fermentable carbon sources such as glycerol and ethanol (Lambrechts, 1996; Gagiano, 1999a, b; Lorenz et al., 2000; Cullen and Sprague, 2001), it is highly likely that *MUC1* is repressed through the main glucose repression pathway and that this repression requires *HXK2*. This, however, remains to be shown.

1.1.3.1 The hexose kinases Hxk1p, Hxk2p and the glucose kinase, Glk1p

The genes encoding the three *S. cerevisiae* hexose kinases, *HXK1*, *HXK2* and *GLK1*, were originally identified and cloned for their role in glycolysis, i.e. the irreversible phosphorylation of the monosaccharides, glucose, fructose and mannose, prior to their entry into glycolysis (Frölich et al., 1985; Kopetzki et al., 1985; Albig and Entian, 1988) (Fig. 4). However, the *HXK2* gene that encodes hexokinase II was also identified as *HEX1* and *GLR1* in several screens for genes involved in the carbon catabolite or glucose repression of genes

required for the utilisation of galactose, maltose and sucrose, as well as genes required for respiration and gluconeogenesis (Entian 1980; 1981; Entian and Mecke, 1982; Michels et al., 1983). Hxk2p is the most abundant kinase when glucose is the sole carbon source. It is constitutively expressed on all media, but induced (up to 30-fold) in the presence of glucose (Herrero et al., 1995). Hxk1p and Glk1p are adequate for growth on glucose, but the levels of expression are the highest in the absence of glucose on carbon sources such as ethanol, glycerol and galactose (Herrero et al., 1995).

The genes encoding the hexose kinases are under complex transcriptional control. The *HXK2* gene has two downstream repressing sequences within its coding region (Herrero et al., 1996; Martínez-Campa et al., 1996). The transcription factors that operate through these sequences, repress *HXK2* transcription when glucose is depleted or when ethanol is used as carbon source (Herrero et al., 1996). Expression of the *GLK1* gene is under the combined control of three regulatory elements: a stress-responsive element (STRE), an ethanol-repression autoregulation/TA box (ERA/TAB) and a sequence through which the glucose regulatory protein, Gcr1p, functions (Liesen et al., 1996; Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996; Uemura et al., 1997). Like *GLK1*, *HXK1* is repressed when cells are grown on glucose, fructose or mannose. The repression occurs via an ERA element and, upon glucose depletion, activation occurs through several STRE elements (Rodriguez et al., 2001). Hxk2p is required for the repression of *GLK1* and *HXK1*, with the consequence being that it plays the dominant role in the glucose repression pathway (Rodriguez et al., 2001).

The hexokinases are similar, but not identical, in sequence and structure. Hxk2p exists as phosphorylated monomers or unphosphorylated dimers in the cell. The Hxk2p monomers have a higher affinity for glucose than the dimers and the dimerisation does not seem to be required for their function. The phosphorylation state of Hxk2p, however, seems to be important for its function (Randez-Gil et al., 1998a, b). The nuclear localisation of Hxk2p is also critical for its role in establishing glucose repression. An *HXK2* mutant, with a 30 bp deletion between nucleotides 19 and 48 and that was unable to confer glucose repression, was isolated. The catalytic activity of the encoded truncated Hxk2p, however, remained intact (Herrero et al., 1998). It was demonstrated that the nuclear localisation of this specific mutant was affected, which implies that the nuclear localisation of Hxk2p is required for its repressive function (Herrero et al., 1998).

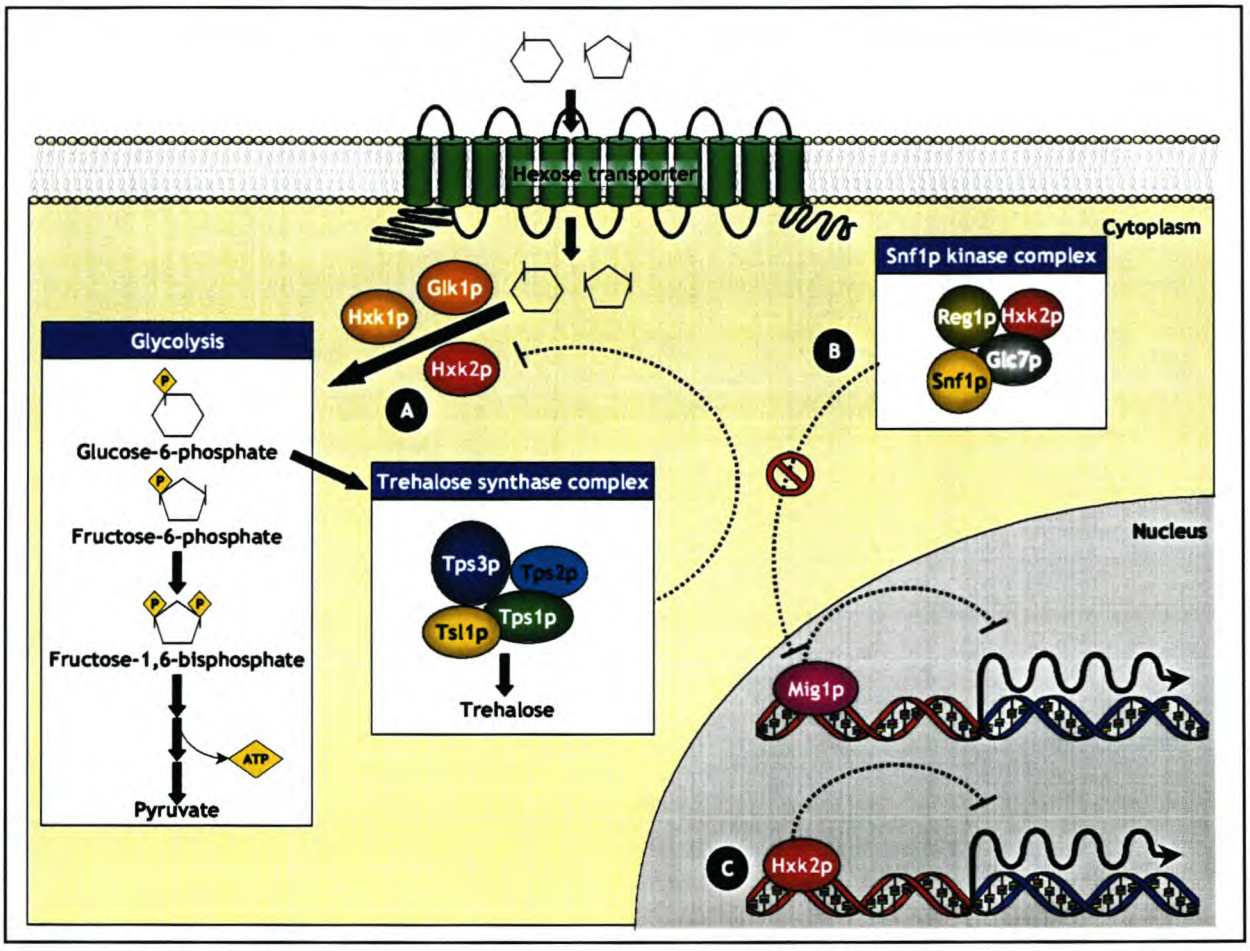

Figure 4. A diagrammatic representation depicting the proposed models for the sensing and transmission of glucose signals by the hexokinases, Hxk1p and Hxk2p, and the glucokinase, Glk1p, in yeast (see text for details). A.) The metabolic mechanisms through which regulatory functions can be exerted are shown. The products of hexose kinase catalytic activity can exert a regulatory function via the trehalose synthase, Tps1p. Metabolic products of glycolysis, e.g. ATP, or the intermediary metabolites can also act as secondary messengers to transmit the glucose signal. B.) Hxk2p exerts a regulatory role indirectly via the Snf1p protein kinase complex. C.) Hxk2p plays a direct role by interacting with *cis*-elements in the promoter of the *SUC2* gene (see text for details).

1.1.3.2 The regulatory mechanisms and signals generated by the hexose kinases

Despite almost two decades of research, the mechanisms through which the hexokinases regulate the induction and repression of genes in the presence of rapidly fermentable carbon sources still remain elusive. Substantiated by a number of observations, hypotheses have been proposed to explain both the nature of the signal and the mechanisms through which the signal is transmitted to the transcriptional regulatory machinery (reviewed in Johnston and Carlson, 1992; De Winde et al., 1996; Gancedo, 1998; Gonçalves and Planta, 1998; Carlson, 1999, Rolland et al., 2001). These include metabolic mechanisms whereby the hexose kinases would play an indirect role in regulation through the products of their catalytic activity, as well as more specific roles whereby Hxk2p would act directly at the DNA level or through regulating the main glucose repression pathway of Snf1p.

Hexose phosphates, the catalytic products of the hexose and glucose kinases, might act as signal transmitters, since a correlation has been reported between hexokinase activity and

the degree of repression (Rose et al., 1991). Also, a specific requirement exists for hexose kinase activity in establishing glucose repression (Ma et al., 1989). This would suggest that regulation occurs via a metabolic control and that a system is in place that monitors the level of phosphorylated sugars (e.g. glucose-6-phosphate and fructose-6-phosphate). One such a system might function via the trehalose-6-phosphate synthase, Tps1p, which was shown to restrict the influx of glucose into glycolysis by inhibiting hexokinase activity (Fig. 4) (Ernandes et al., 1998). Another metabolic regulatory system, influenced by the catalytic activity of the hexose kinases, might function through changes in the ATP:AMP ratio (Carlson, 1998, 1999). This was proposed because some correlation was observed between the ATP:AMP ratio and the extent of glucose repression (Wilson et al., 1996). However, under many conditions, e.g. in the presence of non-fermentable carbon sources, this correlation between the ATP:AMP ratio and repression is absent (Bañuelos et al., 1977).

Overexpression of *GLK1* in *hxk1 hxk2* mutants does not restore glucose-induced repression of *SUC2*, suggesting a specific requirement for the hexokinases and not the hexokinase activity (Herrero et al., 1995). Also, mutants were identified in which the two processes are uncoupled and which therefore suggests a more direct role for the hexokinase protein (Hohmann et al., 1999). Recent evidence suggests that Hxk2p can be localised to the nucleus and that this nuclear localisation is required for the repressive function. It was furthermore also demonstrated that Hxk2p forms part of a DNA-binding complex that binds to the promoter of the *SUC2* gene to establish the repressed state (Herrero et al., 1998).

Hxk2p also mediates a regulatory effect via the main glucose repression pathway (see section 2.4 for a detailed discussion of this pathway). The main component of this pathway is the Snf1p protein kinase that regulates transcription in response to a glucose signal by either inhibiting transcriptional repressors (e.g. Mig1p) or stimulating transcriptional activators (e.g. Cat8p) (reviewed in Carlson, 1998, 1999). The Snf1p protein kinase exists in an autoinhibited state in the presence of glucose and in an active state in the absence of glucose (Carlson, 1998, 1999). The functionality of the Snf1p protein kinase complex is determined, at least in part, by the protein phosphatase, Glc7p, and the regulatory subunit, Reg1p (Carlson 1998, 1999). Hxk2p was shown to regulate the activity of the Snf1p pathway via Glc7p-Reg1p and also to physically interact with Reg1p (Sanz et al., 2000). The exact mechanism by which it regulates the Snf1p function via Reg1p is unknown, but it could either be through stimulating the binding/phosphorylation of Reg1p or by preventing the dephosphorylation of Reg1p by Glc7p (Sanz et al., 2000).

The sensing of sugars by sugar kinases seems to be evolutionarily conserved, since it is not restricted to *S. cerevisiae* alone. The mammalian pancreatic glucokinase, hexokinase IV, is highly similar to the yeast hexokinase and is required for glucose sensing to adjust insulin

secretion and the transcriptional activation of glucose-induced genes (reviewed in German, 1993; Gidh-Jain et al., 1993). Hexokinases also play a central role in the sensing of sugars and the subsequent signal transmission, induced by these sugars, in maize and higher plants (Sheen et al., 1999). The products of the hexokinase-catalysed reactions, i.e. phosphorylated sugars, stimulate various responses in the cell. However, the further metabolism of these sugars is not required for this signalling. As in *S. cerevisiae*, the exact nature of this signal and how it is transmitted by the hexose kinases are unknown.

1.1.4 Other carbon source sensing mechanisms

The three mechanisms discussed in detail in the previous sections are the only ones identified in *S. cerevisiae* to date. This, of course, does not exclude the possibility that other, as yet unidentified, mechanisms might also exist. As stated before, most of the work to date only focused on the rapidly fermented carbon sources and the mechanisms through which the yeast cells sense and respond to these. A vast amount of work therefore remains required to establish how yeast cells sense and respond to alternative carbon sources such as glycerol, starch, xylose, etc. The connection between the carbon sensing mechanisms, the signals generated by these and the resulting responses such as pseudohyphal differentiation and invasive growth are therefore far from fully understood.

1.2 The sensing of nitrogen sources

S. cerevisiae is able to utilise a wide range of nitrogen sources, but, as with carbon sources, not all of these are utilised with equal efficiency (reviewed in Wiame et al., 1985; Marzluf, 1997; Ter Schure et al., 2000). In order to utilise any nitrogen-containing compound as a source of nitrogen, the yeast cell first has to convert it into either glutamine or glutamate. All nitrogen-containing compounds produced in the yeast cell can be synthesised using the degradation products of any carbon source and either glutamate or glutamine as the nitrogen donors (Magasanik, 1992; Ter Schure et al., 2000). Ammonia acts as a nitrogen donor for the synthesis of glutamate and glutamine: glutamate dehydrogenase converts ammonia and α-ketogluterate into glutamate, whereas glutamine synthetase converts ammonia and glutamate into glutamine (Fig. 5). Other nitrogen-containing compounds are also catabolised to yield ammonia, glutamate or glutamine. Asparagine is converted into aspartate and ammonia by asparaginases. Glutamate is produced from proline by a three-step process localised in the mitochondria. Urea is degraded in a two-step process to yield CO_2 and ammonia. All other nitrogen-containing compounds are also converted into glutamine and glutamate by similar mechanisms before they can be utilised by the yeast cell. Most amino

acids can be catabolised and subsequently used as sources of nitrogen, or can be directly incorporated into proteins during protein biosynthesis (Ter Schure et al., 2000).

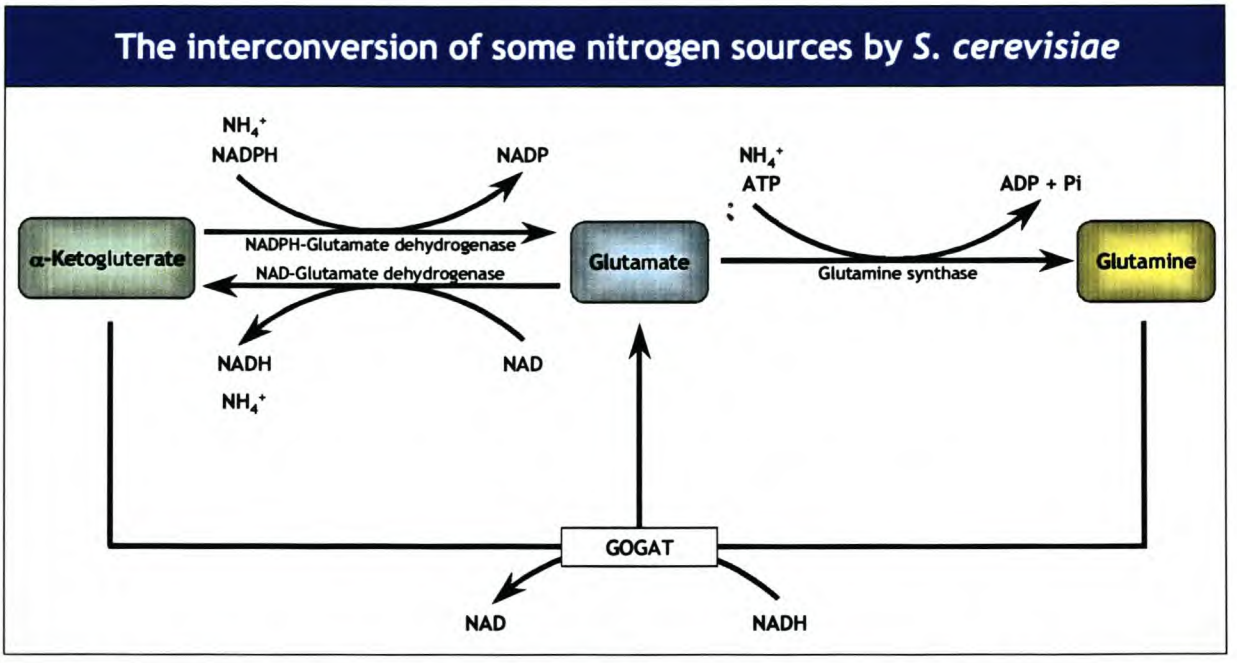

Figure 5. A diagrammatic representation depicting the interconversion between some of the nitrogen sources utilised by yeast cells.

As with carbon sources, *S. cerevisiae* has developed mechanisms that allow more optimal nitrogen sources to be utilised rapidly and optimally, while the utilisation of the less optimal nitrogen sources is repressed. This phenomenon and the underlying mechanism have been termed nitrogen catabolite repression (Wiame et al., 1985) or nitrogen regulation (Magasanik, 1992). Nitrogen sources such as ammonia, glutamate and glutamine are described as good nitrogen sources and support much higher growth rates than nitrogen sources considered to be poor, such as proline, arginine or urea. Similar to the sensing of carbon sources, the ability to discriminate against poor nitrogen sources in favour of better nitrogen sources suggests that yeast cells have mechanisms that allow the sensing of different nitrogen sources that are available in the environment and the implementation of a selective hierarchy for rapid and optimal nitrogen uptake and utilisation. Until recently, however, the selective regulation of genes in response to different nitrogen sources was assumed to be the result of variations in the intracellular concentrations of metabolites, produced during the utilisation of available nitrogen sources (Iraqui et al., 1999).

Pseudohyphal differentiation and invasive growth was initially described as a response to the limitation of nitrogen sources, in particular ammonia, but also to the availability of a poor nitrogen source, proline (Gimeno et al., 1992; Lorenz and Heitman, 1998b). The Gpr1p-Gpa2p G-protein-coupled receptor system and the cAMP cascade regulated by this system were

subsequently shown to be an important regulatory mechanism in response to the availability of the nitrogen source. The ammonium transporters, Mep1p, Mep2p and Mep3p, were also shown to be important regulators of the pseudohyphal and invasive growth phenotypes in response to the availability of the nitrogen source (Lorenz and Heitman, 1998a, b; Gagiano et al., 1999b). The amino acid transporter homologue, Ssy1p, was recently identified as a sensor for amino acids, and also as a regulator of invasive growth (Klasson et al., 1999; Forsberg and Ljungdahl, 2001). The involvement of amino acid permeases in the sensing of nitrogen sources, however, complicates the issue, since some amino acids can be utilised for protein biosynthesis and as a nitrogen source. It therefore becomes necessary to separate amino acid starvation from nitrogen starvation. In addition to the membrane-associated sensing components mentioned, several other components involved in the regulation of nitrogen metabolism have been implicated in the regulation of the invasive growth and pseudohyphal phenotypes, albeit to a very limited extent. These include the GATA factors Npr1p, Gln3, and Dal80p, as well as the non-GATA factor involved in nitrogen regulation, Ure2p (Lorenz and Heitman, 1998b).

The transcription of *MUC1* was shown to be regulated by factors acting downstream of the ammonium transporter *MEP2* (Gagiano et al., 1999b) as well as the GPCR sensor comprised of Gpr1p and Gpa2p (Lorenz and Heitman, 1997; Lorenz et al., 2000). The promoter region of *MUC1*, as well as several of the flocculation genes involved in pseudohyphal and invasive growth, contains a putative Gcn4p binding site in the far upstream region and several GATA boxes, that are binding sites recognised by the GATA factors that regulate the nitrogen response, closer to the open reading frame (ORF) (Gagiano et al., unpublished). The relevance of these putative binding sites in mediating the nitrogen-regulated transcription of *MUC1* is unknown at this stage.

1.2.1 Sensing of nitrogen sources through a G-protein-coupled receptor system

In addition to a clear role in the sensing of glucose and structurally related sugars (see section 1.1.1), the G-protein-coupled receptor system consisting of the sensor, Gpr1p, and the associated α-subunit, was also shown to regulate pseudohyphal differentiation and invasive growth in response to nitrogen starvation (Lorenz et al., 2000). The invasive and pseudohyphal phenotypes were shown to require *MUC1*, *GPA2* as well as *GPR1*, when any of a number of nitrogen sources were limiting, including ammonium, glutamine, proline, aspartate, asparagine and serine. The defects in invasive and pseudohyphal growth observed in *gpa2* and *gpr1* strains correlated well with the decreased transcriptional activity of *MUC1* (Lorenz et al., 2000). The *gpr1* and *gpa2* filamentation defects could furthermore be suppressed by the introduction of the constitutive *RAS2* allele, $RAS2^{val19}$, or the addition of

external cAMP (Kübler et al., 1997; Lorenz and Heitman 1997,1998a, b; Lorenz et al., 2000; Tamaki et al., 2000). This would suggest that the nitrogen starvation signal perceived by the Gpr1p-Gpa2p GPCR generates a cAMP signal that ultimately results in the pseudohyphal and invasive growth phenotypes. As with the different carbon sources reportedly sensed by the GPCR system, none of the nitrogen sources that the system responds to have been shown to physically interact with the Gpr1p sensor. The mechanisms by which limitations in the nitrogen source elicit a cAMP signal via the GPCR system therefore remain to be shown.

1.2.2 Sensing of nitrogen sources through transporter proteins

1.2.2.1 The ammonium transporter, Mep2p

The genes encoding the ammonium transporters, *MEP1*, *MEP2* and *MEP3*, were cloned through complementation in a yeast strain deficient in ammonium uptake (Marini et al., 1994). The characterisation of the encoded proteins revealed that *MEP1* and *MEP2* encode high affinity and *MEP3* encodes lower affinity ammonium transporters (Marini et al., 1994, 1997). These ammonium transporters are very similar in sequence and structure, with Mep3p being 79% identical to Mep1p and 39% identical to Mep2p (Marini et al., 1997). *MEP2* encodes the highest affinity ammonium permease, with a K_m of 1-2 μM for ammonium, whereas the *MEP1* encoded permease has a high K_m of 5-10 μM and the *MEP3*-encoded permease the highest K_m of 1.4-2.1 mM. Deletion of all three ammonium permeases renders the yeast inviable when grown on ammonium as sole nitrogen source.

The deletion of *MEP2*, but not of *MEP1* or *MEP3*, results in a severe filamentation defect on media containing limiting amounts of ammonium as nitrogen source. Nitrogen metabolism in *mep2* strains is not affected, suggesting a specific role for Mep2p in regulating pseudohyphal growth. Mep1p also plays a role in the sensing process, but seemingly as a negative regulator of filamentation (Lorenz et al., 2000). The deletion of *MEP2* does not confer a filamentation defect on any other nitrogen source but ammonium, suggesting ligand specificity (Lorenz and Heitman, 1998b).

Mep2p was subsequently shown to regulate the transcription of *MUC1* via two transcription factors, Mss11p and Msn1p, to establish the invasive growth and pseudohyphal phenotypes (Gagiano et al., 1999b). If expressed from a multiple copy plasmid, both Msn1p and Mss11p can suppress the transcriptional defect of *MUC1* in a *mep2* background (Gagiano et al., 1999b). Several other genes were also identified as multiple copy suppressors of the filamentation defect in a *mep1/mep1 mep2/mep2* strain (Lorenz et al., 2000). These include genes that encode the well-known pseudohyphal and invasive growth regulators, *PHD1 and TEC1*, several that encode the known transcriptional regulators, *MGA1, SKN7 and DOT6*, as

well as two novel genes, *HMS1* and *HMS2*, which encode products with similarity to known transcription factors (Lorenz et al., 2000). Other genes that do not encode transcription factors were also isolated in the same screen. *MSN5*, which encodes a protein required for the nuclear localisation of transcription factors, and *CDC6*, a gene encoding a component of the origin recognition complex required for DNA synthesis, were also isolated as multiple copy suppressors of *mep2*. The connection between these factors and the nitrogen signal generated by Mep2p is unclear at this stage.

The signalling defect conferred by the deletion of *MEP2* could be rescued by the addition of cAMP or the introduction of constitutively active alleles of *RAS2* and *GPA2*, both of which result in increased cAMP levels. Some of the factors identified as multiple copy suppressors of *mep2*, Msn1p and Mss11p, were also shown to participate downstream of the cAMP signal. It would therefore seem that Mep2p also signals via the Gpap2p-Rasp2-cAMP pathway. The signalling function that regulates the pseudohyphal and invasive growth responses was localised to a region of Mep2p between the first and third intracellular domains (Lorenz and Heitman, 1998b). No proteins that physically interact with Mep2p, and specifically with the domain required for the signalling function, have been identified to date.

1.2.2.2 The amino acid permease homologue, Ssy1p

The amino acid permease family of *S. cerevisiae* includes 24 relatively conserved proteins, all consisting of a central hydrophobic core of 12 transmembrane domains flanked by hydrophilic N- and C-terminal regions. The majority of these proteins are required for the uptake of amino acids from the surroundings to serve as either sources of nitrogen or for incorporation into proteins during protein biosynthesis (Horak, 1997; Paulsen et al., 1998; Regenberg et al., 1999). This transport process is active and is driven by a proton gradient across the plasma membrane. With the exception of the general amino acid permeases, Agp1p and Gap1p, most of the amino acid permeases are more or less specific for the transport of structurally similar amino acids (Iraqui et al., 1999; Regenberg et al., 1999). Some members of the family, however, are required for the transport of compounds other than the 20 L-α-amino acids. Hnm1p is required for the uptake of choline (Nikawa et al., 1990), Uga4p is required for the uptake of 4-amino-butyric acid (Andrè et al., 1993) and Ypl274p is required for the uptake of S-adenosyl-methionine (Regenberg et al., 1999). No function could be assigned to six other members of the family (Regenberg et al., 1999).

Ssy1p is the largest member of the amino acid permease protein family and also the only member of the family that does not have a transport function. However, it was shown to control the transcriptional regulation of some of the genes encoding amino acid permeases. It furthermore has an extended N-terminus, ~200 residues in length, which makes it significantly

larger than any of the other amino acid permeases. These attributes are reminiscent of those that distinguish the glucose sensors, Rgt2p and Snf3p, from the rest of the hexose transporter family (Iraqui et al., 1999).

The regulatory effect conferred by Ssy1p occurs in response to the availability of extracellular amino acids, thereby implying a direct amino acid sensing function (Iraqui et al., 1999; Klasson et al., 1999). Ssy1p is required for the transcriptional activation of several genes in response to the presence of all amino acids, except proline (Iraqui et al., 1999). These include the genes that encode the amino acid permeases *AGP1*, *BAP2*, *BAP3*, *TAT1*, *TAT2* and *VAP2*, the peptide transporter, *PTR2*, and the arginase, *CAR1* (Didion et al., 1998; Iraqui et al., 1999; Klasson et al., 1999). Ssy1p is furthermore also required for the transcriptional repression of the gene encoding the general amino acid permease, *GAP1*, in the presence of amino acids in media containing ammonium (Klasson et al., 1999).

Ssy1p is part of a multi-component membrane-associated signalling complex of which two other proteins have been identified and characterised, namely Ptr3p and Ssy5p (Forsberg et al., 2001; Forsberg and Ljungdahl, 2001). Ptr3p is a hydrophilic protein whose only significant characteristic is that it contains a domain of unknown function that shares homology with similar domains in the amino acid permeases and in the transcriptional activator of amino acid biosynthesis genes, Gcn4p (Klasson et al., 1999). Ssy5p is also a hydrophilic protein, but lacks any distinguishing characteristics (Forsberg and Ljundahl, 2001). Like Ptr3p, it is also plasma membrane-associated (Klasson et al., 1999; Forsberg and Ljungdahl, 2001). Ssy1p and Ptr3p were shown to undergo extensive post-translational modifications and could therefore exist in multiple forms inside the cell. The nature and significance of these modifications remain to be determined (Forsberg and Ljungdahl, 2001).

Some of the components situated downstream of the multi-component amino acid sensor have been identified, but no clear signalling cascade that transmits the amino acid signal has been identified to date. Abf1p, Stp1p, Stp2p, Grr1p and Dal81p have all been identified as factors required for the transcription of Ssy1p-regulated genes (De Boer et al., 1998, 2000; Iraqui et al., 1999). Stp1p and Stp2p were originally identified as being required for pre-tRNA maturation (Wang and Hopper, 1988). Abf1p is a general transcription factor required for global gene activation and repression (Biswas et al., 1990; Rivier et al., 1999), while Grr1p is an F-box-containing protein that is part of the SCF ubiquitin ligase complex that targets protein degradation via the proteasome (Li and Johnston, 1997; Patton et al., 1998). Dal81p is the only one of these factors with a specific role in nitrogen metabolism and functions as a non-specific transcriptional activator required for the full induction of the genes involved in nitrogen utilisation (Vissers et al., 1990; Bricmont et al., 1991). None of the components of

known signalling cascades have been identified as being downstream of, or interacting with, the Ssy1p-Ptr3p-Ssy5p amino acid sensor.

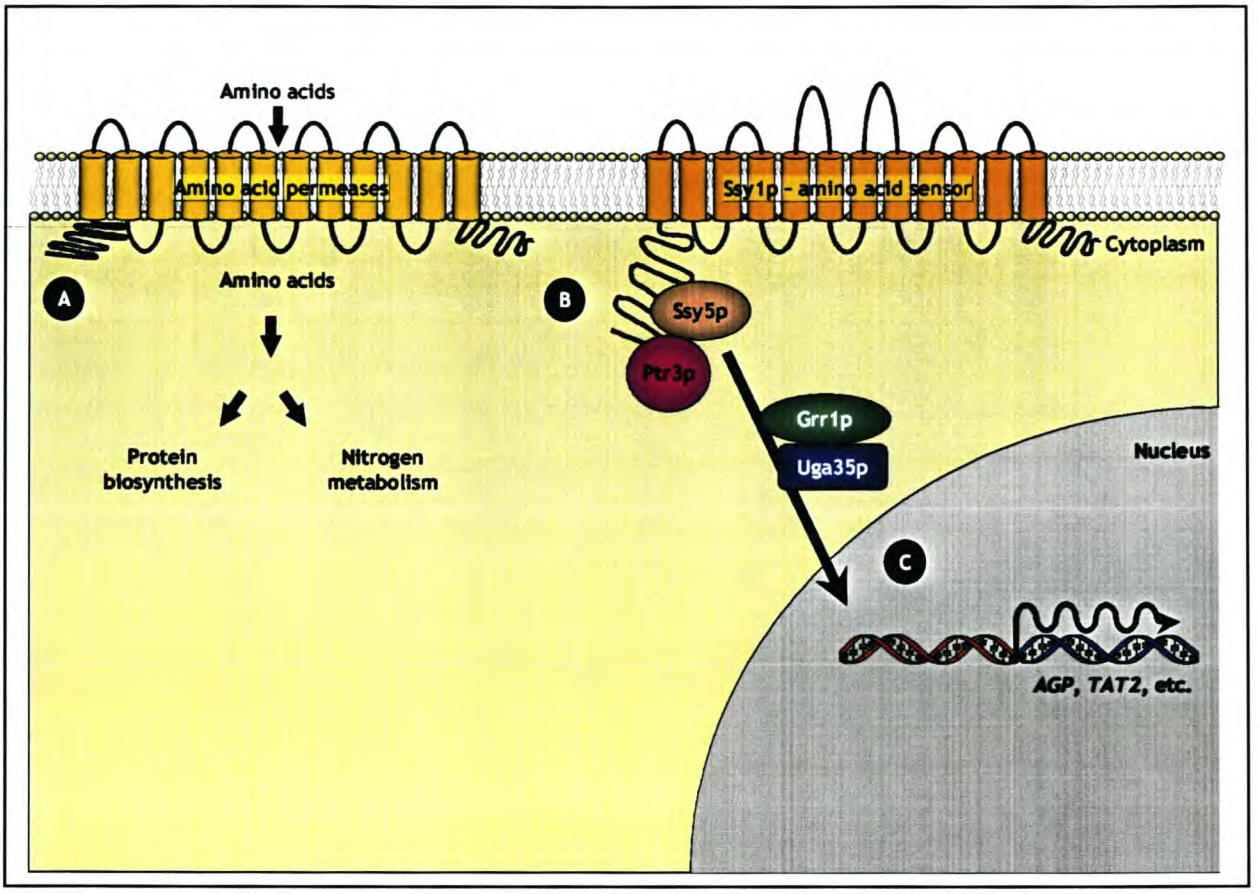

Figure 6. A diagrammatic representation depicting the sensing of nitrogen sources via the Ssy1p amino acid permease system (see text for details).

The deletion of *PTR3* or *SSY1* causes constitutive haploid invasive growth but not diploid pseudohyphal differentiation, providing more evidence that the two processes can be uncoupled and that, despite large overlaps, different mechanisms might be required for the manifestation of each (Klasson et al., 1999). The invasive growth phenotype conferred by these deletions could depend on *FLO8*, since it could only be observed in the Σ1278b background and not in the S288C background (Klasson et al., 1999). One of the major differences between these two genetic backgrounds is the absence of the gene that encodes a transcription factor of the flocculins and adhesins, *FLO8*, in S288C-derived strains. This absence renders such strains unable to undergo filamentous growth (Liu et al., 1996). Flo8p is required for the transcriptional regulation of Muc1p in response to a cAMP signal (Lorenz et al., 2000), suggesting that a cAMP signal could be involved in relating the Ssy1p-Ptr3p-Ssy5p induced amino acid signal to the invasive growth phenotype.

