CELL DIFFERENTIATION IN RESPONSE TO NUTRIENT AVAILABILITY: THE REPRESSOR OF MEIOSIS, *RME1*, POSITIVELY REGULATES INVASIVE GROWTH IN *SACCHAROMYCES CEREVISIAE*

by

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DECLARATION

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

Guy R. Hansson

SUMMARY

Yeasts, like most organisms, have to survive in highly variable and hostile environments. Survival therefore requires adaptation to the changing external conditions. On the molecular level, specific adaptation to specific environmental conditions requires the yeast to be able: (i) to sense all relevant environmental parameters; (ii) to relay the perceived signals to the interior of the cell *via* signal transduction networks; and (iii) to implement a specific molecular response by modifying enzyme activities and by regulating transcription of the appropriate genes.

The availability of nutrients is one of the major trophic factors for all unicellular organisms, including yeast. *Saccharomyces cerevisiae* senses the nutritional composition of the media and implements a specific developmental choice in response to the level of essential nutrients. In conditions in which ample nutrients are available, *S. cerevisiae* will divide mitotically and populate the growth environment. If the nutrients are exhausted, diploid *S. cerevisiae* cells can undergo meiosis, which produces four ascospores encased in an ascus. These ascospores are robust and provide the yeast with a means to survive adverse environmental conditions. The ascospores can lie dormant for extended periods of time until the onset of favourable growth conditions, upon which the spores will germinate, mate and give rise to a new yeast population. However, *S. cerevisiae* has a third developmental option, referred to as pseudohyphal and invasive growth. In growth conditions in which nutrients are limited, but not exhausted, the yeast can undergo a morphological switch, altering its budding pattern and forming chains of elongated cells that can penetrate the growth substrate to forage for nutrients.

The focus of this study was on elements of the signal transduction networks regulating invasive growth in *S. cerevisiae*. Some components of the signal transduction pathways are well characterised, while several transcription factors that are regulated via these pathways remain poorly studied. In this study, the *RME1* gene was identified for its ability to enhance starch degradation and invasive growth when present on a multiple copy plasmid. Rme1p had previously been identified as a repressor of meiosis and, for this reason, the literature review focuses on the regulation of the meiotic process. In particular, the review focuses on the factors governing entry into meiosis in response to nutrient starvation and ploidy. Also, the transcriptional regulation of the master initiator of meiosis, *IME1*, and the action of Ime1p are included in the review.

The experimental part of the study entailed a genetic analysis of the role of Rme1p in invasive growth and starch metabolism. Epistasis analysis was conducted of Rme1p and elements of the MAP Kinase module, as well as of the transcription factors, Mss11p, Msn1p/Mss10p, Tec1p, Phd1p and Flo8p. Rme1p is known to bind to the promoter of *CLN2*, a G_1-cyclin, and enhances its expression. Therefore, the cell

cyclins *CLN1* and *CLN2* were included in the study. The study revealed that Rme1p functions independently or downstream of the MAP Kinase cascade and does not require Cln1p or Cln2p to induce invasive growth. *FLO11/MUC1* encodes a cell wall protein that is required for invasive growth. Like the above-mentioned factors, Rme1p requires *FLO11* to induce invasive growth. We identified an Rme1p binding site in the promoter of *FLO11*. Overexpression of Rme1p was able to induce *FLO11* expression, despite deletions of *mss11*, *msn1*, *flo8*, *tec1* and *phd1*. In the inverse experiment, these factors were able to induce *FLO11* expression in an *rme1* deleted strain. This would indicate that Rme1p does not function in a hierarchical signalling system with these factors, but could function in a more general role to modify transcription.

OPSOMMING

Die natuur is hoogs veranderlik en alle organismes, insluitende gis, moet by die omgewing kan aanpas om te kan oorleef. Baie eksterne faktore beïnvloed die ontwikkeling van die gissel. Vir die gis om by spesifieke omgewingstoestande aan te pas, moet die gis op 'n molekulêre vlak: (i) al die omgewingsparameters waarneem; (ii) die waargenome omgewingsparameters as seine na die selkern deur middel van seintransduksieweë gelei; en (iii) transkripsie van gene aktiveer of onderdruk en ensiemaktiwiteit reguleer om sodoende die gepaste molekulêre respons te implementeer.

Die beskikbaarheid van voedingstowwe in die omgewing is een van die belangrikste omgewingseine wat eensellige organismes moet kan waarneem. *Saccharomyces cerevisiae* kan spesifieke ontwikkelingsopsies, na gelang van die voedingstowwe wat beskikbaar is, uitoefen. In groeiomstandighede waar daar 'n oorvloed van voedingstowwe is, verdeel *S. cerevisiae* d.m.v. mitose en vesprei dit deur die omgewing. Sodra die voedingstowwe uitgeput is, word mitose onderdruk. Diploïede *S. cerevisiae* inisieer meiose, wat aanleiding tot die vorming van vier spore gee. Hierdie spore bevat slegs die helfte van die ouer se chromosome en kan gevolglik met 'n ander spoor paar om weer 'n diploïede gissel te vorm. Die spore is bestand teen strawwe omgewingstoestande en kan vir lang tye oorleef. Wanneer die spoor aan gunstige groeitoestande blootgestel word, ontkiem dit om aan 'n nuwe giskolonie oorsprong te gee. *S. cerevisiae* het egter 'n derde ontwikkelingsopsie, naamlik pseudohife-differensiëring. Wanneer die beskikbaarheid van voedingstowwe in die omgewing afneem, maar nog nie uitgeput is nie, ondergaan die gis 'n morfologiese verandering. Hierdie verandering word gekenmerk deur selverlenging, nl. botselle wat slegs aan die een punt van die gissel vorm en dogterselle wat aan die moederselle geheg bly. Dit lei tot die vorming van kettings van selle wat van die giskolonie af weggroei. Voorts kan die selkettings ook die groeisubstraat binnedring. Dit staan as penetrasie-groei bekend en laat die gis toe om na nuwe voedingsbronne te soek.

Hierdie studie het op die elemente van seintransduksieweë, wat by penetrasiegroei betrokke is, gefokus. Sekere komponente van die seintransduksieweë is reeds goed gekarakteriseer, terwyl ander komponente nog grootliks onbekend is. In hierdie studie, word 'n rol vir *RME1* in die verbetering van styselafbraak en penetrasiegroei geïdentifiseer. Aangesien Rme1p voorheen as 'n onderdrukker van meiose geïdentifiseer is, is 'n litetaruurstudie oor die inisiasie van meiose saamgestel. Die faktore wat meiose induseer, naamlik 'n gebrek aan voedingstowwe en die sel se ploïedie, word bespreek. Die regulering van die meester inisieerder van meiosie, *IME1*, asook die proteïene waarmee Ime1p reageer, is ook in die studie ingesluit.

Die eksperimentele deel van die studie behels die genetiese analise van Rme1p tydens penetrasiegroei en styselhidroliese. 'n Epistase-analise tussen Rme1p en elemente van die MAP-Kinasemodule, asook van die transkripsie faktore Mss11p, Msn1p/Mss10p, Tec1p, Phd1p en Flo8p, is onderneem. Rme1p is bekend om aan die promotor van *CLN2* te bind en transkripsie te induseer. Daarom is die selsikliene *CLN1* en *CLN2* in die studie ingesluit. Die studie dui daarop dat Rme1p onafhanklik van die MAP-Kinasemodule funksioneer en nie Cln1p en Cln2p benodig om penetrasiegroei te induseer nie. *FLO11/MUC1* kodeer vir 'n selwandproteïen wat noodsaaklik vir pentrasiegroei is. Soos in die geval van die bogenoemde faktore, benodig Rme1p *FLO11* om penetrasiegroei te kan induseer. Ten spyte van *mss11-*, *msn1-*, *flo8-*, *tec1-* en *phd1-* delesies, kan ooruitdrukking van Rme1p die transkripsie van *FLO11* induseer. In die omgekeerde eksperiment kon die bogenoemde faktore *FLO11*-transkripsie ten spyte van 'n *rme1* delesie induseer. Die resultate dui daarop dat Rme1p nie in 'n hiërargiese pad funksioneer nie, maar dat dit waarskynlik 'n meer algemene rol deur transkripsiemodifisering vervul.

This thesis is dedicated to my parents.
Hierdie tesis word aan my ouers opgedra.

BIOGRAPHICAL SKETCH

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PREFACE

This thesis is presented as a compilation of four chapters. Each chapter is introduced separately.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**
The regulation of the initiation of meiosis

Chapter 3 **Research Results**
Cellular differentiation in response to nutrient availability: the repressor of meiosis, *RME1*, positively regulates invasive growth in *Saccharomyces cerevisiae*

Chapter 4 **General Discussion and Conclusions**

A modified version of Chapter 3 has been submitted for possible publication in *Proceedings of the National Acadamy of Sciences of the USA (PNAS)*.

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GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

In a growth environment containing all the nutritional requirements, *Saccharomyces cerevisiae* divides mitotically and populates the environment. The cells form buds that separate from the mother cell and then grow to reach a critical mass and start to form buds of their own. As long as the growth environment contains sufficient nutrients, the mitotic cell cycle continues. If nutrients become exhausted, diploid *S. cerevisiae* can undergo a reductional division called meiosis. During meiosis, four ascospores are formed that are contained in the cell wall of the parental yeast, called the ascus (Kron and Gow, 1995). These ascospores encased in the ascus are remarkably resistant to the harsh environmental conditions and can survive in a dormant state for extended periods of time. If the ascospores are exposed to favourable growth conditions, haploid cells will emerge, start to multiply and mate to form diploid cells.

If nutrients become limited in the yeast's growth environment, or if the nutrients are not easily metabolised, *S. cerevisiae* can form pseudohyphae and/or grow invasively into the growth substrate. In this process, the yeast cells alter their morphology from an ovoid to an elongated shape, and the daughter cells remain attached to the mother cells. As new buds are formed, chains of cells are formed that grow away from the colony and into the growth substrate in order to forage for nutrients (Gimeno *et al.*, 1992).

The three growth patterns described above ensure the survival of the yeast in its variable and hostile environment. For the yeast to follow the appropriate developmental pathway, it must sense the environment, in particular the nutritional status of the growth medium. Receptors are situated on the cell surface and respond to the availability or lack of a specific nutrient, such as the membrane-bound Mep2p, which generates an ammonium-specific signal (Lorenz and Heitman, 1998). Such signals are transmitted to the interior of the cell via a complex signal transduction network that relays the information to specific cellular constituents or compartments. Effector proteins are activated or inactivated, and, in turn, some of these proteins will modulate the expression of specific genes to elicit the appropriate cellular response to the nutritional status of the environment. With such complex regulatory systems, many regulatory proteins are involved. Although a significant amount of data concerning these regulatory pathways has been generated, much remains to be elucidated.

Pseudohyphal development and invasive growth are controlled by at least two distinct signalling pathways (Mösch *et al.*, 1996; Gagiano *et al.*, 1999b; Pan and Heitman, 1999). The first of these two pathways appears to be controlled by Ras2p, a small G-protein, that relays the signal via several proteins, including Cdc24, Bem1p, Ste20 and a MAP kinase cascade (Gimeno *et al.*, 1992; Liu *et al.*, 1993; Leberer *et al.*,

1997; Roberts and Fink, 1994). The MAPK Kss1p activates a heterodimeric transcription complex encoded by *STE12* and *TEC1* (Gavrias *et al.*, 1996). This complex is able to regulate the expression of *FLO11/MUC1*.

The second pseudohyphal regulation pathway is controlled by the levels of cAMP (Lorenz and Heitman, 1997). Other proteins involved in this pathway are Gpr1p, Mep2p and Gpa2p. Mep2p is a membrane-bound receptor that is able to induce filamentation in response to ammonium nutritional signals (Lorenz and Heitman, 1998). Gpa2p is a homologue of an α-subunit of heterotrimeric G-proteins and regulates the activity of adenylyl cyclase. Adenylyl cyclase can convert ATP to cyclic-AMP. The cAMP interacts with Bcy1p, resulting in the activation of Tpk2p, one of the three cAMP-dependent kinases. Tpk2p activates Flo8p, which is a transcriptional regulator of the flocculation genes (Pan and Heitman, 1999) and ultimately is an effector of the expression of *FLO11*.

Both pathways converge on the large promoter of the *FLO11* gene (Rupp *et al.*, 1999). Flo11p is required for pseudohyphal development and is involved in the formation of cell aggregates (Lambrechts *et al.*, 1996a; Lo and Dranginis, 1997).

Some evidence has come to light that supports the existence of additional regulatory pathways. Factors have been identified that seem to act independently of the two above-mentioned pathways and are able to induce invasive growth. *PHD1*, *MSN1/MSS10* and *MSS11* are a few of these factors (Gimeno and Fink, 1994; Edgington *et al.*, 1999; Gagiano *et al.*, 1999b). It still remains to be determined with which factors these proteins interact and how these proteins interact with the described pathways or other possible pathways. Extensive further research is required to identify all the elements involved in the regulation of pseudohyphal development.

Starch degradation is facilitated by one of three glucoamylase genes, namely *STA1*, *STA2* and *STA3*. Promoter analysis of *STA2* and *FLO11* revealed that these promoters are unusually large and are 99% homologues (Gagiano *et al.*, 1999a). Many of the regulatory proteins, for example Mss11p and Msn1p are able to effect the transcription of both *FLO11* and *STA2* genes (Lambrechts *et al.*, 1996b; Webber *et al.*, 1997).

The primary initiator of meiosis is *IME1* (Kassir *et al.*, 1988). The expression of *IME1* is indeed directly linked to the onset of meiosis. The *IME1* promoter is extremely large and contains many regulatory regions (Sagee *et al.*, 1998). Rme1p is a repressor of meiosis and binds to the *IME1* promoter and represses its expression.

Mitosis, on the other hand, is initiated by the interaction of G_1 cyclins with the kinase Cdc28p (Cross, 1995). Interestingly, Rme1p is also able to bind to the promoter of *CLN2*, a G_1 cyclin, and induce its expression (Toone *et al.*, 1995). It therefore is possible that *RME1* plays a vital role in the regulation of both meiosis and mitoisis.

This present study shows that Rme1p also plays a role in regulating invasive growth.

1.2 PROJECT AIMS

Two genes identified from a screen for their ability to enhance starch degradation from a multicopy plasmid, namely *MSN1* and *MSS11*, led to the identification of a third gene – *RME1*. Overexpression of *MSN1* and *MSS11* also enabled the yeast colonies to invade the agar surface.

The aims of this study were

(i) to elucidate if Rme1p is involved in invasive growth;

(ii) to determine the relation of Rme1p with the MAPK-module ;

(iii) to determine the genetic relationship between Rme1p and Mss11p, Flo8p, Phd1p, Tec1p and Msn1p by means of epistasis analysis; and

(iv) to assess the role of Cln1p and Cln2p in the ability of Rme1p to induce invasive growth.

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CHAPTER 2

LITERATURE REVIEW

The Regulation of the Initiation of Meiosis

THE REGULATION OF THE INITIATION OF MEIOSIS

2.1 INTRODUCTION TO MEIOSIS

The reproduction and multiplication of a cell requires the full transmission of all the hereditary information contained in the parental cell to its progeny. This cell division is known as mitosis and has been evolutionarily conserved throughout the phylogenetic spectrum.

There is a second type of cell division, in which the genetic complement of the progeny is only half of that of the parental cell. This is known as meiosis and it evolved exclusively to facilitate sexual reproduction. Meiosis is an integral part of the life cycle and species survival of all eukaryotic organisms, from yeasts to plants and vertebrates. The process of meiosis has also been conserved throughout eukaryotic evolution (Haley and Arbel, 1993; Malone, 1990). With the exception of a few species, the chromosomes of all eukaryotic organisms follow the same meiotic programme of duplication, recombination and pairing, reductional division and equational division. Meiosis creates four haploid daughter cells from a single diploid mother cell. These haploid cells are the specialised cells that eukaryotic organisms use for sexual reproduction. The mechanism ensures that genetic information is passed on to the next generation while recombining the genetic material from both the parental organisms to create a new cell with the same quantity of DNA as the parents, but containing a unique mix of genetic information.