1.2.3 Intracellular nitrogen-sensing mechanisms

As mentioned in the previous sections, most amino acids can be utilised as sources of nitrogen or as components incorporated into proteins during protein biosynthesis. An intracellular nitrogen-sensing mechanism, consisting of the tRNA that decodes the glutamine codon, CUG, was shown to regulate pseudohyphal differentiation, invasive growth and sporulation in response to both amino acid and nitrogen availability (Murray et al., 1998; Beeser and Cooper, 1999). Mutations in the CUG anticodon of the tRNA molecule result in pseudohyphal differentiation and invasive growth, as well as sporulation in media rich in nitrogen. The same mutations also eliminated the repression of the *CAR1* gene in media containing ammonia as the nitrogen source (Coffman et al., 1996, 1997; Cunningham et al., 2000; Van der Merwe et al., 2001). This could suggest that the $tRNA_{CUG}$ is required for sensing the quality and availability of nitrogen sources, albeit not through the nitrogen catabolite repression pathway (Beeser and Cooper, 1999). In the presence of sufficient quantities of a nitrogen source, the $tRNA_{CUG}$ could signal this status to inhibit not just pseudohyphal and invasive growth, but also meiosis and sporulation (Murray et al., 1998). Since most nitrogen sources are converted to either glutamine or glutamate to be used as a nitrogen source by yeast cells, the glutamine tRNA provides an elegant intracellular mechanism to sense the quantity and availability of all nitrogen sources and to regulate downstream processes such as pseudohyphal growth, meiosis and sporulation in response to the presence, quality or absence of the nitrogen source.

Strains carrying mutations such as *gln4-1* are defective in $tRNA_{CUG}$ aminoacylation and subsequently translation, but do not exhibit pseudohyphal differentiation (Murray et al., 1998). This indicates a central and specific requirement for the $tRNA_{CUG}$ in signalling that does not involve a role in translation. Specific mutated versions of the $tRNA_{CUG}$ also indicated a specific role for the CUG anticodon in the signalling function. A signalling mechanism involving the interaction of the $tRNA_{CUG}$ anticodon with a specific regulatory protein was therefore proposed (Murray et al., 1998). Uncharged tRNA molecules are known to signal amino acid availability in yeast cells by interacting with a regulatory domain of the Gcn2p protein kinase during the general control of amino acid biosynthesis (for a review, see Hinnebusch, 1997).

When starved for amino acids, the intracellular levels of the Gcn4p transcription factor increase significantly (Hinnebusch, 1997). Gcn4p levels are regulated at the level of translation initiation through the presence of four short ORFs in the leader sequence of the *GCN4* mRNA, upstream of the main coding region. These upstream ORFs prevent efficient translation of the main coding region in the presence of amino acids. When starved for amino acids, however, ribosomes are not prevented from reinitiating at the main ORF and translation of the transcription factor can occur. This translational regulation of Gcn4p

synthesis is dependent on the Gcn2p protein kinase. In cells starved for amino acids, uncharged tRNA molecules bind to a tRNA synthase domain, which, in turn, activates a neighbouring kinase domain. This kinase phosphorylates the translation initiation factor, eIF-2α, which inhibits the GDP-GTP exchange factor, eIF-2B. This exchange factor is required for the exchange of GTP for GDP on eIF-2 after each round of translation initiation. Since only the GTP-bound form of eIF-2 can deliver $tRNA^{Met}$ to the ribosome, restricting the activity of eIF2B results in a lesser amount of active eIF-2 that is available for translational initiation at the upstream ORFs in the *GCN4* mRNA leader sequence. Therefore, the consequence is that initiation at the upstream ORFs is suppressed and that the main ORF is used preferentially (Hinnebusch, 1997). Gcn4p binds to the upstream areas of more than 50 target genes, mostly encoding enzymes required for amino acid biosynthesis (Albrecht et al., 1998; Natarajan et al., 2001). The key to the activation of this pathway under conditions of amino starvation is therefore the association of uncharged tRNA molecules with the Gcn2p kinase.

The Gcn2p-initiated Gcn4p response does not seem to be exclusive to amino acid starvation. A recent report indicated that Gcn4p-mediated activation of target genes could also occur in response to the presence of poor nitrogen sources, presenting evidence of physiological interactions between the *GCN4* and the GATA-factor regulatory networks (Valenzuela et al., 2001). Gln3p, Nil1p/Gat1p, Dal80p/Uga43p, and Gzf3p/Nil2p/Deh1p make up the four transcription factors of the yeast GATA-factor family (Coffman et al., 1996, 1997; Cunningham et al., 2000; Van der Merwe et al., 2001). These transcription factors form the central components of nitrogen catabolite repression or nitrogen regulation, which allows yeast cells to discriminate against poor nitrogen sources, such as allantoin or proline, in favour of more optimal nitrogen sources, such as glutamine or ammonia. The GATA factors control the expression of nitrogen catabolic genes by binding to upstream regulatory sequences with the four nucleotides, GATA, at the core (Coffman et al., 1997). The involvement of some of these factors in pseudohyphal differentiation and invasive growth has been demonstrated (Lorenz and Heitman, 1998b). The cellular response to both types of starvation, i.e. the Gcn4p-mediated amino acid starvation as well as the GATA-factor mediated response to poor nitrogen sources, are negatively regulated by the TOR signalling cascade in nutrient-rich conditions (Valenzuela et al., 2001).

Although the involvement of at least some of the GATA-factors in pseudohyphal and invasive growth has been demonstrated, the role of Gcn4p remains unclear. The presence of putative Gcn4p binding sites in the upstream regulatory areas of genes encoding products required for adhesion and filamentation, i.e. the flocculins and also Muc1p, suggests that the regulation of these genes might also be under general amino acid control via Gcn2p and Gcn4p. The deletion of *GCN4* in strains with a constitutively active Ras2p-cAMP pathway had

no effect on the ability of these strains to grow invasively (Stanhill et al., 1999). *RAS2*, however, is a key factor of at least two other signal transduction pathways that result in pseudohyphal and invasive growth, and these data therefore do not exclude some regulatory role for a Gcn4p response in filamentous growth.

1.3 Other extracellular signals resulting in filamentous growth

In addition to the sensing of the carbon and nitrogen sources discussed in the previous sections, it is also very likely that *S. cerevisiae* is able to sense the presence of other nutrients in its environment. The selective preference that *S. cerevisiae* exhibits towards, e.g. specific phosphate sources, suggests that sensing mechanisms should be in place to ensure the rapid and optimal utilisation of the preferred nutrient sources, while keeping the mechanisms for utilisation of the lesser-preferred sources down-regulated. The effect of starvation for nutrients other than nitrogen and carbon on filamentous growth, however, remains to be determined.

Oxygen availability is also sensed by yeast cells and ultimately regulates a heme-activated, glucose-repressed protein complex consisting of Hap2p, Hap3p and Hap4p, which, in turn, dictates the transcription levels of oxygen-regulated genes (reviewed in Zitomer and Lowry, 1992). The transcription of *MUC1* was shown to be up-regulated in cells deprived of oxygen (Ter Linde et al., 1999), suggesting that filamentous growth could be induced by oxygen starvation. Although the transcription of the co-regulated *STA2* gene was shown to involve Hap2p (Kartasheva et al., 1996), the involvement of this complex and therefore the mechanism through which oxygen levels regulate *MUC1* transcription, and ultimately filamentous growth, remain to be determined.

Nutrient starvation could be interpreted as a type of environmental stress. Indeed, some evidence exists that suggests that nutrient starvation and environmental stresses might be physiologically connected and that they elicit similar and overlapping responses (Park et al., 1997a, b; Pascual-Ahuir et al., 2001). Filamentous growth might be such a commonly elicited response by yeast cells to direct growth towards more optimal, stress-free substrates. In addition to this nutrient-starvation-induced stress response, other environmental stresses were also shown to result in pseudohyphal and invasive growth. Mild heat (37°C) and osmotic shock (1 M NaCl), were shown to induce filamentous growth (Zaragoza and Gancedo, 2000). Furthermore, compromising the integrity of the cell wall and plasma membrane, e.g. by addition of aliphatic alcohols that affect the lipid bilayer of membranes, or congo red that affects the glucan, also stimulates pseudohyphal growth (Zaragoza and Gancedo, 2000). Although the specific genes and mechanism through which this occurs have not been identified, it is very likely to involve at least Msn1p, Muc1p and Flo1p. *MSN1* encodes a

transcription factor of *MUC1* (Gagiano et al., 1999a, b) and was shown to be induced 10-fold in cells of which the integrity of the cell wall was compromised (Braley and Chaffin, 1999). In the same cells, transcription of *FLO1* and *MUC1* was shown to increase five-fold (Braley and Chaffin, 1999).

The members of the flocculin and adhesin family of genes, shown to be specifically required for filamentous growth, have some of the largest and most complex upstream regulatory regions in the yeast cell and contain a multitude of *cis*-acting regulatory sequences (Gagiano et al., 1999a; Rupp et al., 1999). As such, the transcription levels of genes such as *MUC1* can be determined by a very large number of different signals. These signals are initiated at the cell wall in response to specific environmental cues and, inside the cell, transmitted via one or more signalling cascades to converge at the upstream areas of these genes to elicit the filamentous growth response. The nature and function of these signalling cascades and how they regulate the transcription of the genes encoding the flocculins and adhesins are discussed in the following sections.

2. The signalling of nutritional status and the relationship to filamentous growth in *Saccharomyces cerevisiae*

The identification of connections between the nutritional signals generated through the sensing mechanisms discussed in the previous section and the downstream signal transduction pathways that transmit these signals has proven to be difficult. Despite the fact that a large number of the components comprising these signalling pathways have been isolated and characterised to date, the exact mechanisms of communication between the upstream sensing mechanisms and the downstream signal transmission pathways remain elusive. A complicating factor in studying the pseudohyphal/invasive growth signal transduction pathways is that at least some of the components are shared between different pathways. This suggests that signalling networks, consisting of cross-talking signalling pathways, are responsible for transmitting the nutritional signals generated by the different sensing mechanisms, rather than singular isolated signalling cascades. For the sake of this literature review, however, the components and the mechanisms of signal transmission of the different signalling cascades will be discussed as separate sections.

2.1 The cAMP-PKA pathway

The correlation between intracellular cAMP levels and the nutritional status of cells is well established. Cells with constitutively high intracellular cAMP levels are sensitive to stress and nutrient starvation, are unable to grow on non-fermentable carbon sources, are unable to

sporulate (diploids) and only manage to accumulate low concentrations of the storage carbohydrates, glycogen and trehalose (reviewed in Broach, 1991a, b; Thevelein, 1992, 1994; Thevelein and De Winde, 1999). Also, the addition to the growth substrate of rapidly metabolised fermentable sugars such as glucose, fructose, mannose and galactose, triggers a rapid, transient increase in cAMP levels (Eraso and Gancedo, 1984; Yun et al., 1998). This fermentable sugar-induced cAMP signal is transmitted via a distinct signalling cascade, the cAMP-PKA cascade (reviewed in Broach, 1991a, b; Thevelein, 1992, 1994; Thevelein and De Winde, 1999). Due to the central role of this pathway in yeast metabolism and the involvement of some of the conserved components in a variety of mammalian cancers and tumours, the cAMP-PKA cascade has received extensive attention to date. This resulted in the identification of a large number of components involved in cAMP signalling.

Several cell wall-associated receptors that provide input into the cAMP-PKA pathway have been identified in recent years and have been shown to regulate filamentous growth via cAMP levels (Fig. 7). The G-protein-coupled receptor system, comprising the G-protein-coupled receptor, Gpr1p and the associated α-subunit, Gpa2p, as well as the ammonium permease, Mep2p, was shown to regulate the transcription of *MUC1* and consequently filamentous growth via this pathway (Lorenz and Heitman, 1997, 1998a, b; Lorenz et al., 2000). Although the exact mechanisms have not been identified to date, the amino acid sensing Ptr3p-Ssy1p-Ssy5p complex more than likely also regulates filamentous growth via this pathway (see section 1.2.2). It was demonstrated that high intracellular cAMP levels or the addition of exogenous cAMP could also stimulate filamentous growth and that the increased filamentous growth phenotype correlated with expression levels of *MUC1* (Lorenz et al., 2000). Several other components of cAMP signalling in yeast were also shown to regulate pseudohyphal growth via transcription of *MUC1*, e.g. Ras2p (Pan and Heitman, 1999), Ira1p (Rupp et al., 1999), the protein kinase A subunits, Tpk1p, Tpk2p and Tpk3p (Robertson and Fink, 1998) and the regulatory subunit, Bcy1p (Pan and Heitman, 1999). In addition to these well-characterised components of the cAMP-PKA pathway, transcription factors were identified that regulate the transcription of target genes such as *MUC1* in response to cAMP levels. These include the negative regulator, Sfl1p (Robertson and Fink, 1998; Guo et al., 2000), and the transcriptional activator, Flo8p (Gagiano et al., 1999a; Rupp et al., 1999). The components of the pathway, the process of signal transmission and the transcription of the target gene *MUC1* will be discussed in the following section.

2.1.1 Key components of the cAMP-PKA pathway

2.1.1.1 The small guanine nucleotide binding protein, Ras2p

S. cerevisiae Ras2p and the homologous counterpart, Ras1p, are part of the ras superfamily of small guanine nucleotide binding proteins (G-proteins). These proteins are highly conserved between species that range in complexity from yeast to mammals (Garcia-Ranea and Valencia, 1998). Much like the ras proteins from other organisms, the yeast Ras proteins are required for, and are involved in, a large number of cellular processes, but are best known for their roles in respiratory and fermentative metabolism and, most significantly, in signal transduction. Despite their involvement in these important cellular processes, neither *RAS1* nor *RAS2* is essential. Yeast strains with either gene deleted are still viable, but disruption of both is lethal (Kataoka et al., 1984). Although Ras1p and Ras2p are functionally interchangeable, they are differentially expressed in fermentable and non-fermentable carbon sources, as well as in different growth phases, which suggests a more specific role for each (Breviario et al., 1988; Jiang et al., 1998).

The Ras proteins are very similar in sequence and structure, so much so that human *ras* can overcome the defects of *ras2* strains. The N-termini of *S. cerevisiae* Ras1p and Ras2p are 86% identical over the first 180 aa after which they start to diverge significantly. The N-termini of the yeast Ras proteins are also highly homologous (90%) to the 80 N-terminal aa of mammalian Ras proteins but also diverge significantly after this point (DeFeo-Jones et al., 1983; Powers, 1984; Kataoka, 1985). Both Ras proteins are localised to the plasma membrane. This localisation is not required for its biological activity, but is still required for glucose-induced cAMP signalling (Bhattacharya et al., 1995).

Ras2p was the first key regulator of filamentous growth in *S. cerevisiae* to be identified. It was shown to control the expression of *MUC1* through at least two distinct pathways to regulate filamentous growth. The first of these pathways to be characterised was the mating pheromone-responsive MAPK pathway (Gimeno et al., 1992) and the second pathway, the cAMP-PKA cascade (Mösch et al., 1999). Epistasis analyses with the hyperactive Ras2p mutant, $Ras2^{v19}p$, showed that it could complement the filamentous growth defect of deletion mutants of the nutrient receptors that signal via cAMP (Lorenz and Heitman, 1998b; Pan and Heitman, 1999). This suggests that Ras2p is located downstream thereof in signalling cascades. Whereas the downstream components of such signalling pathways are very well characterised and relatively complete, the signal inputs into this pathway and specifically Ras2p, i.e. the mechanism that stimulates Ras activity, remain elusive.

Like other members of the ras superfamily, Ras2p stimulates target proteins when complexed with GTP. This stimulus is switched off when GTP is hydrolysed to GDP and re-

initiated when the GDP is exchanged for GTP. The GTP-binding domain and the intrinsic GTPase activity of the proteins are localised to the conserved N-terminal region. The switching of GDP for GTP associated with Ras2p is mediated by the guanidine nucleotide exchange factors (GEFs) Cdc25p and Sdc25p. Cdc25p only binds to Ras2p when it is associated with GDP. Dominant active versions of Ras2p, such as $Ras2^{v19}p$, are always in the GTP-bound conformation and therefore do not require Cdc25p to function (Gibbs et al., 1987). Like Ras2p, Cdc25p is also localised to the plasma membrane, where it was shown to interact physically with Ras2p, and probably with adenylate cyclase, in a complex (Engelberg et al., 1990; Gross et al., 1992). *SDC25* was isolated as a suppressor of *CDC25* mutations and shown to have a C-terminal domain highly homologous to that of *CDC25*. Unlike *CDC25*, *SDC25* is transcribed differentially in that expression levels are only detectable in post-diauxic cultures or when grown on non-fermentable carbon sources, and not in glucose media (Boy-Marcotte et al., 1996). The function of Ras2p is also regulated through an association with Ira1p and Ira2p. These proteins stimulate the GTPase activity of Ras2p to hydrolyse GTP to GDP and are therefore essential for Ras2p function. Consequently, the deletion of *IRA1* and *IRA2* results in constitutive activation of the cAMP pathway.

The only downstream target identified for Ras2p is the adenylate cyclase, Cyr1p. This relationship has only been established in yeast, since no evidence for interactions between adenylate cyclase and ras proteins exists in other organisms (Beckner et al., 1985, Birchmeier et al., 1985). Ras2p activates adenylate cyclase in response to a glucose signal and intracellular acidification and it was therefore suggested that in yeast, Ras proteins replace G-proteins as regulators of adenylate cyclase (Thevelein, 1992, 1994). Recent findings, however, challenge this view and the α-subunit of the G-protein-coupled receptor system, Gpa2p, was shown to be essential for glucose-induced activation of adenylate cyclase. This mechanism is similar to the manner by which G-proteins mediate the induced activation of adenylate cyclase in higher eukaryotes (Colombo et al., 1998). The exact role and mechanism for Ras2p in cAMP signalling is therefore unclear.

2.1.1.2 Yeast adenylate cyclase, Cyr1p

The gene encoding the adenylate cyclase of *S. cerevisiae* was cloned through the complementation of *cdc35* and *cyr1* mutants (Casperson et al., 1983; Matsumoto et al., 1984). The large encoded product of 2 606 aa has high levels of homology to mammalian adenylate cyclase, but this is mostly restricted to the C-terminal domain (Krupinski et al., 1989). Several distinct functional domains have been identified in this protein. The C-terminal region was shown to contain the catalytic domain (Kataoka et al., 1985) and also identified as the binding site for the cyclase associated protein, CAP (De Vendittis et al., 1986; Mintzer and

Field, 1994). The central leucine-rich repeat domain was identified as the Ras-responsive region of the protein (Uno et al., 1987) and also shown to interact physically with Ras2p (Minato et al., 1994). Yeast adenylate cyclase exists as a multimer in yeast cells, where it can be present in the cytosol, as well as be associated with the plasma membrane. Ras2p and the GEF, Cdc25p, associate with adenylate cyclase in this membrane-bound complex and the localisation thereof to the plasma membrane is dependent on Cdc25p (Pardo et al., 1993).

Besides the Ras proteins, the other important protein to associate with adenylate cyclase is CAP/Srv2p. It associates with the C-terminal domain of adenylate cyclase through its N-terminal region and is involved in the activation of adenylate cyclase by Ras. The C-terminal part of CAP is required for normal cellular morphology and is necessary for the yeast cell to be able to respond to nutrient deprivation and excess (Field et al., 1990; Gerst et al., 1991). CAP mutations result in randomly budding cells that are also defective in their actin distribution patterns (Vojtek et al., 1991). These defects can be suppressed by the overexpression of profilin and it was therefore proposed that CAP and profilin provide a link between growth signals and cytoskeleton remodelling (Vojtek et al., 1991). CAP was subsequently shown to interact physically with actin and also to sequester monomeric actin, a mechanism with which it can prevent the formation of actin filaments and consequently regulate cytoskeletal changes (Freeman et al., 1995, 1996).

Whatever the stimulus, be it directly via Gpa2p, Ras and CAP or indirectly through some other means, adenylate cyclase exhibits elevated levels of Mg^{2+}- and ATP-dependent adenylate cyclase activity, which results in increased levels of intracellular cAMP. This cAMP, in turn, stimulates the cAMP-dependent protein kinases (cAPK).

2.1.1.3 The yeast cAMP-dependent protein kinases, Tpk1p, Tpk2p, Tpk3p

The target of cAMP in yeast is the cAMP-dependent protein kinase, protein kinase A (PKA). The structure and mode of action of yeast PKA is similar to that of mammalian PKA. It consists of a regulatory subunit encoded by a single gene, *BCY1*, and three catalytic subunits, encoded by the *TPK1*, *TPK2* and *TPK3* genes (Cannon and Tatchell, 1987; Toda et al., 1987a, b). The three Tpk's are very similar in sequence and structure and the C-termini are highly conserved over ~300 residues. The shorter N-terminal domains are, however, distinct.

In resting cells, PKA is an inactive tetramer composed of two regulatory subunits bound to two catalytic subunits. In response to extracellular signals that increase cAMP levels, cAMP binds to the regulatory subunits. This causes conformational changes in the regulatory subunits that decrease their affinity for the catalytic subunits and subsequently trigger the release thereof. The catalytic subunits are now in a free active state. Hydrolysis of cAMP through the cAMP phosphodiesterases restores PKA to the resting, inactive state. These cAMP

phosphodiesterases are encoded by *PDE1* and *PDE2* in yeast (Sass et al., 1986; Nikawa et al., 1987).

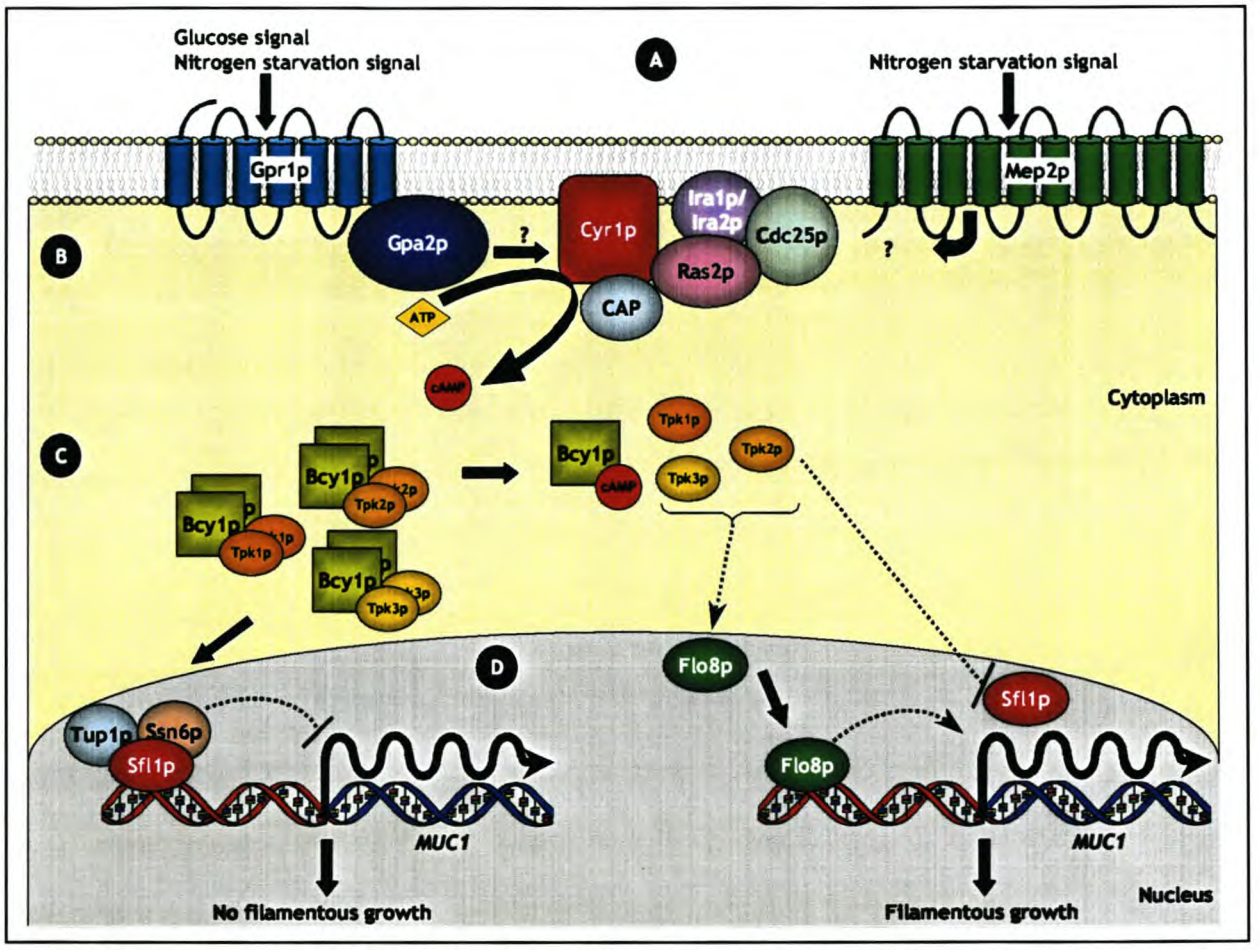

Figure 7. The role of the cAMP-PKA signalling pathway in the filamentous growth response in yeast. A.) A nutritional signal is generated via specific receptors. To date, only Gpr1p and Mep2p have been conclusively shown to provide a filamentation signal via the cAMP-PKA pathway. B.) The receptors indirectly stimulate the adenylate cyclase, Cyr1p, resulting in an increase in cyclic AMP levels. As indicated by the question marks, the exact mechanism behind this is unknown. However, Ras2p is required. C.) The elevated cAMP levels result in the dissociation of the PKA regulatory subunit, Bcy1p, from the kinases, Tpk1p, Tpk2p and Tpk3p. D.) The Tpk's activate the transcriptional repressor, Flo8p, to result in the transcriptional activation of *MUC1*, amongst others. Tpk2p specifically associates with Sfl1p, preventing it from repressing *MUC1* transcription.

Until recently, it was thought that the three PKA catalytic subunits, Tpk1p, Tpk2p and Tpk3p, might be redundant. Triple mutants lacking all three genes encoding these catalytic subunits are unviable, but the expression of any one of the three genes in such a strain rescues this growth defect. The three Tpk's, however, play very different roles with respect to filamentous growth. The deletion of *TPK2* eliminates the phenotype, whereas the deletion of *TPK3* results in enhanced filamentous growth (Robertson and Fink, 1998). Some contradicting reports exist on the role of *TPK1* in the process, since it was initially reported to have no effect (Roberston and Fink, 1998), but was later shown to have a negative effect (Pan and Heitman, 1999). The reasons for this discrepancy are unclear, since yeast strains from the

same genetic background, i.e. Σ1278b were used in both sets of experiments. The distinct roles for the three Tpk's in filamentous growth cannot be attributed to differential expression, since there are no differences in the transcription levels of the three genes encoding Tpk1p, Tpk2p and Tpk3p. Any differences in the effect on filamentous growth should therefore be attributed to divergences in sequence.

2.1.1.4 The transcription factors mediating the cAMP response

Two transcription factors, Flo8p and Sfl1p, act antagonistically to mediate the transcription levels of *MUC1* in response to cAMP signals and thus, ultimately, to regulate the filamentous phenotype in response to nutritional signals (D'Souza and Heitman, 2001). Both factors were shown to act downstream of Tpk2p (Robertson and Fink, 1998). *FLO8* encodes a transcriptional activator of *MUC1* (Gagiano et al., 1999b; Rupp et al., 1999), but was identified initially as a transcriptional activator of the major flocculation gene, *FLO1*, and for its ability to establish the flocculation phenotype in non-flocculent strains. It was shown not to encode a structural flocculation gene, but rather a transcriptional activator of the flocculation genes, specifically *FLO1* (Kobayashi et al., 1996, 1999a, b). The *FLO8* sequence was shown to be present in most yeast strains, but a mutation in the *FLO8* ORF was identified in most commonly used laboratory strains, resulting in the premature termination of translation and the production of a non-functional peptide (Liu et al., 1996). It is this mutated form of *FLO8* that renders laboratory yeast strains unable to flocculate and to grow filamentously (Liu et al., 1996; Kron, 1997).

FLO8 encodes a 729 aa protein with limited homology only to Mss11p, another transcriptional regulator of *MUC1* (Gagiano et al., 1999a, b). Sequences, through which Flo8p exerts its regulatory effect, were identified in the upstream regions of the *MUC1* (Gagiano et al., 1999b; Rupp et al., 1999) and *STA2* promoters (Gagiano et al., 1999b). Putative binding sites for Flo8p were subsequently identified through gel-retardation analysis of the *FLO1*, *STA1* and *MUC1* promoters (Kobayashi et al., 1999a). The exact mechanisms through which Flo8p stimulates the transcriptional activation of its target genes are unknown, but involve the binding of this factor to the promoters of these genes.

SFL1 encodes a 767 aa helix-turn-helix DNA-binding protein, similar in structure to some of the heat shock transcription factors (Fujita et al., 1989). It was initially identified for its ability to suppress flocculation and was later shown to encode a negative regulator of not just *MUC1* (Robertson and Fink, 1998), but also of *SUC2* (Song and Carlson, 1998). The deletion of *SFL1* results in increased levels of *MUC1* transcription, which consequently enhances flocculation and pseudohyphal growth (Robertson and Fink, 1998).

Sfl1p was shown to interact specifically with Tpk2p, but not with Tpk1p or Tpk3p. This interaction prevents Sfl1p from repressing *MUC1* transcription (Robertson and Fink, 1998). Sfl1p contains five putative phosphorylation sites for the cAMP-dependent protein kinases and phosphorylation of Sfl1p by Tpk2p was shown to prevent binding to DNA (Conlan and Tzamarias, 2001). Sfl1p furthermore also associates with the Ssn6p-Tup1p repression complex (Conlan and Tzamarias, 2001). Taken together, the data suggest that unphosphorylated Sfl1p could bind to the *MUC1* promoter and recruit the Ssn6p-Tup1p repressor complex. When Tpk2p is activated, it phosphorylates Sfl1p and prevents binding to the *MUC1* promoter. In these conditions, the Flo8p transcriptional activator results in increased transcription of *MUC1*, resulting in filamentous growth.

Another candidate for a cAMP-stimulated transcription factor that regulates pseudohyphal growth was identified in a two-hybrid screen, using Tpk2p as bait (Robertson and Fink, 1998). Mga1p contains a helix-turn-helix DNA-binding motif and two phosphorylation sites for the cAMP dependent kinases. The deletion of *MGA1* did not interfere with cellular elongation upon nitrogen starvation, but results in random budding, which impacts negatively on pseudohyphal growth (Robertson and Fink, 1998; Lorenz and Heitman, 1998a). It is likely to be a transcriptional activator, but its effect on the transcription of *MUC1* or any of the other adhesin-encoding genes has not been established.

2.2 The pheromone-responsive MAP kinase cascade

The mating pheromone-responsive MAP kinase cascade was the first signalling cascade implicated in the transmission of a nutritional signal (i.e. nitrogen limitation) that resulted in filamentous growth in *S. cerevisiae* (Liu et al., 1993). The components of this pathway were initially isolated and characterised for their roles in the transmission of the pheromone-induced signal during the yeast mating process. Activation of this pathway, stimulated by the binding of the mating pheromone to the cognate receptor, leads to the transcriptional regulation of a large number of genes that ultimately results in cell cycle arrest in the G1 phase, reorientation of cell polarity towards the perceived mate and the actual growth towards the perceived mate (reviewed in Bardwell et al., 1994; Errede et al., 1995; Leberer et al., 1997; Chant, 1999). This reorientation of cellular polarity is a common physiological feature between filamentous growth and the mating process. The most upstream components of the mating pheromone-responsive pathway, i.e. Ste2p and Ste3p (the α- and a-factor receptors, respectively) and the associated G-protein subunits, Gpa1p (α-subunit), Ste4p (β-subunit) or Ste18p (γ-subunit), as well as the scaffold protein, Ste5p, are not involved in establishing the filamentous growth phenotypes (Liu et al., 1993). Only the core module,

comprising Ste7p, Ste11p, Ste20p and Kss1p, as well as the transcription factor, Ste12p, were shown to be required (Fig. 8). The small G-protein, Cdc42p, and its GEF, Cdc24p, were also implicated in regulating filamentous growth via the mating pheromone-responsive cascade, in a similar way as it regulates the mating process (Simon et al., 1995; Zhao et al., 1995). Whereas the upstream components of the pathway during the mating process are known, those regulating the filamentous growth response are not. The only component identified upstream of Cdc24p and Cdc42p is Ras2p (Mösch et al., 1996, 1999). The receptors and upstream mechanisms that serve as input signals to this cascade therefore remain unidentified.

Some of the components of this cascade are also involved in the transmission of other signals, e.g. Ste11p is also activated by high osmolarity (Posas and Saito, 1997). The ability to transmit diverse signals resulted in a number of questions regarding the mechanisms employed by the yeast cell to guarantee a specific response to a specific signal while using common factors. Some recent work illustrated that the specificity of the pathway is partially determined by the different MAP kinases: Fus3p activates specifically in response to the mating pheromone, Hog1p specifically in response to high osmolarity and Kss1p specifically in response to nutritional signals (Cook et al., 1997; Madhani et al., 1997; Bardwell et al., 1998a, b; Gustin et al., 1998; Elion, 2000). These MAP kinases are also partially redundant, and Kss1p can be recruited in the absence of Fus3p (Madhani and Fink, 1997). Similarly, in a strain defective in the *HOG* pathway, Kss1p can be activated in response to hyperosmotic shock (Davenport et al., 1999). It also seems that the MAP kinases inhibit the effects of the other pathways with which it shares modules. Hog1p seems to inhibit the mating pheromone-responsive pathway and *hog1* mutant strains are reported to be more filamentous than the wild-type strains.

A common requirement for changing cellular polarity as well as cellular adhesion properties exists in the mating and filamentous growth phenotypes (Guo et al., 2000). The benefit of using a single pathway to result in the expression of adhesin-encoding genes, such as *MUC1*, therefore seems obvious. The following section will discuss the components of the mating pheromone-response cascade that are involved in the filamentous growth response in some detail, as well as the mechanisms through which the signal is transmitted to result in the expression of *MUC1*.