Haploid cells of some single cell organisms, like yeast, are able to survive in the haploid state and divide mitotically until mating can take place. Higher eukaryotes, like birds and mammals, on the other hand have developed haploid cells for sexual reproduction that are so specialised that they are unable to survive outside the parental body for even a short period of time (Weaver and Hedrick, 1992).

During meiosis, the chromosome number is halved through a process involving two cell divisions, meiosis I and meiosis II. Meiosis I is also called the reductional division, as it reduces the chromosome number to the haploid number. Meiosis II, the equational division, is similar to mitosis, as the centromere of the chromosomes separate and the two sister chromatids are separated (Weaver and Hedrick, 1992).

Although the exact cytological details of meiosis vary between species, the following general description of meiosis can be given.

Typically, meiosis can only be observed in higher eukaryotes, as the chromosomes of yeast do not condense sufficiently to be visualised under a light microscope. Meiosis I is preceded by the interphase, during which the DNA of the cell is duplicated. Meiosis I is divided into four stages based on the position of the chromosomes, namely prophase I, metaphase I, anaphase I and telophase I. Prophase I is the most complex of these and is itself divided into five different stages

as the chromosomes condense. The first stage is called leptotene. During this phase, the chromosomes become visible under a light microscope as long, thin strands. Zygotene follows, during which homologous chromosomes pair side by side. Each set of homologous chromosomes is referred to as a bivalent. During pachytene, the third stage, the bivalent chromosomes condense further and portions of the arms of the homologous chromosomes may cross over and recombine. This crossing-over between homologous chromosomes results in the recombination of genetic information and adds to the variation of the genetic information that will be transferred to each haploid cell by the end of meiosis. The fourth stage, diplotene, is characterised by the separation of the bivalent chromosomes. The sister chromatids remain attached to each other. The areas where the homologous recombination has occurred is known as the chiasmata. Generally, at least one chiasmata can be observed per chromosome arm, but several chiasmata can be detected in larger chromosomes. During the last stage of prophase I, diakinesis, the chromosomes shorten even further and the bivalent chromosomes separate fully, with the chiasmata appearing to slip off the ends of the chromosomes. The nuclear membrane also disappears (Weaver and Hedrick, 1992).

Metaphase I follows prophase I and is characterised by bivalent chromosomes aligning on the equatorial plate. Next follows anaphase I, during which the homologous chromosomes of the bivalent separate – one intact chromosome, with both the chromatids, to each pole. During telophase I, the nuclear membrane reforms around the chromosomes and two daughter cells form, each containing only a haploid complement of chromosomes (Weaver and Hedrick, 1992).

Between meiosis I and meiosis II there is only a short interphase, called interkinesis. No DNA synthesis takes place during this phase. Meiosis II starts off with prophase II, during which the chromosomes condense. Metaphase II follows and the chromosomes arrange along an equatorial line in the centre of the cell. During anaphase II, the centromeres divide and separate. The two sister chromatids separate and each is moved to a different pole. Meiosis II completes with telophase II, when the nuclear membrane forms around the chromatids at each pole and the four haploid daughter cells are formed from the original parental cell (Weaver and Hedrick, 1992). Nearly all eukaryotic organisms follow this meiotic programme.

Yeast has long been used as a model organism to study eukaryotic organisms on a molecular level. The study of meiosis has been no exception. Sporulation and meiosis were studied mainly through the analysis of mutants that are no longer able to initiate meiosis or to cause a termination of meiosis during some point of the meiotic programme. More recently, Chu *et al.* (1998) analysed the transcription of genes during meiosis by micro-array. This study revealed that yeast expresses more than 500 genes during the process of meiosis. The focus of this review is mainly on the initiation of meiosis in yeast. The environmental conditions that induce yeast to initiate

meiosis, the role of the mating type of a cell and the primary meiosis activation genes will be discussed.

2.2 MEIOSIS IN YEAST

In nature, *Saccharomyces cerevisiae* occurs predominantly as diploid cells, but is able to exist as a haploid cell and divide mitotically. Haploids are seldom found in nature, however as they mate readily and return to the diploid state. As in all organisms, meiosis forms the basis of sexual reproduction. For yeast, however, meiosis has the additional function of providing a means to survive adverse environmental conditions through the formation of spores. These spores are remarkably resistant to environmental stress and are capable of surviving for extended periods of time (Haley and Arbel, 1993).

The primary trigger of meiosis is the exhaustion of nutrients in the growth environment. In a nutrient-deprived environment, diploid yeasts are induced to undergo meiosis and form four ascospores contained in the cell wall of the diploid, called the ascus (Kron and Gow, 1995). The cell wall of the ascospores are reinforced and, additionally, contain a dityrosine layer that is resistant to non-polar solvents, adding to the resilience of the ascospores. The ascospores lie dormant until the onset of suitable growth conditions, upon which the spores germinate. The haploid cells will start to multiply and, if opposite mating types are available, two haploids will mate and give rise to a diploid. The diploid will grow, divide mitotically and repopulate the environment.

The two mating types of haploid *S cerevisiae* are differentiated as **a** and α haploid cells. Mating can only occur between an **a** haploid and an α haploid, giving rise to an **a**/α diploid. Haploid yeast have the ability to switch mating type. Therefore, if only one haploid mating type is available, switching from **a** to α or vice-versa will allow mating to occur (Malone, 1990).

The availability of nutrients arguably is the most important trophic factor for yeast, and different developmental options can be taken during the G_1 phase of the cell cycle according to the nutritional status of the environment. If ample nutrients are available in the immediate environment of the yeast, it will initiate mitosis. If nutrients are limited but not exhausted, or if the nutrients are not readily utilisable, yeast can form elongated cells that remain attached to the mother cells, forming chains of cells that grow invasively, a phenomenon known as pseudohyphal growth (Gimeno *et al.*, 1992; Lambrechts *et al.*, 1996). Pseudohyphal growth enables yeast to penetrate its media and forage for new or better nutrients. If the nutrients are completely exhausted, diploid yeast undergoes meiosis and forms spores in order to survive. Haploid yeast cannot undergo meiosis and arrests in the G_1 phase, entering a quiescent phase termed G_0 (Gallego *et al.*, 1997).

Initiation of meiosis therefore is regulated mainly by two factors: the ploidy and nutrient availability (Malone, 1990; Mitchell, 1994). To ensure meiosis is precisely controlled, it is governed by a highly regulated and complex regulatory network. On a molecular level, this control involves several steps: firstly, sensing of the nutritional status of the growth environment and other external signals; secondly, the transduction of the perceived signals (Madhani and Fink, 1998); and, thirdly, the regulation of gene expression and protein activity. The individual components of this regulatory network can interact with each other to either enhance or reduce the signal to elicit the correct cellular response. Ultimately, control of meiotic-specific gene expression is achieved by several mechanisms, such as (i) the modulation of the availability of regulator proteins or factors, (ii) the control over the ability of these factors to bind to DNA, (iii) the modification of DNA accessibility, (iv) the regulation of protein function (e.g. phosphorylation) (Rubin-Bejerano *et al.*, 1996 ; Foulkes and Sassone-Corsi, 1992) and (v) post-transcriptional regulation (e.g. mRNA splicing and mRNA stability) (Engebrecht *et al.*, 1991).

If the conditions that induce meiosis are met, a regulatory protein cascade is activated. This results in the precisely timed expression of all the meiosis-specific genes and, ultimately, in completed meiosis. Failure to properly execute any step during the meiotic programme results in a checkpoint mechanism blocking the expression of downstream genes and meiosis being terminated (Vershon and Pierce, 2000). For the purpose of this review, only the regulation of the initiation of meiosis will be examined. Vershon and Pierce (2000) published a review on the complete transcriptional regulation of meiosis.

2.3 MITOSIS AND MEIOSIS ARE MUTUALLY EXCLUSIVE

Mitosis and meiosis are two crucially important processes in the cell that cannot occur at the same time. Both processes demand a large amount of metabolic energy and an overlap would be lethal to the cell. To avoid their concomitant occurrence, several regulators have an inductive activity for mitosis, while repressing meiosis (Colomina *et al.*, 1999).

To initiate mitosis, the G_1 cyclins (Cln1p, Cln2p and Cln3p) interact with Cdc28p kinase in order to execute Start. Start is defined as a point in the cell cycle when the cell commits to mitotic cell division, which begins with the duplication of the spindle pole body and initiation of DNA replication (Cross, 1995; Dirick *et al.*, 1995; Parviz and Heideman, 1998). In mitotically dividing cells, the Swi4-Swi6p complex activates transcription of *CLN1* and *CLN2* and drives the cell cycle from G_1 to the mitotic S phase (Koch *et al.*, 1993). Cln2p and Cln1p are functionally redundant, as only one of the cyclins is required to interact with Cdc28p to initiate mitosis (Cross, 1995; Edgington *et al.*, 1999; Toone *et al.*, 1995; reviewed in Cross, 1995). The initiation of meiosis is not as clearly defined as that of mitosis. Ime2p, a meiosis-specific kinase,

appears to replace Cdc28p in a meiosis-specific role. As both Clb5p and Clb6p cyclins, as well as Ime2p, are required for meiotic DNA replication, a complex may be formed that facilitates G_1-S transition to initiate meiosis, similar to Cdc28p and the cell cyclins during mitosis (Dirick *et al.*, 1998). In addition, Sagee *et al.* (1998) demonstrated that the Swi4p/Swi6p complex inhibits meiosis, making mitosis and meiosis incompatible.

The initiation of the meiotic S-phase has evolved different constituents to that of the mitotic S-phase, ensuring that mitosis and meiosis cannot occur simultaneously. The mitotic SBM/MBF transcription programme, triggered by the G_1 cyclins/Cdc28p complex, has been replaced, although not completely, by a meiosis-specific transcription programme, in which Ime2p replaces Cdc28p and Ime1p controls the meiotic gene transcription (Dirick *et al.*, 1998).

The gene primarily responsible for the induction of meiosis is *IME1* (**I**nducer of **me**iosis) and its expression is linked to the onset of meiosis (Kassir *et al.*, 1988). Colomina *et al.* (1999) showed that the mitosis-inducing G_1 cyclins (Cln1p, Cln2p and Cln3p) have a negative effect on the expression of *IME1*. Furthermore, the G_1 cyclins also prevent the accumulation of Ime1p in the nucleus.

Colomina *et al.* (1999) also demonstrated that a yeast culture with a *CLN3* null mutation sporulates with higher efficiency and enters the pre-meiotic S-phase earlier than the isogenic wild type yeast strain. Mutation analysis of the regulation of mitosis and meiosis further revealed that overexpression of the G_1 cyclins prevents entry into meiosis in nutritional conditions which induce sporulation and drives the yeast cell through mitosis. In the inverse scenario, a *Δcln1Δcln2* strain, which carries an inducible *GAL1p-CLN3* and *IME1* expressed under the constitutive *Schizosaccharomyces pombe adh* promoter – the yeast cells stops division in rich acetate-based media (as expression from P_{GAL1}-*CLN3* is not induced), initiates meiosis and sporulates with high efficiency. The sporulation efficiency of the *Δcln1Δcln2-* P_{GAL1}*CLN3* strain is comparable to wild type cells starved of nitrogen on sporulation medium. The same mutant strains grown on galactose-containing media do not sporulate, despite the continuous expression of *IME1*. Colomina *et al.* (1999) demonstrated that a G_1 cyclin-deficient yeast displayed similar sporulation levels in the presence and absence of nitrogen sources. This indicates that nitrogen starvation may exert its regulation of meiosis via the down-regulation of G_1 cyclins, and not by preventing the accumulation of Ime1p or Ime1p function directly.

The same strain, *Δcln1 Δcln2* P_{GAL1}-*CLN3*, with a wild type *IME1* gene controlled by its native promoter, sporulates in media rich in acetate, but with markedly lower efficiency (only 15% compared to 40% in sporulation medium). The data show that the absence of G_1 cyclins allows low levels of sporulation, which correlates with the hypothesis that Cln3p (or Cln1p or Cln2p) represses *IME1* transcription or down-regulates Ime1p activity post-transcriptionally (Colomina *et al.*, 1999). Yeast strains that are deficient in the G_1-specific cyclins, *cln1*, *cln2* and *cln3*, cannot undergo mitosis, but can form viable spores to the same extent as wild type cells (Dirick *et al.*, 1998). If

the Δ*cln1* Δ*cln2* P_{GAL1}-*CLN3* yeast is transferred from galactose to glucose-based rich media, no sporulation is observed, despite the absence of G_1 cyclins. This indicates that *IME1* expression is directly repressed by glucose (Colomina *et al.*, 1999; Sagee *et al.*, 1998).

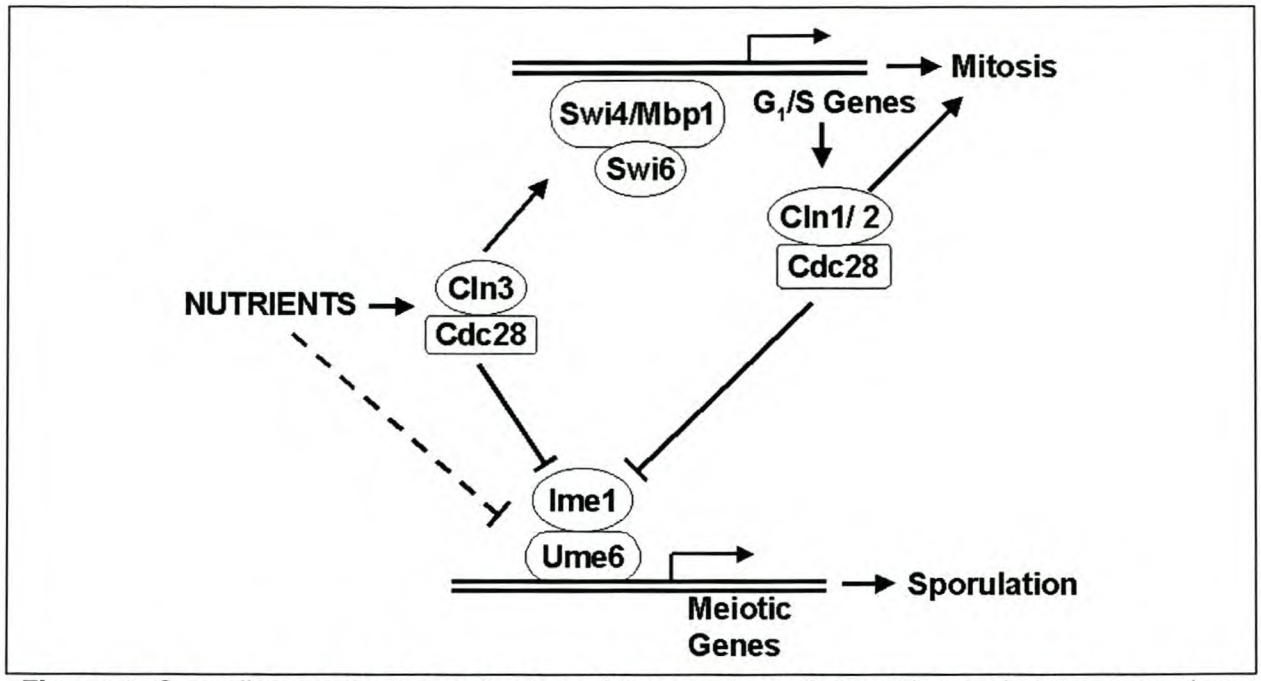

Figure 1 G_1 cyclins are the key activators of the mitotic G_1-S transition and at the same time, prevent meiosis. In the presence of nutrients, the G_1 cyclin levels are high and activate SBF (Swi4p/Swi6p) and MBF (Mbp1p/Swi6p), which drive the cells through mitosis. High G_1 cyclin levels also down-regulate *IME1* expression and prevent Ime1p accumulation in the nucleus, and thus inhibit meiotic gene expression. Upon the depletion of nitrogen and the absence of a fermentable carbon sources, the G_1 cyclin levels fall rapidly, resulting in the arrest of the mitotic cell cycle and removing the inhibition on *IME1*. The cells can enter the pre-meiotic S-phase, as Ime1p is able to interact with a meiotic-specific transcription regulator, like Ume6p, and induce meiosis.