2.2.1 The small guanine nucleotide binding protein, Ras2p

The core module of the mating pheromone-responsive pathway receives the nutritional signal from the small guanine nucleotide binding protein, Ras2p, via Cdc42p (Mösch et al., 1996). As discussed in the previous sections, the exact mechanism by which the sensing components

communicate with the signalling modules via Ras2p is unclear. However, it is clear that Ras2p is an essential component for the signalling of nutritional status through the mating pheromone responsive MAP kinase cascade. Ras2p was shown to be required for the transmission of both nitrogen and carbon source limitation signals via the mating pheromone-responsive MAP kinase cascade and it could therefore receive nutritional signals generated by any one or more of the sensing mechanisms discussed in the previous section (Gimeno et al., 1992; Mösch et al., 1996, 1999; Lorenz and Heitman, 1997, 1998a; Gagiano et al., 1999b; Pan and Heitman, 1999). The permanently active form of Ras2p, encoded by *RAS2*^{val19}, signals constitutively via the mating pheromone-induced pathway to result in pseudohyphal growth. Mutations in any one of the genes encoding the signalling cascade eliminate or severely reduce the filamentous phenotypes.

2.2.2 Cdc42p and its associated regulatory proteins

Rho-type GTPases play critical roles in regulating the signal transduction pathways that generate and maintain cell polarity in eukaryotic cells and are pivotal in the reorientation and polarisation of the actin cytoskeleton (reviewed in Cabib et al., 1998). Cdc42p of *S. cerevisiae* is no exception to this. It has a high degree of homology to similar Rho-type GTPases identified in a wide range of organisms, ranging in complexity from yeast to mammals (reviewed in Johnson, 1999). Indicative of a critical and central function, Cdc42p is essential for viability, not just in *S. cerevisiae* but also in *S. pombe* (Miller and Johnson, 1994). *S. cerevisiae* strains carrying temperature-sensitive alleles of *CDC42* exhibit blocked bud formation at the restrictive temperature, but still allow cell mass and volume to increase (Zhang et al., 1999). Cell division is arrested, but DNA and nuclear division continue. These phenotypes ultimately result in large, unbudded multinucleate yeast cells. The cortical actin distribution towards the areas of growth (in this case the bud) of such mutant strains are disrupted and chitin and other cell surface materials are deposited uniformly throughout the enlarging cell wall, instead of the normal polarised deposition. All of these phenotypes are clear indications that Cdc42p, either directly or indirectly, controls polarisation in *S. cerevisiae* and, as such, is critical for processes such as mating and filamentous growth that depend on polarisation. This importance of Cdc42p in establishing the filamentous growth phenotypes can be illustrated by the dominant negative alleles of *CDC42* that inhibit Ras2p-dependent filamentous growth and, in the inverse scenario, through constitutively activated *CDC42* alleles that induce filamentous growth via the mating pheromone responsive MAP kinase cascade (Mösch et al., 1996, 1999).

The function of Cdc42p is dependent on its association with a large number of proteins or upstream effectors that regulate its guanine nucleotide-bound state (reviewed in Johnson,

1999). These include the Guanine nucleotide Exchange Factor (GEF), Cdc24p, the Guanine nucleotide Dissociation Inhibitor (GDI), Rdi1p, and the GTPase Activating Proteins (GAPs), Bem3p, Rga1p and Rga2p. It also interacts with a large number of downstream effectors that, in turn, propagate signals received from Cdc42p. The most notable of these are the members of the p21-activated kinase (PAK) family, Cla4p, Skm1p and Ste20p. Both Cla4p and Ste20p can transduce a Cdc42p signal to the cytoskeleton, suggesting partial redundancy. The physiological relevance and function of the third kinase, Skm1p, is unknown at this stage, but it was reported to play some role in filamentous growth (Martin et al., 1997).

The domains required for association with the large and diverse group of effectors were identified through studies on a number of temperature-sensitive mutants, as well as by obtaining the crystal structure of Cdc42p (reviewed in Johnson, 1999). Cdc42p contains four domains for the binding and hydrolysis of GTP, similar to those identified in Ras proteins. It also contains a Rho insert domain that distinguishes the Rho-type GTPases from the rest of the Ras superfamily. This domain in the human equivalent of Cdc42p has been implicated in interacting with the GDIs. However, in terms of providing a basis for the regulation of the Cdc42p-dependent processes, the most important domain is an N-terminal effector domain with which the upstream effector, Cdc24p, and the downstream effectors, Cla4p, Skm1p and Ste20p interact. These effector proteins all contain a consensus Cdc42p/Rac Interactive Binding (CRIB) domain with which they interact with the Cdc42p effector domain. Lastly, Cdc42p also contains distinct domains required for localisation to the plasma membranes or internal membrane structures in areas of polarised growth (Ziman et al., 1993; Ayscough et al., 1997). The mechanism by which Cdc42p is targeted to the membranes at areas of polarised growth is unknown at this stage.

The upstream components that stimulate Cdc24p to generate a filamentous growth signal have not been identified to date. The simplest and most elegant scenario would be if any of the membrane-associated nutrient-responsive receptors discussed in the previous section could be linked physically to Cdc24p, in a manner similar to the association of the mating pheromone GPCR β-subunit, Ste4p (Butty et al., 1998). However, there is no evidence to support such a physical link between Cdc24p and any of the upstream receptors. The only potential upstream factors identified so far are the Ras-family proteins, Ras2p and Rsr1p (Mösch et al., 1996, 1999; Park et al., 1997a, b). A physical link between Rsr1p and Cdc24p was identified, but the relevance of this in establishing the filamentous growth phenotype is unknown, and only a genetic connection between Ras2p and Cdc42p has been identified to date. Interestingly, this link was identified through assessing the filamentous growth phenotypes in strains carrying constitutively active *RAS2* alleles in combination with either dominant negative or constitutively-active *CDC42* alleles. The signalling between Ras2p and

Cdc42p is mediated, to some extent, by the seemingly redundant 14-3-3-proteins, Bmh1p and Bmh2p (Roberts et al., 1997). Any physical interaction between Ras2p and Cdc24p or Ras2p and Cdc42p, however, remains to be discovered.

Cdc42p is regulated by its upstream effectors, the GEF, Cdc24p, and the GAP, Bem3p, in response to specific extracellular signals. In response to the mating signal, the GEF, Cdc24p, interacts directly with the β-subunit of the mating pheromone GPCR, Ste4p (Butty et al., 1998). This physical interaction between the receptor and Cdc24p therefore provides a direct mechanism by which Cdc24p is activated. Cdc24p facilitates the active GTP-bound state of Cdc42p that, in turn, transmits the mating signal to downstream effectors, specifically the Cla4p, Skm1p and Ste20p kinases (Johnson, 1999). The GAPs, Bem3p, Rga1p and Rga2p, stimulate the hydrolysis of the Cdc42p-bound GTP to return it to an inactive GDP-bound state. The mechanism and details of this process in response to extracellular mating pheromone have been characterised reasonably well. The mechanism for filamentous growth, however, is not so clear and still requires some elucidation. It is possible that the mechanism of action for the mating response and the filamentous response is similar, but involves different proteins.

2.2.3 The MAP kinase kinase kinase kinase, Ste20p

Ste20p is a member of the PAK family of serine\threonine kinases, and contains three distinct domains - a large non-catalytic region in the amino-terminal half, a kinase domain in the COOH-terminal half and a short, non-catalytic sequence, C-terminal to the kinase domain. For the filamentous growth phenotypes, the specific interaction of Cdc42p with a domain in the amino-terminal half of Ste20p is required (Leberer et al., 1997). This domain is short (36 aa) and is conserved among members of the Ste20p protein kinase family. The interaction of Cdc42p with Ste20p furthermore displaces a negative regulator, Hsl7p, which also interacts with the amino-terminal domain of Ste20p. This association occurs either adjacent to, or overlapping with, the area with which Cdc42p associates (Fujita et al., 1989; Dan et al., 2001). The interaction of Cdc42p directs Ste20p towards the site of growth but does not seem to affect the activity of the kinase domain. The specific role for the association with Cdc42p therefore seems to be the localisation of the Ste20p kinase activity to sites of polarised growth and the stabilisation of Ste20p at these sites, and not by any modifications. The identity of the protein(s) that activate Ste20p through phosphorylation is unknown at this stage.

The only conclusive statement that can be made is that, upon association with Cdc42p, Ste20p is free to activate the core MAP kinase cascade through phosphorylation to propagate the signal received via Cdc42p. Ste20p can, however, also play a more direct role in establishing cell polarity and the reorientation of the actin cytoskeleton in response to

extracellular signals, since it was demonstrated to interact physically with the β-subunit of the pheromone-induced receptor (Leeuw et al., 1998). It is therefore possible that Ste20p can directly receive signals from upstream receptors. Ste20p was also shown to associate with and directly phosphorylate myosin-I (Myo3p) and it would therefore also be possible for Ste20p to directly regulate components of the actin cytoskeleton (Wu et al., 1997). The main function of Ste20p, however, is proposed to be the phosphorylation of Ste11p. Although Ste20p phosphorylates Ste11p, this phosphorylation is not required for the activation of Ste11p kinase activity. The exact relevance of the Ste20p-to-Ste11p phosphorylation step, as well as the mechanism of Ste11p activation, remain unclear (Gustin et al., 1998).

2.2.4 The MAP kinase kinase kinase, Ste11p

Ste11p is the MAP kinase kinase kinase of the pheromone responsive (Chant 1999), filamentous growth (Liu et al., 1993) and high osmolarity sensing pathways (Posas and Saito, 1997). Its main function in the filamentous growth and mating pheromone response is to phosphorylate the MAP kinase kinase of these pathways. As such, it associates with, and phosphorylates, Ste7p to transmit the filamentous growth and mating pheromone signals (reviewed in Gustin et al., 1998, Posas et al., 1998a; Elion, 2000). It also phosphorylates Pbs2p to transmit the high osmolarity signal (Posas and Saito, 1997). The phosphorylation of Ste7p in the transmission of the mating pheromone signal requires the interaction of Ste11p with both Ste5p and Ste50p (Xu et al., 1996; Ramezani Rad et al., 1998; Wu et al., 1999). Ste5p is the scaffold protein for the mating pheromone-responsive pathway and associates with the components of the core MAP kinase cascade. The functional relevance of a scaffold protein is unclear at this stage, but it is hypothesised to facilitate specific interactions between the members of the core MAP kinase cascade and thereby enhancing specific signal transmission by minimising the interactions of the associated kinases with kinases from other pathways (Whitmarsh and Davis, 1998). A scaffold protein for the filamentous growth and high osmolarity sensing pathways has not been identified yet. Ste50p, on the other hand, plays a minor role in both the mating pheromone-responsive and filamentous growth signalling pathways (Xu et al., 1996; Ramezani Rad et al., 1998). The deletion of *STE50* results in defects in filamentous growth and minor defects in mating, but the relevance and exact role of Ste50p in these processes are unclear. The association of Ste11p with Ste50p is, however, critical for the Ste11p-mediated activation of the HOG pathway (Posas et al., 1998b). The interaction of Ste50p with Ste11p, regardless of the environmental conditions, suggests that Ste50p is an accessory to Ste11p. This interaction might be a prerequisite to receive and modulate signals from different upstream components to mediate mating, filamentous growth or osmotolerance (Jansen et al., 2001).

2.2.5 The MAP kinase kinase, Ste7p, and the MAP kinase, Kss1p

Ste7p interacts physically with the MAP kinases of both the filamentous growth pathway, i.e. Kss1p, and the mating pheromone-responsive pathway, i.e. Fus3p (reviewed in Gustin et al., 1998; Posas et al., 1998a; Elion, 2000). In response to a mating pheromone or filamentous growth signal, this serine/threonine/tyrosine kinase is phosphorylated and thus activated by Ste11p. Ste7p, in turn, phosphorylates the corresponding MAP kinase to transmit the specific signal. The MAP kinase that is phosphorylated by Ste7p will determine the cellular response - if it is Kss1p, the cell will commit to filamentous growth, and if it is Fus3p, the cell will continue with the mating process. The MAP kinases can, however, functionally substitute for each other, albeit to a minimal extent.

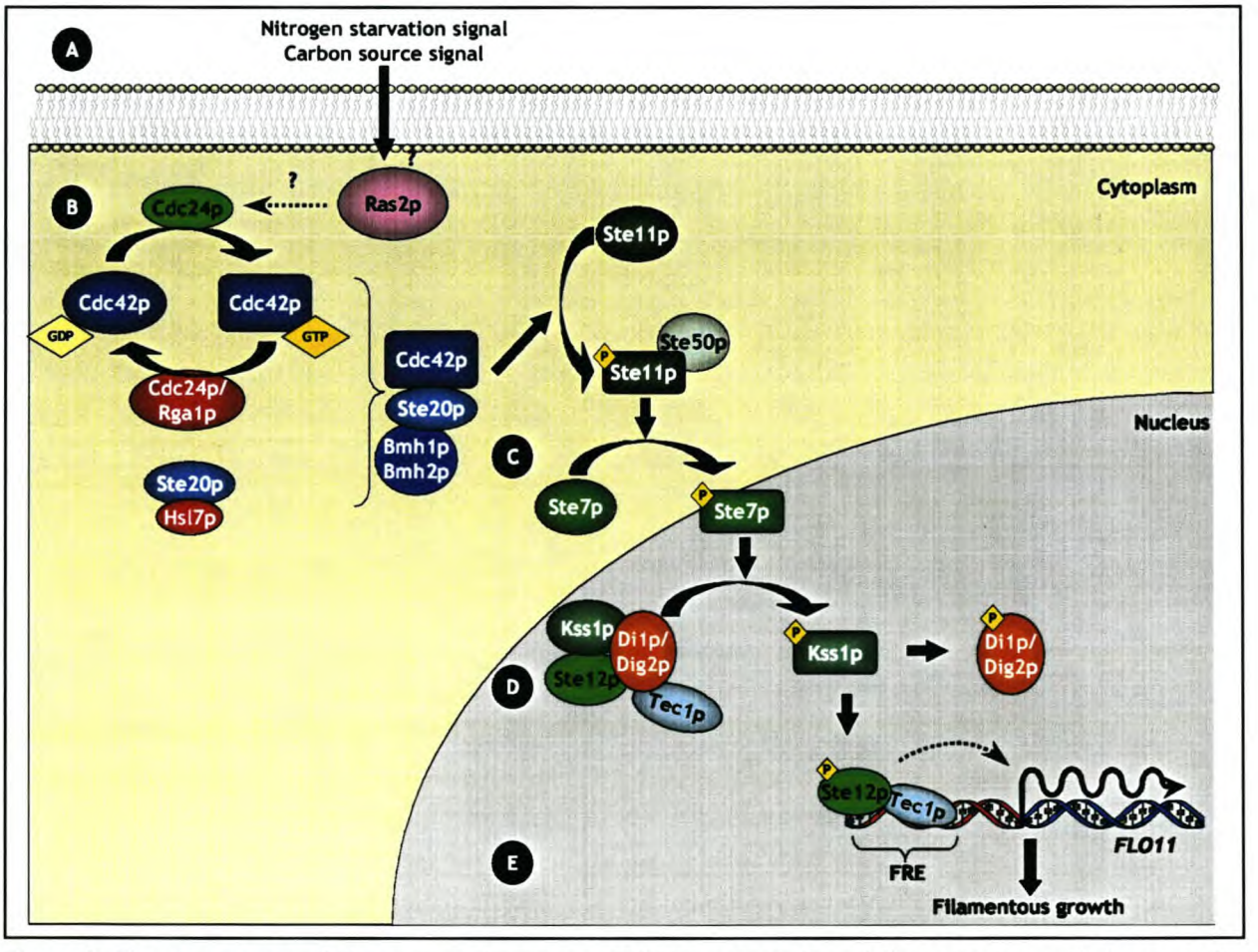

Figure 8. The components of the mating pheromone MAP kinase cascade and filamentous growth (see text for details).

The MAP kinase for the filamentous growth response was, until recently, unknown. The deletion of *STE7* suggested that it was the most downstream component of the cascade that played a significant role in the filamentous growth response (Bardwell et al., 1998b). The deletion of neither Fus3p nor Kss1p, the two MAP kinases known to interact with, and that are

phosphorylated by, Ste7p, had similar negative effects on filamentous growth. It was therefore proposed that Ste7p had other targets than these MAP kinases, or even non-MAP kinase targets (Hunter and Plowman, 1997). Only when *KSS1* was deleted in combination with *STE7* did the mechanism of action become clear (Cook et al., 1997; Madhani et al., 1997; Bardwell et al., 1998a, b). This dual deletion restored invasive growth in a non-invasive strain and suggested that unphosphorylated Kss1p acts to inhibit invasive growth. Ste7p therefore acts to alleviate the Kss1p-mediated inhibition on invasive growth in response to the specific upstream signals, since phosphorylated Kss1p also has a stimulatory role in invasive growth. This positive role in invasive growth requires its kinase activity and involves the transcription factors, Ste12p and Tec1p, which were shown to activate the genes required for the filamentous growth phenotype, specifically Muc1p (Fig. 8).

2.2.6 The transcription factors, Ste12p and Tec1p

Ste12p is a transcriptional activator, required for the activation of a large number of mating pheromone-responsive genes (reviewed in Bardwell et al., 1994; Errede et al., 1995; Leberer et al., 1997; Chant, 1999), as well as genes required for pseudohyphal differentiation and invasive growth (reviewed in Kron, 1997; Madhani and Fink 1998; Borges-Walmsley and Walmsley, 2000; Pan et al., 2000; Gancedo, 2001). Critical for its functionality, it contains an N-terminal DNA-binding domain with some homology to homeodomains and a C-terminal transcriptional activation domain (Pi et al., 1997; Crosby et al., 2000). In response to the a-mating pheromone, Fus3p phosphorylates and activates Ste12p. Activated Ste12p interacts with another transcriptional activator, Mcm1p, and these proteins then bind co-operatively to Pheromone Response Elements (PREs) in the promoters of a-specific genes such as *MFA2*. In response to the α-mating pheromone, it interacts not only with Mcm1p, but also with α1, to regulate α-specific genes such as *MFα1* (reviewed in Bardwell et al., 1994; Errede et al., 1995; Leberer et al., 1997; Chant, 1999).

Tec1p is a lesser-known transcription factor with some homology to the ATTS/TEA family of transcription factors that regulates fungal morphology (Gavrias et al., 1996). It was originally cloned and characterised for its role in regulating Ty1-mediated transcription (Laloux et al., 1990, 1994), but later was shown to induce filamentous growth when overexpressed (Gavrias et al., 1996). It was shown not just to bind co-operatively with Ste12p at the promoters of target genes (Gavrias et al., 1996; Madhani and Fink, 1997), but also to physically interact with Ste12p (Norman et al., 1999).

In the absence of a specific filamentous growth signal, unphosphorylated Kss1p interacts with Ste12p. This binding of inactive, unphosphorylated Kss1p to Ste12p inhibits the function of Ste12p as a transcriptional activator (Bardwell et al., 1998a). Ste12p also interacts with

two other negative regulators, Dig1p (Rst1p) and Dig2p (Rst2p), which have additional inhibitory roles on Ste12p function (Cook et al., 1996; Pi et al., 1997, Bardwell et al., 1998a; Olson et al., 2000). In response to the specific upstream signal, Ste7p phosphorylates Kss1p and Kss1p, in turn, phosphorylates Ste12p, Dig1p and Dig2p. The phosphorylation of the Dig proteins results in their dissociation (Bardwell et al., 1998a), which renders Ste12p free to not just activate the transcription of *TEC1* (Oehlen and Cross, 1998), but also to interact with the encoded protein (Bardwell et al., 1998a). Dig1p and Dig2p also interact with Tec1p. Ste12p and Tec1p bind cooperatively to Filamentous growth Response Elements (FREs) that have been identified in the promoters of several genes, but have to date only been shown to be functional in the *MUC1* promoter (Lo et al., 1997b; Madhani and Fink, 1997; Mösch and Fink, 1997; Lo and Dranginis, 1998; Mösch et al., 1999; Rupp et al., 1999). These FRE sequences consist of binding sites for Tec1p (CATTC$^{T}/_{C}$) and Ste12p (TGAAACA) in close proximity to one another in order for Ste12 and Tec1p to bind cooperatively (Madhani and Fink, 1997). Ste12p and Tec1p were shown to be required for the activation of *MUC1* transcription from far upstream binding sites, located at -800 to -1200, -1400 to -2200 and -1600 to -2000 of the *MUC1* promoter, in response to different nutritional signals (Rupp et al., 1999). Since these binding sites are much further upstream than in genes normally activated by Ste12p, the exact mechanism of transcriptional activation is unclear. The transcription of *MUC1* is, however, enhanced several-fold in the presence of overexpressed or multiple copies of *STE12* or *TEC1*, and it would therefore seem that Ste12p and Tec1p constitute the last part of the filamentous growth MAP kinase cascade, which is required to switch on *MUC1*, but not any of the other flocculins or adhesins (Lo et al., 1997b; Lo and Dranginis, 1998; Pan and Heitman, 1999; Rupp et al., 1999; Guo et al., 2000).

2.3 The TOR cascade

The TOR (Target Of Rapamycin) signalling cascade of *S. cerevisiae* controls a major cell-growth programme in response to nutrient availability (reviewed in Thomas and Hall, 1997; Dennis et al., 1999). The pathway seems to be conserved, at least to some extent, between eukaryotic organisms, since some of the components required for TOR signalling in yeast and mammalian systems are similar, e.g. the mammalian protein mTOR and the yeast proteins, Tor1p and Tor2p. The phenotypes displayed upon disruption of the TOR pathway in these systems are also very similar and include cell cycle arrest, inhibition of translation initiation and others (Thomas and Hall, 1997; Dennis et al., 1999). This section will discuss the identified components of the TOR pathway of *S. cerevisiae* and the mechanisms by which it regulates cellular responses in response to nutritional status (Fig. 9).

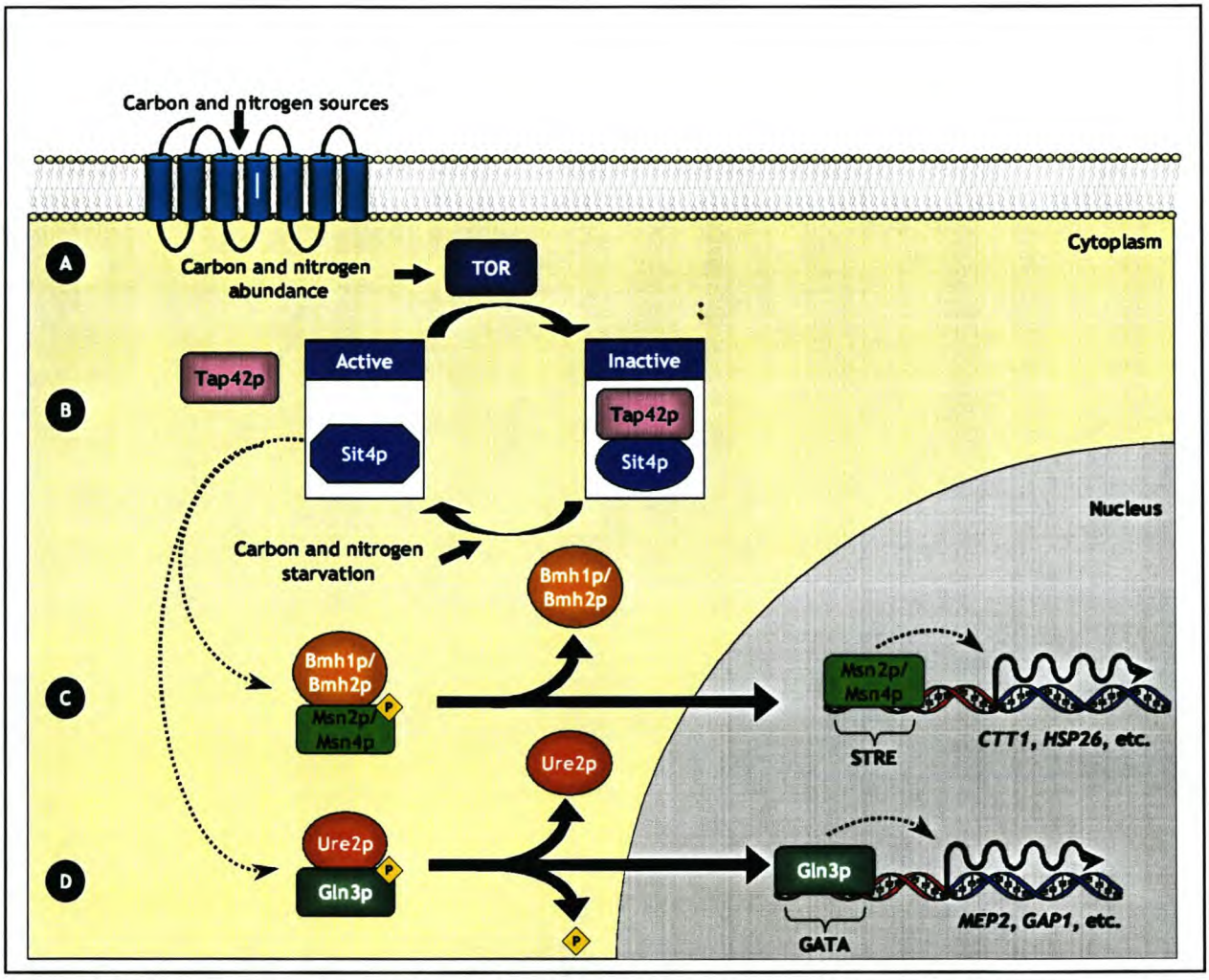

Figure 9. A diagrammatic representation of the TOR signalling cascade and the mechanism through which it regulates target genes.

2.3.1 Components of the TOR pathway

The main constituents of the yeast TOR pathway are two phosphatidyl-inositol kinase homologues encoded by *TOR1* and *TOR2*, and an effector or mediator protein encoded by *TAP42* (Fig. 9). The TOR genes were first identified as dominant mutations that confer resistance to the immunosuppressant and fungicidal drug, rapamycin (Heitman et al., 1991). Subsequent work showed that the disruption of *TOR1* and *TOR2* causes arrest in the G1-phase of the cell cycle, a phenotype similar to wild-type cells treated with rapamycin or wild-type cells subjected to nutrient starvation (Barbet et al., 1996). Although both Tor1p and Tor2p were shown to regulate translation initiation, Tor2p was shown to have an additional function in regulating cytoskeletal organisation through a Rho1p-Rho2p GTPase switch (Schmidt et al., 1996, 1997). Tor1p and Tor2p act via Tap42p to control cytoplasmic protein synthesis (through translation initiation), protein degradation and G1-phase progression (Thomas and

Hall, 1997; Helliwell et al., 1998). Upon phosphorylation by the TOR kinases, Tap42p binds to and inhibits type 2A and 2A-related phosphatases. TOR also controls nuclear events, such as the global repression of starvation-specific transcription, by mediating the retention of specific transcription factors in the cytoplasm.

2.3.2 Controlling translation initiation and cell cycle progression

TOR controls translation initiation and cell cycle progression in response to the carbon source, and probably other nutrients, through the type 2A-related phosphatase, Sit4p, and other type 2A phosphatases. The process requires the type 2A phosphatase-associated protein, Tap42p, the translation initiation factor, eIF4E, and the ribosomal protein, S6 (Di Como and Arndt, 1996; Thomas and Hall, 1997). The TOR cascade stimulates the association of Tap42p with the phosphatases required for translation initiation, but the exact mechanism by which Tap42p affects translation initiation remains to be identified. The cell cycle arrest caused by the deletion or inhibition of Tor is a secondary effect of translational arrest (Barbet et al., 1996).

2.3.3 Repression of starvation-specific transcription

Upon nitrogen limitation, two partially redundant GATA-type transcription factors, Gln3p and Gat1p, are activated (Coffman et al., 1996, 1997; Valenzuela et al., 1998; reviewed in Hofman-Bang, 1999; Cunningham et al., 2000; Van der Merwe et al., 2001). However, when sufficient amounts of a nitrogen source are present, the TOR signalling pathway will prevent transcription of genes expressed under nitrogen limitation. This is accomplished through the association of the GATA transcription factor, Gln3p, with the cytoplasmic protein Ure2p and requires the TOR-dependent phosphorylation of Gln3p (Beck and Hall, 1999). The phosphorylation and cytoplasmic retention of Gln3p are dependent on the effector of the TOR pathway, Tap42p and are antagonised by the type 2A-related protein phosphatase, Sit4p (Beck and Hall, 1999). TOR promotes complex formation between Tap42p and Sit4p. This complex formation renders the phosphatase inactive, while Gln3p remains phosphorylated and subsequently associates with Ure2p, which prevents it from being localised to the nucleus. Upon inactivation of the TOR pathway by rapamycin, the transcript levels of several target genes of Gln3p and Gat1p, e.g. *MEP2*, *GAP1* and *GLN1*, increase 10-fold (Beck and Hall, 1999).

Under a variety of stress conditions, including carbon limitation, the zinc-finger transcription factors, Msn2p and Msn4p, are localised to the nucleus, where they are responsible for the activation of a large number of stress-related genes, e.g. *CTT1*, *HSP26* and *SSA3* (Rep et al., 1999, 2000). In non-stress conditions, however, the TOR cascade prevents

the nuclear localisation of the Msn2p and Msn4p transcription factors through a mechanism similar to the one observed for Gln3p and Gat1p. The 14-3-3 protein, Bmh2p, was shown to associate with both Msn2p and Msn4p and this association was shown to be dependent on a functional TOR pathway (Beck and Hall, 1999). The association of Bmh2p with Msn2p/Msn4p was also shown to be dependent on the presence of sufficient amounts of a carbon source, since withdrawal of the carbon source (glucose) from the growth media resulted in the termination of the Bmh2p-Msn2p/Msn4p association. It is therefore clear that the TOR pathway recruits the 14-3-3 protein, Bmh2p, to retain Msn2p/Msn4p in the cytoplasm when sufficient amounts of a carbon source are present (Beck and Hall, 1999). However, this carbon source dependent sequestration of transcription factors is not dependent on the Tap42p effector or the Sit4p phosphatase. The exact mechanism of action by which the TOR cascade responds to nutrient limitation, as well as the relationship between the other nutrient-responsive signalling pathways such as cAMP-PKA, remains to be identified. All the recent evidence, however, points to the inhibition of transcription factor function, specifically transcription factors such as Gln3p, Dal80p, Msn2p, Msn4p, etc., that were shown to be activated by nutrient starvation or limitation (Coffman et al., 1996, 1997; Valenzuela et al., 1998; Beck and Hall, 1999; Hofman-Bang, 1999; Rep et al., 1999, 2000; Cunningham et al., 2000; Van der Merwe et al., 2001).

2.4 Glucose signalling

As discussed in a previous section (see section 1), glucose is the most abundant monosaccharide and preferred carbon source for *S. cerevisiae*. In addition to the elaborate sensing and transport mechanisms that *S. cerevisiae* employs to sense the availability of glucose and to facilitate its rapid and exclusive uptake, equally complicated signalling mechanisms are employed that extend the sensing of the glucose to the transcriptional regulation of specific genes. The genes encoding products that are required for the metabolism of lesser-preferred carbon sources, mitochondrial biosynthesis, etc., are generally repressed in the presence of glucose, whereas genes that encode products required in the metabolism of glucose, e.g. glycolytic enzymes, are induced (reviewed in Trumbly, 1992; Gancedo, 1998; Carlson, 1999). The presence of glucose therefore results in two distinct signals - a repression signal for the former sets of genes and an induction signal for the latter sets of genes. These signals, however, occur in parallel to the cAMP-PKA pathway, which transmit a cAMP signal that assists the cell in adapting to the availability of glucose (reviewed in Rolland et al., 2001). In contrast to the reasonably well-characterised cAMP-PKA pathway, the mechanisms by which the glucose induction and glucose repression signals are generated and the exact nature of the signals are not well understood. A gap currently exists

as to how the presence or absence of glucose is physically sensed, and how this information is translated into a signal that can be transmitted to a signalling module. Several glucose sensors have been identified (see section 1.1), but the mechanisms of sensing the glucose in these systems are not currently understood. It is, for example, unknown whether these sensors physically bind the glucose and whether this sensing action occurs intracellularly or extracellularly. Despite these information gaps, the far downstream signalling process in response to glucose has been well characterised and the effects of glucose on the transcription of several genes are thoroughly understood.

From the transcription levels of the genes essential for filamentous growth, particularly *MUC1*, it is clear that they are severely repressed in the presence of glucose, at least in some media (Gagiano et al., 1999a; Rupp et al., 1999). Considering that filamentous growth might be a stress phenotype in response to nutrient limitation and starvation, it should be repressed under conditions of glucose abundance. In line with this reasoning, several transcriptional regulators of *MUC1* have been identified to date and include, amongst others, some of the components of glucose repression mechanisms, most notably, Hxk2p (Kartasheva et al., 1996), Grr1p (Palecek et al., 2000), Tup1p and Ssn6p (Conlan and Tzamarias, 2001). The particulars of these regulatory mechanisms are discussed in the following section.

2.4.1 The main glucose repression pathway

The repression of genes in the presence of glucose requires phosphorylated glucose but not further metabolism. Glucose transport therefore seems to contribute by supplying a source of glucose to be phosphorylated (Rolland et al., 2001). This suggests that glucose repression is initiated at the level of the enzymes required for the phosphorylation of glucose, i.e. the hexokinases and the glucokinase. A physical connection between the hexokinase, Hxk2p, and the downstream components that mediate the long-term repressive effect of glucose on glucose-repressed genes, was made recently (Sanz et al., 2000). The downstream components and the mechanism of action are well characterised and revolve around the Snf1p kinase, protein phosphatase I, the transcriptional repressor, Mig1p, and the co-repressors, Tup1p and Ssn6p (Fig. 10) (reviewed in Gancedo, 1998; Carlson, 1999).

2.4.1.1 The Snf1p-Snf4p kinase complex and the Glc7p-Reg1p phosphatase complex

The Snf1p protein kinase is the best characterised of the glucose repression mechanisms identified to date and is a key element in the signalling of glucose repression. It is a highly conserved kinase and Snf1p-homologues have been identified and characterised in several mammalian and plant systems, where they were shown to perform similar functions (Carling

et al., 1994; Mitchelhill et al., 1994; reviewed in Hardie et al., 1998). In S. *cerevisiae*, Snf1p associates with a number of other proteins in a high molecular weight complex. The most important of these proteins are the activating subunit, Snf4p, and the scaffold components, Sip1p, Sip2p and Gal83p (Jiang and Carlson, 1997). The activating subunit, Snf4p, is required for optimal Snf1p activity (Celenza and Carlson, 1989) and the Sip1p, Sip2p and Gal83p scaffold proteins are required to optimise the response to glucose starvation. Unlike Snf4p, the scaffold proteins are not essential for the function of the Snf1p kinase repression pathway.

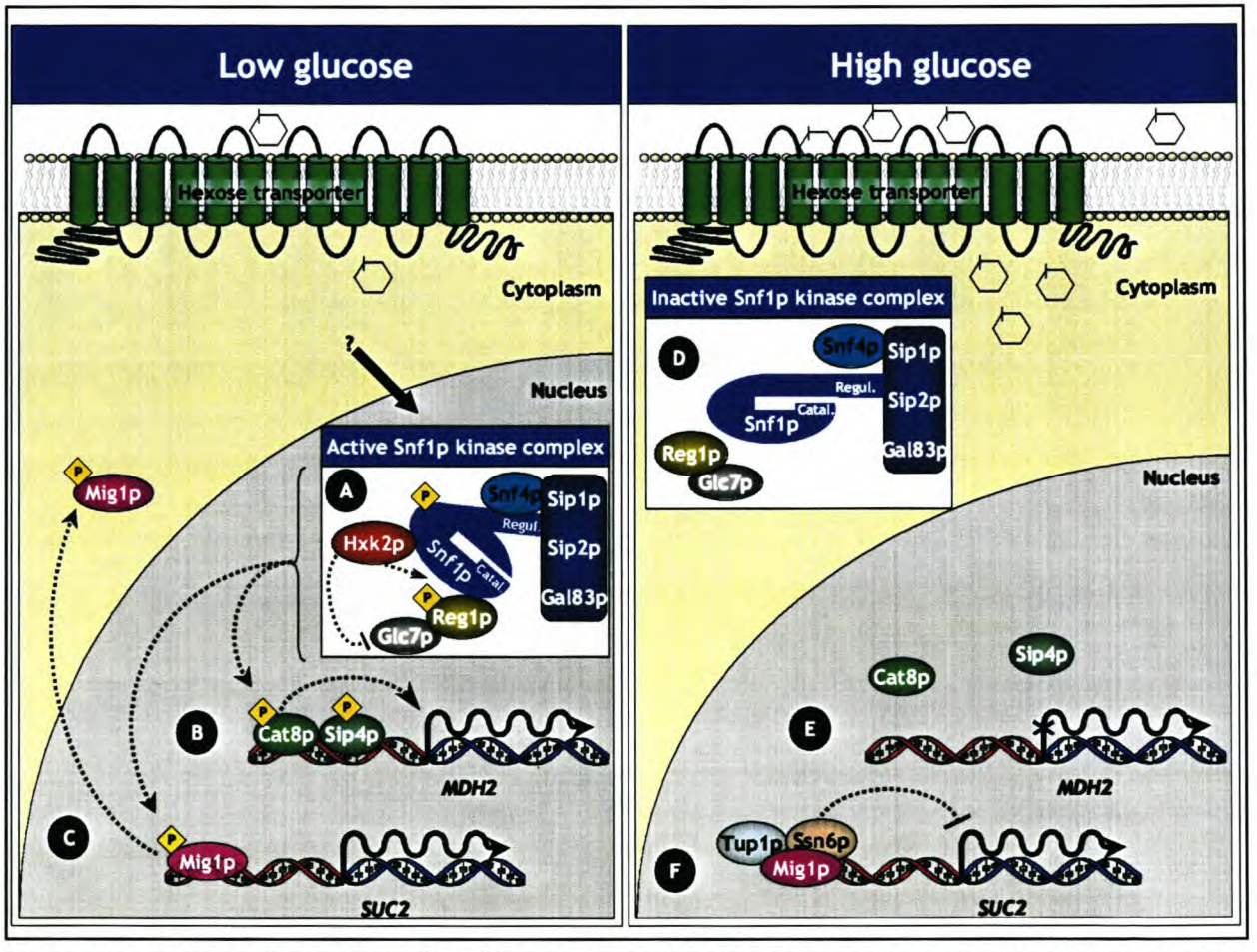

Figure 10. A diagrammatic representation of the role of the Snf1p complex in glucose signalling pathways in yeast.

The activity and localisation of the Snf1p complex are regulated by the carbon sources. Snf1p is inhibited in the presence of glucose and activated upon glucose limitation (reviewed in Carlson, 1998, 1999). Furthermore, it localises to the nucleus in response to the carbon source present and this localisation requires the different subunits of the Snf1p-complex (Vincent et al., 2001). In glucose media, the regulatory domain of Snf1p is required for the autoinhibition of the catalytic domain. In media lacking glucose, the Snf4p activating subunit binds to the Snf1p regulatory domain and counteracts this autoinhibition. The modifications

and changes in the activity of the Snf1p-Snf4p complex are regulated via phosphorylation (Celenza and Carlson, 1986; Jiang and Carlson, 1997). The activation loop is phosphorylated on a conserved threonine residue and this phosphorylation event is required for Snf1p activation (Ludin et al., 1998). The identities of the Snf1p kinase kinase and other upstream components are unknown at this stage (Carlson, 1998, 1999).

The *S. cerevisiae* protein phosphatase 1 (PP1), made up by Glc7p and its regulatory subunit, Reg1p, interacts with the activated Snf1p catalytic domain (Tu and Carlson, 1995). This interaction results in the dephosphorylation of Snf1p, which facilitates the conformational change from the active state to the inactive state. Therefore, if glucose is abundant, the Snf1p kinase is inactive and transcriptional repression of the glucose-repressed genes occurs via the transcriptional repressor, Mig1p (Vallier and Carlson, 1994; Treitel and Carlson, 1995; Ostling and Ronne, 1998). The major function of the activated Snf1p protein kinase therefore, is to inhibit the function of the Mig1p repressor in the absence of glucose.

2.4.1.2 The transcriptional repressor, Mig1p, and the co-repressors, Ssn6p and Tup1p

Mig1p is a DNA-binding transcription factor that binds to the consensus $^{G}/_{C}$ $^{C}/_{T}$ G G $^{G}/_{A}$ G binding site in the upstream areas of most glucose-repressed genes to inhibit transcription (Lundin et al., 1994). It seems to be conserved, at least amongst fungal species, since a number of proteins with some homology and with similar functions have been identified in *Aspergillus*, *Candida*, *Kluyveromyces* and *Schizosaccharomyces* species (Cassart et al., 1995, 1997). It uses a zinc-finger DNA-binding domain to bind to its recognition sites in the upstream areas of a large number of glucose-repressed genes and exerts a repressive effect on the transcription of such genes (Lundin et al., 1994). The ability of Mig1p to perform the glucose repression function is dependent on its localisation, which, in turn, is dependent on its phosphorylation status (Treitel and Carlson, 1995). In limited glucose media, Mig1p is phosphorylated by the active Snf1p kinase, and localises specifically to the cytoplasm. In high glucose media, the Snf1p kinase is inactive, Mig1p remains unphosphorylated and localises specifically to the nucleus where it can perform its repressor function. Mig1p, however, does not act alone in repressing the transcription of glucose-repressible genes - it recruits assistance in the form of the co-repressors, Tup1p and Ssn6p (Treitel and Carlson, 1995).

The co-repressors associate with Mig1p in a multimeric complex consisting of four Tup1p subunits and one Ssn6p subunit (reviewed in Smith and Johnson, 2000). This complex can also be recruited by other DNA-binding proteins and, as such, is involved in the repression of a large number of very diverse genes. It is therefore not exclusive to glucose repression pathways. The exact mechanism of function more than likely involves the modification of

chromatin structure and nucleosome positioning in some of the promoters to which it is recruited to (Gavin et al., 2000; Watson et al., 2000). Tup1p was also shown to interact directly with histones H3 and H4, and some biochemical evidence suggests that this association is required for the repression of some of the Tup1p-Ssn6p repressed genes. It can, however, also prevent transcription by directly interacting with components of the RNA polymerase II transcription initiation complex (Conlan and Tzamarias, 2001). Whatever the mode of action or the mechanism, the outcome remains the same, i.e. the transcriptional repression of glucose-repressible genes.

Although Tup1p and Ssn6p were both shown to be required for the repression of Muc1p and for negatively regulating filamentous growth, it is unclear whether this repressive function is due to these factors acting in response to a glucose signal. As mentioned above, these factors can also act in response to other signals and are, as such, not exclusively dedicated to the main glucose repression pathway. The recruitment of Tup1p and Ssn6p to the *MUC1* promoter is furthermore probably mediated by Sfl1p (Conlan and Tzamarias, 2001) and not by Mig1p. Mig1p was demonstrated not to be required for the glucose-mediated repression of *STA2*, a gene co-regulated with *MUC1*, because of high levels of homology between the upstream regulatory areas (Gagiano et al., 1999a). These observations cast doubt on a role for the main glucose repression pathway in negatively regulating *MUC1* transcription in response to a glucose signal, but to date this has not been demonstrated clearly.

3. Conclusion

In conclusion, a vast number of environmental conditions can elicit the formation of pseudohyphae or invasive growth in yeast. The identification of such conditions is still in the initiation phase and consequently the mechanisms through which yeast cells sense these environmental cues are not characterised very well. However, the intracellular signalling pathways that transmit these signals are far better characterised, but a major task still lies ahead in connecting such signal transduction pathways to specific upstream sensors (sensing mechanisms such as membrane-associated receptors) and downstream effectors (target genes specifically required/responsible for the filamentous growth phenotypes), of which only *MUC1* has been characterised to some extent.

Some basic questions on the relationship between the presence of glucose and filamentous growth also exist. The expression of genes such as *MUC1*, that was shown to be required for filamentous growth in both haploid and diploid cells, is repressed by glucose and other rapidly fermentable carbon sources, at least in some conditions. In agreement with this, the depletion of glucose seems to result in increased transcription of *MUC1* (Gagiano et al.,

1999a; Rupp et al., 1999). However, haploid invasive growth seems to occur in the presence of rich glucose media. The elevated cAMP levels associated with the filamentous growth phenotype are also stimulated by the addition of rapidly fermentable carbon sources to starved cells. The exact nature of the carbon source signal, specifically glucose, and the relationship between that and filamentous growth therefore require some investigation.

The sensing and signalling modules discussed in this literature review do not by any means constitute the full complement of sensing and signalling mechanisms employed by *S. cerevisiae* to link nutritional status to filamentous growth. These merely represent the examples that have been identified to date and specifically the better characterised ones. These examples, for the time being, seem to play the most important roles in the manifestation of the filamentous growth phenotypes, at least as far as the severity of the phenotype is concerned. This, however, does not apply to the TOR pathway, which has never been implicated in the filamentous growth response. This is surprising, since a number of the factors that are directly negatively regulated by the TOR cascade (e.g. Gln3p, Gat1p) are modulators of filamentous growth. The absence of TOR from the literature on filamentous growth is probably due to the genetic background of the yeast strains commonly used to study TOR-related phenotypes. If these strains are from the S288C and W303 backgrounds, it is unlikely that a connection will be made between TOR and filamentous growth.

This literature review, although not attempting to be detailed or complete, provides an overview of the literature that currently exists on nutritional signalling and its relationships to the filamentous growth phenotypes. In the process, it also identified several questions or gaps in the filamentous growth study field. One of the gaps that needed to be addressed was the function of a recently identified and seemingly critical transcriptional regulator, Mss11p. The identification of Mss11p as a transcriptional regulator of both *MUC1* and *STA2* and attempts to place it in context to known signal transduction pathways are described in Chapter 3. Its role in transcription, specifically where it acts on the *MUC1* promoter is discussed in Chapter 4. The further characterisation thereof, i.e. the identification of its functional domains, is discussed in Chapter 5 and some concluding remarks are given in Chapter 6.

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Chapter 3

Research results I*

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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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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1. Abstract

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.

2. Introduction

Pseudohyphal differentiation and invasive growth of diploid and haploid cells of the yeast *Saccharomyces cerevisiae* has 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; Roberts and Fink, 1994; Lambrechts et al., 1996a). 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 α-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 idirectly 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. Experimental procedures

3.1 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 contain 50 μM of ammonium sulphate as sole nitrogen source, were prepared as described by Lorenz and Heitman (1997).

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 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 Δste7::*LEU2* and Δste12::*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).

3.3 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.

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

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.

An 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.

3.4 Invasive growth and pseudohyphal development assays

Yeast strains were transformed with plasmids bearing *MSN1*, *MSS11*, *STE7*, *STE12* and RAS2val19, 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 OD600 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.).

3.5 Plate assays to determine starch utilisation

The *STA2* gene encodes an extracellular glucoamylase that 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.

3.6 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 *Bal*I-*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 labeled with P^{32} dATP using the Prime-It II random primer labeling kit (Stratagene).

4. Results

4.1 *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.

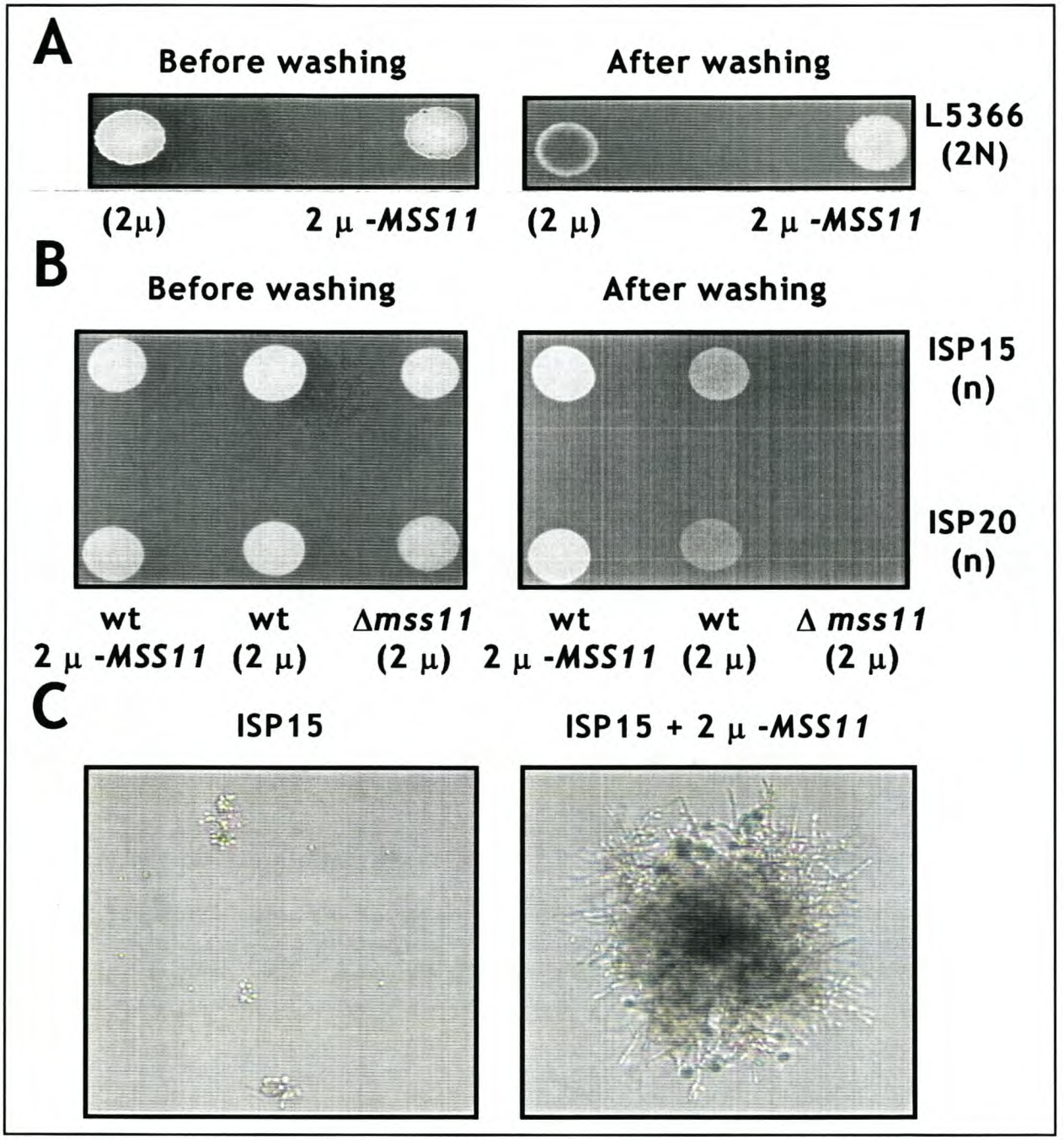

Figure 1. Role of Mss11p in invasive and filamentous growth. A.) The effect of multiple copies of *MSS11* on the invasive growth of a Σ1278 diploid strain, L5366, transformed with plasmid YEpLac195-MSS11. The control consists of L5366 transformed with YEpLac195 without insert. B.) The effect of multiple 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.

To ascertain whether *MSS11* played an important role in the invasive growth process, the gene was disrupted in several genetic backgrounds. The *Δmss11* 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.

4.2 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.

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.

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.

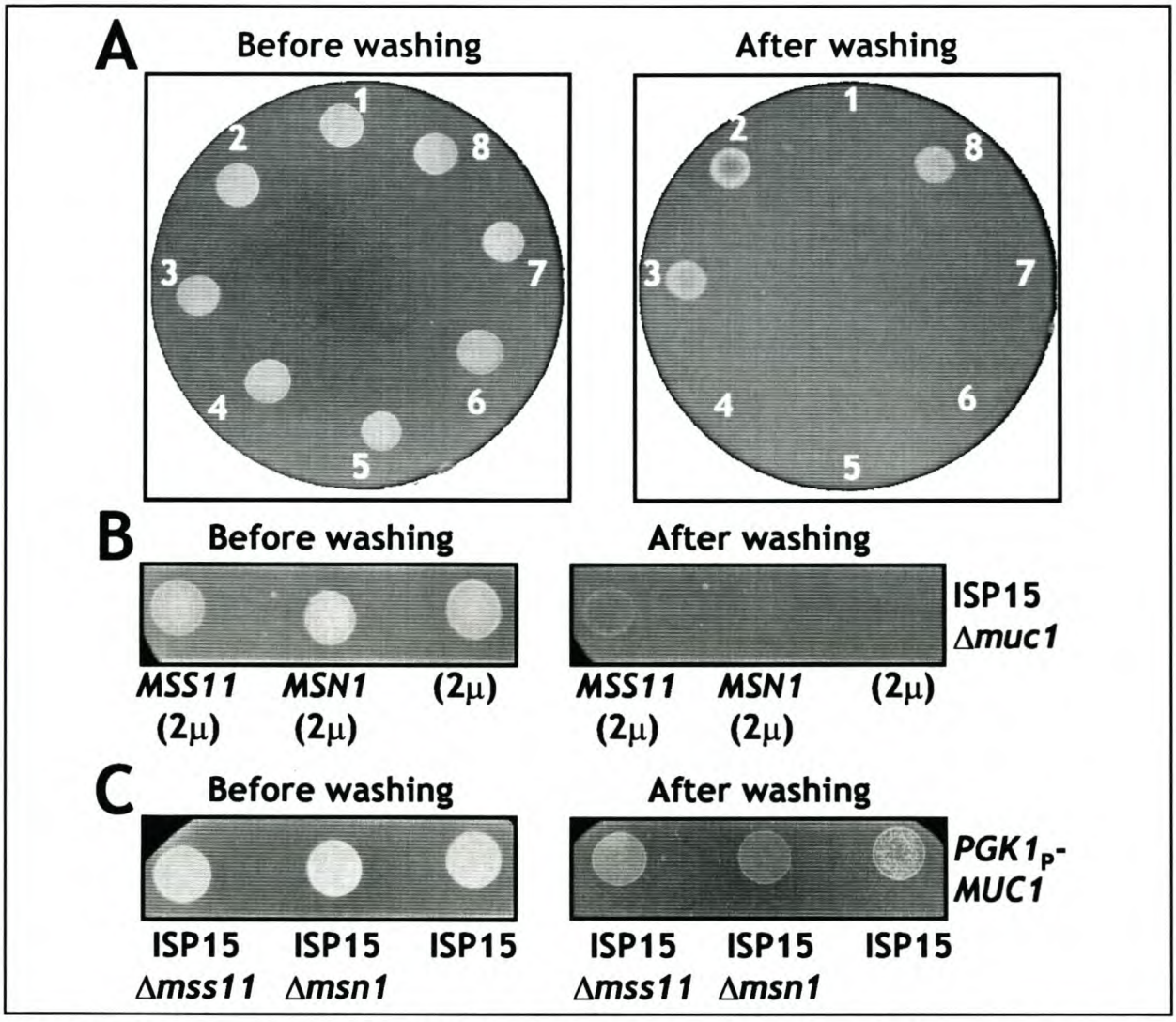

Figure 2. Epistasis analysis of *MSN1*, *MSS11* and *MUC1*. Photographs show strains spotted on SLAD medium as described in Experimental Procedures. Surface growth was washed off after incubating the plates for 3 days at 30°C. A.) Relation between *MSN1* and *MSS11*. The strains spotted are: (1) ISP15 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.

4.3 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.

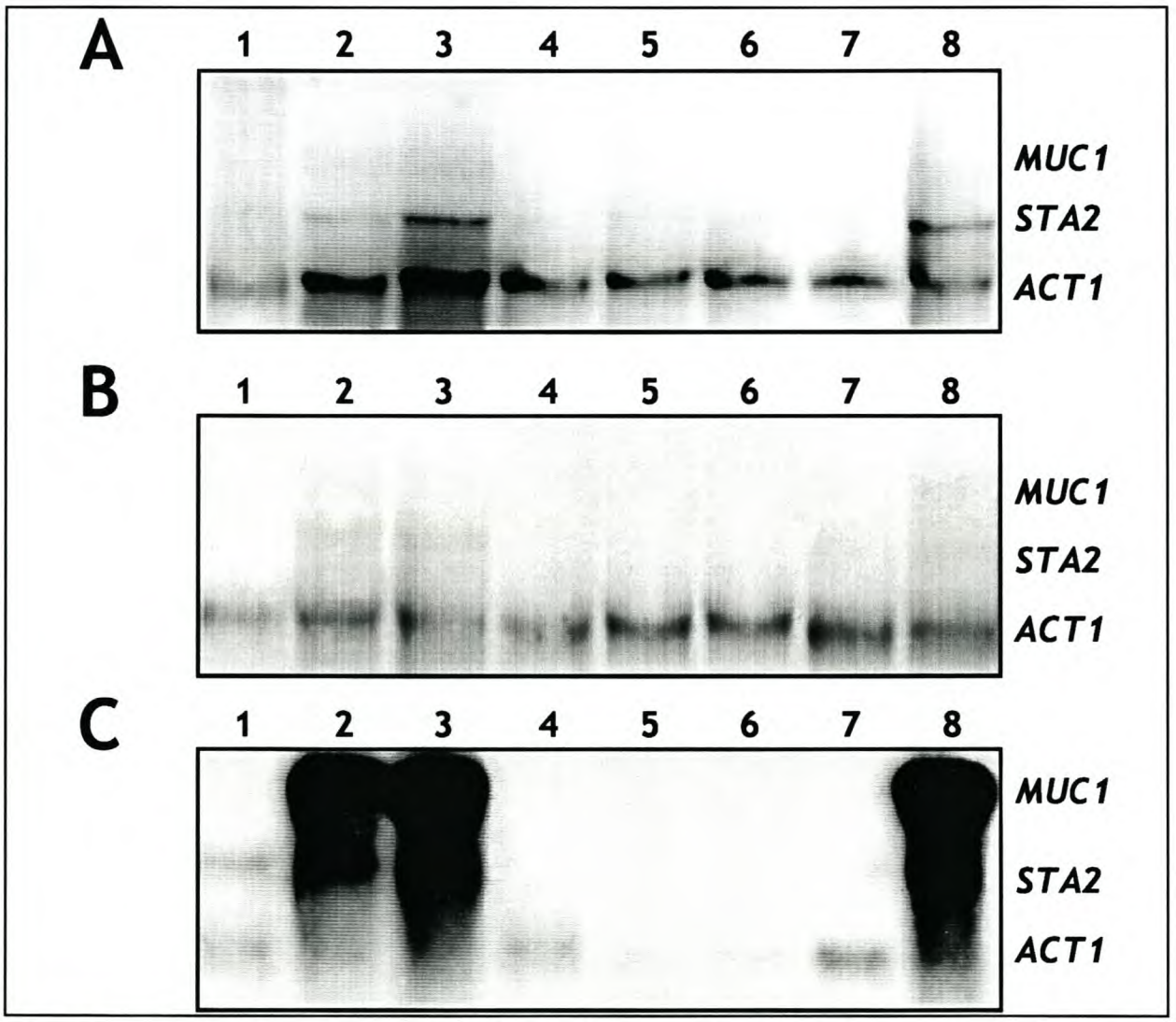

Figure 3. Northern blot analysis of the different ISP15 strains on different carbon sources. Each lane contains 10 µg of total RNA from the different strains in the following order: (1) ISP15 transformed with YEpLac112; (2) ISP15 with YEpLac112-MSN1; (3) ISP15 with YEpLac112-MSS11; (4) ISP15Δ*msn1* with 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.

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.

4.4 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).

4.5 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 signaling (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).

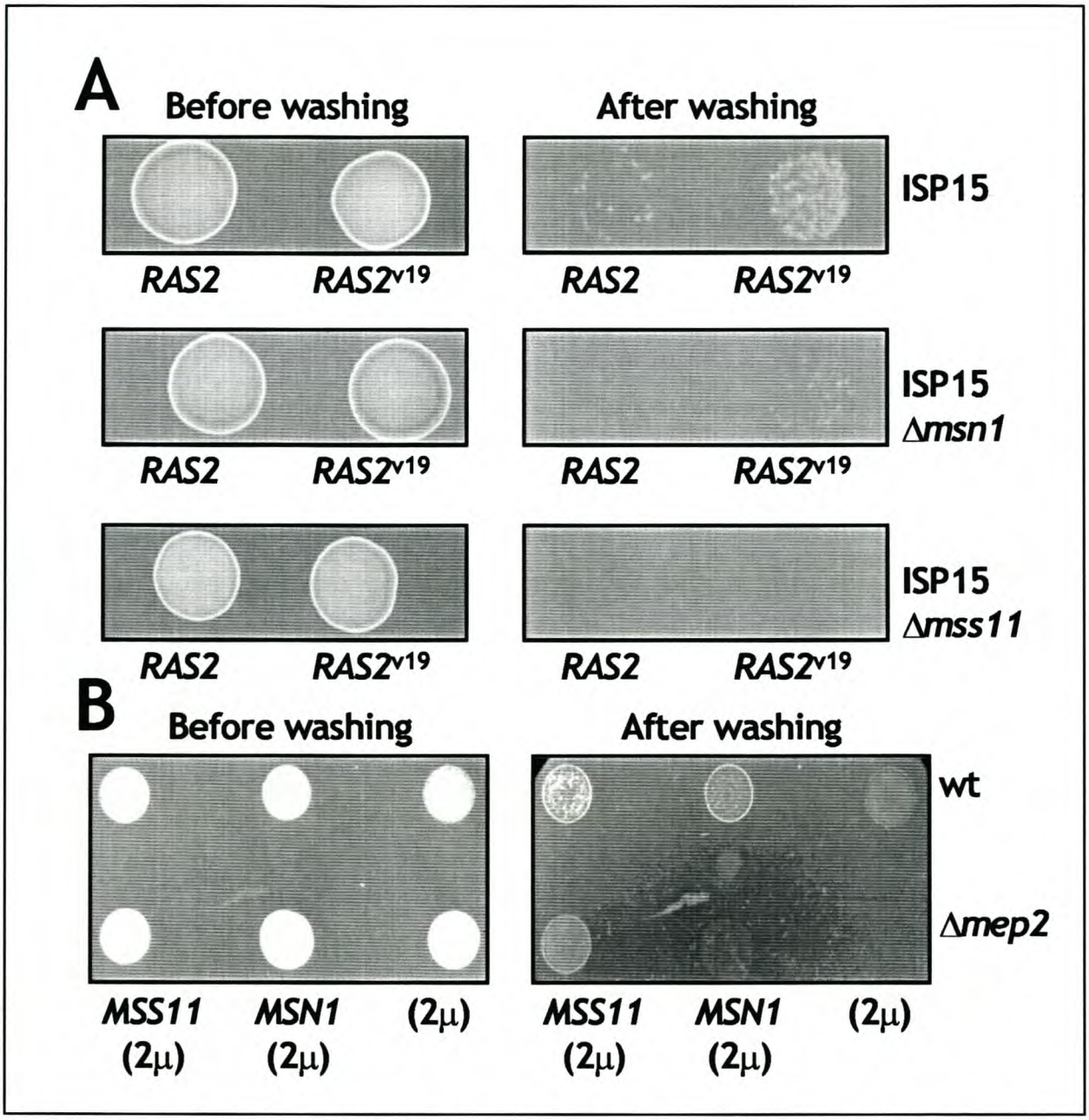

Figure 4. Msn1p and Mss11p act downstream of Mep2p and Ras2p in a pathway resulting in invasive growth. A.) The effect of the hyperactivated *RAS2* allele, *RAS2*^{val19} on the ability to grow invasively in a strain with a disrupted *MSS11* locus. ISP15 and ISP15Δ*mss11* were transformed with YCpLac22-RAS2^{val19}, and, as control, YCpLac22-RAS2 bearing the wild-type RAS2 allele. Strains were spotted on SCS medium and surface growth washed off after 3 days. Increased invasive growth can be seen at the colony periphery and the halo around the washed off *RAS2*^{val19} colony is indicative of increased glucoamylase production. No starch degradation or invasive growth can be seen in strains with disrupted *MSS11* loci. Similar results were obtained with ISP20. Invasive growth phenotypes was similar on SLAD medium for strains ISP15, ISP20 and FY23 (data not shown). 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.

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.

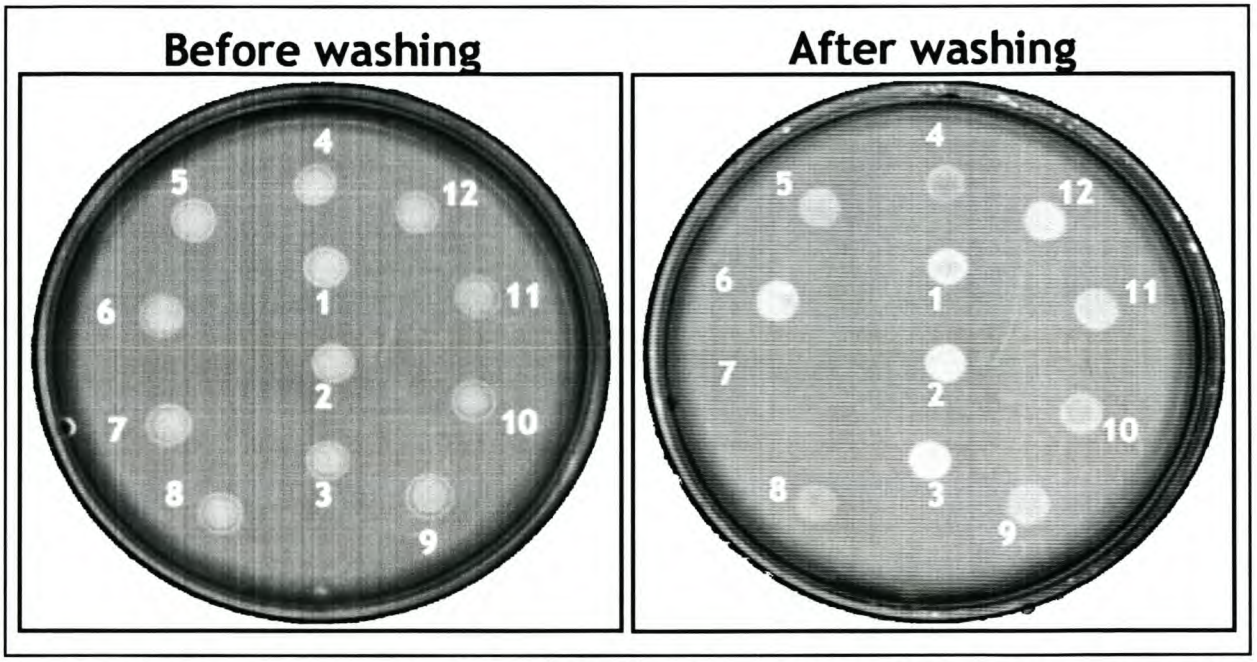

Figure 5. Effect of multiple copies of *MSN1* and *MSS11* in wild-type, Δ*ste20*, Δ*ste11 and* Δ*ste7* strains on SLAD medium. The strains were transformed with YEpLac195-MSN1, YEpLac-MSS11 and YEpLac195 as control and spotted onto the plates in the following order: (1) L5366h (wt) transformed with YEpLac195; (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* reestablish invasive growth in the Δ*ste20*, Δ*ste11* and Δ*ste7* strains.

4.6 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 Δste11, 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* reestablished 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.