In the same G_1 cyclin-deficient strain carrying *IME1* under the *ADH1*-promoter, *IME1* is expressed and the *IME1* protein is detected in the nucleus, but sporulation in not induced. Furthermore, the transcription of meiosis-specific genes, such as *SPO13*, is not induced, despite the presence of Ime1p (Colomina *et al.*, 1999). This indicates that glucose has a second regulatory effect, other than the prevention of *IME1* expression, that prevents Ime1p from functioning. Furthermore, as no G_1 cyclins are expressed by the mutant on glucose media, the G_1 cyclins do not appear to play a role in preventing the interaction between Ime1p and Ume6p, a repressor of meiotic genes, which is altered to an activation complex by the binding of Ime1p to Ume6p (Colomina *et al.*, 1999).

A regulator that makes meiosis and mitosis mutually exclusive is Rme1p. Rme1p binds to UAS1 in the *CLN2* promoter and activates its expression, thereby inducing mitosis. Rme1p also binds to the *IME1* promoter, represses its expression and thus prevents meiosis from occurring (Toone *et al.*, 1995).

Similarly, Sok2p is a positive regulator of mitosis. In the presence of glucose, Sok2p is phosphorylated by protein kinase A and associates with Msn2p, a transcription regulator, thus preventing transcription of *IME1*. In the absence of glucose and the presence of Ime1p, Sok2p is converted to a weak activator (Shenhar and Kassir, 2001).

In conclusion, the nutritional status of the growth environment dictates whether mitosis or meiosis should occur. Some genes expressed during mitosis prevent the initiation of meiosis and thus prevent the two processes from occurring at the same time (Colomina *et al.*, 1999; Sherman *et al.*, 1993).

2.4 NUTRITIONAL CONTROL

As described above, nutrients are the "life blood" of yeast and the availability of different nutrients dictates which developmental pathway a specific yeast cell will follow. It is obvious that the various nutritional signal transduction pathways must be able to interact to regulate the transcription of the appropriate genes, and to ensure that the required metabolic and developmental pathways can be activated to utilise the available nutrients.

For the yeast cell to undergo meiosis and sporulate effectively, the cell must be starved for at least one essential nutrient, e.g. nitrogen (Freese *et al.*, 1982), and only nonfermentable carbon sources, such as acetate or ethanol, may be present (Mitchell, 1994). Kassir *et al.* (1988) reported the results of Northern analysis of various yeast strains grown on acetate medium and showed that 35-70 *IME1* mRNA were produced per cell that underwent meiosis. On the other hand, if a fermentable carbon source, such as glucose, is present and nitrogen is in short supply, the yeast will not undergo meiosis, but rather initiate pseudohyphal growth (Gimeno *et al.*, 1992).

With the onset of starvation, the level of G_1 cyclins in the cell is reduced and the transcription of genes that repress meiosis is inhibited, while meiosis-inducing genes are activated. This culminates in the initiation of meiosis (Covitz *et al.*, 1994). As *IME1* is the primary meiosis-inducing gene, most of the starvation signals affect the transcription of *IME1* or the stability or function of Ime1p. The exact mechanisms of how these nutritional signals are transmitted to the promoter of *IME1* are still unknown, but several pathways have been implicated in the regulation of meiosis. Matsuura *et al.* (1990) reported that the Ras-cyclic AMP pathway is connected to the regulation of meiosis. It was also reported, however, that meiosis can be initiated independently of a decrease in cyclic AMP (Olompska-Beer and Freese, 1987). Interestingly, Lee and Honingberg (1996) reported that the nutritional regulation of early and late meiotic events follows two different pathways, indicating that meiosis is regulated at its onset and during completion by nutritional starvation signals. Unfortunately, the exact mechanism by which these pathways affect meiosis is still unknown.

Glucose is the preferred carbon source utilised by the yeast *S. cerevisiae*. Glucose functions as an inducer of the expression of the genes required to metabolise glucose, as well as a repressor for a vast array of other genes that are involved in other metabolic pathways. A protein that connects nutritional control to meiosis is Snf1p kinase (Honigberg and Lee, 1998). Snf1p kinase is required to achieve sufficient expression of *IME1* and *IME2* to initiate meiosis. Glucose prevents meiosis from occurring by repressing Snf1p, which in turn prevents sufficient expression of *IME1* and *IME2* (Colomina *et al.*, 1999; Honigberg and Lee, 1998). Yeast that is induced to initiate meiosis on sporulation medium and then moved to rich media after the early stages of meiosis are completed (DNA replication and meiotic recombination) will not complete the meiotic division, but instead will re-enter the mitotic growth cycle. This indicates a checkpoint mechanism that blocks the expression of downstream meiotic genes if glucose is reintroduced into the growth environment (Vershon and Pierce, 2000). Meiotic completion only becomes obligatory after meiotic chromosome segregation is initiated. Honigberg and Lee (1998) suggested that Snf1p kinase may connect the regulation of the early stages of meiosis to the regulation of the latter stages of meiosis. Glucose is further able to interfere with Ime1p post-transcriptionally and prevent the association of Ime1p with Ume6p – therefore preventing the activation of meiotic genes (Vidan and Mitchell, 1997).

The exact mechanism by which the different nutrient signals effect meiosis are not clear. Certain nutrients have been shown to affect different components of the meiotic machinery, but these will be discussed together with the relevant factors.

2.5 MATING-TYPE CONTROL

As only diploid strains of *S. cerevisiae* are able to undergo meiosis, the mating type has a vital role to play in the initiation of meiosis. The **a**/α diploid yeast undergoes meiosis and gives rise to two **a**-haploids and two α-haploids. The mating type of a yeast is determined by the genes expressed from the *MAT* locus. **a**-haploid cells express the *MAT***a***1* gene, while α-haploid cells express both *MATα1* and *MATα2* genes. Diploid yeast expresses both *MAT***a***1* and *MATα2* genes (Malone, 1990). The products of both *MAT***a***1* and *MATα2* combine to form an **a**1-α2 heterodimer, which is required for the initiation of meiosis (Shah and Clancy, 1992). Without the **a**1-*α2* heterodimer, meiosis cannot be executed – thus preventing both the *MAT***a***1* and *MATα* haploids from entering meiosis. It also prevents diploids of the genotypes *MATa/MATa, MATα /MATα*, *mata1/MATα*, *MATa/matα1* and *mata1/matα* from entering meiosis (Kassir *et al.*, 1988). I will henceforth refer to these as non-**a**/α diploids.

The control by the **a**1-*α2* heterodimer is exerted at the transcriptional level by repression of a negative regulator of meiosis, *RME1*. For meiosis to occur, the transcription of *RME1* has to be suppressed (Mitchell and Herskowitz, 1986). In

haploid cells, *RME1* is expressed continuously, which prevents haploids from entering meiosis. If haploid yeasts are starved of nutrients, the expression of *RME1* is induced a further 10-fold to ensure that starved haploid yeast do not initiate meiosis (Covitz *et al.*, 1994; Shimizu *et al.*, 1997). In diploid yeast, Rme1p prevents meiosis until the environmental conditions are unsuitable for vegetative growth, upon which the expression of *RME1* reduces 20- to 100-fold and its repressive effects are alleviated (Malone, 1990; Mitchell, 1994).

Kassir *et al.* (1988) isolated and identified a gene that, when overexpressed on a high copy number plasmid, allowed a diploid yeast lacking *MAT***a***1* to sporulate and named it *IME1*. *IME1* is not expressed when the yeast is grown on rich, fermentable medium, but there is an eight-fold increase in transcription of *IME1* upon a shift to a nonfermentable carbon source. An increase in *IME1* transcription is also preceded by a significant decrease in *RME1* gene product (Malone, 1990). Two observations suggest that repression by *RME1* is not the only mechanism by which the **a**1-α*2* heterodimer regulates meiosis. In yeast strains lacking a functional *RME1* gene, **a**/α-diploids sporulate more efficiently than non-**a**/α diploids, indicating that the **a**1-α*2* heterodimer could induce expression of *IME1* directly (Mitchell and Herskowitz, 1986). Secondly, *RME1* expression blocks sporulation more effectively in **a**/α-diploids than in non-**a**/α diploids (Mitchell, 1994). More direct evidence of an *RME1*-independent pathway to induce meiosis came from the identification of *RES1* and *IME4*, which have been identified as positive regulators of meiosis. A partially dominant mutation, *RES1-1*, is able to induce meiosis in a yeast strain containing multiple copies of *RME1*. It was also seen that the effects of Δ*rme1* and Δ*res1* are additive in non-**a**/α diploids (Kao *et al.*, 1990). These factors will be discussed later.

Although the regulation of meiosis by the **a**1-α*2* heterodimer is not fully understood, it is clear that the mating type ensures that only wild type diploid yeast can sporulate.

2.6 STRUCTURE OF EARLY MEIOTIC PROMOTERS

The execution and completion of meiosis require the precisely timed expression of about 500 genes (Chu *et al.*, 1998). The time at which these genes are expressed after the initiation of meiosis, was used to classify them into four groups: early, middle, mid-late and late sporulation genes. The early genes are expressed 0.5 to 2 hours after the cells are introduced to sporulation conditions and are required for DNA replication, chromosome paring and the formation of synaptonemal complex, a structure that facilitates recombination between homologous chromosomes. The middle meiotic genes are expressed between 2 and 5 hours after the initiation of meiosis, starting near the end of the meiotic prophase. These genes are required for meiotic divisions and the initiation of the synthesis of spore wall components. The mid-late genes are expressed 5 to 7 hours after meiotic initiation and are responsible for

the formation of the chitin / chitosan and dityrosine layers of the spore wall. The late genes are expressed after 7 hours and appear to be required for the maturation of the yeast spores (Chu *et al.*, 1998; Vershon and Pierce, 2000). Analysis of the promoters of genes expressed during a particular stage indicated that the promoters contain common regulatory elements (Chu *et al.*, 1998; Vershon and Pierce, 2000). The focus of this study is on the initiation of meiosis and therefore only the early meiotic promoters will be discussed in detail. For a review, see Mitchell (1994).

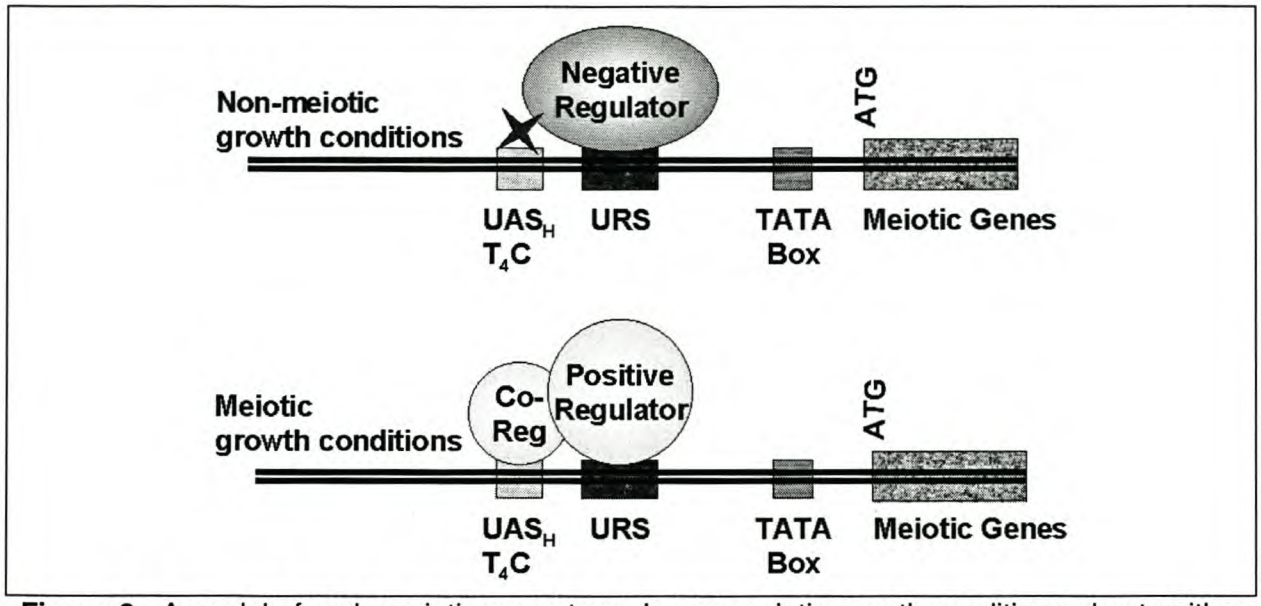

Figure 2 A model of early meiotic promoters. In non-meiotic growth conditions, due to either the presence of fermentable carbon or the absence of the **a**1-α2 heterodimer, a negative regulator binds to the URS and prevents the expression of meiotic genes. The negative regulator also prevents the binding of a positive regulator (co-reg) to UAS_H or T_4C. If the environment is depleted of nitrogen, no fermentable carbon sources are present and the **a**1-α2 heterodimer is present, a positive regulator will bind to the URS and a second positive regulator can bind to the UAS_H or T_4C site.

2.6.1 PROMOTERS OF EARLY MEIOTIC GENES

Early meiotic genes are expressed in the first 2 hours after meiosis is induced and comprise about 204 genes. Functional analysis of the promoters of the early meiotic genes revealed several similarities between them (Fig. 2) (Strich *et al.*, 1989; Vershon and Pierce, 2000). Firstly, a regulatory sequence was identified near the minimal promoter sequences (TATA-box). Secondly, many promoters contain a conserved nine base pair upstream repressor sequence (URS) (TCGGCGGCT), generally situated between position -200 and the TATAbox. Repression occurs from the URS in nonmeiotic conditions (Chu *et al.*, 1998). Also, in conditions that induce meiosis, activation occurs from the same URS sequence. This two-fold regulation of meiosis ensures expression during meiosis only (Buckingham *et al.*, 1990; Mitchell, 1994). Thirdly, many early meiotic promoters contain a site in close proximity to the URS, which acts synergistically with the URS to activate transcription during meiosis. There are two associated transcriptional activation sites, UAS_H and T_4C, but most meiotic

promoters contain only one of these. Although their DNA sequences differ, UAS_H (TGGAAGTG) and T_4C (TTTTCTTCG) are functionally similar. Mitchell (1994) suggested that the URS functions as an on/off switch, with UAS_H or T_4C controlling the overall level of expression of the different genes. This mechanism of regulation would facilitate the expression requirements of the large meiotic gene family, with the individual genes having varying levels of expression levels in order to complete meiosis.

The proteins responsible for the repression of meiotic genes under non-meiotic growth conditions and in non-**a**/α diploids are Ume1p,2p,3p,5p (Strich *et al.*, 1989) and Ume6p (Bowdish and Mitchell, 1993). Of these, only Ume6p is known to be required for the induction of meiotic genes in sporulation conditions (Rubin-Bejerano *et al.*, 1996), but this will be discussed at a later stage.

Promoters of early meiotic genes that will be discussed in more detail are *SPO13*, *HOP1* and *IME2*. Spo13p functions during meiosis I, while Hop1p forms part of the meiotic recombination system, specifically during prophase I. It was the analysis of the promoter of *SPO13*, *HOP1* and *IME2* that revealed that the repressor sequences, URS1, also acts as an activator sequence under sporulation conditions (Buckingham *et al.*, 1990). A *lacZ* fusion to the *SPO13* promoter shows early meiotic expression. In nonlimiting growth conditions, as well as in non-**a**/α yeast strains, no β-galactosidase activity could be detected – indicating complete repression under these conditions. Deletion of the URS1 caused a six-fold decrease in P_{SPO13}-*lacZ* expression in sporulation conditions, while some expression was observed in non-meiotic conditions (Buckingham *et al.*, 1990).