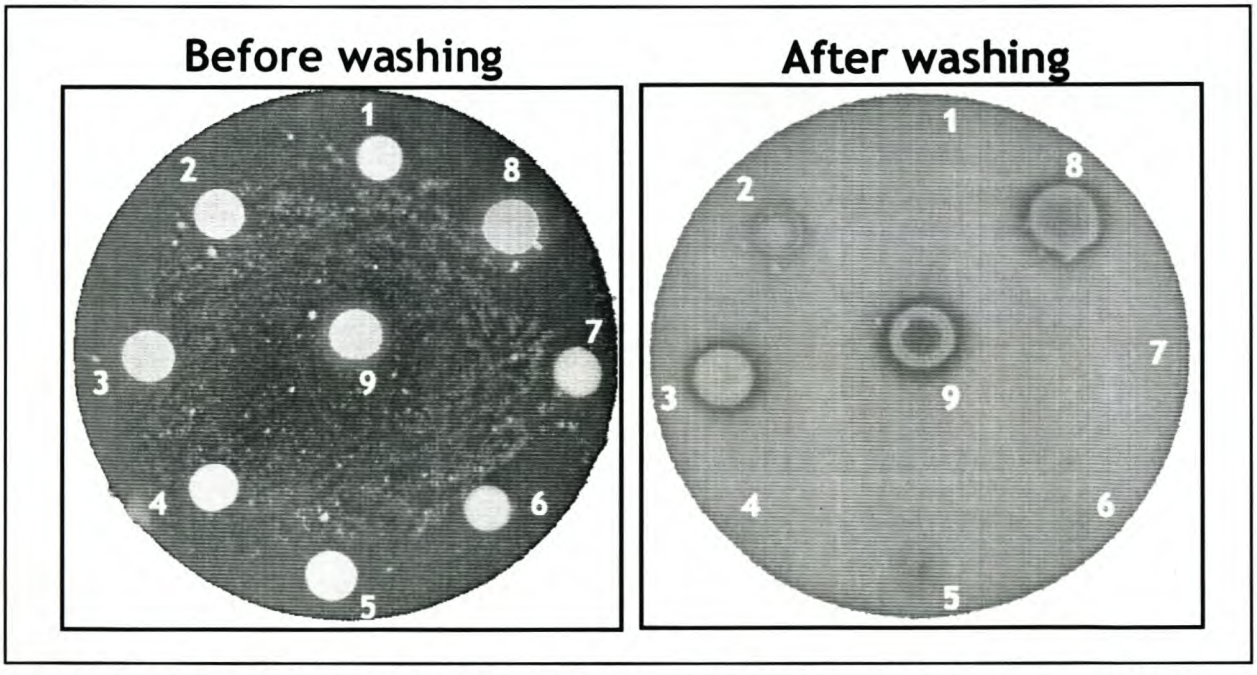

Figure 6. Effect of multiple copies of *MSN1* and *MSS11* in a Δ*ste12* background. Strains were spotted onto SCS plates and incubated for 6 days. The following strains were spotted: (1) ISP15Δ*ste12* transformed with YEpLac112; (2) ISP15Δ*ste12* with YEpLac112-MSN1; (3) ISP15Δ*ste12* with YEpLac112-MSS11; (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).

4.7 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 signaling 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* are resulting 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.

4.8 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).

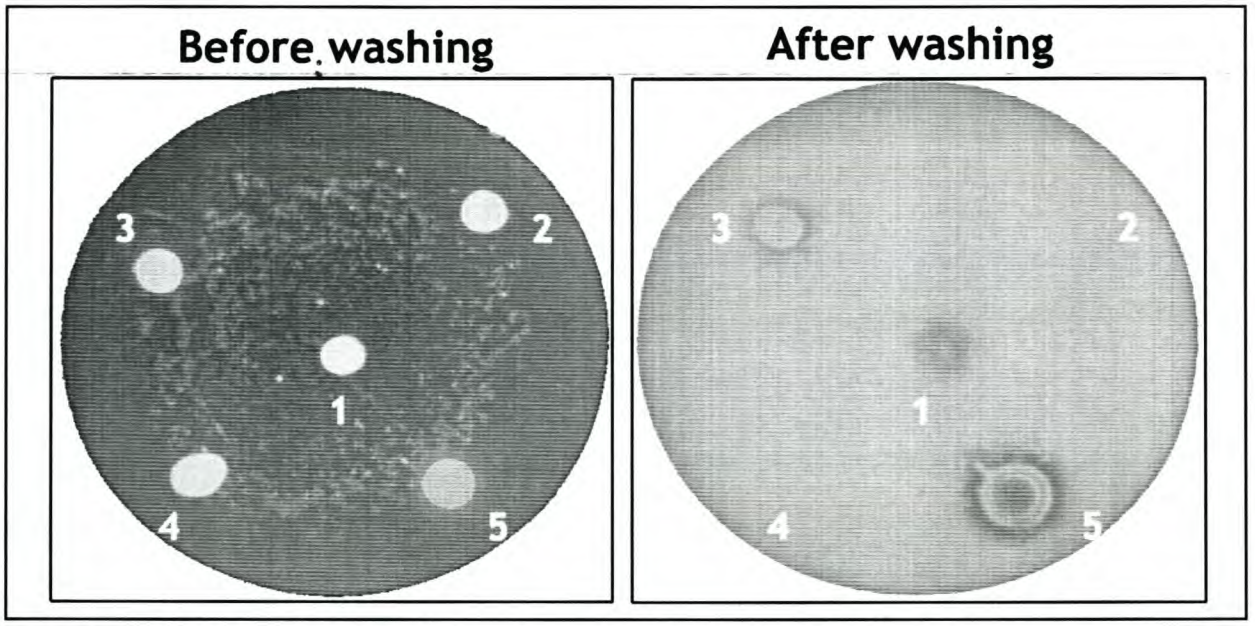

Figure 7. The MAP kinase cascade activates glucoamylase activity. The following strains were spotted onto SCS medium: (1) ISP15 transformed with YEpLac112; (2) ISP15Δste7 with YEpLac112; (3) ISP15 with YEpLac112-STE7; (4) ISP15Δste12 with YEpLac112 and (5) ISP15 with YEpLac112-STE12. Surface growth was 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.

4.9 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 (1.5×10^5) 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 phenoytype 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).

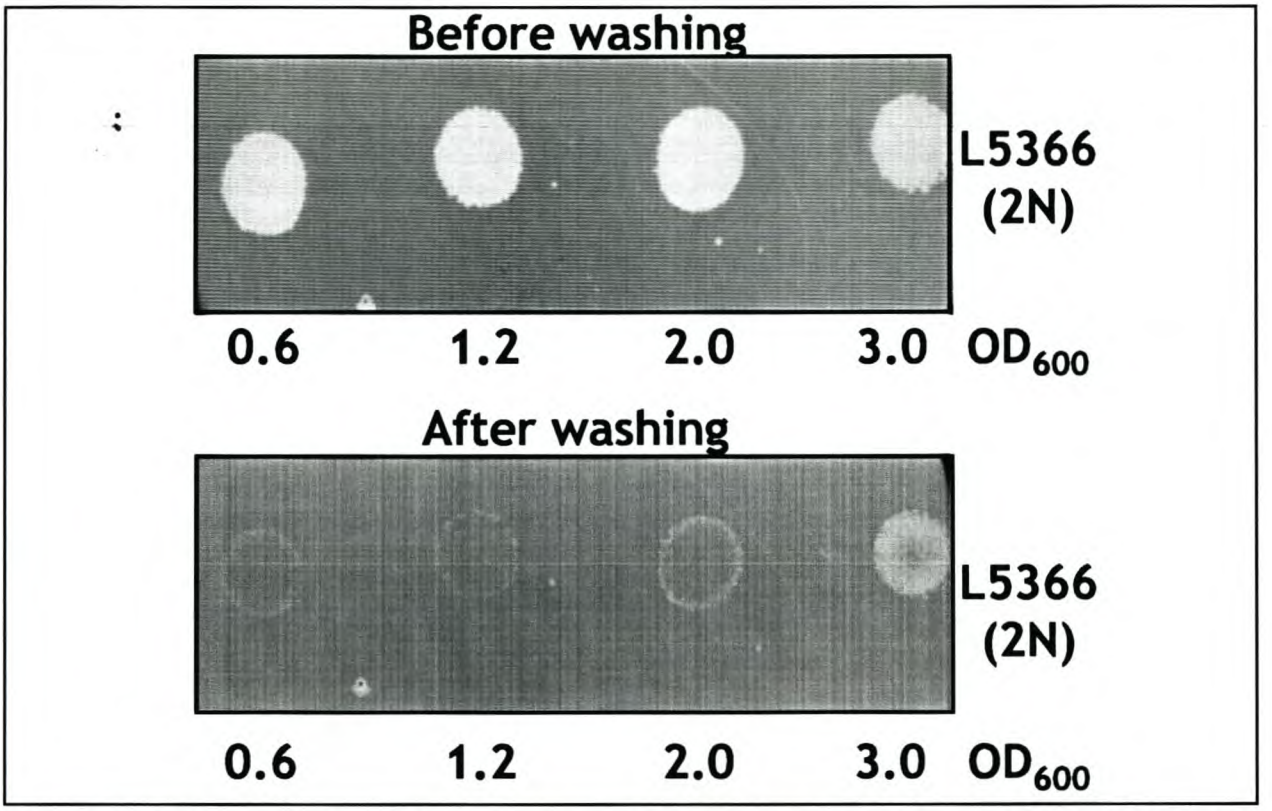

Figure 8. Effect of growth phase on invasive growth. The same number of cells (1.5×10^5) of the diploid Σ1278 strain, L5366, was spotted onto SLAD medium from cultures grown in rich media (YPD) to different growth phases. Surface growth was washed off after 3 days. Similar results were obtained with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

5. 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 reestablish 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 either encode 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.

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 signaling 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.

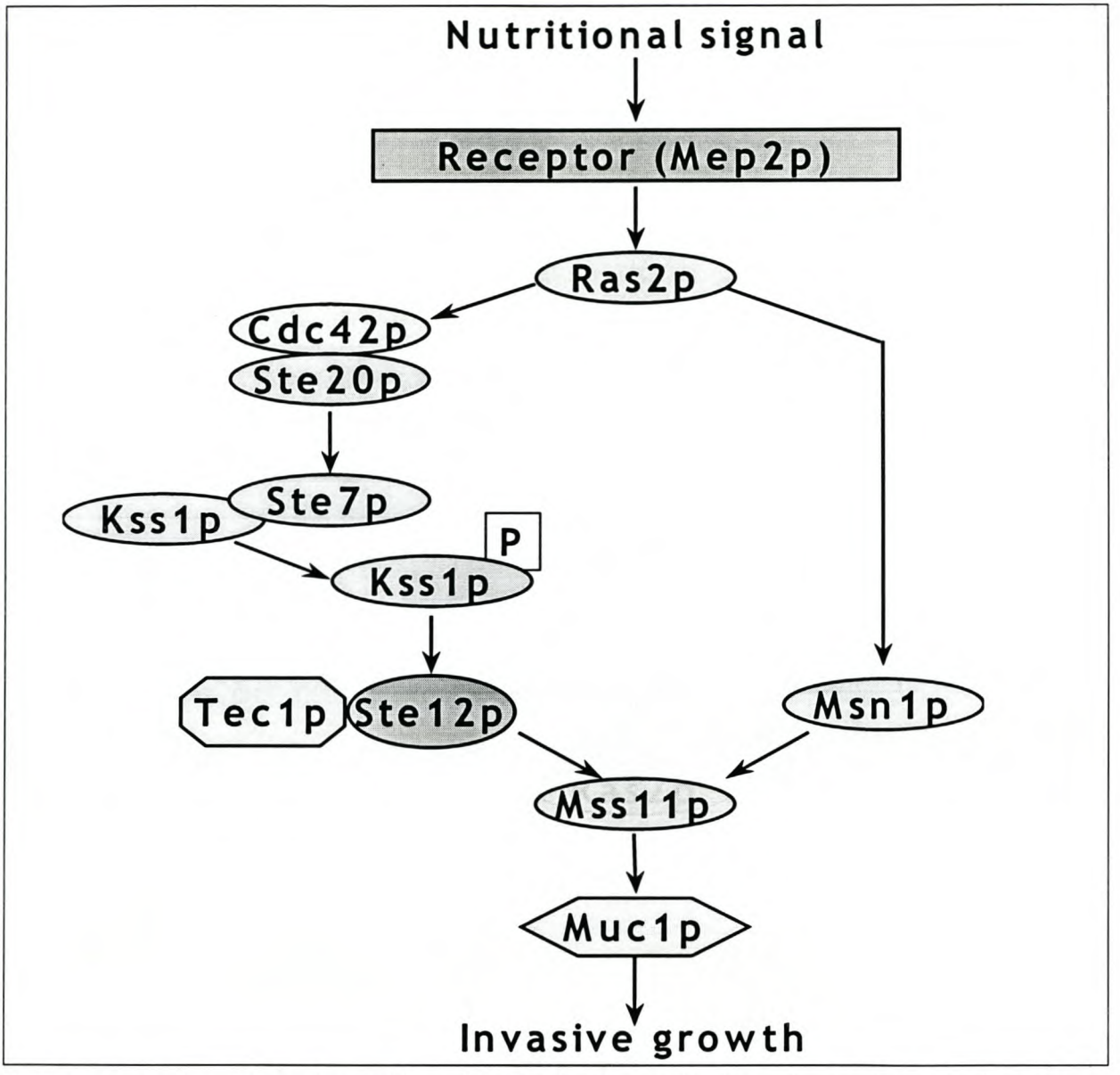

Figure 9. Proposed model for the positions of Msn1p, Mss11p and Muc1p in the signal transduction pathways resulting in invasive growth.

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Chapter 4

Research results II*

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

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

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1. Abstract

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 as well as evolutionary point of view. Transcription of the genes seems indeed dependent on numerous transcription factors that integrate the information of a complex network of signalling pathways, while the very limited sequence differences between them should allow studying 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*.

2. Introduction

The *STA1*, *STA2* and *STA3* genes encode extracellular glucoamylase isozymes that enable *Saccharomyces cerevisiae* cells to utilise starch as a carbon source (reviewed in Pretorius et al., 1991; Pretorius, 1997; Vivier et al., 1997). 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 Teunissen and Steensma, 1995), *SUC* (reviewed in Johnston and Carlson, 1992) and *MAL* (reviewed in Needleman, 1991) 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 (Lambrechts et al., 1996a; Lo and Dranginis, 1996, 1998; Gagiano et al., 1999). 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 (Vivier et al, 1999). 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 (Lambrechts et al., 1996a). 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 (Yamashita et al., 1985; Yoshimoto et al., 1991). 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* (Yamashita et al., 1985, 1987; Carstens et al., 1998) and *SGA1* (Yamashita et al., 1985, 1987) in all *S. cerevisiae* strains investigated so far and (iii) the existence of homologous repeated sequences on either side of the proposed junctions (Yamashita et al., 1985, 1987).

Analyses of the upstream areas of *STA1* (Shima et al., 1989; Ahn et al., 1995), *STA2* (Lambrechts et al., 1994) and *MUC1* (Rupp et al., 1999) 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 (Gagiano et al., 1997; Rupp et al., 1999). The *STA* and *MUC1* upstream regions are therefore of particular interest from both an evolutionary as well as 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.* (1996a, b) and Gagiano *et al.* (1999) 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 (1998) demonstrated that *MUC1* is regulated by Ste12p, a transcription factor responsible for both pheromone-specific (reviewed in Kurjan, 1992), and, in combination with the TEA/ATTS family transcription factor, Tec1p, filamentation-specific gene regulation (Gavrias et al., 1996; Madhani and Fink, 1997). Gagiano *et al.* (1999) 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 that controls invasive growth and pseudohyphal differentiation (Lo and Dranginis, 1998; Gagiano et al., 1999; Rupp et al., 1999). This network combines inputs from at least three interacting signal transduction modules, including (i) the filamentation-specific MAP kinase cascade (Liu et al., 1993; Roberts and Fink, 1994; Mösch et al., 1996), (ii) the cAMP and cAMP-dependent kinase (Lorenz and Heitman, 1998; Robertson and Fink, 1998), and (iii) the cyclin-dependent kinase Cdc28p (Edgington et al, 1999). 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 (Rupp et al., 1999), a transcription factor initially identified for its role in flocculation (Kobayashi et al., 1996). 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 (Robertson and Fink, 1998).

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 (Pretorius et al., 1986b; Dranginis, 1989; Suntsov et al., 1991; Kuchin et al., 1993; Kartasheva et al., 1996). Carbon catabolite repression was reported to involve two separate pathways of which one requires *HXK2* and the other *HAP2* (Kartasheva et al., 1996). 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 (Gagiano et al., 1999; Lo and Dranginis, 1996), 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* (Pretorius et al., 1986b; Dranginis, 1989). The mechanism through which repression occurs is not defined, since the removal of the putative a1/α2 binding sites from the *STA2* promoter does not relieve the repressive effect observed in diploid strains (Lambrechts et al., 1994). 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 (Lo and Dranginis, 1998; Rupp et al., 1999).

Most laboratory strains of *S. cerevisiae* contain an undefined repressor, *STA10*, which reduces transcription of the *STA1-3* genes at least 20-fold (Polaina and Wiggs, 1983; Pretorius et al., 1986b). It was reported that the repressive effect of *STA10* results from interaction between two unlinked genes, *IST1* and *IST2* (Park and Mattoon, 1987), but this was not confirmed. The negative effect of several other genes i.e. *INH1* (Yamashita and Fukui, 1984), *SGL1* (Patel et al., 1990), *SNS1* and *MSS1* (Ahn et al., 1995) 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 (Yamashita, 1993). Furthermore, transcription of *STA1-3* also requires the presence of components of the SWI-SNF global activation complex (Inui et al., 1989; Okimoto et al., 1989; Yoshimoto and Yamashita, 1991; Yoshimoto et al., 1991, 1992; Kuchin et al., 1993), which associates with the RNA polymerase holoenzyme at specific promoters and relieves the repressive effect of chromatin on transcription (Kruger et al., 1995; Wilson et al., 1996).

Cis-acting promoter elements in several regions within the *STA1* (Shima et al., 1989; Ahn et al., 1995), *STA2* (Lambrechts et al., 1994) and *MUC1* (Rupp et al., 1999) 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 (Lambrechts et al., 1994). URS1 was found to reside in the area between nucleotides -1 390 and -1 074 that also host 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 (Shima et al., 1989; Ahn et al., 1995). A recent, more systematic, analysis of the *MUC1* promoter (Rupp et al., 1999), revealed a vast array of regulatory elements that 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* (Rupp et al., 1999).

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 (Shima et al., 1989; Lambrechts et al., 1994; Ahn et al., 1995; Rupp et al., 1999). 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 (Lambrechts et al., 1994), (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*.

3. Materials and methods

3.1 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 (Ausubel et al., 1994). The one-step gene replacement method (Ausubel et al., 1994) was used to disrupt the *FLO8* loci with the *flo8*::*URA3* cassette, pΔ*flo8*, in the genomes of strains ISP15 and ISP20, to generate strains ISP15Δ*flo8* and ISP20Δ*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 ISP15Δ*flo8* and ISP15Δ*msn1* was regenerated through transformations with the *ura3*::*kan*^{R} disruption cassette, pΔ*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 (Winston et al., 1995) is isogenic to the S288C genetic background and L5366 (Liu et al., 1993) and L5366h (Gagiano et al., 1999) to the Σ1278b 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.

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*	Gagiano et al., 1999
ISP20	*MATa STA2 thr1 trp1 leu2 ura3*	This laboratory
ISP20Δ*msn1*	*MATa STA2 thr1 trp1 leu2 msn1::URA3*	Gagiano et al., 1999
ISP20Δ*mss11*	*MATa STA2 thr1 trp1 ura3 mss11::LEU2*	Gagiano et al., 1999
ISP20Δ*flo8*	*MATa STA2 thr1 trp1 leu2 flo8::URA3*	This work
JM2508	*MATa leu2 ura3*	Julius Marmur
FY23	*MATα leu2 ura3 flo8*	Winston et al., 1995
L5366	*MATa/MATα ura3/ura3*	Liu et al., 1993
L5366h	*MATa ura3*	Gagiano et al., 1999

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 (Gimeno and Fink, 1994). 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-sterilized proline as sole nitrogen source.

E. coli strain DH5α (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.* (1989).

3.2 Construction of plasmids.

FLO8 was isolated as a 3 252 bp *Sph*I-*Eco*RV fragment from plasmid pF415-1 (Kobayashi et al., 1996) and ligated to plasmids YEplac112 and YEplac181 (Gietz and Sugino, 1988), digested with *Sph*I and *Sma*I, to generate plasmids YEplac112-*FLO8* and YEplac181-*FLO8*. YEplac112-*FLO8* was subsequently used to construct pΔ*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 (Jones and Prakash, 1990).

YCplac33-*STA2* was constructed by inserting a *Xho*I-*Eco*RV fragment from plasmid pSP*STA2* (Lambrechts et al., 1994) into the unique *Sal*I-*Sma*I sites of YCplac33 (Gietz and Sugino, 1988). 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 (Lambrechts et al., 1996a). 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.

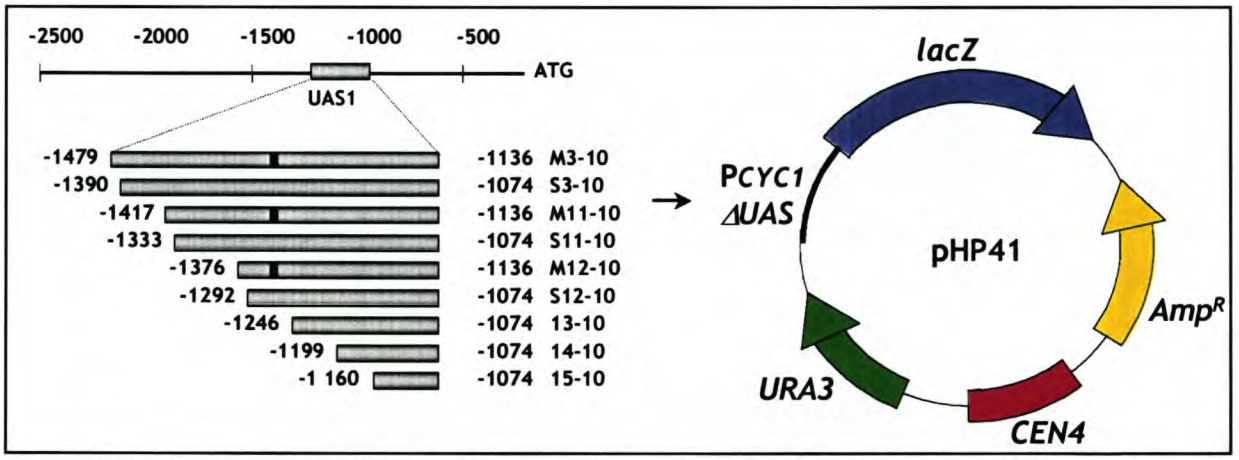

Figure 1. Construction of a series of plasmids containing sequential deletions of the *STA2* and *MUC1* UAS1, upstream of the *lacZ* reporter gene in plasmid pHP41. The position of UAS1 and UAS2 relative to the translation initiation codon (ATG) of the *STA2* and *MUC1* ORFs are indicated and the positions 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.

A 1 675 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 plasmid YEplac181 (Gietz and Sugino, 1988) to generate YEplac181-*MSN1*. A 3 326 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 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 (Lambrechts et al., 1996a) 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 hybridized to generate fragment M20, the 20 bp *MUC1* promoter insert, and primers F-M64 and R-M64 hybridized 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.

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**tccgagcgtttagaaggtgattgtaggcagaaattaactttgcggtaaa agaatgacattctttcc-3′	-	-869 to -933
R-M64	5′-**tcgag**gaaagaatgtcattcttttaccgcaaagttaatttctgcctacaatcacc ttctaaacgctcggag-3′	-	-869 to -933
P*MUC1*-FX	5′-gatc**tctaga**gaaatgtgggtcatcttttt-3′	-437 to -463	-439 to -465
P*MUC1*-RB	5′-ttaa**ggatcc**ggtcatagtgtgcgtatatg-3′	-	-1 to -14
P*STA2*-RB	5′-cgc**gggatcc**ggtcatagtgtgcgtatatggatt-3′	-1 to -18	-

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.

Plasmids pHP41 (Park et al., 1992), pLG670-Z (Guarente and Ptashne, 1981) and pLGΔ312 (West et al., 1987) 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 3. Plasmids and constructs used in this study

Plasmid	Relevant genotype	Source/reference
p*STA3*-6-4	2μ *URA3 STA3*	Yamashita et al., 1985
pSP*STA2*	*STA2*	Lambrechts et al., 1994
PMS2A	*MSN1*	Lambrechts et al., 1996b
pMUU	2μ *URA3 MUC1*	Lambrechts et al., 1996a
pF415-1	*CEN4 LEU2 FLO8*	Kobayashi et al., 1996
pJJ242	*URA3*	Jones and Prakash, 1990
pUG6	kan^R	J. H. Hegemann
YEplac112	2μ *TRP1*	Gietz and Sugino, 1988
YEplac112-*MSN1*	2μ *TRP1 MSN1*	Gagiano et al., 1999
YEplac112-*MSS11*	2μ *TRP1 MSS11*	Gagiano et al., 1999
YEplac112-*FLO8*	2μ *TRP1 FLO8*	This work
YEplac181	2μ *LEU2*	Gietz and Sugino, 1988
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*	Gietz and Sugino, 1988
YCplac22-*FLO8*	*CEN4 TRP1 FLO8*	Gagiano et al., 1999
YCplac33	*CEN4 URA3*	Gietz and Sugino, 1988
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*	Park et al., 1992
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*	Guarente and Ptashne, 1981
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*	West et al., 1987
pLGΔ312 + M20	2μ *URA3* P*CYC1-lacZ* + M20	This work
pLGΔ312 + M64	2μ *URA3* P*CYC1-lacZ* + M64	This work

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.* (1989).

3.3 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 (1985) 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.

3.4 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.* (1994). Error margins were calculated for each set of assays and were usually less than 7.5% and never higher than 15%.

3.5 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 taken with a Matrox Intellicam 2 (Matrox Electronics Inc.).

3.6 Plate assays to determine starch utilisation.

The *STA2* gene encodes an extracellular glucoamylase that 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 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, is indicative of the amount of glucoamylase secreted (Yamashita et al., 1985; Pretorius et al., 1986a). The expression of *STA2* in yeast strains were 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 and 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.

3.7 Sequence analysis and homology searches.

Homology searches in the yeast genome subdivision of GenBank were done with the BLAST software (Altschul et al., 1997). Sequence fragment assembly and individual alignments between the *STA* genes and *MUC1* were done using the OMIGA v1.1 package (Oxford Molecular Ltd, UK).

4. Results

4.1 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 p*MUC1-lacZ* and p*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 50 μM $(NH_4)_2SO_4$		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
p*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
p*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 p*MUC1-lacZ* and p*STA2-lacZ* were low in most conditions, similar to those reported for genes transcribed at low levels, e.g. P*HIS3*-lacZ (Ausubel et al., 1994). *MUC1* promoter-dependent expression levels were however consistently lower than *STA2* promoter-dependent levels.

The data indicate that in haploid strains both p*MUC1-lacZ* and p*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), p*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 p*MUC1-lacZ* construct increased 3-fold. Interestingly, this increase is nearly completely absent in diploid strains, where p*STA2-lacZ* expression was only increased 2-fold, and no increase at all was observed for p*MUC1-lacZ*. A 2.6-fold increase in expression

levels of p*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 p*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 (Lo and Dranginis, 1996, 1998; Rupp et al., 1999) or media containing glucose as carbon source (Gagiano et al., 1999) whereas *STA1* and *STA2* were reported to be repressed in all media containing readily metabolised carbon sources such as glucose (Pretorius et al., 1986b; Dranginis, 1989; Suntsov et al., 1991; Kuchin et al., 1993; Kartasheva et al., 1996; Gagiano et al., 1999).

Despite the high homology between the *STA2* and *MUC1* promoters, p*MUC1-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*MUC1-lacZ* was observed, whereas p*STA2-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 heat shock (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*MUC1-lacZ* and p*STA2-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*STA2-lacZ* in the Σ1278b haploid strain, only a 3.7-fold difference was observed for ISP20. A similar effect was seen for p*MUC1-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.

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
p*STA2-lacZ*	1.10	4.03	12.76	13.31	20.67	13.30	36.42	19.62
p*STA2*-ΔUAS1-*lacZ*	2.03	2.66	19.63	6.32	0.82	1.81	7.83	34.50
p*MUC1-lacZ*	0.33	0.51	1.41	3.15	0.66	0.36	6.33	2.88
p*MUC1*-ΔUAS1-*lacZ*	0.55	0.34	1.64	1.06	0.49	0.23	1.22	3.81

4.2 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 (Pretorius et al., 1986b). This phenomenon was believed to be due to the presence of a repressor, designated *STA10* (Polaina and Wiggs, 1983). 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 (Liu et al., 1996). Due to the extensive homology between the *STA2* and *MUC1* promoter regions and since *FLO8* was shown to be required for transcription of *MUC1* (Rupp et al., 1999), we investigated whether a genetic relationship between *STA10* and *FLO8* exists.

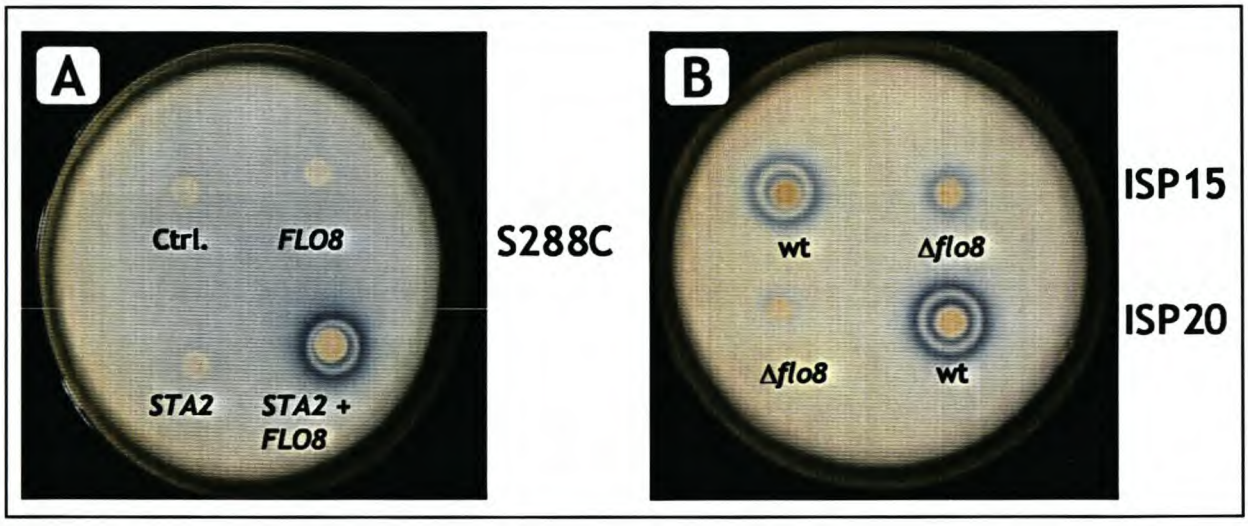

Figure 2. A.) The S288C-derived *STA10* strain, FY23, transformed with the centromeric plasmids YCplac22 and YCplac33 (Ctrl.), YCplac33 and YCplac22-*FLO8* (*FLO8*), YCplac22 and YCplac33-*STA2* (*STA2*) and YCplac22-*FLO8* and YCplac33-*STA2* (*STA2* + *FLO8*) on plates containing potato starch as sole carbon source (SCS). Cells that are unable to express *STA2* are unable to grow, whereas cells that do express *STA2* sufficiently produce extracellular glucoamylase that enable them to grow. The clear zone around the Sta^+ colony is due to the hydrolysis of the starch in the media. B.) 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.

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 (Winston et al., 1995), 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*.

4.3 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 converges at the promoter of the *MUC1* (Gagiano et al., 1999; Rupp et al., 1999) and *STA1-3* (Gagiano et al., 1999) 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.

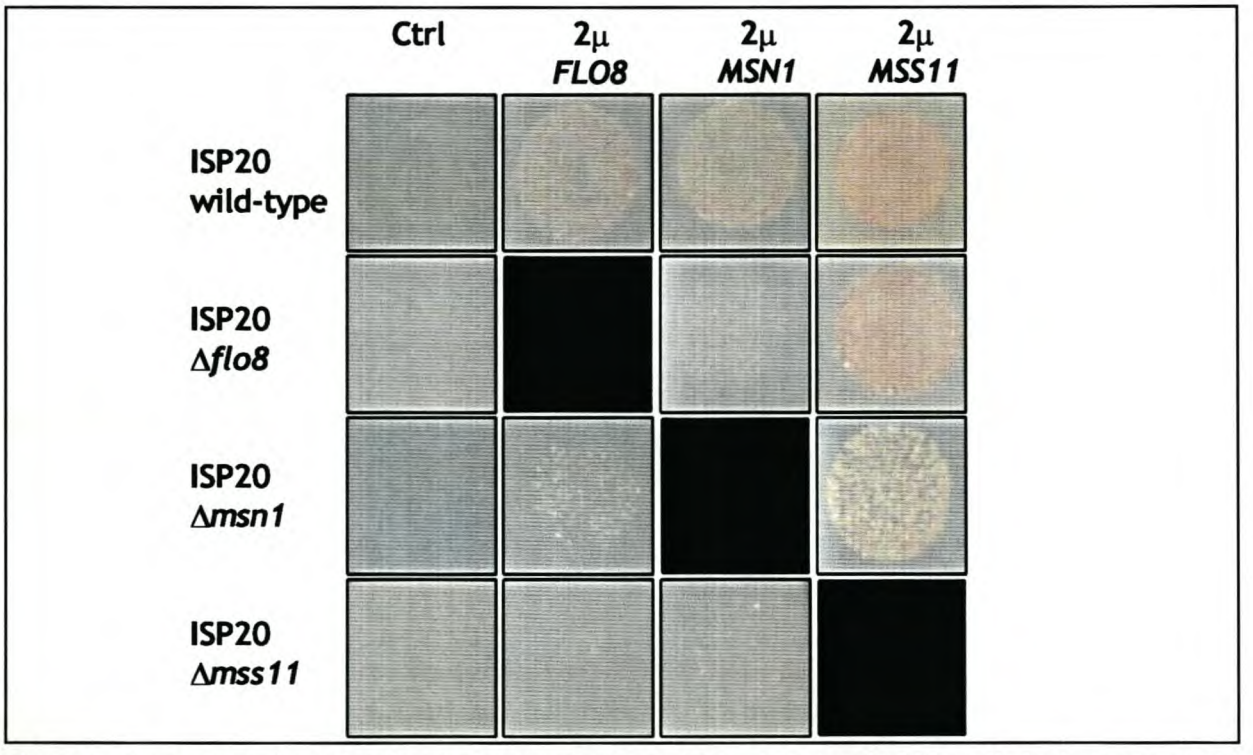

Figure 3. Determining the epistatic relationships between *MSN1*, *MSS11* and *FLO8* on limited nitrogen (SLAD) media in strain ISP20. Wild-type ISP20, ISP20Δ*flo8*, ISP20Δ*msn1* and ISP20Δ*mss11* were transformed with YEplac112 without insert (Ctrl.), YEplac112-*FLO8* (2μ *FLO8*), YEplac112-*MSN1* (2μ *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 was 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 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).

4.4 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 p*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.

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 that 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.

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.

4.5 Identification of regulatory regions within the UAS1 area.

Both a previous report (Rupp et al., 1999) 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.

To locate the sequences in UAS1 through which *FLO8*, *MSS11* and *MSN1* confers 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 represents 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 than 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*.

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*	Δ*flo8*
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

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

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

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 are 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.

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

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 was 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. such as in SCD media. It was shown that Mig1p is not involved in repression of the *STA* genes (Kartasheva et al., 1996), 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.

4.6 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 (Altschul et al., 1997) 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.

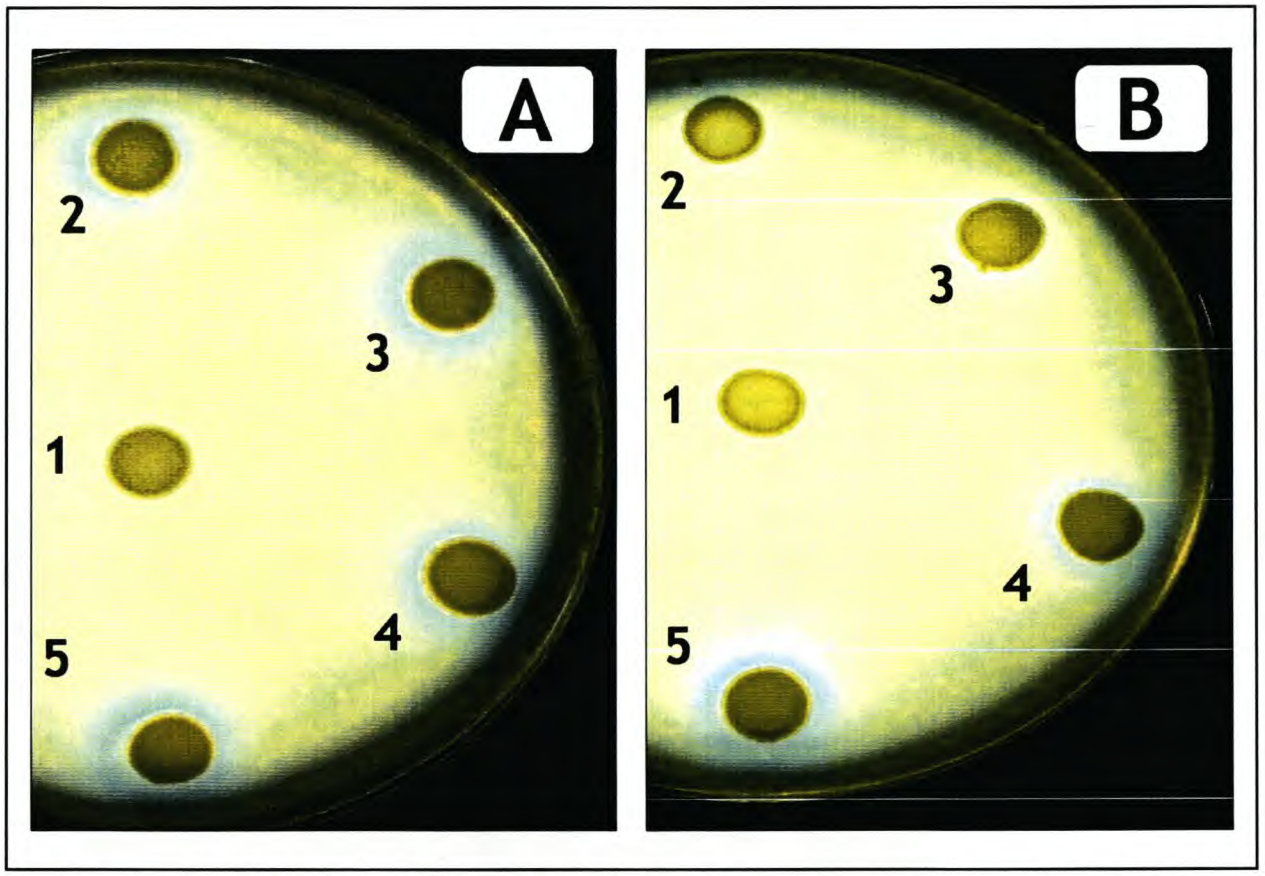

Figure 4. A). SCS plate (containing 2 % corn starch as carbon source) to investigate the starch utilisation of *S. cerevisiae* JM2508 transformed with (1) plasmids YCplac33 and YEplac112 without any inserts; (2) YCplac33-*STA2* and YEplac112; (3) YCplac33-*STA2* and YEplac112-*FLO8*; (4) YCplac33-*STA2* and YEplac112-*MSN1* and (5) YCplac33-*STA2* and YEplac112-*MSS11*. B.) 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 (Vivier et al., 1998). 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.

5. 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 that 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 (Lambrechts et al., 1996a; Lo and Dranginis, 1996; Gagiano et al., 1999). From an evolutionary perspective, the changes between the two promoters have therefore allowed the yeast to retain 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 that 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 targets 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.* (1999) 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 (Jiang et al., 1998; Thevelein, 1992), 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 that 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 -1 160 and -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 than observed in naturally occurring starch-degrading strains. *FLO8* was shown to be required for invasive growth in S288C-derived strains (Liu et al., 1996), 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*, also has mutations in other activators required for invasive growth and pseudohyphal differentiation (Liu et al., 1996) 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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Chapter 5

Research results III*

The functional dissection of Mss11p, a transcription factor regulating pseudohyphal differentiation, invasive growth and starch metabolism in *Saccharomyces cerevisiae*

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The functional dissection of Mss11p, a transcription factor regulating pseudohyphal differentiation, invasive growth and starch metabolism in *Saccharomyces cerevisiae*

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1. Abstract

The cell surface proteins required for the adhesion of cells, either to each other or to the growth substrate, were shown to be key components in establishing the pseudohyphal and invasive growth phenotypes in *Saccharomyces cerevisiae*. Of these proteins, the flocculin, Muc1p, was shown to be critical for both invasive growth and pseudohyphal differentiation in response to specific nutritional signals, most notably nitrogen and carbon source limitation. In response to these signals, the expression levels of *MUC1* are ultimately determined by the interplay of a multitude of transcriptional regulators that include, among others, Ste12p, Tec1p, Flo8p, Msn1p and Mss11p. Epistasis analyses of these factors would suggest that Mss11p functions at the convergence of at least two of these signalling cascades, the filamentous growth MAPK cascade (Ste20p, Ste7p, Ste11p, Kss1p, Ste12p) and the cAMP-PKA pathway (Gpr1p, Gpa2p, Tpk2p, Flo8p). Furthermore, Mss11p was also shown to activate the transcription of *MUC1* and the co-regulated *STA2* glucoamylase gene via a nucleotide sequence in the far upstream regulatory regions of these genes. Despite a clear role in regulating filamentous growth and starch metabolism via the expression levels of *MUC1* and *STA2*, the exact molecular function of Mss11p is unknown. We therefore subjected Mss11p to a detailed molecular analysis and report here on its role in the transcriptional regulation of *MUC1* and *STA2*, as well as on the identification of specific domains required to confer transcriptional activation in response to specific nutritional signals. We identify the transactivation domain of Mss11p as a highly conserved sequence, found in several mammalian and invertebrate organisms, and identify conserved amino acids as being critical for the activation function.

2. Introduction

Upon nutrient limitation, normal cells of *Saccharomyces cerevisiae* undergo a transition from normal ovoid cells that bud in an axial (haploid) or bipolar (diploid) fashion, to elongated

cells that bud in a unipolar fashion (reviewed in Kron, 1997; Madhani and Fink 1998; Borges-Walmsley and Walmsley, 2000; Pan et al., 2000; Bauer and Pretorius, 2001; Gancedo 2001). In this case, the daughter cells tend to stay attached to the mother cells, resulting in chains of cells referred to as pseudohyphae. These filaments can grow invasively into the agar or away from the colony and are hypothesised to be an adaptation of yeast cells that enables them to search for nutrient-rich substrates (Kron, 1997). A large number of genes that play a role in this phenotype have been isolated and most of these were shown to participate in distinct signalling cascades to regulate the dimorphic switch from yeast to hyphal form (reviewed in Kron, 1997; Madhani and Fink 1998; Borges-Walmsley and Walmsley, 2000; Pan et al., 2000; Bauer and Pretorius, 2001; Gancedo 2001). The best characterised of these signalling pathways are the invasive growth response cascade that consists of Ras2p, Cdc42p, Ste20p, Ste11p, Ste7p and Ste12p (Gimeno et al., 1992; Liu et al., 1993; Kron et al., 1994; Roberts and Fink, 1994; Mösch et al., 1996; Cook et al., 1996, 1997; Madhani and Fink, 1997, 1998; Madhani et al., 1997; Mösch and Fink, 1997; Bardwell et al., 1998a, b; Rupp et al., 1999) and the Gpa2p-cAMP-PKA pathway that consists of Gpa2p, the protein kinases Tpk1p, Tpk2p and Tpk3p and the transcription factors, Flo8p and Sfl1p (Ward et al., 1995; Kübler et al., 1997; Lorenz and Heitman, 1997, 1998a, b; Roberts et al., 1997; Robertson and Fink, 1998; Mösch et al., 1999; Pan and Heitman, 1999; Rupp et al., 1999; Lorenz et al., 2000; Tamaki et al., 2000). In addition to the components of the established regulatory signalling cascades, several other factors have also been identified for their roles in determining pseudohyphal and invasive growth. These include Phd1p (Gimeno and Fink, 1994; Lorenz and Heitman, 1998a), Sok2p (Ward et al., 1995; Pan and Heitman, 1999), Elm1p (Blacketer et al., 1993; Garret, 1997; Koehler and Myers, 1997), Msn1p and Mss11p (Gagiano et al., 1999a, b), but these factors have either not been placed in the context of known signal transduction pathways, have not been characterised sufficiently or seem to function through alternative pathways.

MUC1 (also known as *FLO11*) is a member of the adhesin- or flocculin-encoding genes, and is regulated via the signalling pathways that determine filamentous growth (Guo et al., 2000). It encodes a large, cell wall-associated, GPI-anchored threonine/serine-rich protein with structural resemblance to mammalian mucins and yeast flocculins (Lambrechts et al., 1996a, Lo and Dranginis, 1996, 1998; Guo et al., 2000). Deletion analyses demonstrated that *MUC1* is critical for pseudohyphal differentiation and invasive growth, whereas overexpression of the gene results in flocculating yeast strains in liquid media and pseudohyphal/invasive growth on solid media (Lambrechts et al., 1996a; Lo and Dranginis, 1996, 1998; Guo et al., 2000).

The upstream regulatory region of *MUC1* is one of the largest yeast promoters identified to date and areas as far as 2.4 kb upstream of the transcription start site were shown to be

required for regulation on *MUC1* expression (Gagiano et al., 1999a; Rupp et al., 1999). The *MUC1* upstream region is almost identical to that of the *STA2* gene (Gagiano et al., 1999b), which encodes for an extracellular glucoamylase that enables the yeast cell to utilise starch as a carbon source (reviewed in Pretorius et al., 1991; Vivier et al., 1997).

Genetic evidence suggests that of all the genes encoding factors that regulate filamentous growth and starch metabolism, *MSS11* appears to have the most central role. Overexpression thereof results in highly elevated levels of *MUC1* and *STA2* transcription in all strains tested including strains with single or multiple deletions of genes encoding other factors that activate *MUC1* and *STA2* transcription (Gagiano et al., 1999a, b). On the other hand, the deletion of the *MSS11* locus results in the complete absence of these phenotypes, which cannot be suppressed by overexpressing any of the factors identified to date, including Ste12p, Flo8p and Msn1p (Gagiano et al., 1999a, b).

Despite a clear role in regulating filamentous growth and starch metabolism via the transcription levels of *MUC1* and *STA2*, the exact molecular function of Mss11p is unknown. Although it was shown to regulate expression levels of *MUC1* and *STA2* at a transcriptional level (Gagiano et al., 1999a, b), and that this activation occurs via specific areas within the *MUC1* and *STA2* promoters (Gagiano et al., 1999a), it is unclear whether it confers this activation directly, i.e. by acting as a transcriptional activator, or indirectly, i.e. by interacting with or recruiting other transcription factors. It has no significant homology to any yeast protein, with the exception of some limited homology to the Flo8p transcriptional activator (Gagiano et al., 1999a). Mss11p also contains distinctive poly-glutamine and poly-asparagine domains that are similar to, but significantly larger than, the domains observed in the repressor, Ssn6p. It also contains a putative ATP- or GTP-binding domain, commonly found in ATP- or GTP-binding proteins such as the kinases, ATPases or GTPases (Saraste et al., 1990). The functional relevance and significance of all these domains has not been demonstrated yet. Furthermore, Mss11p has been implicated only in the regulation of *MUC1* and *STA2* transcription and it is therefore unknown whether any other target genes exist or whether Mss11p also plays a role in cellular processes other than filamentous growth or starch metabolism. These information gaps do not allow any speculation on the possible physiological roles of Mss11p or its exact molecular function.

In this paper, we identify Mss11p as a transcription factor by fusing it to the Gal4p DNA-binding domain and monitoring the ability of the fusion-protein to activate a reporter gene under specific conditions. We delineate a minimal activation domain by means of domain mapping and show that is sufficient for the activation of a reporter gene, as well as of the *MUC1* and *STA2* genes. We go on to show that the activation activity of this domain depends on a few conserved amino acids. Interestingly, this newly identified domain is highly

conserved in mammalian and invertebrate proteins of unknown function. We also report on the function of the other domains in Mss11p.

3. Materials and Methods

3.1 Yeast strains, genetic methods and media

The yeast strains used in this study, along with the relevant genotypes, are listed in Table 1. The ISP15 and ISP20 strains are from different genetic backgrounds and were selected for their ability to degrade starch and to grow invasively. Both strains have been used extensively in the characterisation of starch metabolism and pseudohyphal differentiation (Lambrechts et al., 1994; Lambrechts et al., 1996a, b; Webber et al., 1997; Gagiano et al., 1999a, b). Strain pJ69-4A is commonly used in the analysis of two-hybrid interactions and was generously provided by P. James (James et al., 1996).

Table 1. The yeast strains used in this study.

Strain	Genotype	Source or reference
ISP15	*MATa STA2 his3 thr1 trp1 leu2 ura3*	Gagiano et al., 1999a
ISP15Δmss11	*MATa STA2 his3 thr1 trp1 leu2 ura3 Δmss11::LEU2*	Gagiano et al., 1999a
ISP20	*MATa STA2 thr1 trp1 leu2 ura3*	Gagiano et al., 1999a
ISP20Δmss11	*MATa STA2 thr1 trp1 leu2 ura3 Δmss11::LEU2*	Gagiano et al., 1999a
PJ69-4A	*MATa his3 trp1 leu2 ura3 gal4 gal80 LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James et al., 1996

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

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

Media	Nitrogen Source	Carbon Source
YPD	1% yeast extract, 2% peptone	2% glucose
YPLD	1% yeast extract, 2% peptone	0.1% glucose
SCD	1.7% yeast nitrogen base', 40 mM $(NH_4)_2SO_4$	2% glucose
SCLD	1.7% yeast nitrogen base', 40 mM $(NH_4)_2SO_4$	0.1% glucose
SLAD	1.7% yeast nitrogen base', 20 μM $(NH_4)_2SO_4$	2% glucose
SLALD	1.7% yeast nitrogen base', 20 μM $(NH_4)_2SO_4$	0.1% glucose

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

3.2 Plasmid construction

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

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

The promoter region of *MSS11* was PCR-amplified using primers MSS11-PF and MSS11-PR, together with plasmid YEplac112-MSS11 (Gagiano et al., 1999a) as template. The reverse primer, MSS11-PR, was designed to contain an *Eco*RI site after the *MSS11* start codon. This fragment was digested with *Eco*RI and *Sca*I and inserted into the unique *Eco*RI and *Hin*dII sites of plasmid YEplac112. The terminator region was PCR-amplified using primers MSS11-TF and MSS11-TR, together with YEplac112-MSS11 as template. The forward primer, MSS11-TF, was designed to contain a *Sal*I restriction site immediately 5' to the stop codon and the reverse primer a *Hin*dIII restriction site for cloning the fragment into the unique *Sal*I and *Hin*dIII sites of plasmid YEplac112. The resulting plasmid, YEplac112-MSS11exp, therefore contained the full-length *MSS11* promoter, start codon, stop codon and terminator region, as well as unique *Eco*RI and *Sal*I sites for insertion of the different *MSS11* ORF fragments.

Different combinations of the primers listed in Table 3 were used to generate the truncated ORF fragments by means of PCR. Plasmid YEplac112-MSS11 was used as a template in all PCR reactions. All forward primers were designed to contain an *Eco*RI restriction site and all reverse primers to contain a *Sal*I restriction site for cloning the different fragments in-frame into plasmid YEplac112-MSS11exp, described above. The resulting plasmids are listed in

Table 4. All plasmids were sequenced to verify that the expected deletions were correct and that no mutations were introduced through PCR.

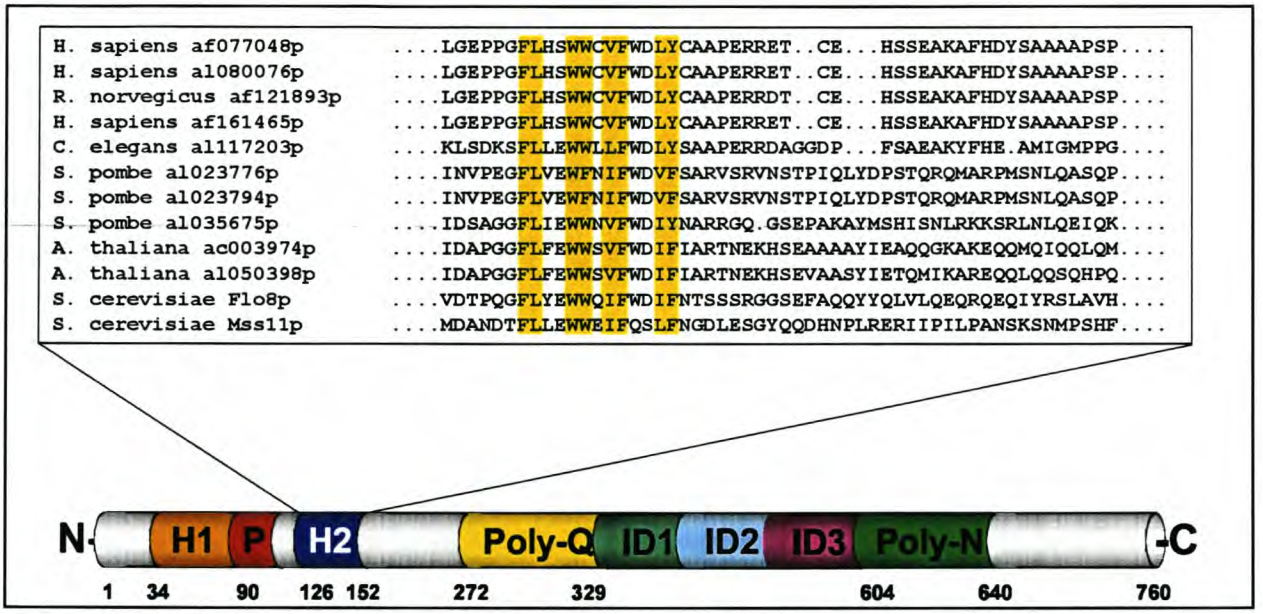

Figure 1. A diagrammatic representation of Mss11p that illustrates the position and length of the different domains. Domains H1 and H2 represent the domains with homology to *S. cerevisiae* Flo8p. The alignment of the second homology domain, H2, with Flo8p and proteins with unknown function from other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

The poly-glutamine and poly-asparagine domains were deleted by replacement with an *Eco*RI restriction site. Primer MSS11-OF was used in a PCR reaction, together with primer MSS11-QReco, which is designed to contain an in-frame *Eco*RI site. Plasmid YEPlac112-MSS11 was used as template to generate a fragment stretching from the ATG initiation codon of *MSS11* to before the poly-glutamine domain, ending in an *Eco*RI site. This fragment was digested with *Eco*RI and ligated into plasmid YEplac112-MSS11-QF-OR, then opened with *Eco*RI to generate an *MSS11* ORF of which an *Eco*RI site replaced the area encoding the poly-glutamine domain. The correct orientation was selected through restriction analysis and the construct was sequenced for confirmation. The poly-asparagine domain was deleted through a similar strategy, using YEplac112-MSS11 as template and primers MSS11-NReco and MSS11-OF in a PCR reaction. This fragment was digested with *Eco*RI and ligated into plasmid YEplac112-MSS11-NF-OR, then opened with *Eco*RI to generate an *MSS11* ORF of which an *Eco*RI site replaced the area encoding the poly-asparagine domain.

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

*Eco*RI-*Sal*I fragments into the unique *Eco*RI and *Sal*I sites of plasmid pGBD-C2 (James et al., 1996). The resulting plasmids are listed in Table 5.

3.3 Site-directed mutagenesis

ATP- and GTP-binding proteins from a number of different organisms were shown to carry a glycine-rich motif known as the P-loop, required for the binding of ATP and/or GTP and generally critical for the function of the protein (reviewed in Saraste et al, 1990). The consensus sequence of this domain was determined as Gly_1-X_2-X_3-X_4-X_5-Gly_6-Lys_7-Ser/Thr_8 by mutation analysis of a common sequence found in myosin and many other nucleotide-binding enzymes (Saraste et al., 1990). Mutation analyses of a very large number of ATP- and GTP-binding proteins suggested that the critical amino acids are indeed Gly_1, Gly_6 and Lys_7 (invariant), as well as Ser_8, which can be replaced only with a functionally equivalent Thr (Saraste et al., 1990). The putative P-loop (Gagiano et al., 1999a; Fig. 1) of Mss11p was eliminated by mutating amino acids that were shown to be critical for its function (Saraste et al., 1990): a glycine at position 113 and a lysine at position 114, to alanine and arginine, respectively. This was achieved by designing a forward primer that contained the desired nucleotide changes. The primer was extended to span a native *Xba*I site in the *MSS11* ORF that would aid in the cloning of the fragment. This primer, MSS11-PloopF, was used together with the reverse primer, MSS11-OR, to generate a fragment that contained the desired sequence alterations. The fragment was digested with *Xba*I and *Sal*I before ligation into plasmid MSS11-OF-OR, of which the corresponding fragment was removed. The construct was sequenced to verify that the correct alterations were made and that no additional mutations were introduced through PCR.

A small stretch of amino acids in Mss11p was shown to have some homology to a similarly sized domain in Flo8p (Gagiano et al., 1999a). This domain, dubbed H2, was subsequently found to be conserved between a number of eukaryotic proteins of unknown function. An alignment of the relevant protein sequences, with the conserved amino acids highlighted, is shown in Fig. 1. To establish whether these amino acids contribute to the functioning of Mss11p, the amino acids pairs, i.e. the isoleucine and phenylalanine, the phenylalanine and leucine, the leucine and phenylalanine, as well as the two tryptophanes, were all mutated to glycine and alanine, respectively. This was achieved through a PCR-based mutagenesis strategy. Forward and reverse primers containing the desired nucleotide changes were designed and by changing the nucleotides to encode for glycine and alanine, a unique *Cfr*10I restriction site was introduced. Using YEplac112-MSS11 as template, the different forward primers were used together with primer MSS11-OR, and the reverse primers together with primer MSS11-OF to generate fragments that contain the desired mutations. The smaller

fragments, generated by using primer MSS11-OF with the reverse primers, were digested with *Eco*RI and *Cfr*10I. The larger fragments, generated by using the forward primers together with primer MSS11-OR, were digested with *Cfr*10I and *Sal*I. The fragments were ligated in the necessary combinations, together with YEplac112-MSS11exp digested with *Eco*RI and *Sal*I, to form full-length *MSS11* fragments containing the desired mutations.

3.4 RNA isolation and Northern analysis

The impact of *MSS11* on *STA2* and *MUC1* transcription was assessed in different nutritional conditions to determine if the Mss11p-mediated regulation of *STA2* and *MUC1* varies between different nutritional conditions. Strains ISP15 and ISP15Δmss11 were transformed with the unmodified plasmid, YEplac112, to complement the *TRP1* marker, and strain ISP15 was also transformed with YEplac112-MSS11 for the overexpression of *MSS11*. Colonies from each transformation were inoculated from the selective media into 5 ml liquid SCD media and grown to an optical density (OD) of ~1 to serve as starter cultures. These starter cultures were used to inoculate 50 ml flasks of media containing varying concentrations and types of nitrogen and carbon sources (Table 2). All media were inoculated to an initial OD of 0.05 and incubated on a rotary shaker to reach a final OD of ~1.0. Total RNA from each strain was isolated and separated on a 1.2% formaldehyde agarose gel, using the Bio101 FastRNA RedKit according to the specifications of the supplier.

The RNA was transferred and fixed onto Hybond-N nylon membranes (Amersham Pharmacia Biotech, Uppsala, Sweden), according to the specifications of the manufacturer. *ACT1*, *MUC1* and *STA2* transcripts were detected using gene-specific probes prepared with the DIG PCR labelling kit (Roche Diagnostics) according to the specifications of the manufacturer. Hybridisations were done at 42°C for 16 h in standard formaldehyde buffer, containing 50% formamide.

3.5 β-Galactosidase liquid and plate assays

The pGBD-C2-based constructs contain the *MSS11* fragments ligated in-frame to the part of the *GAL4* ORF that encodes the DNA-binding domain. Strain pJ69-4A contains the *GAL7* promoter fused to the *lacZ* reporter gene. All the Gal4p-Mss11p fusion constructs were therefore transformed into this strain to determine whether Mss11p or specific parts thereof can act as activation domains in an artificial system. After transformation, three independent colonies of each transformation were grown in 5 ml of selective SCD media to an OD of ~1.0. From each of these starter cultures, a 5 ml culture of SCD was inoculated to an OD of 0.05 and incubated to grow at 30°C to an OD of ~1.0. β-Galactosidase assays were performed as

described in Ausubel et al. (1994). Assays were performed on all three transformants, and in each case the mean value is presented. The standard deviation did not exceed 15% and was usually less than 8%.

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

Primer Name	Position relative to ORF	Sequence
MSS11-P-F	-581 to -600	5'-ACAGGGCGCAATCAGCTACC-'3
MSS11-P-R	+3 to -21	5'-cgtgaattcCATATCTTTATCATGCACCTTTTT-3'
MSS11-T-F	+2 275 to +2 304	5'-atctgtcgacCTTAAAACCTATTAAACAACAAAAAGTGTTTC-3'
MSS11-T-R	+ 2 717 to +2 736	5'-gatcaagcttTGGCCAGATAGCTTGCTTAC-3'
MSS11-OF	+4 to +30	5'-atcgaattcGATAACACGACCAATATTAATACAAAT-3'
MSS11-OR	+2 250 to +2 274	5'-gcaggtcgac*a*GCTATCCATTAGATCAGGAGAAAAG-3'
MSS11-H1F	+103 to +126	5'-gatcgaattcTTTGATGCGGATTCTCGAGTTTTC-3'
MSS11-H1R	+254 to +276	5'-tcaggtcgac*a*ACCCGAAGCAGATCCGTTTATTC-3'
MSS11-H2F	+376 to +396	5'-gatcgaattcCTGATGGACGCTAATGACACG-3'
MSS11-H2R	+421 to +444	5'-tcaggtcgac*a*GTCTCCATTGAACAATGATTGAAA-3'
MSS11-PH2F	+442 to 465	5'-atggaattcGACCTAGAATCTGGGTACCAACAG-3'
MSS11-QF	+988 to +1 011	5'-atcgaattc*a*CACCGTATCCTATTGTCAACCCA-3'
MSS11-QR	+794 to +816	5'-caggtcgac*a*TGCTGGTGATTGCAAATCATTGA-3'
MSS11-QxF	+817 to +837	5'-atggaattcCAGCCCCAGCAATCATCTCAA-3'
MSS11-QxR	+961 to +984	5'-gcaggtcgac*a*TTGCTGCTGTTGATGTTGTTGCTG-3'
MSS11-ID1R	+1 240 to +1 260	5'-gatgtcgac*a*TTGCTGTAGTGCTTGCTGCTG-3'
MSS11-ID2F	+1 240 to +1 260	5'-gatgaattcCAGCAGCAAGCACTACAGCAA-3'
MSS11-ID2R	+1 510 to +1 530	5'-gatgtcgac*a*TAATTGCTGGTTAGCCGCCAT-3'
MSS11-ID3F	+1 510 to +1 530	5'-gatgaattcATGGCGGCTAACCAGCAATTA-3'
MSS11-NF	+1 921 to +1 944	5'-atggaattcACACCCACAGTATCACAACCATCA-3'
MSS11-NR	+1 789 to +1 812	5'-caggtcgac*a*AGGCAAAGGAAAGACGGAGGTAGA-3'
MSS11-NxF	+1 810 to +1 839	5'-atggaattcCCTAACAATAACAATAACAATAACAACAAC-3'
MSS11-NxR	+1 897 to +1 926	5'-gcaggtcgac*a*GGGTGTATTATTACTATTATTATTATTATT-3'
MSS11-QReco	+796 to +816	5'-atcgaattcTGCTGGTGATTGCAAATCATT-3'
MSS11-NReco	+1 789 to +1 811	5'-atcgaattc*a*GGCAAAGGAAAGACGGAGGTAGA-3'
MSS11-PloopF	+247 to +288	5'-TTA*TCTAGA*ATAAACGGATCTGCTTCGGGT***GCGAGAA***CTAGC-3'
MSS11-WW-F	+409 to +432	5'-gaaGCCGGCGAAATTTTTCAATCATTG-3'
MSS11-WW-R	+391 to +414	5'-ttcGCCGGCTTCCAGTAAAAACGTGTC-3'
MSS11-IF-F	+418 to +441	5'-gaaGCCGGCCAATCATTGTTCAATGGA-3'
MSS11-IF-R	+400 to 423	5'-ttgGCCGGCTTCCCACCATTCCAGTAA-3'
MSS11-FL-F	+403 to +417	5'-acgGCCGGCCTGGAATGGTGGGAAATT-3'
MSS11-FL-R	+379 to +402	5'-cagGCCGGCCGTGTCATTAGCGTCCAT-3'
MSS11-LF-F	+436 to +453	5'-tcaGCCGGCAATGGAGACCTAGAATCT-3'
MSS11-LF-R	+412 to +435	5'-attGCCGGCTGATTGAAAAATTTCCCA-3'

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

Strain ISP20Δmss11 was transformed with the plasmids p*MUC1-lacZ* and p*STA2-lacZ* (Gagiano et al., 1999b), to obtain two reporter strains for assessing the impact of the Mss11p deletions and modifications on *MUC1* and *STA2* transcription. These strains were subsequently transformed with the different deletion and mutation constructs and the unmodified vector, YEplac112, as negative control. Three colonies from each transformation were grown in 5 ml of selective SCD media to an OD of ~1.0 at 600 nm. From each of these starter cultures, 15 μl was dropped onto solid YPD, YPLD, SCD, SCLD, SLAD and SLALD plates (see Table 2 for media components). These plates also contained X-gal, added according to Ausubel et al. (1994), for

the optical assessment of the activity conferred by the different Mss11p derivatives on the transcription levels of the reporter genes.

Table 4. The list of plasmids used in this work. For the plasmids carrying *MSS11* fragments, the encoded area is indicated in subscript, giving the first and last amino acids of the Mss11p-derivative encoded by the respective insert.