Vershon *et al.* (1992) identified UAS_H in the promoter of *HOP1*, situated in close proximity to the URS1. In a mutated URS1, UAS_H drives transcription in non-meiotic conditions. UAS_H deletion results in a four- to eight-fold reduction in *HOP1* expression levels during meiosis, with detrimental effects on the completion of meiosis (Vershon *et al.*, 1992). In a wild type cell that is grown in non-meiotic growth conditions, repressors bind to the URS1 and prevent the activation from UAS_H, resulting in repression of *HOP1* expression. In sporulation conditions, the repressors are removed from the URS1, the activators bind to URS1 and UAS_H enhances the expression of *HOP1*.

The URS element of *IME2* is situated more upstream, at positions -584 and -442, and not close to the TATA box as in the case of *SPO13* and *HOP1* (Bowdish and Mitchell, 1993). The *IME2* URS also displays inductive and repressive capabilities, but contains two UAS sequences. An analysis of these UAS regions showed a requirement for Ime1p for *IME2* expression. The kinases Rim11p and Rim15p are required for optimal expression of *IME2*. Deletion of these kinases has a detrimental effect on *IME2* expression, as Ime1p is not able to bind to the *IME2* promoter. The role of Rim11p and Rim15p will be discussed at a later stage. Additionally, a second regulatory element that contributes to the activation activity was identified next to the

URS, namely the T_4C site. This site was found to modulate the overall expression level of *IME2* and functions independently of Ime1p (Bowdish and Mitchell, 1993). In non-meiotic conditions, the T_4C site is inaccessible to a positive regulator due to the negative regulators bound to URS1.

2.6.2 PROMOTERS OF MIDDLE MEIOTIC GENES

Near the end of the meiotic prophase, the transcription of more than 158 genes is activated. The promoters of 70% of these genes contain a conserved middle sporulation element (MSE), which functions as a strong activator site during meiosis (Chu *et al.*, 1998; Ozsarac *et al.*, 1997).

2.6.3 PROMOTERS OF MID-LATE MEIOTIC AND LATE MEIOTIC GENES

About 61 genes are expressed during the mid-late stage of meiosis. Many of these promoters contain the MSE, but also contain additional negative regulatory elements that prevent expression during the middle meiotic programme, thus delaying their expression to mid-late meiosis. These sites are called Negative Regulatory Elements (NREs) (Friesen *et al.*, 1997). Ufano *et al.* (1999) reported that mutations in *SWM1* caused a significant reduction in the expression levels of mid-late and late meiotic genes. Swm1p is a mid-sporulation nuclear protein required for the formation of the spore wall.

URS and MSE are absent in the promoters of the late meiotic genes,. A common regulatory element for these few genes has not been identified (Vershon and Pierce, 2000).

2.7 MEIOTIC REGULATORY GENES

As described above, *IME1* is the master regulator of meiosis, with its expression causing the onset of meiosis (Kassir *et al.*, 1988). Most of the regulatory genes involved during meiosis regulate the expression of *IME1* or interact with Ime1p. The regulation of *IME1* expression and the factors involved in the regulation will be discussed first. This will be followed by a discussion the role and function of Ime1p.

2.7.1 *IME1*

IME1, the primary regulator of meiosis, encodes for a 360 amino acid protein containing two tyrosine-rich segments (Smith *et al.*, 1993). Ime1p is a nuclear protein and is composed of at least two domains, namely a transcriptional activation domain and a protein interaction domain. The interaction domain is required for the specific interaction with meiotic targets (Mandel *et al.*, 1994). The transcriptional activation domain, situated within the last 90 C-terminal amino acids of Ime1p, is required for interaction with Ume6p, the kinase Rim11p and for either self-association or interaction

with other meiosis-specific transcriptional modulators (Rubin-Bejerano *et al.*, 1996). The C-terminal region also regulates the activation ability of Ime1p and may provide specificity to direct Ime1p to the regulatory locations where it is required (Smith *et al.*, 1993).

Diploid yeast homozygous for null mutations of the *IME1* gene cannot enter meiosis in starvation conditions and arrests as unbudded cells that do not undergo pre-meiotic DNA synthesis or meiotic recombination. The *IME1* deletion has no effect on mitotic cell division and the cells do not lose viability. *IME1* overexpression overcomes the requirement for the **a**1-α2 heterodimer and all cell types, both haploids and diploids, enter meiosis (Kassir *et al.*, 1988).

2.7.1.1 *IME1* PROMOTER

The promoter of *IME1* is among the largest and most complex promoters identified to date. Both the cell-type signal and the nutritional signal converge on the promoter of *IME1* to regulate its expression (Colomina *et al*, 1999).

An extremely large 4122 bp area upstream of *IME1* is devoid of any open reading frame (Sagee *et al.*, 1998). The exact length of the promoter is not known for certain, but regulatory sites have been identified up to position -2100. However, it appears that regions further upstream also contain regulatory elements and the *IME1* promoter could extend to -3800 bp. Furthermore, sequence analysis of the *IME1* promoter revealed three putative TATA boxes at positions -353 (TATATTA), -330 (TATTTAA) and -158 (TATAAT). Sequential deletions of these TATA boxes revealed that the functional TATA box is located at -330 (Sagee *et al.*, 1998).

Sagee *et al.* (1998) made systematic deletions of different areas of the *IME1* promoter and fused them to a *lacZ* reporter system. Four regulatory regions of the *IME1* promoter were identified and arbitrary named UCS1 to UCS4 (Fig. 3). UCS1, USC3 and UCS4 function as negative regulators, whereas USC2 has a more positive regulatory role and is absolutely required for the expression of *IME1*. UCS1 and UCS2 respond to the various nutritional signals, whereas UCS3 and USC4 suppress transcription in *MAT*-insufficient cells and ensure that only **a**/α-diploids sporulate (Sagee *et al.*, 1998). This indicates that the promoter contains several modulators for the expression of *IME1*. Most of the identified regulatory areas have shown function only at a genetic level and defined binding sites for the proteins have not been identified (Sagee *et al.*, 1998).

UCS1 represses *IME1* transcription in response to the presence of sufficient nitrogen and allows mitosis to continue. The meiotic repression is mediated by the G_1 cyclins (Colomina *et al.*, 1999; Park *et al.*, 1996). If nitrogen is depleted, a starvation signal is transmitted to UCS1 via the RAS-PKA pathway. Mutations in the pathway that lower the PKA activity, like *ras2*, cause meiosis to occur despite the presence of nitrogen (Matsuura *et al.*, 1990; Sagee *et al.*, 1998; Smith and Mitchell, 1989).

UCS2 responds to the availability of different carbon sources. The analysis of USC2 revealed different areas within USC2 that responds to the various carbon signals and these consequently were subdivided into several different regulatory regions (Fig. 3).

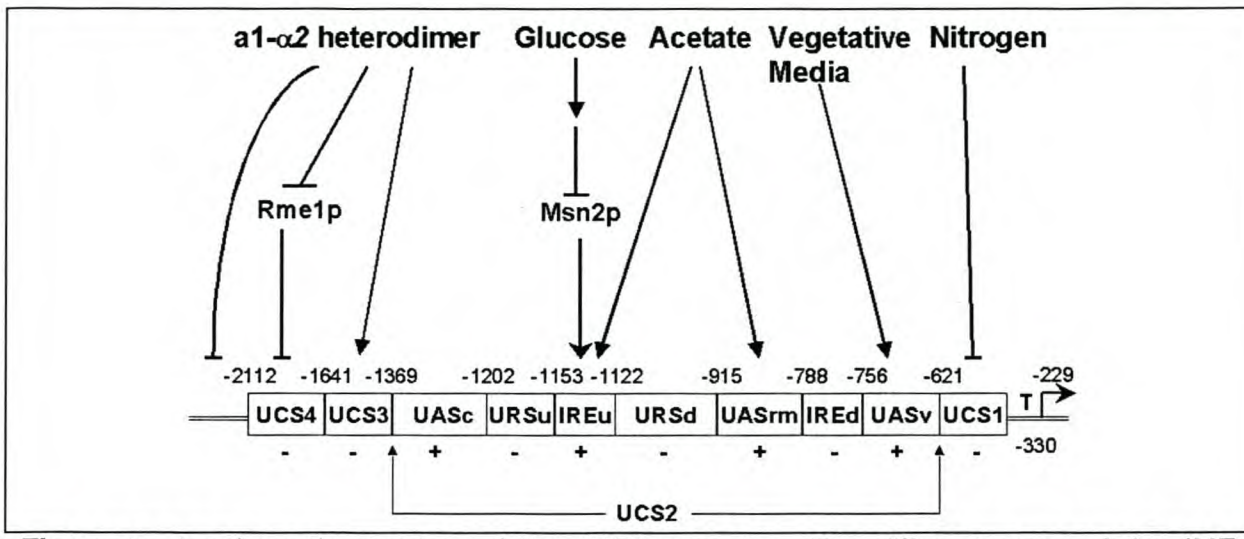

Figure 3 A schematic structure of the *IME1* promoter. The different areas of the *IME1* promoter that respond to *MAT*, glucose, vegetative media, acetate and nitrogen are indicated. The arrows indicate induction or a positive role, whereas a negative role is indicated by a line.

IREu and IREd are almost identical repeats within UCS2, but have quite different functions. IREd functions as a constitutive URS element. IREu contains a stress-responsive STRE element and a cell cycle box (SCB). STRE elements are known to function as activation sequences in response to stress conditions (Marchler *et al.*, 1993). In response to the absence of glucose, Msn2p/Msn4p binds to the STRE elements and activates the expression of *IME1* (Martinez-Pastor *et al.*, 1996; Sagee *et al.*, 1998). Deletions of *msn2* and *msn4* significantly reduce activation by the STRE element. In the presence of glucose, the RAS-cAPK pathway prevents activation by IREu and functions as a negative element. Shenhar and Kassir (2001) demonstrated that Sok2p associates with Msn2p and mediates repression by IREu.

The IREu further contains the sequence TTTTGCTC, which is virtually identical to the known cell cycle box (SCB) (Sagee *et al.*, 1998). The SCB is present in the promoters of the cell cyclins *CLN1* and *CLN2*, as well as *HO*, and is known to serve as a UAS to exit G_1 arrest and as a position to which the Swi4p-Swi6p complex bonds (Breeden and Nasmyth, 1987; Koch *et al.*, 1993). Sagee *et al.* (1998) showed that null mutations of *swi4* and *swi6* cause an increase in expression from the IREu element, indicating the negative effect of the Swi4p-Swi6p complex on the initiation of meiosis and explaining why transcription of *IME1* only occurs after G_1 arrest.

UASv contains a 28 bp binding site for Yhp1p at -701 to -675. In the presence of glucose, *IME1* expression is repressed by Yhp1p (Kuno *et al.*, 2000). UASrm activates transcription in the presence of acetate. UASc promotes expression under all growth

conditions, whereas URSu and URSd function as URS elements. IREu and UASrm promote the expression of *IME1* in the presence of acetate (Sagee *et al.*, 1998).

USC3 and USC4 respond to the **a**1-α*2* heterodimer by prevent transcription of *IME1* in the absence of the **a**1-α*2* heterodimer, thus ensuring that only **a**/α diploids initiate meiosis. USC4 contains an RRE (Rme1p Responsive Element) to which the negative regulator, Rme1p, binds (Benni and Neigeborn, 1997). The RRE is a 21 bp element and is situated at -2044 to -2024 (Fig. 3). The 233 bp surrounding the 21 bp binding site are essential for repression by Rme1p and are probably responsible for directing the repressive effect of Rme1p (Covitz *et al.*, 1994). Interestingly, the Rme1p repression acts over very large distances, as the Rme1p binding site is about 1000 bp upstream of a UAS element (Shimizu *et al.*, 1997; Smith *et al.*, 1990). Rme1p may function as a repressor through the alteration of chromatin structure, thereby preventing the binding of positive regulators (Fig. 4).

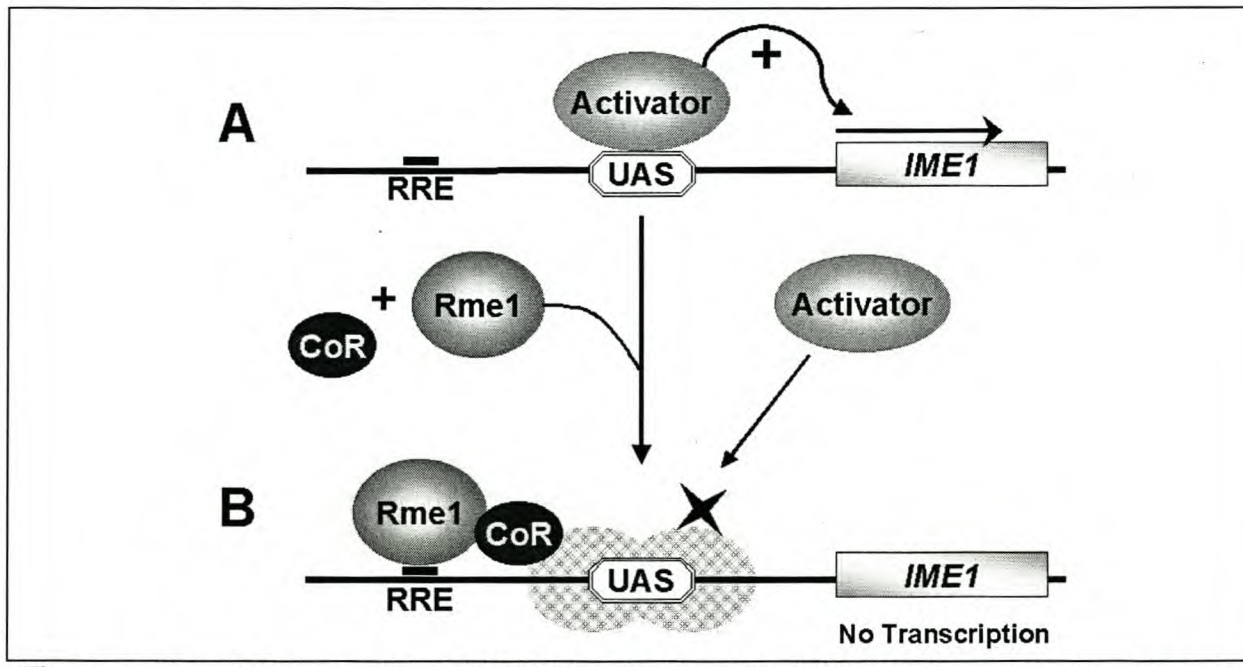

Figure 4 A model of repression of *IME1* by Rme1p. When Rme1p is absent, an activator is able to bind to the UAS and activate the transcription of *IME1* (A). When Rme1p is present, it interacts with its co-repressors, Sin4p and Rgr1p (indicated by CoR), and binds to the RRE (Jiang and Stillman, 1992). The RRE is a 22 bp element to which Rme1p can bind. The binding of Rme1p and its co-repressors alters the chromatin structure and makes the DNA inaccessible for activator binding.

Sakai *et al.* (1990) showed that Rme1p requires Rgr1p and Sin4p to repress *IME1* expression. Rgr1p and Sin4p are known to maintain high density chromatin and make the DNA inaccessible to activator proteins and the RNA polymerase II (Covitz *et al.*, 1994; Jiang *et al.*, 1995; Mizuno and Harashima, 2000). The flanking sequence around the RRE is required to establish a repressive structure and may contain a binding site for a co-repressor (Shimizu *et al.*, 1997). A chromatin structural alteration can exert repression over large distances – as would be required for the repression of *IME1* by Rme1p (Covitz *et al.*, 1994; Jiang *et al.*, 1995; Sagee *et al.*, 1998). UCS3

repression functions independently of Rme1p, as it does not contain an Rme1p binding site. How the non-**a**/α diploid signal is transmitted to USC3 remains to be elucidated (Sagee *et al.*, 1998).

Shah and Clancy (1992) described a gene by which *MAT* controls the initiation of meiosis in an Rme1p-independent manner, namely *IME4* (Fig. 5). Ime4p is a positive regulator of meiosis and induces the expression of *IME1*. *IME4* is only expressed in **a**/α diploid yeasts that are starved of nutrients. No mRNA transcripts of *IME4* can be detected in vegetatively growing cells or in *MAT*-insufficient cells grown in sporulation conditions. No binding site for Ime4p has been identified on the *IME1* promoter. It is possible that Ime4p could act through another unknown effector to induce the expression of *IME1*. Deletion of *ime4* prevents the transcription of *IME1* and meiosis does not occur. Overexpression of *IME1* is able to bypass the requirement for *IME4* during sporulation, while overexpression of *IME4* cannot induce sporulation in *ime1*-deleted yeast. Thus, *IME4* is able to induce the expression of *IME1* in the presence of the **a**1-α*2* heterodimer (Sagee *et al.*, 1998; Shah and Clancy, 1992). Ime4p also facilitates the accumulation of Ime1p.

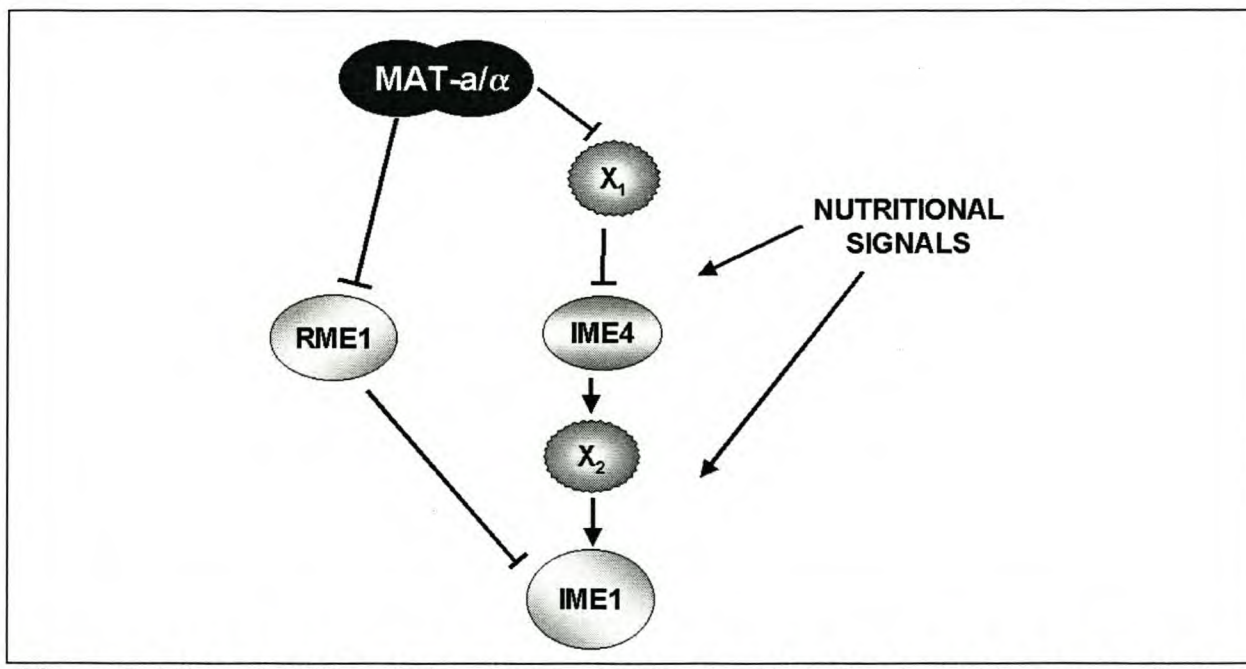

Figure 5 Effect of *MAT* control on Rme1p and Ime4p. Both Rme1p and Ime4p are effectors of *IME1* expression; Rme1p is a repressor and Ime4p acts as an activator. *MAT* functions via an unidentified factor(s), indicated by X_1 on Ime4p. Additionally, Ime4p is able to communicate the nutritional signal to *IME1*, although Ime4p does not interact with Ime1p directly, but via an unidentified factor X_2. Rme1p is repressed by the **a**/α heterodimer. In *MAT*-insufficient cells, *RME1* is expressed and prevents the expression of *IME1*. The expression of *IME1* results in meiosis.

Mck1p, a dosage-dependent repressor of centromeric mutations, is required for the full induction of *IME1*, although the mechanism is not understood as yet. If the Mck1p kinase is mutated, sporulation is deficient. Furthermore, Rim1p, Rim8p, Rim9p and Rim13p are also involved in increasing the level of *IME1* expression, but it remains

unknown how this is achieved (Li and Mitchell, 1997; Vershon and Pierce, 2000). *MDS3* and *PMD1* are negative regulators of meiosis and function synergistically to repress *IME1*. However, neither Mds3p or Pmd1p contains a known DNA-binding motif and they appear to affect *IME1* expression indirectly. The exact mode of action of these proteins remains to be elucidated (Benni and Neigeborn, 1997).

An interesting aspect of *IME1* expression is that the expression level of *IME1* is somehow linked to the expression of the target genes, although this mechanism is also unknown. In conclusion, the regulation of *IME1* expression is clearly far from being understood. With the development of new techniques, it should be possible to unravel the *IME1* regulation.

2.7.2 *RME1*

Rme1p is a zinc finger protein capable of binding to the RRE sequence in the *IME1* promoter. The DNA-binding domain of Rme1p consists of three zinc finger segments, as well as the C-terminal 16 amino acid segment called C-TR, which is vital for Rme1p function (Covitz *et al.*, 1994; Shimizu *et al.*, 1999; Shimizu *et al.*, 2001).

RME1 is expressed strongly in **a** haploids, because the *MATα2* gene product is absent. The same applies to an α haploid, where the *MAT***a***1* is absent. In **a**/α diploid yeast in which both *MAT***a***1* and *MATα2* are present, the level of *RME1* transcription is reduced 10-20 fold, but not repressed totally (Mitchell and Herskowitz, 1986). This reduction in *RME1* transcription is not observed in non-**a**/α diploids, as both the *MAT***a***1* and *MATα2* gene products are not present. Null mutation of *rme1* allows starved non-**a**/α diploid yeast to undergo meiosis and do sporulate efficiently. Haploid yeast lacking a functional copy of *RME1* and subjected to sporulation conditions will engage in premeiotic DNA synthesis and recombination and will initiate spore formation. These cells, however, cannot complete meiosis due to the lack of homologous chromosomes and will perish (Mitchell and Herskowitz, 1986; Toone *et al.*, 1995).

Toone *et al.* (1995) determined that *RME1* expression is linked to the cell cycle and that the gene is expressed late in the G_1 phase, from where Start can be executed. Synchronised yeast cultures showed that the mRNA of *RME1* peaked at the M/G_1 boundary (Toone *et al.*, 1995). This indicates that the expression of *RME1* is itself regulated by the cell cycle and cannot initiate Start itself. The expression of *RME1* is further regulated by additional factors. Swi5p/Ace2p transcription factors are known to activate a range of genes at specific stages of the cell cycle and also to regulate *RME1* expression. The role of Rme1p in the activation of Start was reported in haploid yeast only (Toone *et al.*, 1995).

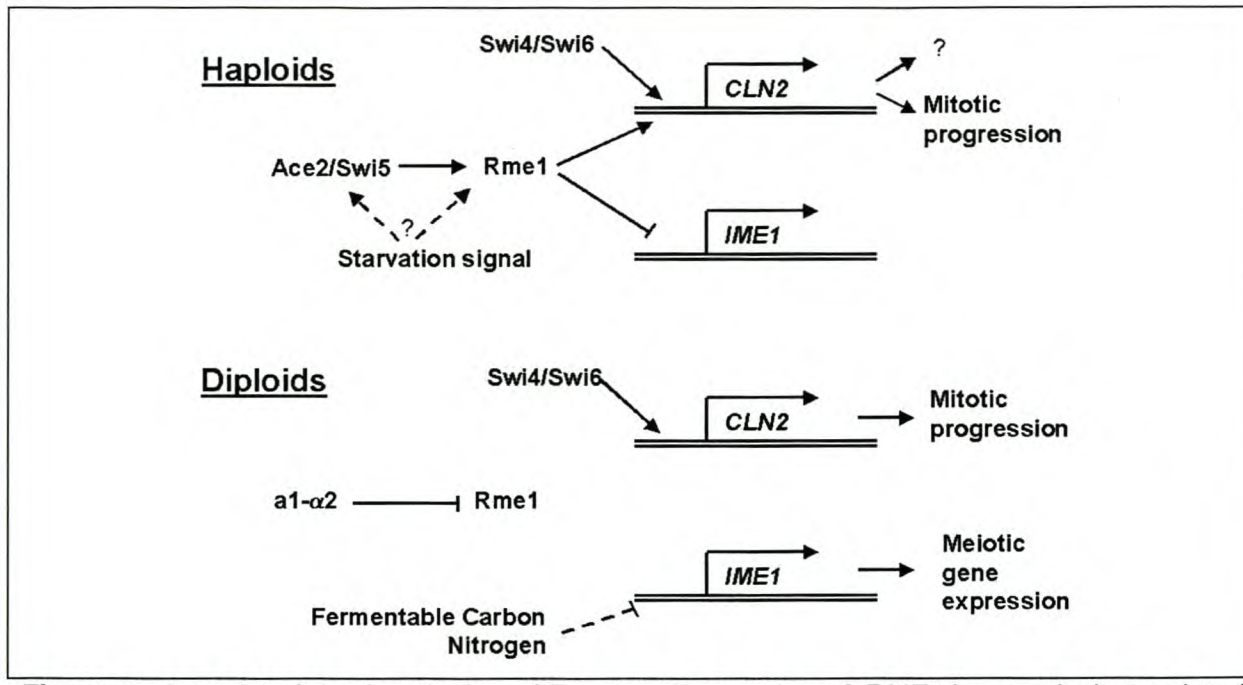

Figure 6 A model of the functioning of Rme1p. Expression of *RME1* is negatively regulated by the **a**/α-heterodimer. In diploid yeast, it is questionable if Rme1p has any role regarding *CLN2* expression. In haploid yeast, the **a**/α-heterodimer is absent and *RME1* is expressed, and preventing the expression of *IME1* and inhibiting meiosis. Rme1p is also able to bind to the promoter of *CLN2*, where it acts as an activator. Ace2p/Swi5p transcription factors are responsible for periodic expression of *RME1*. In conditions in which nutrients are limited, the expression of *RME1* is induced. The mechanism for this induction is unknown. It is unknown if Ace2p and/or Swi5p are involved or if the *RME1* induction is achieved through a totally different pathway.

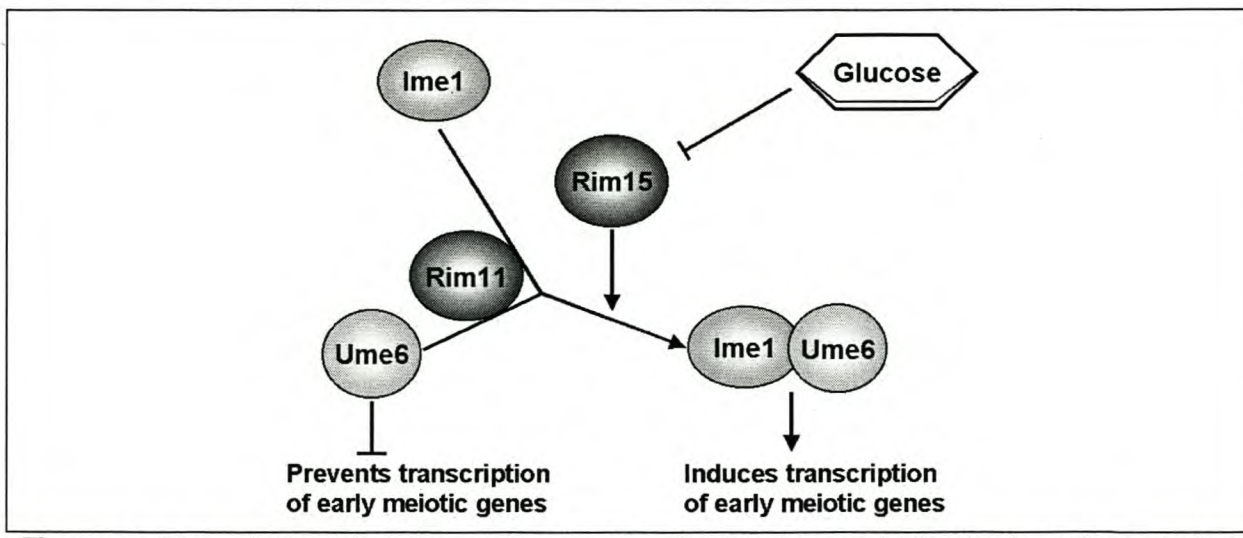

Figure 7 The relationship between Ime1p, Ume6p, Rim11p and Rim15p in order to induce meiosis-specific transcription. In the absence of Ime1p, Ume6p acts as a repressor and prevents the transcription of most meiotic genes. In the presence of Ime1p, Ume6p induces meiotic expression (Bowdish *et al.*, 1995; Steber and Esposito, 1995). This activation of expression is achieved through Ime1p-Ume6p complex formation. Rim11p is vital for the formation of the Ime1p-Ume6p complex (Rubin-Bejerano *et al.*, 1996). Rim15p also is involved in the formation of the Ime1p-Ume6p complex. Whether it plays a direct role or an indirect role (through an as yet unidentified protein) remains to be determined (Vidan and Mitchel, 1997). Glucose prevents Rim15p accumulation and Ime1p-Ume6p complex formation.

2.8 *IME1*-ASSOCIATED PROTEINS

The regulation of genes expressed only during meiosis is achieved through Ime1p, Ume6p and Rim11p. In non-meiotic conditions, Ume6p is bound to the URS of most meiotic promoters and represses their expression (Bernstein *et al.*, 2000). Several proteins associate with Ume6p to form a repression complex. With the onset of meiosis, the repression must be alleviated. This is achieved by Ime1p and the protein kinase Rim11p. Rim11p phosphorylates both Ime1p and Ume6p. Ime1p binds to Ume6p and this alters Ume6p from being a repressor to being an Ime1p/Ume6p activation complex, which allows transcription of the meiotic genes to take place (Fig. 7). The protein kinase Rim15p is subject to glucose repression and is also required for the formation of the Ime1p-Ume6p complex.

2.8.1 *RIM11*

Rim11p is a homologue of vertebrate glycogen synthase kinase 3 and belongs to a protein kinase subfamily with diverse functions (Malathi *et al.*, 1997; Zhan *et al.*, 2000). The kinase activity of Rim11p (Mds1p and ScGSK3) is essential for meiosis in yeast (Malathi *et al.*, 1999; Puziss *et al.*, 1994). An analysis of *RIM11* null mutations indicates an absolute sporulation defect, with transcripts of early meiotic genes failing to accumulate (Bowdish *et al.*, 1994). *Rim11* mutation otherwise displays no obvious phenotypical growth defect or any defect during mitosis. It appears that Rim11p function is unique to meiosis (Bowdish *et al.*, 1994; Puziss *et al.*, 1994).