Plasmid	Relevant genotype	Source/reference
PP*MUC1-lacZ*	*CEN4 URA3 PMUC1-lacZ*	This work
PP*STA2-lacZ*	*CEN4 URA3 PSTA2-lacZ*	This work
YEplac112	2μ *TRP1*	Gietz and Sugino, 1988
YEplac112-*MSS11*	2μ *TRP1 MSS11*	Gagiano et al., 1999a
YEplac112-MSS11exp	2μ *TRP1 PMSS11 TMSS11*	This work
YEplac112-MSS11-OF-OR	2μ *TRP1 MSS11*$_{1\text{-}758}$	This work
YEplac112-MSS11-OF-NxR	2μ *TRP1 MSS11*$_{1\text{-}640}$	This work
YEplac112-MSS11-OF-NR	2μ *TRP1 MSS11*$_{1\text{-}604}$	This work
YEplac112-MSS11-OF-ID2R	2μ *TRP1 MSS11*$_{1\text{-}511}$	This work
YEplac112-MSS11-OF-ID1R	2μ *TRP1 MSS11*$_{1\text{-}420}$	This work
YEplac112-MSS11-OF-QR	2μ *TRP1 MSS11*$_{1\text{-}272}$	This work
YEplac112-MSS11-OF-H2R	2μ *TRP1 MSS11*$_{1\text{-}168}$	This work
YEplac112-MSS11-OF-H1R	2μ *TRP1 MSS11*$_{1\text{-}112}$	This work
YEplac112-MSS11-H1F-OR	2μ *TRP1 MSS11*$_{35\text{-}758}$	This work
YEplac112-MSS11-H1F-NR	2μ *TRP1 MSS11*$_{35\text{-}604}$	This work
YEplac112-MSS11-H1F-ID2R	2μ *TRP1 MSS11*$_{35\text{-}511}$	This work
YEplac112-MSS11-H1F-ID1R	2μ *TRP1 MSS11*$_{35\text{-}420}$	This work
YEplac112-MSS11-H1F-QR	2μ *TRP1 MSS11*$_{35\text{-}272}$	This work
YEplac112-MSS11-H1F-H2R	2μ *TRP1 MSS11*$_{35\text{-}168}$	This work
YEplac112-MSS11-H1F-H1R	2μ *TRP1 MSS11*$_{35\text{-}112}$	This work
YEplac112-MSS11-H2F-OR	2μ *TRP1 MSS11*$_{146\text{-}758}$	This work
YEplac112-MSS11-H2F-NR	2μ *TRP1 MSS11*$_{146\text{-}604}$	This work
YEplac112-MSS11-H2F-ID2R	2μ *TRP1 MSS11*$_{146\text{-}511}$	This work
YEplac112-MSS11-H2F-ID1R	2μ *TRP1 MSS11*$_{146\text{-}420}$	This work
YEplac112-MSS11-H2F-QR	2μ *TRP1 MSS11*$_{146\text{-}272}$	This work
YEplac112-MSS11-H2F-H2R	2μ *TRP1 MSS11*$_{146\text{-}168}$	This work
YEplac112-MSS11-PH2F-OR	2μ *TRP1 MSS11*$_{169\text{-}758}$	This work
YEplac112-MSS11-PH2F-NR	2μ *TRP1 MSS11*$_{169\text{-}604}$	This work
YEplac112-MSS11-PH2F-ID2R	2μ *TRP1 MSS11*$_{169\text{-}511}$	This work
YEplac112-MSS11-PH2F-QR	2μ *TRP1 MSS11*$_{169\text{-}272}$	This work
YEplac112-MSS11-QxF-OR	2μ *TRP1 MSS11*$_{274\text{-}758}$	This work
YEplac112-MSS11-QxF-NxR	2μ *TRP1 MSS11*$_{274\text{-}640}$	This work
YEplac112-MSS11-QxF-NR	2μ *TRP1 MSS11*$_{274\text{-}604}$	This work
YEplac112-MSS11-QxF-ID2R	2μ *TRP1 MSS11*$_{274\text{-}511}$	This work
YEplac112-MSS11-QxF-ID1R	2μ *TRP1 MSS11*$_{274\text{-}420}$	This work
YEplac112-MSS11-QxF-QxR	2μ *TRP1 MSS11*$_{274\text{-}329}$	This work
YEplac112-MSS11-QF-OR	2μ *TRP1 MSS11*$_{330\text{-}758}$	This work
YEplac112-MSS11-QF-ID2R	2μ *TRP1 MSS11*$_{330\text{-}511}$	This work
YEplac112-MSS11-QF-ID1R	2μ *TRP1 MSS11*$_{330\text{-}420}$	This work
YEplac112-MSS11-ID2F-OR	2μ *TRP1 MSS11*$_{414\text{-}758}$	This work
YEplac112-MSS11-ID2F-NR	2μ *TRP1 MSS11*$_{414\text{-}604}$	This work
YEplac112-MSS11-ID2F-ID2R	2μ *TRP1 MSS11*$_{414\text{-}511}$	This work
YEplac112-MSS11-ID3F-OR	2μ *TRP1 MSS11*$_{504\text{-}758}$	This work
YEplac112-MSS11-ID3F-NR	2μ *TRP1 MSS11*$_{504\text{-}604}$	This work
YEplac112-MSS11-NxF-OR	2μ *TRP1 MSS11*$_{605\text{-}758}$	This work
YEplac112-MSS11-NxF-NxR	2μ *TRP1 MSS11*$_{605\text{-}640}$	This work
YEplac112-MSS11-NF-OR	2μ *TRP1 MSS11*$_{641\text{-}758}$	This work
YEplac112-MSS11-ΔP	2μ *TRP1 MSS1* $_{1\text{-}758;\ G113\rightarrow A;\ K114\rightarrow R}$	This work
YEplac112-MSS11-ΔQ	2μ *TRP1 MSS11*$_{1\text{-}758;\ \Delta 272\text{-}329}$	This work
YEplac112-MSS11-ΔN	2μ *TRP1 MSS11*$_{1\text{-}758;\ \Delta 605\text{-}640}$	This work
YEplac112-MSS11-FL	2μ *TRP1 MSS11*$_{1\text{-}758\ F153\rightarrow G;\ L154\rightarrow A}$	This work
YEplac112-MSS11-WW	2μ *TRP1 MSS11*$_{1\text{-}758\ W157\rightarrow G;\ W158\rightarrow A}$	This work
YEplac112-MSS11-IF	2μ *TRP1 MSS11*$_{1\text{-}758\ I160\rightarrow G;\ F161\rightarrow A}$	This work
YEplac112-MSS11-LF	2μ *TRP1 MSS11*$_{1\text{-}758\ L164\rightarrow G;\ F165\rightarrow A}$	This work

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

Plasmid	Relevant genotype	Source/reference
pGBD-C2	2μ *TRP1* $GAL4_{1-147}$	James et al., 1996
pGBD-C2-MSS11-OF-OR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{1-758}$	This work
pGBD-C2-MSS11-OF-NxR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{1-640}$	This work
pGBD-C2-MSS11-OF-NR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{1-604}$	This work
pGBD-C2-MSS11-OF-ID2R	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{1-511}$	This work
pGBD-C2-MSS11-OF-ID1R	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{1-420}$	This work
pGBD-C2-MSS11-OF-QR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{1-272}$	This work
pGBD-C2-MSS11-OF-H2R	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{1-168}$	This work
pGBD-C2-MSS11-OF-H1R	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{1-112}$	This work
pGBD-C2-MSS11-H1F-OR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{35-758}$	This work
pGBD-C2-MSS11-H1F-NR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{35-604}$	This work
pGBD-C2-MSS11-H1F-ID2R	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{35-511}$	This work
pGBD-C2-MSS11-H1F-ID1R	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{35-420}$	This work
pGBD-C2-MSS11-H1F-QR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{35-272}$	This work
pGBD-C2-MSS11-H1F-H2R	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{35-168}$	This work
pGBD-C2-MSS11-H1F-H1R	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{35-112}$	This work
pGBD-C2-MSS11-H2F-OR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{146-758}$	This work
pGBD-C2-MSS11-H2F-NR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{146-604}$	This work
pGBD-C2-MSS11-H2F-ID2R	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{146-511}$	This work
pGBD-C2-MSS11-H2F-ID1R	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{146-420}$	This work
pGBD-C2-MSS11-H2F-QR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{146-272}$	This work
pGBD-C2-MSS11-H2F-H2R	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{146-168}$	This work
pGBD-C2-MSS11-PH2F-OR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{169-758}$	This work
pGBD-C2-MSS11-PH2F-NR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{169-604}$	This work
pGBD-C2-MSS11-PH2F-ID2R	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{169-511}$	This work
pGBD-C2-MSS11-PH2F-QR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{169-272}$	This work
pGBD-C2-MSS11-QxF-OR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{274-758}$	This work
pGBD-C2-MSS11-QxF-NxR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{274-640}$	This work
pGBD-C2-MSS11-QxF-NR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{274-604}$	This work
pGBD-C2-MSS11-QxF-ID2R	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{274-511}$	This work
pGBD-C2-MSS11-QxF-ID1R	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{274-420}$	This work
pGBD-C2-MSS11-QxF-QxR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{274-329}$	This work
pGBD-C2-MSS11-QF-OR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{330-758}$	This work
pGBD-C2-MSS11-QF-ID2R	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{330-511}$	This work
pGBD-C2-MSS11-QF-ID1R	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{330-420}$	This work
pGBD-C2-MSS11-ID2F-OR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{414-758}$	This work
pGBD-C2-MSS11-ID2F-NR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{414-604}$	This work
pGBD-C2-MSS11-ID2F-ID2R	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{414-511}$	This work
pGBD-C2-MSS11-ID3F-OR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{504-758}$	This work
pGBD-C2-MSS11-ID3F-NR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{504-604}$	This work
pGBD-C2-MSS11-NxF-OR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{605-758}$	This work
pGBD-C2-MSS11-NxF-NxR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{605-640}$	This work
pGBD-C2-MSS11-NF-OR	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{641-758}$	This work
pGBD-C2-MSS11-ΔP	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{1-758;\ G113\rightarrow A;\ K114\rightarrow R}$	This work
pGBD-C2-MSS11-ΔQ	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{1-758;\ \Delta 272-329}$	This work
pGBD-C2-MSS11-ΔN	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{1-758;\ \Delta 605-640}$	This work
pGBD-C2-MSS11-FL	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{1-758\ F153\rightarrow G;\ L154\rightarrow A}$	This work
pGBD-C2-MSS11-WW	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{1-758\ W157\rightarrow G;\ W158\rightarrow A}$	This work
pGBD-C2-MSS11-IF	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{1-758\ I160\rightarrow G;\ F161\rightarrow A}$	This work
pGBD-C2-MSS11-LF	2μ *TRP1* $GAL4_{1-147}$ $MSS11_{1-758\ L164\rightarrow G;\ F165\rightarrow A}$	This work

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

3.6 Computer-aided analyses and homology searches

Homology searches with Mss11p were done using the WWW-based BLASTP function (Altschul et al., 1997). Optimised sequence alignments between Mss11p domains and the domains of proteins identified through BLASTP (Fig. 1) were done using the BESTFIT and PILEUP functions of the GCG Wisconsin package. Access to the software was generously provided by the South African National Bioinformatics Institute (SANBI).

4. Results

4.1 Mss11p differentially regulates *MUC1* and *STA2* transcription levels in response to nutritional conditions

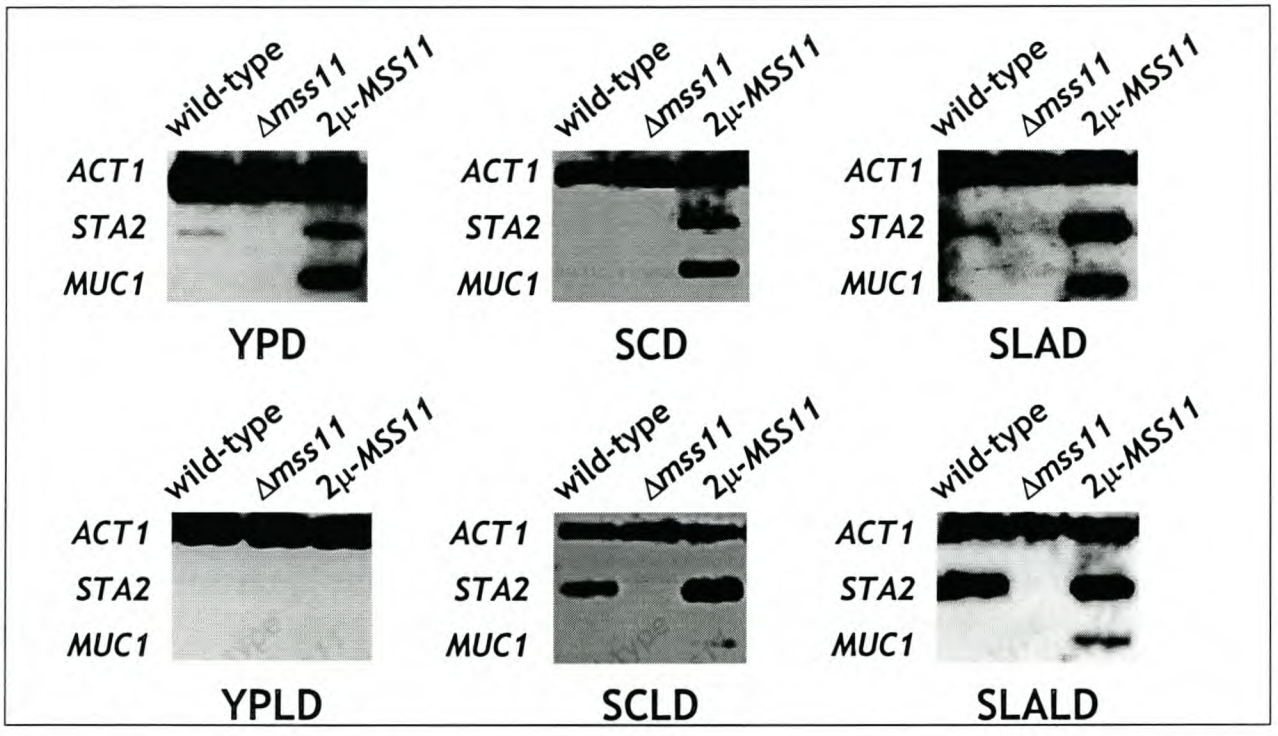

Figure 2. A Northern blot analysis on the effect of single and multiple copies of *MSS11*, as well as the deletion thereof, on the transcript levels of *STA2* and *MUC1* in different nutritional conditions. The concentrations and components of the different media are described in detail in Table 2.

To determine if there are variations in the Mss11p-mediated transcription levels of *MUC1* and *STA2* in different nutritional conditions, we isolated RNA from cells grown in rich media containing high (2%) or low concentrations (0.1%) of glucose as carbon source. We also isolated RNA from cells grown in synthetic media containing high (2%) or low concentrations (0.1%) of glucose as carbon source and $(NH_4)_2SO_4$ as nitrogen source. The wild-type strain, ISP15, was transformed with the 2μ-plasmid bearing *MSS11*, YEplac112-MSS11, or the unmodified vector, YEplac112, as negative control, to study the effect of multiple and single copies of *MSS11* on the transcription of *MUC1* and *STA2* in the different nutritional conditions. The effect of a deletion of *MSS11* was assessed in strain ISP15Δmss11, transformed with the unmodified vector, YEplac112. The results are presented in Fig. 2.

From the Northern blots, it is clear that both *MUC1* and *STA2* are expressed at lower levels in the wild-type strain under most nutritional conditions, with *MUC1* transcription levels being at undetectable low levels in all media tested, including under nitrogen and carbon limitation. The transcription levels of *STA2* in the wild-type background are, however, clearly detectable in all media except the synthetic media containing 2% glucose (SCD) and the rich

media containing 0.1% glucose (YPLD). This discrepancy in the transcription levels of the two genes with almost identical regulatory regions has been described before and can be attributed to the presence of two inserts in the promoter region of *MUC1*, which are absent from that of *STA2* (Gagiano et al., 1999a).

Both nitrogen and carbon limitation result in increased transcription of *STA2*, since stronger signals can be observed when comparing the transcript levels of *STA2* in SLAD [20 μM $(NH_4)_2SO_4$, 2% glucose] or SCLD (40 mM $(NH_4)_2SO_4$, 0.1% glucose) media to those in SCD media [40 mM $(NH_4)_2SO_4$, 2% glucose]. The two signals appear to have a cumulative effect, since a significantly stronger signal can be observed when both nitrogen and carbon (SLALD) are limiting.

Although the transcription levels vary significantly, the overexpression of *MSS11* from the multiple copy plasmid has the same effect on the transcription of *MUC1* than what it has on the transcription of *STA2*, reaffirming previous observations regarding the co-regulation of the two genes (Gagiano et al., 1999a, b). The nutritional conditions, however, continue to exert control over the relative expression levels of *MUC1* and *STA2* in the presence of multiple copies of *MSS11*. In these conditions, the expression of *MUC1* is always higher in media containing high (2%) glucose concentrations (YPD, SCD, SCLD) than in media containing low (0.1%) glucose (YPLD, SCLD, SLALD). In the synthetic minimal media (SCD, SCLD, SLAD and SLALD) *STA2* transcript levels are lower on media containing high (2% glucose - SCD, SLAD) than in media containing low (0.1% - SCLD, SLALD) glucose. However, in rich media this does not apply, since *STA2* transcripts can be observed in YPD (2% glucose) but not in YPLD (0.1% glucose). The observation that *MUC1* transcript levels, in the presence of multiple copies of *MSS11*, are higher in high glucose concentrations than in low glucose concentrations can be explained by findings from other groups that identified *MUC1* as being downstream of the Gpr1p-Gpa2p glucose receptor that senses high glucose concentrations and transmits the signal via intracellular cAMP levels to *MUC1* (Lorenz et al., 2000). The fact that the transcript levels are higher in the rich media with high glucose concentrations than in the rich media with low glucose concentrations also supports the involvement of the Gpr1p-Gpa2p glucose sensor and the cAMP-signalling pathway. This pathway was shown to require glucose as well as complex media for sustained activation (Colombo et al., 1998) and the key component in this, the Sch9p protein kinase, was shown to regulate *MUC1* transcription in response to cAMP levels (Lorenz et al., 2000). The observation that overexpression of the Flo8p transcription factor is unable to suppress the invasive growth defect of an *mss11* strain, but that overexpression of *MSS11* is able to restore the invasive growth defect of a *flo8* strain (Gagiano et al., 1999a) supports a role for Mss11p in mediating the Gpr1p-Gpa2p-PKA signal to transcription of *MUC1*.

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

The Northern blot analyses presented in Fig. 2 suggests that Mss11p mediates the expression levels of *MUC1* and *STA2* in response to specific nutritional signals. However, it is unclear whether Mss11p confers this transcriptional activation directly by stimulating the transcription of *MUC1* and *STA2* by itself, or indirectly by recruiting or interacting with transcriptional activators that stimulate transcription of *MUC1* and *STA2*. For Mss11p to act directly as a transcription factor, it would be expected to harbour at least one distinct activation domain. Transcriptional activation domains mediate the activity of transcriptional activators by making direct contact with the RNA polymerase II-associated transcription machinery bound at the TATA box in the regulatory regions of target genes (Mahanta et al., 1997).

Activation domains from several transcriptional activators have been shown to functionally substitute for deleted or mutated activation domains of different transcription factors, even from other organisms (Gill et al., 1994; Triezenberg, 1995; Askovick and Baumann, 1997; Pongubala and Atchison, 1997). We exploited this modular characteristic of activation domains to identify the domain(s) in Mss11p that would be required for the transcriptional activation of target genes. A series of fusions between Mss11p and the Gal4p transcription factor, of which the activation domain was deleted were created. The resulting constructs included full-length Mss11p fused to the Gal4p DNA-binding domain, as well as sequential deletions of Mss11p fused to this domain. These constructs were transformed into a strain containing an integrated reporter gene, *lacZ*, under expression from the *GAL7* promoter. The *GAL7* promoter contains binding sites for the Gal4p transcriptional activator and, if Mss11p contained transcriptional activation domains, the fusion protein would mediate the transcriptional activation of the reporter gene (James et al., 1996). Both liquid and plate β-galactosidase assays were used to identify such activation domains. The results of these assays are presented in Figs. 3, 4, 5 and 6.

From the liquid β-galactosidase assays (Fig. 3), it can be observed that, relative to the vector containing only the Gal4p DNA-binding domain as negative control, full-length Mss11p resulted in a 15-fold increase in reporter gene activity in the liquid media (SCD). This observation was confirmed by the plate assays on the synthetic media containing limiting concentrations of glucose (SCLD), nitrogen (SLAD) or both (SLALD), but not on synthetic media containing high concentrations of glucose (2% - SCD) or rich media (YPD, YPLD or YPGE) (Fig. 5).

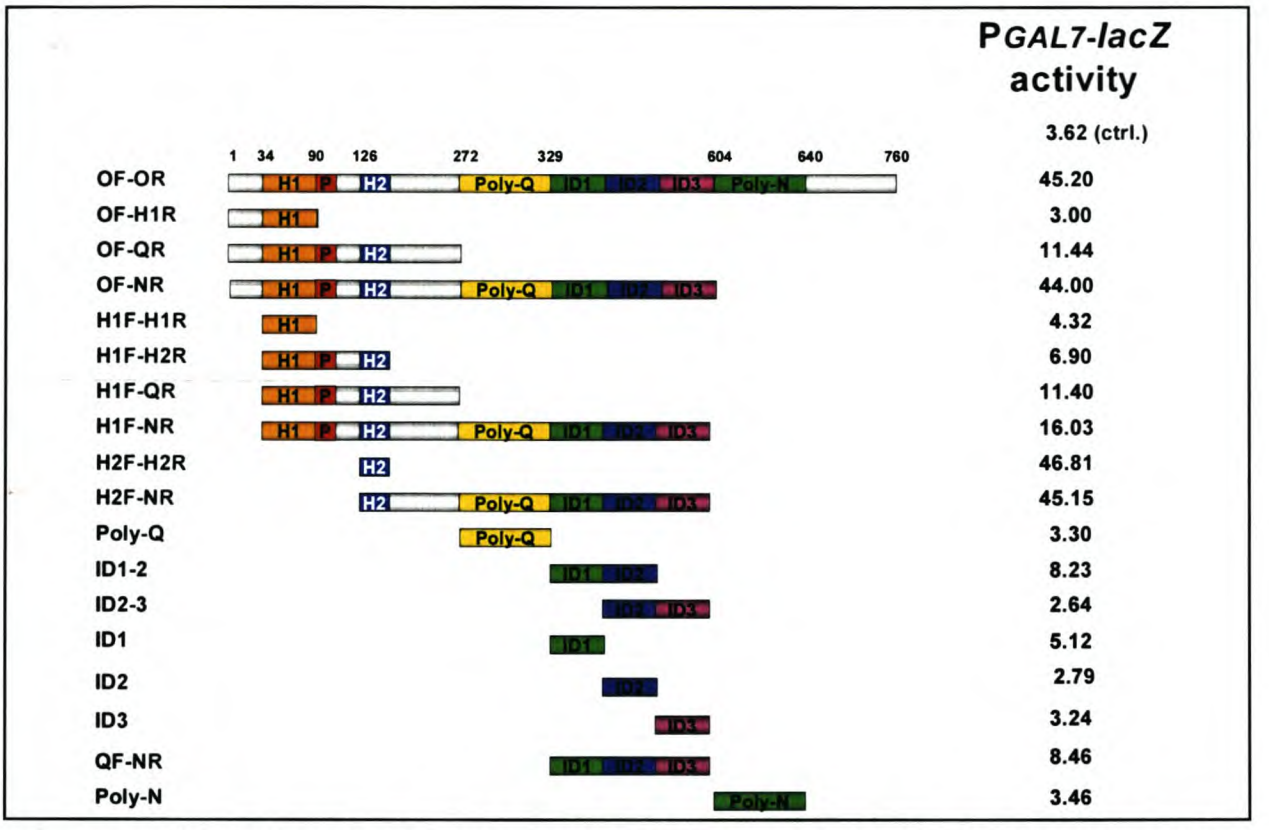

Figure 3. The identification of Mss11p as a transcriptional activator and the identification of specific activation domains in Mss11p. The different Mss11p fragments fused to Gal4p are represented diagrammatically and the levels of reporter gene activity conferred in liquid SCD media by each, as measured through β-galactosidase activity, are indicated next to the relevant construct.

Since the constitutively active *ADH1* promoter would result in high levels of transcription of the different genes encoding the fusion proteins under all nutritional conditions, one would expect to have high levels of β-galactosidase on all media. The differences in β-galactosidase levels observed on the different media (Fig. 4.) would therefore suggest that the ability of Mss11p to activate transcription is regulated at a post-transcriptional level in response to the specific nutritional conditions. The expression levels of the reporter gene on the different media also correspond to the transcription levels observed for *MUC1* and *STA2* in the different nutritional conditions, supporting the observation that the ability of Mss11p to regulate *MUC1* and *STA2* transcription occurs at a post-transcriptional level. The ability of Mss11p to trans-activate a reporter gene, out of its normal context, furthermore also suggests that Mss11p harbours at least one activation domain and that the role of Mss11p in mediating *MUC1* and *STA2* transcription is through the direct stimulation of transcription.

4.3 The poly-glutamine and poly-asparagine domains of Mss11p are not required for transcriptional activation

Glutamine-rich domains have been identified as the activation domains of transcription factors in a number of organisms, ranging in complexity from yeast (e.g. Mcm1p) to humans

(e.g. Oct1 and Oct2) (Johnson et al. 1993). The difference between these prototypical glutamine-rich activation domains and the poly-glutamine domain of Mss11p, however, is the dispersion of hydrophobic amino acids such as leucine, valine and phenylalanine between the glutamine residues (Johnson et al., 1993; Triezenberg, 1995), a characteristic that is absent from the Mss11p poly-glutamine domain. These hydrophobic amino acids were shown to be critical for the activation function of the transcription factors (Gill et al., 1994). The Mss11p poly-glutamine domain consists of 30 glutamine residues, a single histidine residue, followed by a further stretch of five glutamine residues is significantly different from the glutamine-rich activation domains of the characterised eukaryotic transcription factors. A poly-glutamine domain significantly shorter than that of Mss11p (12 glutamine residues) was identified in the yeast protein, Pgd1p (Brohl et al., 1994). Although the exact function of the poly-glutamine domain is unknown at this stage, Pgd1p was subsequently shown to be a component of the mediator complex between transcriptional activators and the RNA polymerase II complex (Gustafsson et al., 1998; Myers et al., 1998).

The function of the poly-asparagine domains in proteins is unknown at this stage. Asparagine-rich domains have been described only for two other *S. cerevisiae* proteins. *S. cerevisiae* Azf1p and Swh1p both include short, asparagine-rich domains (Schmalix and Bandlow, 1994; Stein et al., 1998), but the relationship between the function and the presence of these domains has not been investigated. The *Candida albicans* geranylgeranyltransferase, CaCdc43p, also contains a poly-asparagine stretch, the size of which varies between 6 and 17 amino acids in different strains, as well as between different alleles in a single strain. The functional relevance of the poly-asparagine domain in this protein was also not established (Mazur et al., 1999).

To determine if the Mss11p poly-glutamine and poly-asparagine domains are involved in the transcriptional activation function, we fused each of the two domains to the Gal4p DNA-binding domain and assessed whether the fusion proteins were able to activate the P*GAL7-lacZ* reporter. To test the reverse scenario, we also deleted these sequences from Mss11p and assessed the ability of these Mss11p deletion-variants to activate the P*GAL7-lacZ* reporter. The result of this experiment is presented in Figure 4.

Neither the deletion of the poly-glutamine nor of the poly-asparagine domain had any significant impact on the levels of activity conferred by Mss11p in any of the reporter systems used, P*GAL7*-lacZ (Fig. 4), P*MUC1-lacZ* or P*STA2-lacZ* (data not shown). The reporter gene expression levels conferred by the poly-glutamine and poly-asparagine deletion variants of Mss11p (ΔQ and ΔN, respectively) were essentially identical to that conferred by wild-type Mss11p. The deletion of these domains therefore does not impact on the transcriptional activation of *MUC1* or *STA2*. In the reversed scenario, overexpression of the fusion of either of

the two domains (Fig. 5 - QxF-QxR = poly-glutamine domain and NxF-NxR = poly-asparagine domain) to the Gal4p DNA-binding domain failed to result in the transcriptional activation of the reporter gene, P*GAL7-lacZ*, in any of the nutritional conditions tested. It is likely that the Mss11p poly-glutamine or poly-asparagine domains are required for protein-protein interactions or perform a structural role in Mss11p.

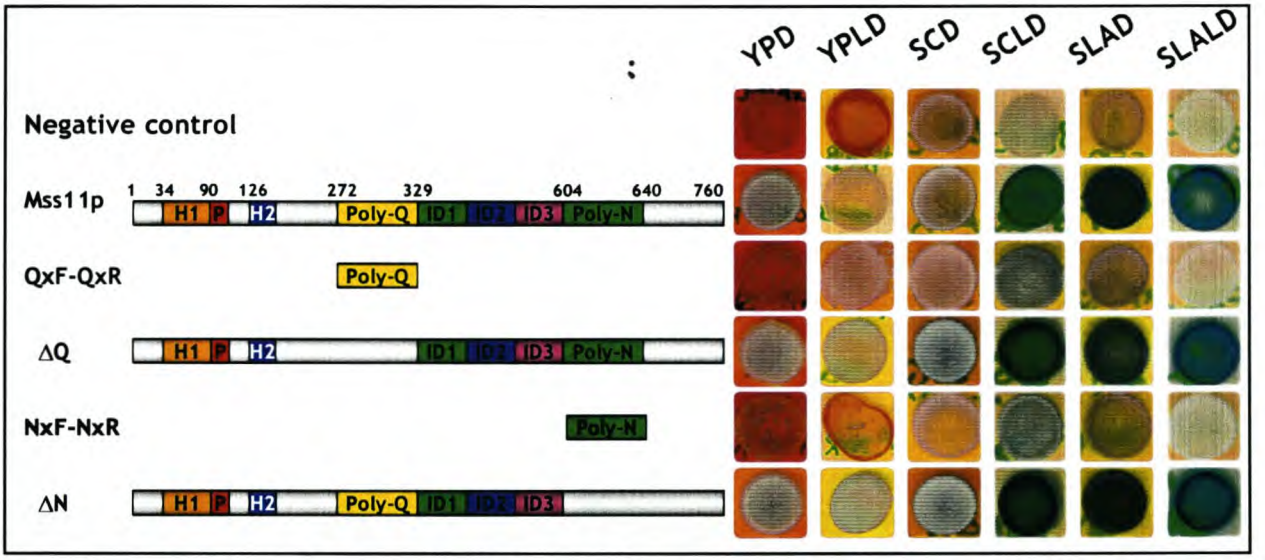

Figure 4. The functional relevance of the Mss11p poly-glutamine (poly-Q) and poly-asparagine (poly-N) domains. The ability of the different Mss11p fragments, fused to the Gal4p DNA-binding domain, to activate the P*GAL7-lacZ* reporter system was assessed in strain pJ69-4A. The Mss11p domains fused to Gal4p are diagrammatically represented and the levels of reporter gene activity conferred by each are represented by the intensity of the blue colour of the colonies in the photographs. The constituents of the media used are listed in Table 2.

4.4 The poly-glutamine and poly-asparagine domains of Mss11p are of identical size in different laboratory strains

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

Due to the presence of such trinucleotide repeats in *MSS11*, we investigated the possibility that the Mss11p poly-glutamine and poly-asparagine domains might vary in size between different strains. We therefore PCR-amplified the *MSS11*-encoding sequences from strains ISP15, ISP52, FY23 (S288C) and W303 and sequenced the resulting fragments. There was no difference in size between the different fragments obtained or the sequences of the fragments obtained from the different genetic backgrounds (results not shown). However, it

is possible that differences might exist between the *MSS11* alleles of feral strains and laboratory strains, but this remains to be shown.

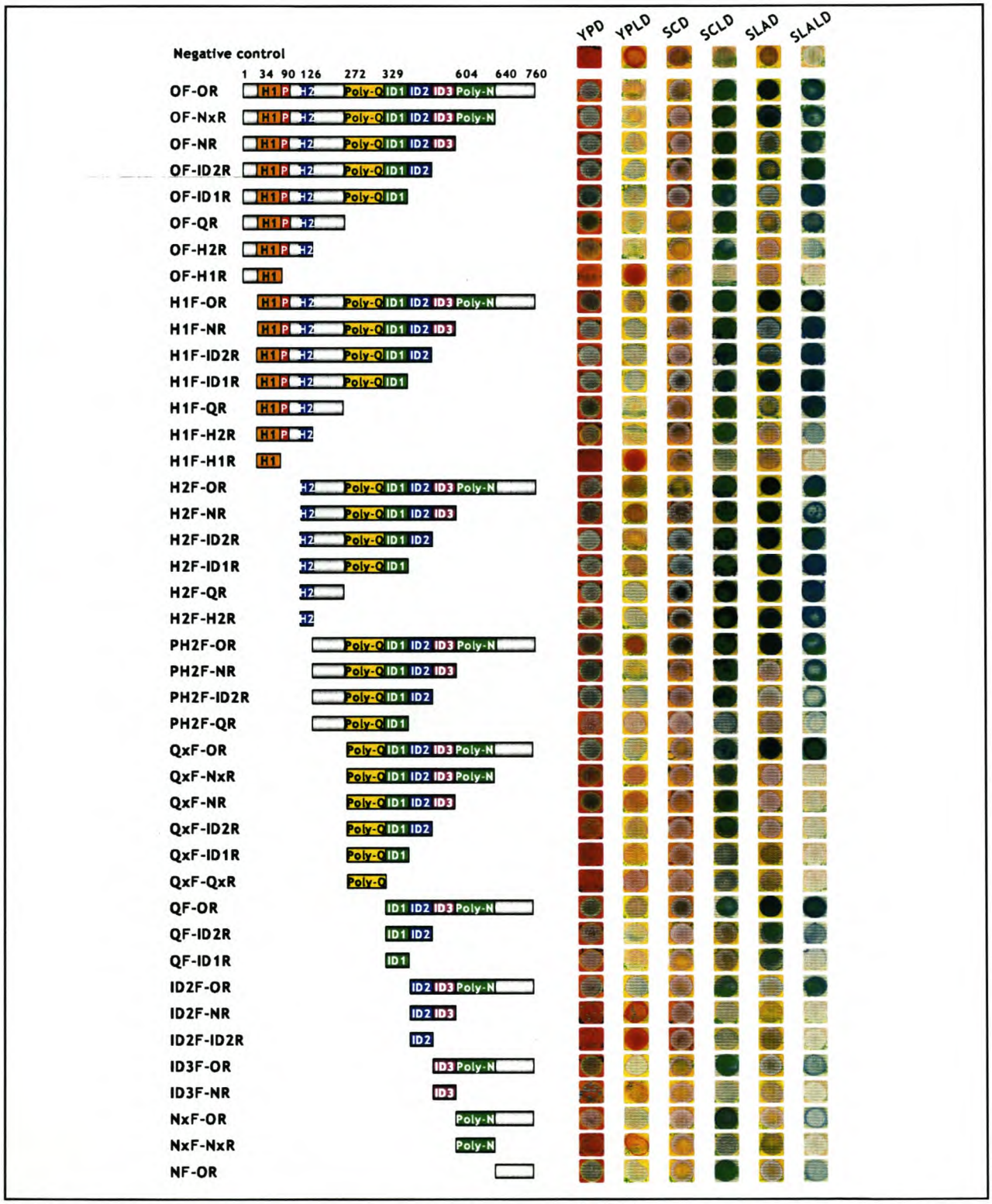

Figure 5. The identification of the Mss11p activation domains. The Mss11p domains fused to Gal4p are represented diagrammatically and the levels of reporter gene activity conferred by each, as measured through β-galactosidase activity, are indicated next to the relevant construct.