Rim11p phosphorylates Ime1p, thereby activating and facilitating the self-association of Ime1p. Co-immunoprecipitation studies have demonstrated that Rim11p and Ime1p precipitate together, which indicates their association (Bowdish *et al.*, 1994; Malathi *et al.*, 1997; Rubin-Bejerano *et al.*, 1996; Zhan *et al.*, 2000). Ume6p is also phosphorylated by Rim11p and an activator complex is formed through the interaction of Ime1p. The formation of the activation complex is achieved by one of two suggested models (Fig. 8). Firstly, Rim11p could have a catalytic role, i.e. Rim11p phosphorylates both Ume6p and Ime1p and the phosphorylated Ime1p interacts with the phosphorylated Ume6p to form an activation complex. The second model depicts Rim11p in a more structural role, with an Ime1p-Rim11p complex interacting with Ume6p to form a ternary Ime1p-Rim11p-Ume6p activator complex (Malathi *et al.*, 1999). Data obtained from deletion studies of both *rim11* and *ume6* are consistent with either the catalytic or structural roles of Rim11p and could not be used to distinguish between the two models. For example, catalysis-defective mutants of Rim11p fail to promote Ime1p-Ume6p complex formation. They also fail to interact with either Ime1p or Ume6p (Malathi *et al.*, 1999).

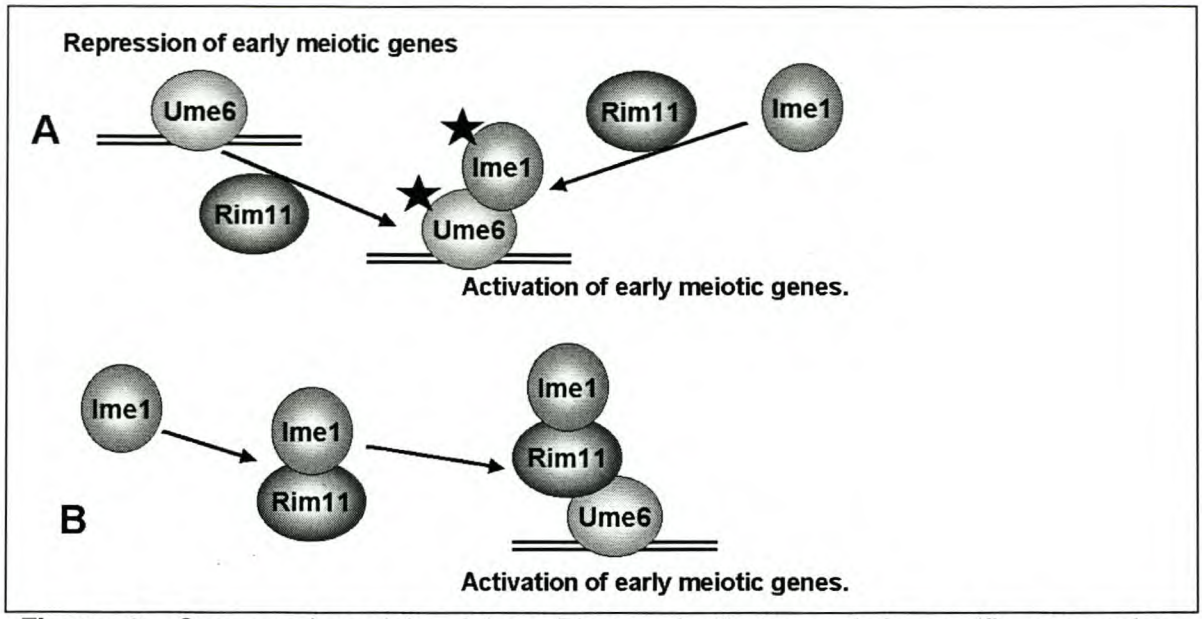

Figure 8 Suggested models of how Rim11p facilitates meiotic-specific expression. A) Catalytic Model. Rim11p phosphorylates both Ime1p and Ume6p and the phosphorylated Ime1p and Ume6p are able to interact and activate expression. The ☆ indicates a phosphorylated protein. B) Structural model. Rim11p first binds to Ime1p and then the Rim11p-Ime1p-complex binds to Ume6p and activates meiotic expression.

Two mutants of Ime1 that are able to bind to Rim11p, but display no detectable phosphorylation, reveal that these non-phosphorylated Ime1p mutants are unable to bind to or interact with Ume6p. This prevents meiosis from occurring and shows that Ime1p has to be phosphorylated to induce meiosis (Malathi *et al.*, 1999). In addition to the Rim11p-dependent phosphorylation of Ime1p and Ume6p, a starvation signal is required for the full activation of meiotic genes (Rubin-Bejerano *et al.*, 1996). As stated previously, Ime1p is affected post-transcriptionally by the nutritional status of the cell. Rim11p could be the facilitator of the starvation signal, as Malathi *et al.* (1997) reported that the phosphorylation activity of Rim11p decreased four-fold in glucose media in comparison to the decrease in acetate media. Nitrogen limitation, on the other hand, had no effect on phosphorylation properties of Rim11p.

2.8.2 *RIM15*

The kinase Rim15p also acts in the Ime1p-Ume6p-Rim11p activation pathway. It is thought to stimulate Ime1p-Ume6p interaction and it is probable that Ume6p is the final target of Rim15p. Alternatively, Rim15p could phosphorylate an undefined gene product that acts in the Ime1p-Ume6p complex formation pathway. *RIM15* null mutation analysis revealed that *IME1* expression is reduced in meiotic conditions and results in a six-fold reduction in Ime1p-Ume6p interaction (Vidan and Mitchell, 1997). In the presence of glucose, Rim15p fails to accumulate and this could explain the inhibitory effect of glucose on Ime1p-Ume6p complex formation (Vidan and Mitchell, 1997).

2.8.3 *UME6*

Ume6p is a zinc cluster protein that binds to the promoters of many genes, including the meiotic genes, and functions as a *cis*-acting repressor in non-meiotic conditions, preventing meiosis from occurring (Bernstein *et al.*, 2000; Bowdish *et al.*, 1995). Repression of meiotic genes by Ume6p is dependent on both Sin3p and Rpd3p (Elkhaimi *et al.*, 2000; Lamb and Mitchell, 2001). Sin3p is a known repressor that functions in cell differentiation and cell-type regulation (Vidal *et al.*, 1991). The co-repressors Sin3p and Sap30p and the histone deacetylase Rpd3p form a complex that is required to regulate silencing in yeast (Sun and Hampsey, 1999). The Sin3p-Sap30p-Rpd3p complex is recruited to the Ume6p binding site to deacetylate the adjacent histone (Fig. 9) (Bernstein *et al.*, 2000; Vogelauer *et al.*, 2000). Lysine 5 of histone H4 is the specific target of Rpd3p (Rundlett *et al.*, 1998). Decreased histone acetylation is correlated with the inaccessibility of chromatin for transcriptional activators or the transcriptional machinery and creates localised regions of repressed chromatin. Vogelauer *et al.* (2000) reported that these repressed chromatin regions can encompass areas of more than 4kb. The Sin3p-Ume6p association is also known to facilitate the repression of other metabolic pathways, such as arginine catabolism. If either *sin3* or *rpd3* are not functional, the repression of the meiotic genes by Ume6p is reduced. Thus, Ume6p repression is achieved mainly through chromatin modification (Kadosh and Struhl, 1997).

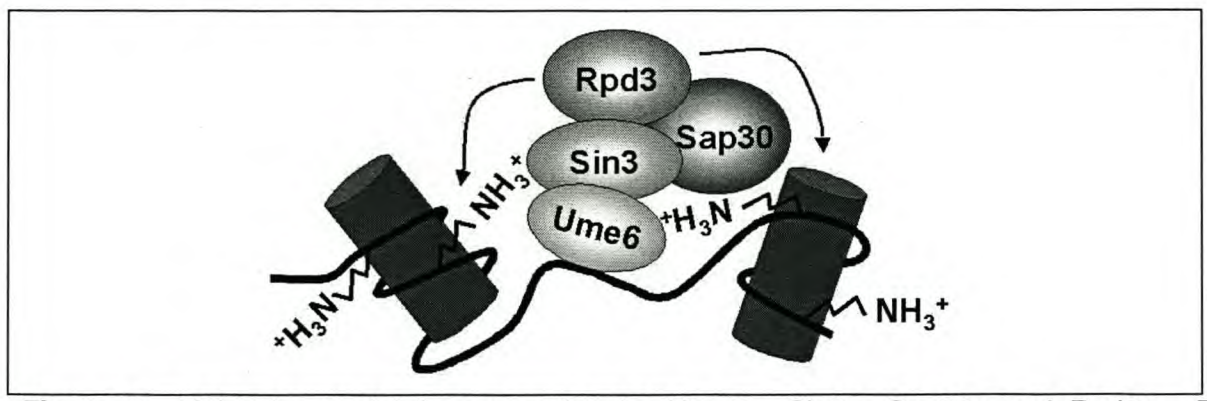

Figure 9 Molecular model for repression by Ume6p, Sin3p, Sap30p and Rpd3p. The repressive complex binds to the DNA and alters the conformation of the chromatin, making it inaccessible to activator molecules.

Chromatin repression must be alleviated in nutritional conditions that induce meiosis. Gcn5p is a histone H3 acetylase and adds an acyl group on histone H3, making the chromatin more accessible to the transcriptional complex. Burgess *et al.* (1999) reported that the transcription of *IME2* is preceded by an increase in H3 acetylation, indicating that chromatin is made accessible. Once the chromatin is accessible, Ime1p and Rim11p interact with Ume6p to create the activation complex (Rubin-Bejerano *et al.*, 1996).

In yeasts carrying null mutations of *UME6* and which are grown in non-meiotic conditions, the repression of meiotic genes is alleviated, while *IME1* is expressed at low levels (Steber and Esposito, 1995). Ume6p is not known to bind to the *IME1* promoter, so it is unknown how *ume6* deletion affects *IME1* expression. Diploid yeasts, grown in sporulation medium and carrying a *UME6* null mutation sporulate very poorly, as the meiotic regulatory proteins, such as Ime2p and Spo1p, are not expressed sufficiently to reach the critical levels required (Steber and Esposito, 1995). It is interesting that, in these mutant yeasts, Ime1p and Rim11p are still required for meiosis. This suggests that Ime1p and Rim11p also function in an Ume6p-independent pathway during meiosis (Malathi *et al.*, 1999). If *IME1* is not repressed after meiosis has been initiated and continues to be expressed, it interferes with the completion of meiosis and prevents the formation of viable spores. Thus, Ume6p is vital to prevent meiosis from occurring until the environmental conditions demand it, and it is responsible to re-establishing the repression of meiotic genes as their respective functions are completed during meiosis (Steber and Esposito, 1995).

2.8.3.1 Regulatory functioning of Ume6p

Malathi *et al.* (1997) showed that Rim11p and the N-terminal region of Ume6p are capable of interaction. The Ume6p N-terminal, specifically residues 99 to 109, serves as an Rim11p phosphorylation site, which is required for interaction with Ime1p (Rubin-Bejerano *et al.*, 1996). Phosphorylation of Ume6p is required for interaction with Ime1p (Lamb and Mitchell 2001). Interestingly, both Ume6p and Rim11p are able to bind to Ime1p – suggesting a trimeric Ume6p-Ime1p-Rim11p complex. Two hybrid assays showed interaction between Ume6p and Rim11p in a Δ*ime1* strain, indicating that their binding is independent of a functional Ime1 protein. Malathi *et al.* (1997) showed that a functional Rim11p protein kinase is required to form the Ume6p-Ime1p complex and that the interaction between Rim11p and Ume6p is necessary to form the Ume6p-Ime1p complex. Whether the interaction between Rim11p and Ime1p is required for the Ume6p-Ime1p complex is not clear - it is more plausible that Rim11p stimulates transcriptional activation or does so indirectly by causing the dimerisation of Ime1p (Malathi *et al.*, 1997). Thus, Rim11p is required for the transcriptional activation activity of both Ume6p and Ime1p by binding to them directly and Rim11p is vital for converting Ume6p from being a repressor to being an activator (Rubin-Bejerano *et al.*, 1996).

2.8.4 UME1 – UME5

These five genes encode trans-acting regulatory proteins that are involved in the regulation of the transcription of later meiotic genes, such as *SPO11*, *SPO13* and *SPO16*. Ume1p - Ume5p act downstream or independently of Ime1p and Ime2p (Strich *et al.*, 1989).

2.8.5 IME2

Ime2p functions as a meiosis-specific kinase. Sequence analysis revealed that Ime2p is similar to Cdc28p (Dirick *et al.*, 1998). It has also been shown that the temperature-sensitive CDK mutation, *cdc28-4*, which blocks the mitotic S-phase, has no effect on the meiotic S-phase, indicating that Cdc28p plays no role during meiosis and that Ime2p functions as Cdc28p would during meiosis.

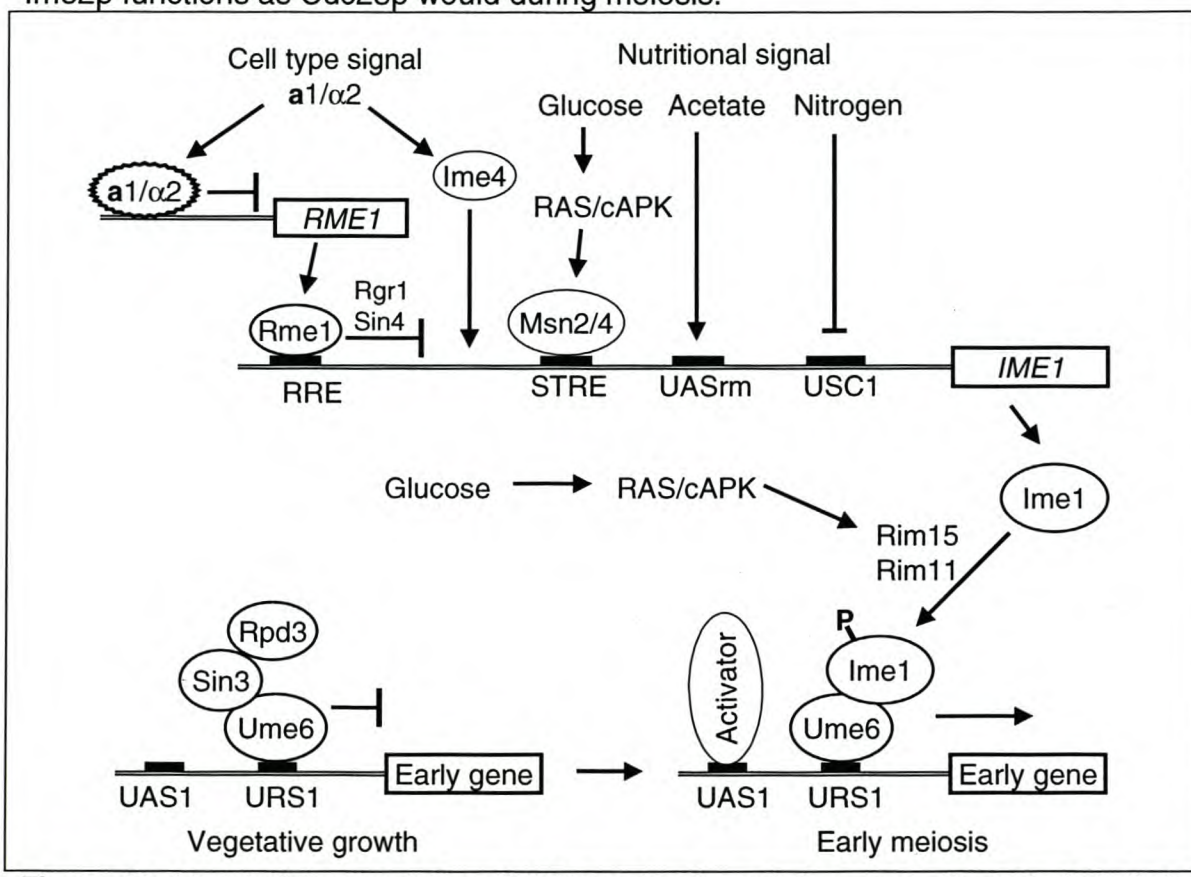

Figure 10 Model of the regulation of *IME1* and early meiotic genes. As meiosis is limited to **a**/α-diploid yeast, the cell-type signal is vital for the regulation of *IME1*. In a diploid, the **a**1/α2-heterodimer prevents the expression of *RME1* and induces *IME1* expression via Ime4p. In haploid yeast, *RME1* is expressed and Rme1p binds to RRE in the promoter of *IME1* and, in conjunction with the co-repressors, Rgr1p and Sin4p, prevents the expression of *IME1*. Low levels of glucose activate the RAS-cAPK pathway to promote the binding of Msn2p and Msn4p to the STRE element and to activate transcription of *IME1*. Acetate signals the UASrm and induces transcription, whereas high levels of nitrogen have a repressive effect on *IME1* transcription through UCS1. Once *IME1* is expressed, Ime1p has to be phosphorylated by Rim11p before it can interact with URS-bound Ume6p. This interaction changes the repressor to an activator and induces the expression of early meiotic genes. In non-meiotic conditions, Ume6p is also bound to the URS, but it recruits the Sin3p-Rpd3 histone deacetylation complex.

It is not clear how Ime2p facilitates G_1-S transition during meiosis. Sic1p is an inhibitor of meiosis and prevents meiosis by blocking entry into the meiotic S-phase. In wild type yeast strains, Sic1p is lost at the time of entry into the S-phase, but in yeast strains deleted of *ime2*, Sic1p is not lost and remains bound to the promoter sequences of the meiotic genes, preventing meiosis. Ime2p therefore may mediate

the destruction or removal of the Sic1p inhibitor to allow entry into the meiotic S-phase (Dirick *et al.*, 1998).

Furthermore, it appears that Ime2p is required to re-establish the repression of early meiotic genes as the cell progresses into sporulation. Ime1p is able to induce *IME2* expression. Ime2p also enhances its own expression and acts on *IME1* expression in a negative feedback loop. It is unknown how Ime2p re-establishes repression or whether the negative feedback loop plays a role. Ime2p thus ensures the balanced expression of all early meiotic genes (Bowdish and Mitchell, 1993; Mitchell *et al.*, 1990; Shah and Clancy, 1992).

2.9 CONCLUSION

Meiosis is a very complex and precisely regulated process. More than 500 genes are induced during meiosis, while an almost equal number of genes are repressed during the sporulation programme. Many of the meiotic regulatory proteins function antagonistically, such as Rme1p which represses *IME1* transcription, while Ime4p activates the expression of *IME1*.

To facilitate the level of control required by meiosis, the primary initiator of meiosis, *IME1*, is regulated by one of the largest identified promoters in yeast. All the different signal transduction pathways that induce or repress meiosis converge on this promoter. Although the *IME1* promoter contains several regulatory regions that respond to all the different transduced signals, only two proteins have been shown to bind to the *IME1* promoter directly, namely Rme1p and Yhp1p. Only some of the contributory proteins that act in the signal transduction pathway are known. The regulation of *IME1* expression is clearly far from understood. Techniques like microarray analysis will provide and have provided a wealth of information on the proteins expressed during meiosis. This will help to understand the regulatory network governing entry into meiosis.

Although a fair amount of data have been generated on the regulation of meiosis, the function of many genes, proteins and regulatory elements is still poorly understood or unknown. Several pathways also contain unknown components and will require further study.

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CHAPTER 3

RESEARCH RESULTS

Cellular differentiation in response to nutrient availability: The repressor of meiosis, *RME1*, positively regulates invasive growth in *Saccharomyces cerevisiae*

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RESEARCH RESULTS

3.1 CONTRIBUTION OF AUTHOR TO THE ARTICLE

A screen of a multiple copy genomic library for DNA fragments that can induce starch degradation in a *STA10* yeast strain resulted in the isolation of three different plasmids containing unknown DNA fragments. It was later discovered that the *STA10* yeast strains are unable to utilise starch as sole carbon source, due to the absence of the transcriptional activator *FLO8*. Previous studies at the laboratory examined two of the fragments obtained from the screen and identified the genes responsible for overcoming the inability to utilise starch as *MSN1/MSS10* and *MSS11* (Lambrechts *et al.*, 1996; Webber *et al.*, 1997). The initial goal of this study was to identify the gene/s contained in the third fragment that was responsible for the phenotype.

With the use of Southern Blot, the possibility that the third fragment contained either *MSN1* or *MSS11* firstly was eliminated. Consequently, a small piece of the fragment was sub-cloned and sequenced. The DNA sequence obtained was compared to the known DNA sequence of *Saccharomyces cerevisiae*. This indicated that the fragment from the genomic library was located on the right arm of chromosome 7. Next, a restriction enzyme digestion analysis was conducted, which indicated that the fragment was about 12 kb in size and contained seven open reading frames (ORFs). These seven ORFs were sub-cloned and screened in starch utilisation assays. The growth on starch phenotype was attributed to the gene *RME1*.

It has been demonstrated that *MSN1* and *MSS11* induce starch degradation as well as invasive growth. To ascertain whether *RME1* can also induce invasive growth, multiple copy plasmids containing *RME1* with different marker genes were constructed. A yeast strain with *RME1* deletion also was constructed. In this study, it was shown that overexpression of *RME1* induces invasive growth, whereas the deletion of *RME1* reduces invasive growth in otherwise wild type strains. These results were confirmed in the yeast strains ∑1278b and ISP15.

The role of *RME1* in relation to the MAP kinase cascade was investigated next. *RME1* was overexpressed in the wild type yeast strain, as well as in yeast containing deletions of *STE7*, *STE11*, *STE12* and *STE20*. These were compared to the control plasmids in the invasive growth plate assays. The results demonstrated that *RME1* functions independently of the MAP kinase cascade to induce invasive growth.

The next set of experiments conducted was a genetic analysis between *RME1* and the transcription factors *MSS11*, *PHD1*, *MSN1/MSS10*, *FLO8* and *TEC1*. Plate assays were conducted on the four different growth media: dextrose media, SCD; glucose-derepressed media, SCGE; limited nitrogen media, SLAD; and starch medium, SCS, to acquire different nutritional signals. *RME1* overexpression was compared to yeast carrying deletions of the above-mentioned factors. The inverse experiments, in which

the effect of overexpression of the above-mentioned factors in a yeast strain deleted of *RME1* was examined, were also conducted. These were compared to the overexpression of the transcription factors in wild type yeast strains. The plate assays were also conducted on the four different growth media.

As Rme1p is known to bind to the promoter of the cell cyclin *CLN2* and to induce its expression, it was necessary to determine the functional relationship between the cell cyclins and the ability of Rme1p to induce invasive growth and starch degradation. For this purpose, *CLN1*, *CLN2* and *CLN1-CLN2* double deletion strains were constructed in the yeast ISP15. *RME1* was overexpressed in the cyclin-deleted yeast strains to assess the effect on invasion. The assays were conducted on the four different growth media. The factors *MSS11*, *PHD1*, *MSN1/MSS10*, *FLO8* and *TEC1* were also included in the study.

3.1.1 REFERENCE LIST

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Cellular differentiation in response to nutrient availability: The repressor of meiosis, *RME1*, positively regulates invasive growth in *Saccharomyces cerevisiae*

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3.2 ABSTRACT

In *Saccharomyces cerevisiae*, limited availability of essential nutrients can result in invasive growth and/or the formation of pseudohyphae. Complete depletion of these same nutrients, on the other hand, may lead to meiosis and spore formation in diploid strains, whereas haploid cells will enter a non-budding, metabolically quiescent state. Rme1p is a three zinc finger-containing DNA-binding protein that inhibits entry into meiosis by repressing the transcription of *IME1*, a gene that is pivotal to the induction of early meiosis-specific genes. In this paper, we present evidence that Rme1p acts as a central genetic switch between invasive growth and sporulation. The protein positively regulates invasive growth and starch metabolism in both haploid and diploid strains, while repressing meiosis. Rme1p induces invasive growth and starch degradation by directly activating the transcription of the *FLO11* gene, which encodes a cell wall-associated protein that is essential for the formation of pseudohyphae and invasive growth, and of the glucoamylase-encoding *STA2* gene, which is required for starch degradation. The data suggest that Rme1p functions independently of identified signalling modules, as well as of several other transcription factors that regulate *FLO11*. We also present evidence that Rme1p does not require the presence of G_1 cyclins. Finally, we show that Rme1p acts via a specific sequence in the promoter of *FLO11* that shows homology to previously identified Rme1p response elements (RREs).

3.3 INTRODUCTION

For most unicellular organisms, the availability of nutrients in the immediate surroundings is a central environmental factor determining the choice of a specific developmental pathway. In the yeast *Saccharomyces cerevisiae*, nutrient-rich environments support the rapid growth and multiplication of single cells, leading to an exponential increase in cell numbers. When essential nutrients, particularly nitrogen and carbon sources, become limiting or cannot be efficiently utilized, both haploid and

diploid cells can undergo a morphological differentiation process, switching from an ovoid to an elongated cell shape to form pseudohyphae and/or grow invasively into the substrate (1, 2, 3). Upon complete depletion of any of several essential nutrients, haploid yeast arrests in the G_1 phase of the cell cycle and enters a quiescent phase referred to as G_0. Diploid yeast, on the other hand, can initiate meiosis to form ascospores (4). In addition, entry into meiosis requires the absence of glucose and the presence of a non-fermentable carbon source.

The meiotic process is tightly regulated and several transcriptional regulators play key roles in controlling the sequential expression of sets of genes (5). Rme1p (Regulator of Meiosis) is a three zinc finger motive-containing protein that inhibits the onset of meiosis by repressing the transcription of the *IME1* gene, which is pivotal to the induction of early meiosis-specific genes (6, 7). Rme1p binds directly to two binding sites, RREs, within the *IME1* promoter (7, 8). In addition to repressing *IME1*, Rme1p positively regulates the *CLN2* gene (9, 10), which encodes a G_1 cyclin and controls cell cycle progression through the initializing phase of a new cell division cycle (11). Thus, Rme1p appears to be able to promote mitosis by inducing *CLN2* transcription and to prevent meiosis by repressing *IME1* (9). It has been suggested that repression and activation by Rme1p are the result of the exclusion of other factors from the promoter, and that this exclusion can occur at large distances from the RRE (12, 13). Some evidence also suggests that this Rme1p-dependent exclusion may be linked to chromatin-dependent regulation (8).

The expression of *RME1* is repressed 10- to 20-fold in diploid strains by the MAT**a**/α heterodimeric repressor (14). In haploid yeast starved of nutrients, the expression of *RME1* is induced to ensure that haploids cannot initiate meiosis (12). Expression in both haploid and diploid strains is also cell cycle dependent, increasing at the M/G1 boundary of the cell cycle (10, 14).

Invasive and pseudohyphal growth are controlled by a network of signaling modules and transcription factors that respond to the limited availability of nutrients. Signaling modules include the nutrient-dependent MAP kinase cascade (15, 16, 17) and the cAMP-PKA pathway (18, 19, 20). Some evidence also implicates the G_1 cyclins in the regulation of this cellular adaptation (21, 22). Deletions of *CLN1* and/or *CLN2* result in a decrease in invasive growth, with the deletion of *CLN2* leading to a less severe reduction. All of the signaling pathways appear to converge on the large promoter of the *FLO11* gene, the expression of which is essential for invasive growth and pseudohyphal differentiation to occur (23, 24, 25, 26). *FLO11* encodes a GPI-anchored cell wall protein and is co-regulated with the *STA2* gene, which encodes a starch-degrading glucoamylase (25, 27).

Here we show that *RME1* acts as a central switch between nutrient-induced cellular differentiation pathways. The data demonstrate that Rme1p activates invasive growth and starch degradation in haploid and diploid cells by directly inducing *FLO11* and *STA2*. We furthermore show that the promoter of *FLO11* contains a functional

RRE, and that mutations within this site render Rme1p incapable of exerting its effect. The activity of Rme1p appears independent of the identified signaling pathways that regulate invasive growth, including the cAMP-PKA pathway, the nutrient-sensing MAP kinase cascade and the G_1 cyclins.

3.4 MATERIALS AND METHODS

3.4.1 YEAST STRAINS AND CULTURE COMPOSITION

All yeast strains are listed in Table 1. Media contained either 2% starch (SCS), 3% glycerol and 3% ethanol (SCGE), 2% glucose (SCD and SLAD), or 0.1% glucose (SCLD X-gal) as carbon source. The SCS, SCGE, SCD and SCLD X-gal media contained 0.67% yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI), and the SLAD medium contained 50 µM ammonium sulfate as sole nitrogen source and 0.67% YNB without ammonium sulfate and amino acids (Difco Laboratories, Detroit, MI). The SCLD X-gal medium was prepared according to Ausubel *et al.* (28).

3.4.2 PLASMID CONSTRUCTION AND RECOMBINANT DNA TECHNIQUES

All plasmids, constructs and primers are listed in Tables 2 and 3. New constructs were created as follows: (i) 1 622 bp *Hpa*I-*Sph*I fragment containing *RME1* from genomic library plasmid YEp24-MSS12, into *Hpa*I-*Sph*I sites of YEplac112, YEplac181 and YEplac195 (29), (ii) pΔgpa2, a 1 774 bp *Spe*I-*Nru*I fragment from pUC118-GPA2 (Joris Winderickx, Katholieke Universiteit Leuven, Belgium), was replaced with *Sma*I-*Nhe*I *LEU2* of pJJ252 (30), (iii) YEpLac112-PHD1 and pΔphd1, a 2 792 bp *PHD1* PCR fragment digested with *Bam*HI-*Hin*dIII and cloned into the corresponding sites of YEplac112, a 2 214 bp *Xba*I-*Bgl*II fragment of YEplac112-PHD1 was replaced with *Xba*I-*Bam*HI *LEU2* of pJJ252, (iv) pΔras2, a 428 bp *Bal*I-*Pst*I fragment of YCplac22-RAS2 (25), was replaced with *Sma*I-*Pst*I *LEU2* from YDp-L (31), and (v) pΔtec1, a PCR-amplified *TEC1* fragment containing primer-generated *Eco*RI sites, into *Eco*RI digested pSPORT1 (Invitrogen Life Technologies) and YEplac112, and the resulting pSPORT-TEC1 was digested with *Xba*I, blunt-ended and redigested with *Nhe*I, to replace 975 bp of *TEC1* ORF with *Sma*I-*Nhe*I *LEU2* of YDp-L. Δ*cln1*::*HIS3* and Δ*cln2*::*LEU2* disruption constructs were supplied by B. Futcher. A Δ*rme1*::*URA3* disruption cassette was generated using RME1-DISR-F and RME1-DISR-R. Both primers consisted of 48 nucleotides homologous to upstream and downstream sequences of the *RME1* ORF and 20 nucleotides homologous to *URA3.* The construction of the other disruption cassettes is described in Gagiano *et al.* (25,27). Reporter cassettes were constructed to monitor *FLO11* and *STA2* expression. P_{FLO11}-*lacZ* and P_{STA2}-*lacZ* were isolated from pPMUC1-lacZ and pPSTA2-lacZ (27) as *Xba*I-*Nco*I fragments, with 461 nucleotides of the respective promoters fused to *lacZ*, and ligated to *Spe*I-*Nco*I-digested pGEM®-T (Promega Corporation, Madison, WI). The resulting constructs were digested with *Nco*I, blunt-ended and ligated to a blunt-ended

HIS3 fragment obtained from YDp-H (31) digested with *Bam*HI. The integration cassettes were PCR amplified with Fp-PMUC1$_{BstEII}$, which binds ~430 bp upstream of *FLO11*/*STA2* ATGs, in combination with Rp-PMUC1-lacZ-pGEM-T and Rp-PSTA2-lacZ-pGEM-T, consisting of 60 nucleotide *FLO11*- and *STA2*-specific sequences and 20 nucleotides of pGEM®-T situated immediately 3' of the reporter cassettes.