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

In a number of transcription factors from different organisms, activation domains can be recognised by the prevalence of specific amino acids (reviewed in Johnson et al., 1993; Triezenberg, 1995). In this way, the acidic activation domains of *S. cerevisiae* Gcn4p and Gal4p and the Herpes simplex virus VP16 are recognised by high levels of glutamate and aspartate, the glutamine-rich domains of *S. cerevisiae* Mcm1p and mammalian Oct-1, Oct-2, Sp1, Sp2 and Sp3 are recognised by high levels of glutamine residues; the proline-rich domains of human CTF/NFI and *Zea mays* Opaque-2 are recognised by the prevalence of proline; and the serine/threonine-rich domains of the mammalian immunoglobulin enhancers ITF-1, ITF-2 and TFE3 are characterised by the prevalence of serine and threonine. However, a very large number of activation domains have been identified without the dominant presence of any specific amino acid (Johnson et al., 1993; Triezenberg, 1995). Since the poly-glutamine and poly-asparagine domains of Mss11p do not seem to assist in the activation function, we made systematic deletions from both the N- and C-termini to identify the domains specifically required for the activation function. The results are presented in Fig. 5.

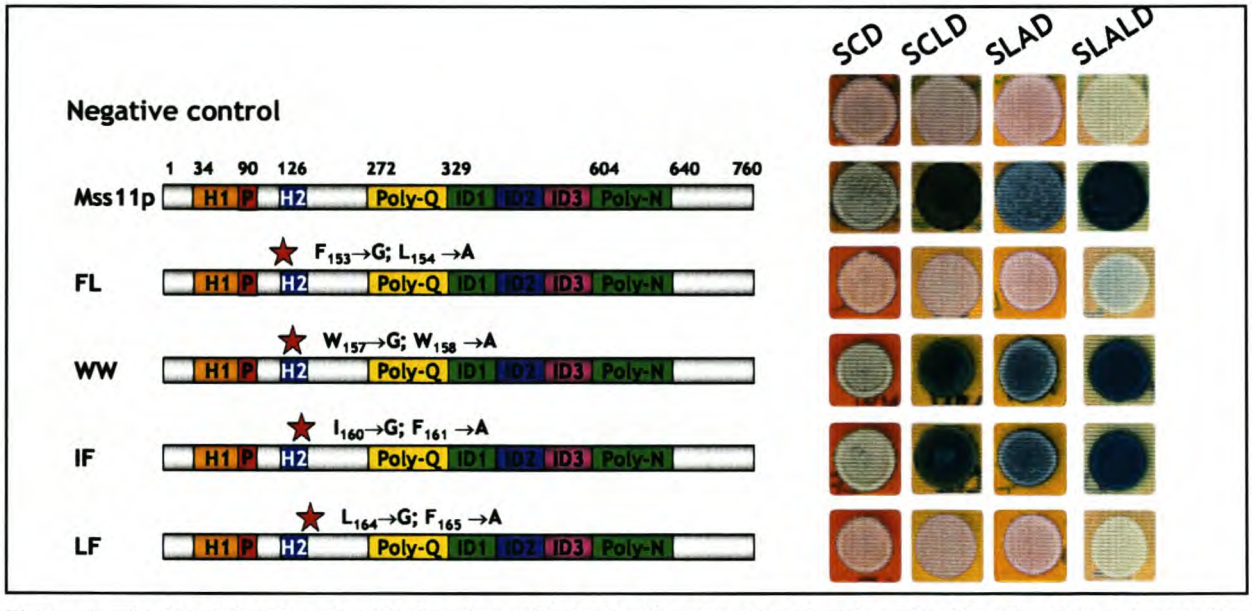

Figure 6. The identification of critical amino acids in the H2 activation domain of Mss11p. The figure depicts the impact of the mutations in the H2 domain on the ability of Mss11p to activate the P*MUC1-lacZ* reporter gene under different nutritional conditions in strain ISP20Δmss11 (see text for details). Results obtained with the P*STA2-lacZ* reporter gene in the same strain and under the same conditions were essentially identical (results not shown).

The data suggest that there are two areas in Mss11p that are required for the transcriptional activation function. Indeed, all sequences containing the most C-terminal domain or the conserved H2 domain are able to stimulate transcription of the reporter gene in all the media tested. The smallest domain that conferred the same levels of activation as

full-length Mss11p was the conserved H2 domain (H2F-H2R in Figs. 3 and 5). However, constructs bearing the N-terminal part of Mss11p in combination with the H2 domain were unable to stimulate transcription of the reporter gene to the same levels as full-length Mss11p. This would suggest that the N-terminal domain has an inhibiting role on the activation function of the H2 domain. It is possible that this is due to an autoregulatory function, similar to what is observed in the Snf1p protein kinase, for example, where a regulatory domain inhibits the function of the catalytic domain in repressive conditions (Carlson, 1998, 1999).

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

Since the H2 domain was shown to be required for the activation function of Mss11p and also to be able to stimulate transcription of the P*GAL7-lacZ* reporter gene when fused to the Gal4p DNA binding domain, we investigated whether the conserved amino acids identified in H2 (Fig. 1) are required for the activation function. We specifically targeted the conserved amino acids pairs Phe_{153}-Leu_{154}, Trp_{157}-Trp_{158}, Ile_{160}-Phe_{161} and Leu_{164}-Phe_{165} (Fig. 1). All of these amino acids were mutated to glycine and alanine, respectively (Table 4 and Fig. 7). The effects of the mutations on the ability of Mss11p to stimulate transcription of the reporter genes the P*STA2-lacZ* and P*MUC1-lacZ* were assessed through β-galactosidase plate assays in strain ISP20Δmss11. The results obtained on the different media were essentially identical for the two reporter constructs and are presented for P*MUC1-lacZ* in Fig. 6.

From the results presented in Fig. 6, it is clear that two of the conserved amino acids pairs are critical for the activation function of Mss11p. The mutation of Phe_{153}-Leu_{154} and Leu_{164}-Phe_{165} to Gly and Ala residues completely eliminated the ability of Mss11p to activate the *lacZ* reporter gene under expression of the *MUC1* (Fig. 6) and *STA2* (results not shown) promoters. The symmetrical distribution of these amino acids in the H2 domain (see Fig. 1) could suggest a structural role in the folding of the domain, which was disturbed by the mutation of the critical amino acids. A more detailed analysis of this domain should reveal the role of each of these amino acids in the transcriptional activation function of Mss11p.

4.7 The putative ATP-GTP binding domain of Mss11p is dispensable for its function as a regulator of *MUC1* and *STA2* transcription.

Many ATP- and GTP-binding proteins have a glycine-rich motif known as the P-loop (reviewed in Saraste et al, 1990). These domains are required for the binding of ATP and/or GTP and are generally critical for their function. A putative P-loop (Gly_{108}-Ser_{109}-Ala_{110}-Ser_{111}-Gly_{112}-Gly_{113}-

Lys_{114}-Thr_{115}-Ser_{116}) was identified in a computer-aided primary structure analysis of Mss11p (Gagiano et al., 1999b). To test whether the P-loop contributed to the activation function of Mss11p, we mutated two of the critical amino acids, Gly_{113} and Lys_{114}, to Ala113 and Arg114, respectively. We tested the ability of the P-loop-mutated allele of *MSS11* to confer transcriptional activation of the P*STA2-lacZ* and P*MUC1-lacZ* reporter genes in strain ISP20Δmss11 on different media. We also fused the *MSS11* ORF, carrying the P-loop mutations, to the *GAL4* fragment encoding the DNA-binding domain to assess if the encoded protein would be able to activate the P*GAL7-lacZ* reporter gene in strain pJ69-4A on different media. The results for the P*STA2-lacZ* reporter gene in strain ISP20Δmss11 are presented in Fig. 6.

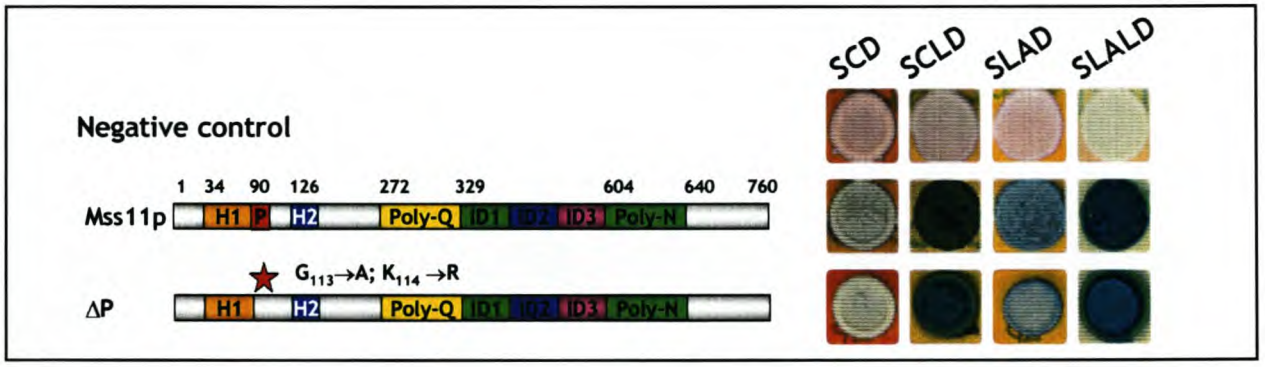

Figure 7. The function of the putative P-loop domain of Mss11p in its role as a transcriptional activator. The figure depicts the impact of the mutated P-loop domain on the ability of Mss11p to activate the P*STA2-lacZ* reporter gene under different nutritional conditions in strain ISP20Δmss11 (see text for details). Results obtained with the P*MUC-lacZ* reporter gene in the same strain and under the same conditions were essentially identical (results not shown).

The data presented in Fig. 7 suggest that the P-loop is dispensable for the Mss11p activation function. In all cases, the mutation of the critical amino acids in the putative Mss11p P-loop did not diminish the ability of the resulting protein to activate the transcription of any of the three reporter genes tested. These negative results could suggest that the putative Mss11p P-loop is not a functional ATP- or GTP-binding domain and that the distribution of the amino acids in the specific order is merely coincidental. Future experiments testing whether this domain physically binds nucleotides should resolve this question. If it is indeed a functional ATP or GTP-binding domain, it is clearly not in the context of Mss11p as a transcriptional activator and, as such, the actual context remains to be identified.

5. Discussion

In this paper we present a molecular analysis of Mss11p, a transcriptional regulator of the *MUC1* and *STA2* genes of *S. cerevisiae*. As a regulator of these two genes, it also is a major

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

It was demonstrated recently that the transcription of the *PGL1* gene is regulated by the same signalling elements that regulate the transcription of *MUC1* in conditions conducive for filamentous growth (Madhani et al., 1999; Gognies et al., 2001). The *PGL1* gene encodes an endopolygalacturonase that enables the yeast cell to hydrolyse pectin. These observations would suggest that the co-regulation of filamentous growth and starch metabolism should be extended to include polysaccharide degradation in general. The role of Mss11p, if any, in regulating the transcription of other members of the adhesin and flocculin gene family, that are required for establishing the filamentous growth phenotype, and genes such as *PGL1*, that encodes enzymes required for polysaccharide metabolism, is not clear. A micro-array analysis to identify the target genes of Mss11p, other than *MUC1* and *STA2*, revealed that Mss11p is very specific in regulating the transcription of *MUC1* and *STA2* and failed to identify genes of which the transcription was increased significantly in the presence of multiple copies of *MSS11* (results not shown).

The molecular analysis of Mss11p presented here conclusively shows that Mss11p is able to activate transcription. We identified two activation domains, one of which seems to be highly conserved amongst several proteins of unknown function. Specific amino acids in this domain are required for the activation function. We also showed that the putative P-loop, poly-glutamine and poly-asparagine domains are not required for the activation function of Mss11p. The role of these domains therefore remains to be identified. Although significantly smaller, a poly-glutamine domain has only been identified in one other *S. cerevisiae* protein, Pgd1p (Brohl et al., 1994). Pgd1p was shown to be a component of the mediator complex between transcriptional activators and the RNA polymerase II complex (Gustafsson et al., 1998; Myers et al., 1998). Considering that Pgd1p functions in a multi-component protein

complex, it is possible that the poly-glutamine domain has a structural role or that it is required for protein-protein interactions.

The genetic evidence presented to date suggests that Mss11p, like Pgd1p, could also have a role as a transcriptional mediator. The results of the epistasis analyses involving *MSS11* demonstrated that all other transcription factors required for the transcriptional activation of *MUC1* and *STA2*, i.e. Ste12, Mss10p and Flo8p, also require Mss11p for their activation function (Gagiano et al., 1999a, b). These results could also be interpreted as evidence that Mss11p is the most downstream component of each of the different signal transduction cascades represented by these transcription factors. However, the fact that Flo8p, Ste12p and Msn1p were all identified as DNA-binding transcription factors (Estruch and Carlson, 1990; Madhani and Fink, 1997; Kobayashi et al., 1999) make this explanation highly unlikely and points strongly towards Mss11p as facilitating the transcriptional activation function of these transcription factors at the *MUC1* and *STA2* promoters.

The strong activation in response to specific nutritional signals, presented in this paper, is not in line with a role for Mss11p as a mediator. It rather would suggest a more direct role as a transcriptional activator, but the dependency of three structurally dissimilar and unrelated transcription factors, Flo8p, Msn1p and Ste12p, on Mss11p, is difficult to reconcile with such a role. It is therefore possible that Mss11p is part of a complex that potentates transcription in response to the specific signals. Considering the amount of genetic evidence that points towards *MUC1* and *STA2* transcription as being repressed by the state of the chromatin over their promoters (Inui et al., 1989; Okimoto et al., 1989; Yoshimoto and Yamashita, 1991; Yoshimoto et al., 1991, 1992; Kuchin et al., 1993; Yamashita, 1993; Park et al., 1999), a role for Mss11p in a complex that reduces this repressive effect, such as a histone acetyltransferase complex (reviewed in Sterner and Berger, 2000), seems possible. Removing or releasing the chromatin barrier over the *STA2* and *MUC1* promoters in response to specific nutritional signals could therefore result in the observed activation, since it would make the promoter accessible to Flo8p, Msn1p, Ste12p as well as other transcription factors. Future efforts will focus on the identification of proteins that interact with Mss11p to assist in identifying a more precise role for Mss11p in regulating *MUC1* and *STA2* transcription.

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Chapter 6

Concluding remarks and future perspectives

1. Concluding remarks and future perspectives

The ability of the yeast *Saccharomyces cerevisiae* to grow towards more optimal growth substrates in response to extracellular cues is determined by the expression of a family of genes that encode large, cell wall-associated proteins, such as Flo1p and Muc1p (Guo et al., 2000). A seemingly unrelated phenotype, the hydrolysis of polysaccharides such as pectin and starch, is dependent on the expression of genes encoding specific enzymes that facilitate the hydrolysis of these polysaccharides. The *PGL1* endopolygalacturonase and the *STA2* glucoamylase genes are required for the utilisation of pectin and starch, respectively, and were both shown to be co-regulated with genes such as *MUC1*, that assist in establishing the filamentous growth phenotype (Lambrechts et al., 1996a; Lo and Dranginis, 1996; Vivier et al., 1997; Gagiano et al., 1999a, b; Madhani et al., 1999; Gognies et al., 2001). This theme formed the primary context of this dissertation. The more specific focus, however, was how Mss11p, a transcriptional regulator of the *MUC1* and *STA2* genes, facilitates this process in response to specific nutritional signals. Chapter 1 reviewed what was known about Mss11p and the regulation of *MUC1* and *STA2* prior to the onset of this study, whereas Chapter 2 provided the informational backdrop to the work presented in Chapters 3-5. Chapter 2 therefore consists of a comprehensive review of nutritional sensing and signalling in *Saccharomyces cerevisiae*, specifically as it relates to the expression of *MUC1* and *STA2* and, ultimately, to filamentous growth and polysaccharide metabolism. Although new questions were raised by this work and some other questions remain, the specific aims of this study, as listed in Chapter 1, were all met. Therefore, to conclude this study, the results obtained in the course of this work as presented in Chapters 3-5 will be discussed and integrated into a current working model for *MUC1* and *STA2* transcription by Mss11p and the transcription factors, Flo8p, Msn1p and Ste12p.

As detailed in the previous chapters, the upstream regulatory regions of *MUC1* and *STA2* are considered to be some of the largest promoters in the yeast genome. By sequencing the upstream areas of *STA2* and *STA3* and comparing them to the sequence of *MUC1*, made available by the *S. cerevisiae* genome-sequencing project, we could show that these upstream areas are 99.7% identical over more than 3 900 base pairs (bp) upstream of the translational start site (Gagiano et al., 1999a - Chapter 4). With the exception of a few minor substitutions, the only significant difference between the *MUC1* and *STA2* promoters is the presence of a 20 bp and a 64 bp sequence, found at positions -1 333 to -1 313 and -933 to -869 of the *MUC1* promoter, respectively, but not in the promoters of any of the *STA1-3* genes (Gagiano et al., 1999a - Chapter 4). As to be expected from two genes with almost identical regulatory regions, transcription of *MUC1* and *STA2* is largely co-regulated. We demonstrated

with Northern analyses, as well as with expression analyses of the *lacZ* reporter gene fused to the *MUC1* and *STA2* promoters, that the two genes are indeed regulated in a similar manner under the same nutritional conditions and by the same transcriptional regulators, i.e. Flo8p, Msn1p and Mss11p (Chapters 3, 4 and 5).

MSN1 and *MSS11* were cloned as multiple copy suppressors of the *STA10* repressor in our laboratory (Lambrechts et al., 1996b; Webber et al., 1997), whereas Kobayashi et al. (1996) cloned *FLO8* as a transcriptional activator of the flocculation genes. We present evidence demonstrating that the repressive effect of *STA10* is actually a phenotype conferred by a *FLO8* mutation in some laboratory strains of *S. cerevisiae* (Gagiano et al., 1999a - Chapter 4). The deletion of either *FLO8*, *MSN1* or *MSS11* results in severe reductions in the transcription levels of *MUC1* and *STA2*, with equally severe reductions in filamentous growth and the ability to hydrolyse starch. On the other hand, overexpression of *FLO8*, *MSN1* or *MSS11* from multiple copy plasmids results in elevated expression levels of both *MUC1* and *STA2* in most nutritional conditions and enhances the filamentous growth phenotypes of the strain, as well as the ability to degrade starch.

A more detailed deletion analysis of a *STA2* promoter sequence, previously described as Upstream Activating Sequence 1 (UAS1) (Lambrechts et al., 1994), demonstrated that an 80 bp sequence present in this area mediates the activating effect of Flo8p, Msn1p and Mss11p, as well as carbon catabolite repression on the transcription of not just *STA2*, but also *MUC1* (Gagiano et al., 1999a - Chapter 4). This sequence is located at -1 160 to -1 070 in the *STA2* and -1 210 to -1 130 in the *MUC1* promoters. Extensive homology searches with the sequence did not reveal any homology with binding sites of characterised transcription factors. Two independent reports confirmed that Flo8p regulates *MUC1* (Kobayashi et al., 1999a, b; Rupp et al., 1999) and *STA1* (Kobayashi et al., 1999a, b) transcription via this sequence. The weak binding of purified Flo8p to this sequence was also demonstrated (Kobayashi et al., 1999a). Several attempts to determine whether Mss11p also binds to this sequence were unsuccessful (results not shown). This would suggest that Mss11p might not be a DNA-binding protein, that Mss11p might be a DNA-binding protein but that its affinity for the specific DNA sequence is too weak to detect, or that the conditions (pH, salt concentrations, etc.) under which we investigated the potential DNA-binding properties of Mss11p were not conducive for physical interaction with the specific DNA sequence. Alternative approaches (e.g. the mono-hybrid assay) will have to be employed to determine with certainty if Mss11p is indeed a DNA-binding protein and if it binds to the specific area identified in the *MUC1* and *STA2* promoters.

Despite the similarities in the expression patterns of *MUC1* and *STA2*, attributed to a high level of identity between the upstream regulatory regions and regulation by common transcription factors, some discrepancies were also shown to exist (Gagiano et al., 1999a -

Chapter 4). The most significant difference is that, in wild-type cells and under all the nutritional conditions tested, *MUC1* transcription is significantly reduced if compared to the transcription levels of *STA2*. This can, to a large extent, be attributed to the presence of the 20- and 64-bp sequences, that are present in the promoter region of *MUC1*, but absent from that of *STA2*. These elements were shown to confer a repressive effect even when placed upstream of a reporter gene out of its native context (Gagiano et al., 1999a - Chapter 4). Although the presence of these elements explains the lower expression levels of *MUC1*, the repressive function of the inserts could not be linked to any specific nutritional condition or any known transcriptional regulator (results not shown).

To place the transcriptional regulators of *MUC1* and *STA2* in the context of known signal transduction pathways, we conducted an epistasis analysis between *MSN1*, *MSS11* and components of the mating pheromone/filamentous response MAP kinase cascade that was shown to be required for the filamentous growth response (Liu et al., 1993). For this purpose, we focused on *STE7*, which encodes the MAP kinase kinase of the pathway, and *STE12*, which encodes the transcription factor of the pathway (reviewed in Sprague and Thorner, 1992). This analysis revealed that *MSN1* functions in a pathway independent of the pheromone response/filamentous growth MAP kinase cascade, but that Mss11p is required for the activation of *MUC1* and *STA2* via this pathway (Gagiano et al., 1999b - Chapter 3). Another epistasis analysis between *FLO8*, *MSN1* and *MSS11* revealed that Msn1p acts in a pathway independent of Flo8p, but that Mss11p functions downstream of Flo8p (Gagiano et al., 1999a - Chapter 4). Several reports (Lorenz and Heitman, 1998a, b; Robertson and Fink, 1998) identified Flo8p as the transcription factor mediating the response of the cAMP-PKA pathway on the transcription of *MUC1*. Considering the results of our epistasis analyses, it is clear that Msn1p functions in a third, as yet uncharacterised, signal transduction pathway, also downstream of Ras2p, but independent of the two identified pathways, i.e. the cAMP-PKA and pheromone response/filamentous growth response MAP kinase pathways. However, Mss11p seems to function downstream of all three the identified pathways (Fig. 1). This suggests a critical and central role for Mss11p in determining the transcription levels of *MUC1* and *STA2*.

To further characterise Mss11p and its role in the transcriptional regulation of *MUC1* and *STA2*, we also subjected it to a detailed deletion and mutation analysis. We present evidence that Mss11p harbours two distinct activation domains required for the activation of *STA2* and *MUC1*, but also able to activate a reporter gene expressed from under the *GAL7* promoter (Gagiano et al., submitted - Chapter 5). The C-terminal domain has a clear activation function in all nutritional conditions tested, but there are no distinguishing characteristics in this domain that would allow one to speculate on a possible mechanism for activation. It furthermore has no homology to any other protein identified to date. A more detailed

deletion and mutation analysis of this area would therefore be required to identify the critical amino acids that might allow one to speculate on a potential activation mechanism.

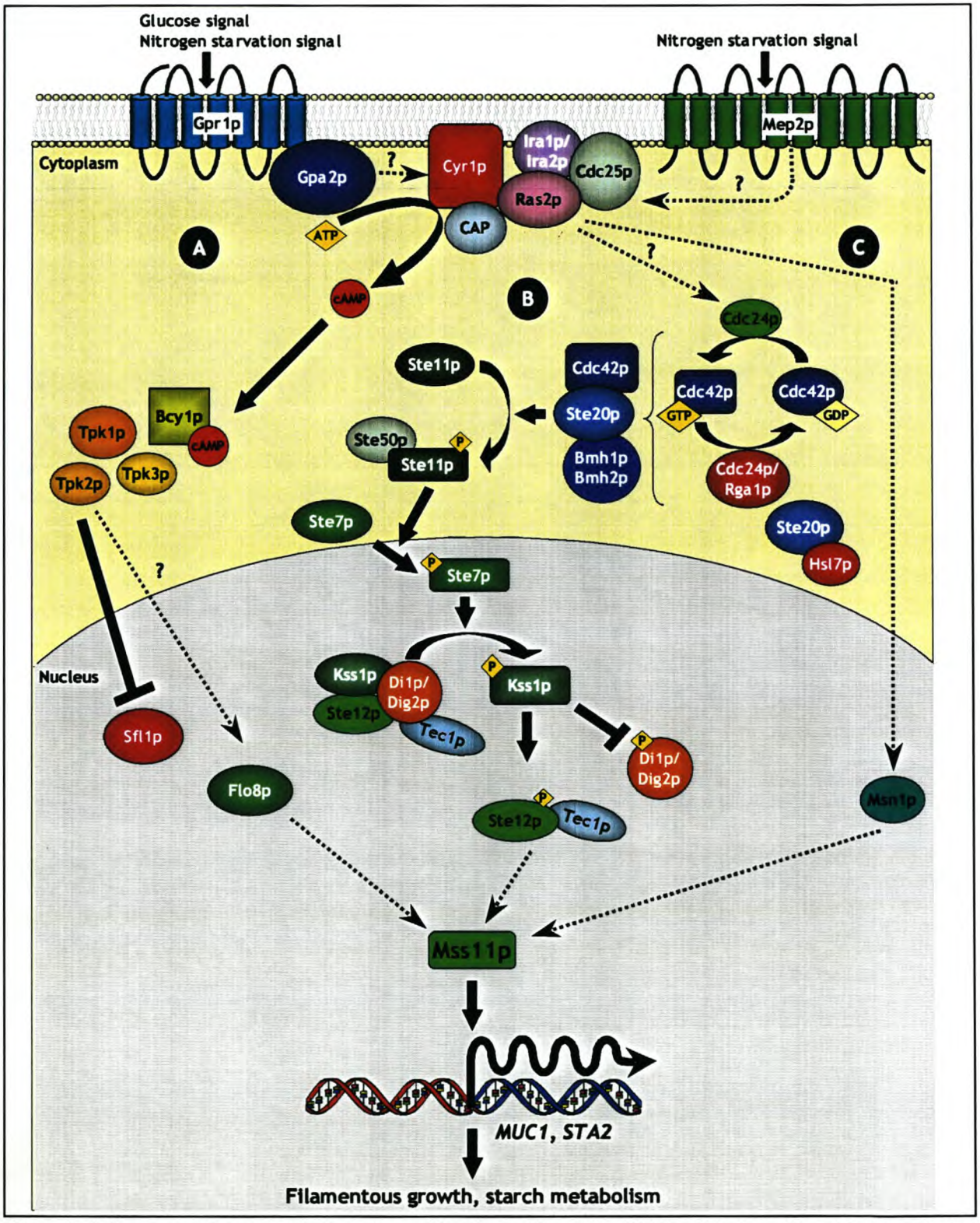

Figure 1. A diagrammatic representation summarising the results of the *MSS11* epistasis analyses (Gagiano et al., 1999a, b). The results would suggest that Mss11p is situated downstream of the A.) PKA-cAMP pathway, B.) the mating pheromone/filamentous growth response signalling cascade and C.) a third unidentified pathway, to date shown to consist of only Msn1p and Ras2p. Solid, bold lines indicate physical links and dotted lines indicate genetic links. Arrows represent activation and solid bars represent repression or inactivation.

The second activation domain of Mss11p was shown to be one of the domains with homology to Flo8p, designated H2 (Gagiano et al., 1999b - Chapter 3). The H2 domain confers the same levels of reporter gene expression as wild-type Mss11p on all media tested and, as such, is the more prominent of the two activation domains. The H2 domain has significant homology to a number of proteins of unknown function from a range of different organisms (Gagiano et al., submitted - Chapter 5). A multi-sequence alignment allowed the identification of conserved amino acids in this domain. Mutations in two of the four conserved amino acid pairs in the H2 domain completely eliminated the activation function. Since these amino acids (phenylalanine and leucine) are located symmetrically within the H2 domain, it is possible that they perform some spatial or structural role critical to the activation function of the domain. It would be interesting to determine if the corresponding domain in Flo8p has a similar function and if the same conserved amino acids are required for its function. To date, no function has been assigned to any of the other proteins that exhibit homology to the Mss11p H2 domain. Since both Flo8p and Mss11p are transcriptional activators, it is tempting to speculate that these could be transcriptional activators as well, but this remains to be shown.

The poly-glutamine and poly-asparagine domains of Mss11p are not required for its activation function. We demonstrated that the deletion of these domains has no impact on the ability of Mss11p to activate *MUC1* or *STA2* or of the Gal4p-Mss11p fusion to activate the *lacZ* reporter gene expressed from under the *GAL7* promoter. Gal4p fusions of either of these domains were also unable to trans-activate the P*GAL7-lacZ* reporter gene. As such we can conclude that neither of these genes performs a function in the role of Mss11p as a transcriptional activator. However, the possibility that these domains might participate in protein-protein interactions or have specific structural roles should be investigated. Since neither of these domains have the ability to activate within the context of the two-hybrid system (results not shown), they could be used as bait for the identification of proteins potentially interacting with Mss11p. The identification of proteins interacting with these domains would assist in clarifying and/or expanding the cellular context in which Mss11p functions. Furthermore, identification of these domains as required for protein-protein interactions would add significant value to the study of yeast proteins in general, since it would be the first report on a clear function for poly-glutamine and poly-asparagine domains in yeast.

We also demonstrated that the putative ATP/GTP-binding domain (P-loop) is not required for the transcriptional activation function of Mss11p (Gagiano et al., submitted - Chapter 5). The relevance of this domain should be investigated, however, to establish with a high degree of certainty whether it is indeed an ATP- or GTP-binding domain. A difference between the

mobility of Mss11p, isolated from a wild-strain, and Mss11p, isolated from a strain carrying the allele with the mutated P-loop, would provide sufficient evidence to suggest that the putative P-loop of Mss11p is functional. The reason why Mss11p would require the binding of ATP or GTP would unfortunately not be resolved by these experiments and remains a case for speculation, at least for the time being.

In an attempt to identify other target genes of Mss11p, we employed the use of micro-arrays to assess the impact of the overexpression and deletion of *MSS11* on the total yeast transcriptome. Our results showed that *MUC1* and *STA2* are the only two genes in the ISP15 genetic background that are significantly (more than 15-fold) enhanced by overexpression of *MSS11* (results not shown). Interestingly, the transcription of several genes, most significantly *DBP2*, *ROM1*, *YPL080C*, *YGR053C*, *YNL179C* and *YGR066C*, was reduced upon overexpression of *MSS11*. The transcription of these genes was also enhanced significantly (more than 13-fold) in the reverse scenario, in which *MSS11* was deleted. Of these genes, only *ROM1* encodes a protein of known function, i.e. a guanine nucleotide exchange factor recently shown to participate in the cell wall integrity pathway (Ozaki et al., 1996). The identification of functions for the other proteins could aid further characterisation of the role of Mss11p in filamentous growth and starch metabolism and will help to establish the greater cellular context in which Mss11p functions.

2. A model for the role of Mss11p in *STA2* and *MUC1* transcription

Considering the genetic evidence from the epistasis analyses presented in Chapters 3 and 4, it seems highly unlikely that Mss11p could be situated downstream of the DNA-binding transcription factors of the three different signal transduction pathways. The data rather suggest that all three of these transcription factors have a functional requirement for Mss11p. One possible explanation for this is that Mss11p performs the role of a transcriptional mediator. The mediator proteins are part of a complex that is essential for basal and regulated expression of nearly all RNA polymerase II-dependent genes in the *Saccharomyces cerevisiae* genome (reviewed in Gustaffson and Samuelsson, 2001). The complex acts as a bridge, conveying regulatory information from upstream regulatory elements to the transcription machinery assembled at the core promoter (Gustaffson and Samuelsson, 2001). However, Mss11p also has a strong activation function and seems to relate the different nutritional signals to the transcription of the *MUC1* and *STA2* genes. The identification of strong activation domains within Mss11p further suggests a more direct role in transcriptional activation and the likelihood that Mss11p might be a transcription factor itself, and not just

perform a bridging function between upstream transcription factors and the transcription machinery assembled at the core promoter. The role as transcription factor, unfortunately, does not explain the dependencies that the three structurally unrelated and dissimilar transcription factors, Flo8p, Msn1p and Mss11p, have for Mss11p.

The currently favoured model suggests that Mss11p potentiates transcription of the *MUC1* and *STA2* promoters as part of a larger complex. A large amount of genetic evidence points towards *MUC1* and *STA2* transcription as being repressed by the state of the chromatin over their promoters (Inui et al., 1989; Okimoto et al., 1989; Yoshimoto and Yamashita, 1991; Yoshimoto et al., 1991, 1992; Kuchin et al., 1993; Yamashita, 1993; Park et al., 1999). A role for Mss11p in a complex that reduces this repressive effect, such as the SWI/SNF, SAGA or HAT chromatin remodelling complexes, seems likely (reviewed in Sterner and Berger, 2001). Removing or releasing the chromatin barrier over the *STA2* and *MUC1* promoters in response to specific nutritional signals could therefore result in the observed nutrient-dependent activation. In this way, Mss11p would facilitate, at least in part, the decondensation of the chromatin over the *MUC1* and *STA2* promoters, thereby making it accessible to Flo8p, Msn1p and Ste12p, as well as other transcription factors, such as Tec1p, to enhance transcription in response to specific signals.

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