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

Strain	Relevant genotype	Source or reference
ISP15	*MAT***a** *his3 leu2 thr1 trp1 ura3 STA2*	This laboratory
ISP15Δ*cln1*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δcln1::HIS3*	This study
ISP15Δ*cln2*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δcln2::LEU2*	This study
ISP15Δ*flo8*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δflo8::URA3*	Ref. 27
ISP15Δ*gpa2*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δgpa2::LEU2*	This study
ISP15Δ*msn1*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δmsn1::URA3*	Ref. 25
ISP15Δ*mss11*	*MAT***a** *his3 thr1 trp1 ura3 STA2 Δmss11::LEU2*	Ref. 25
ISP15Δ*phd1*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δphd1::LEU2*	This study
ISP15Δ*ras2*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δras2::LEU2*	This study
ISP15Δ*rme1*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δrme1::URA3*	This study
ISP15Δ*ste12*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δste12::URA3*	Ref. 25
ISP15Δ*tec1*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δtec1::LEU2*	This study
ISP15Δ*flo11::lacZ*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δflo11::lacZ-HIS3*	This study
ISP15Δ*flo11::lacZ*Δ*rme1*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δflo11::lacZ-HIS3 Δrme1::URA3*	This study
ISP15Δ*flo11::lacZ*RREmu	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δflo11::lacZ-HIS3*	This study
ISP15Δ*sta2::lacZ*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δsta2::lacZ-HIS3*	This study
ISP15Δ*sta2::lacZ*Δ*flo8*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δsta2::lacZ-HIS3 Δflo8::LEU2*	This study
ISP15Δ*sta2::lacZ*Δ*gpa2*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δsta2::lacZ-HIS3 Δgpa2::URA3*	This study
ISP15Δ*sta2::lacZ*Δ*msn1*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δsta2::lacZ-HIS3 Δmsn1::URA3*	This study
ISP15Δ*sta2::lacZ*Δ*mss11*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δsta2::lacZ-HIS3 Δmss11::LEU2*	This study
ISP15Δ*sta2::lacZ*Δ*phd1*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δsta2::lacZ-HIS3 Δphd1::LEU2*	This study
ISP15Δ*sta2::lacZ*Δ*ras2*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δsta2::lacZ-HIS3 Δras2::LEU2*	This study
ISP15Δ*sta2::lacZ*Δ*rme1*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δsta2::lacZ-HIS3 Δrme1::URA3*	This study
ISP15Δ*sta2::lacZ*Δ*ste12*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δsta2::lacZ-HIS3 Δste12::URA3*	This study
ISP15Δ*sta2::lacZ*Δ*tec1*	*MAT***a** *his3 leu2 thr1 trp1 STA2 Δsta2::lacZ-HIS3 Δtec1::LEU2*	This study
L5366h	*MAT***a** *ura3*	P. Sudberry
L5624h	*ura3 Δste20*	P. Sudberry
L5625h	*ura3 Δste11*	P. Sudberry
L5626h	*ura3 Δste7*	P. Sudberry
L5627h	*ura3 Δste12*	P. Sudberry
YHUM271	*MAT***a** *ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG*	H.-U. Mösch
Σ271Δ*rme1*	*MAT***a** *ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG Δrme1::LEU2*	This study
YHUM272	*MATα ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG*	H.-U. Mösch
Σ272Δ*flo11::lacZ*	*MATα ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG Δflo11::lacZ-HIS3*	This study
Σ272Δ*flo11::lacZ*RREmut	*MATα ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG Δflo11::lacZ-HIS3*	This study
Σ272Δ*flo11::lacZ*Δ*flo8*	*MATα ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG Δflo11::lacZ-HIS3 Δflo8::LEU2*	This study
Σ272Δ*flo11::lacZ*Δ*gpa2*	*MATα ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG Δflo11::lacZ-HIS3 Δgpa2::LEU2*	This study
Σ272Δ*flo11::lacZ*Δ*msn1*	*MATα ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG Δflo11::lacZ-HIS3 Δmsn1::URA3*	This study
Σ272Δ*flo11::lacZ*Δ*mss11*	*MATα ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG Δflo11::lacZ-HIS3 Δmss11::LEU2*	This study
Σ272Δ*flo11::lacZ*Δ*phd1*	*MATα ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG Δflo11::lacZ-HIS3 Δphd1::LEU2*	This study
Σ272Δ*flo11::lacZ*Δ*ras2*	*MATα ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG Δflo11::lacZ-HIS3 Δras2::LEU2*	This study
Σ272Δ*flo11::lacZ*Δ*rme1*	*MATα ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG Δflo11::lacZ-HIS3 Δrme1::URA3*	This study
Σ272Δ*flo11::lacZ*Δ*ste12*	*MATα ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG Δflo11::lacZ-HIS3 Δste12::URA3*	This study
Σ272Δ*flo11::lacZ*Δ*tec1*	*MATα ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG Δflo11::lacZ-HIS3 Δtec1::LEU2*	This study
2NΣΔ*flo11::lacZ*	*MAT***a**/*α ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG Δflo11::lacZ-HIS3/FLO11*	This study
2NΣΔ*flo11::lacZ*Δ*rme1*	*MAT***a**/*α ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG Δflo11::lacZ-HIS3/FLO11 Δrme1::URA3/Δrme1::URA3*	This study

Table 2. Plasmids used in this study.

Plasmid	Relevant genotype	Source or Reference
YEpLac112	2μ *TRP1*	Ref. 29
YEpLac181	2μ *LEU2*	Ref. 29
YEpLac195	2μ *URA3*	Ref. 29
YDp-L	*LEU2*	Ref. 31
YDp-U	*URA3*	Ref. 31
YDp-H	*HIS3*	Ref. 31
PJJ252	*LEU2*	Ref. 30
YEpLac112-FLO8	2μ *TRP1 FLO8*	Ref. 27
YEpLac181-FLO8	2μ *LEU2 FLO8*	Ref. 27
YEpLac112-MSN1	2μ *TRP1 MSN1*	Ref. 25
YEpLac112-MSS11	2μ *TRP1 MSS11*	Ref. 25
YEpLac112-PHD1	2μ *TRP1 PHD1*	This study
YEpLac112-RME1	2μ *TRP1 RME1*	This study
YEpLac181-RME1	2μ *LEU2 RME1*	This study
YEpLac195-RME1	2μ *URA3 RME1*	This study
YEpLac112-STE12	2μ *TRP3 STE12*	Ref. 25
YEpLac112-TEC1	2μ *TRP3 TEC1*	This study
YEp24-MSS12	2μ *URA3* genomic library fragment	This laboratory
YCpLac22-RAS2	*CEN4 TRP1 RAS2*	Ref. 25
YCpLac22-RAS2^{val19}	*CEN4 TRP1 RAS2*^{val19}	Ref. 25
pSPORT1		Invitrogen Life Technologies
pSPORT-TEC1	2 055 bp TEC1 fragment in pSPORT1	This study
pGEM®-T		Promega Corporation
pGEM-T-PMUC1-lacZ-HIS3	430 nucleotides of P_{FLO11} fused to lacZ *HIS3*	This study
pGEM-T-PSTA2-lacZ-HIS3	430 nucleotides of P_{STA2} fused to lacZ *HIS3*	This study
pΔcln1	Δ*cln1*::*HIS3*	B. Futcher
pΔcln2	Δ*cln2*::*LEU2*	B. Futcher
pΔflo8	Δ*flo8*::*URA3*	Ref. 27
pΔgpa2	Δ*gpa2*::*LEU2*	This study
pΔmsn1	Δ*msn1*::*URA3*	Ref. 25
pΔmss11	Δ*mss11*::*LEU2*	Ref. 25
pΔphd1	Δ*phd1*::*LEU2*	This study
pΔras2	Δ*ras2*::*LEU2*	This study
pΔste12	Δ*ste12*::*URA3*	Ref. 25
pΔtec1	Δ*tec1*::*LEU2*	This study

3.4.3 YEAST STRAIN CONSTRUCTION

PCR-amplified P_{FLO11}-*lacZ* and P_{STA2}-*lacZ* integration cassettes were transformed into ISP15 and YHUM272 (H.- U. Mösch) to generate ISP15Δ*flo11*::*lacZ*, ISP15Δ*sta2*::*lacZ* and Σ272Δ*flo11*::*lacZ*. Integration was confirmed through Southern blot analysis and subsequently sequenced. All additional gene disruptions were obtained through the one-step gene replacement method (28) in wild type ISP15, in YHUM271, and in the *lacZ* reporter strains. The strains Σ272Δ*flo11*::*lacZ* and Σ272Δ*flo11*::*lacZ*Δ*rme1* were crossed with YHUM271 and Σ271Δ*rme1* to generate the two diploid strains, 2NΣΔ*flo11*::*lacz* and 2NΣΔ*flo11*::*lacz* Δ*rme1*, respectively.

3.4.4 SITE-DIRECTED MUTAGENESIS

The genomic DNA of ISP15Δ*flo11*::*lacZ* and Σ272Δ*flo11*::*lacZ* served as templates for the site-directed mutagenesis of the putative RRE. Primer PMUC1FpRREmut was used to convert GTACCACAAAA to ATATTATAAAA. RRE mutagenesis and the subsequent PCR amplification of the P_{FLO11}-*lacZ-HIS3* cassettes were achieved with primers Fp-PMUC1-RREmut and Rp-MUC1 (+4.0 kb). The mutated *lacZ* reporter cassettes were reintroduced into wild type ISP15 and YHUM272 to generate ISP15Δ*flo11*::*lacZ*RREmut and Σ272Δ*flo11*::*lacZ*RREmut. The desired nucleotide changes were confirmed through sequence analysis.

Table 3. Primers used in this study.

Name	Sequence
Fp-CLN1	5'-CCATAGCATGGAACTTGCCG-3'
Rp-CLN1	5'-CGGTCCCGTGAACACTTGAT-3'
Fp-CLN2	5'-CCTCCGCACTTTTACCCTGA-3'
Rp-CLN2	5'-TTCGCCGGTTGAGTGTATCG-3'
Fp-FLO8	5'-CTTTCCCACCCAATCTTAGGCACCT-3'
Rp-FLO8	5'-CCGGAACAAACCTTTAGCAATTGCG-3'
Fp-GPA2	5'-AGGCTAAGGAAACGGGTAAC-3'
Rp-GPA2	5'-TTGTCTCTTTCTTGGGTGGC-3'
Fp-MSN1	5'-CACCTACAAAGCGTTGATGG-3'
Rp-MSN1	5'-GTTGTTGGCTGACTTCTGAG-3'
Fp-MSS11	5'-GATGCCATAACCGACTAGAC-3'
Rp-MSS11	5'-ACAGGGCGCAATCAGCTACC-3'
Fp-PHD1	5'-GGCCTATCCACGCCAATTTA-3'
Rp-PHD1	5'-TCGAGCTTTGAGCGCAGAGT-3'
Fp-RAS2	5'-AGTGGGTGGTGTGGCTAATC-3'
Rp-RAS2	5'-CATCGTCGTCTTCTTCCTCG-3'
Fp-RME1	5'-GTTTGGACAGGGATAGTGGGT-3'
Rp-RME1	5'-CGTGGTGCCATATTCACGACA-3'
Fp-STE12	5'-CACAGCATTTCTTTTCGGAG-3'
Rp-STE12	5'-AATCTCGCTTTTTCTGGTGG-3'
Fp-TEC1	5'-CCGGAATTCAAACAAGCTGAGCTGGACTCC-3'
Rp-TEC1	5'-CCGGAATTCGCATGGCGCTAGAGAACTTTC-3'
Fp-PMUC1$_{BstEII}$	5'-TCCGTTCTCTTCTGATGAGGTAACC-3'
Rp-PMUC1-lacZ-pGEM-T	5'-AATAACCCATGATATCTAGGCACATTAAGGTTAGCGTGGG GGGACGCGAATAATATAAGCGCCAGGGTTTTCCCAGTCAC-3'
Rp-PSTA2-lacZ-pGEM-T	5'-TGGCAACAAGTTGACACAGGATGAGAAAGTGAAAAGAACT GCAAACGTGGTTGGGCTGGAGCCAGGGTTTTCCCAGTCAC-3'
RME1-DISR-F	5'-GTGTCAACGCATTGGAACTGACATTGTTCTTATCCTATAAGT CATACAGGCCTGACTGCGTTAGCAATT-3'
RME1-DISR-R	5'-GAGTTTCATGGGGTACATTTTTAATGCCTCAACTATTTGGTA TTGTTCCCGTGGAATTCTCATGTTTG-3'
Fp-PMUC1-RREmut	5'-GGTATGGAGTTTTATATTATAAAACTTTAGGAATACCGGATT GTGTGCCT-3'
Rp-MUC1 (+4.0 kb)	5'-GCGACTGCAGAACCAGAAGC-3'

3.4.5 INVASIVE GROWTH, STARCH UTILIZATION AND β-GALACTOSIDASE ASSAYS

The invasive growth, starch utilization and *β*-galactosidase assays were performed according to Gagiano *et al.* (27). All assays were conducted in triplicate on three independent transformants. The differences in *β*-galactosidase values between the different experiments never exceeded 10%. Data represent the average of three independent experiments.

3.4.6 SPORULATION ASSAYS

Cells ($2x10^8$) from liquid SCD ($O.D._{600}$ of 1) were washed and dropped onto sporulation plates (28). After three, six and nine days, the colonies were resuspended in 200 µl of sterile water. Cell suspensions (10 µl) of three different colonies of each strain were analyzed under the 40X magnification of a Nikon Optiphot-2 light microscope. At least 600 cells per strain were counted and the ratio between sporulating and non-sporulating cells was determined. The difference between colonies of the same strain was always below 3%.

3.5 RESULTS

3.5.1 RME1P INDUCES INVASIVE GROWTH AND STARCH DEGRADATION BY REGULATING THE TRANSCRIPTION OF *FLO11* AND *STA2*

RME1 was isolated from a 2µ-based *S. cerevisiae* genomic library, which was screened for genes that, when present in multiple copies, would enhance the ability of *STA2*-containing strains to grow on starch as sole carbon source, as described by Lambrechts *et al.* (23). As can be seen in Fig. 1*A*, multiple copies of *RME1* result in more efficient starch degradation and, as a consequence, in faster growth on starch-containing media, while the deletion of *RME1* leads to a significant decrease in starch utilisation. Since starch degradation and invasive growth are co-regulated phenotypes, we assessed whether *RME1* would also enhance invasive growth in a glucose-based, nitrogen-limited medium. Compared to the strain transformed with the 2µ plasmid without insert, the 2µ-*RME1* strain invaded the agar more effectively (Fig. 1*A* and *B*), whereas the Δ*rme1* mutant exhibited a reduced invasiveness in both conditions. No differences in growth were observed in the glucose-based media (data not shown).

We verified whether *RME1* requires the *FLO11* gene to induce invasion. Fig. 1*C* shows that 2µ-*RME1* was no longer able to induce invasive growth in the absence of an intact *FLO11* gene. To further assess whether *RME1* affects the transcription of *FLO11* and *STA2*, we replaced the ORFs of these genes with the *β*-galactosidase-encoding *lacZ* gene. Figs. 1*D* and 1*E* show that the presence of 2µ-*RME1* led to increased reporter gene activity in strains ISP15Δ*flo11*::*lacZ* and ISP15Δ*sta2*::*lacZ*. As

reported previously (25), the expression levels conferred by the *FLO11* promoter were always significantly lower than those conferred by the *STA2* promoter, and both genes showed lower expression in glucose (SCD) than in glycerol-ethanol (SCGE) media. However, 2μ-*RME1* activated both promoters, P_{FLO11} and P_{STA2}, to a similar extent (8- to 10-fold) in both conditions. The deletion of *RME1*, on the other hand, decreased the expression levels conferred by the promoters.

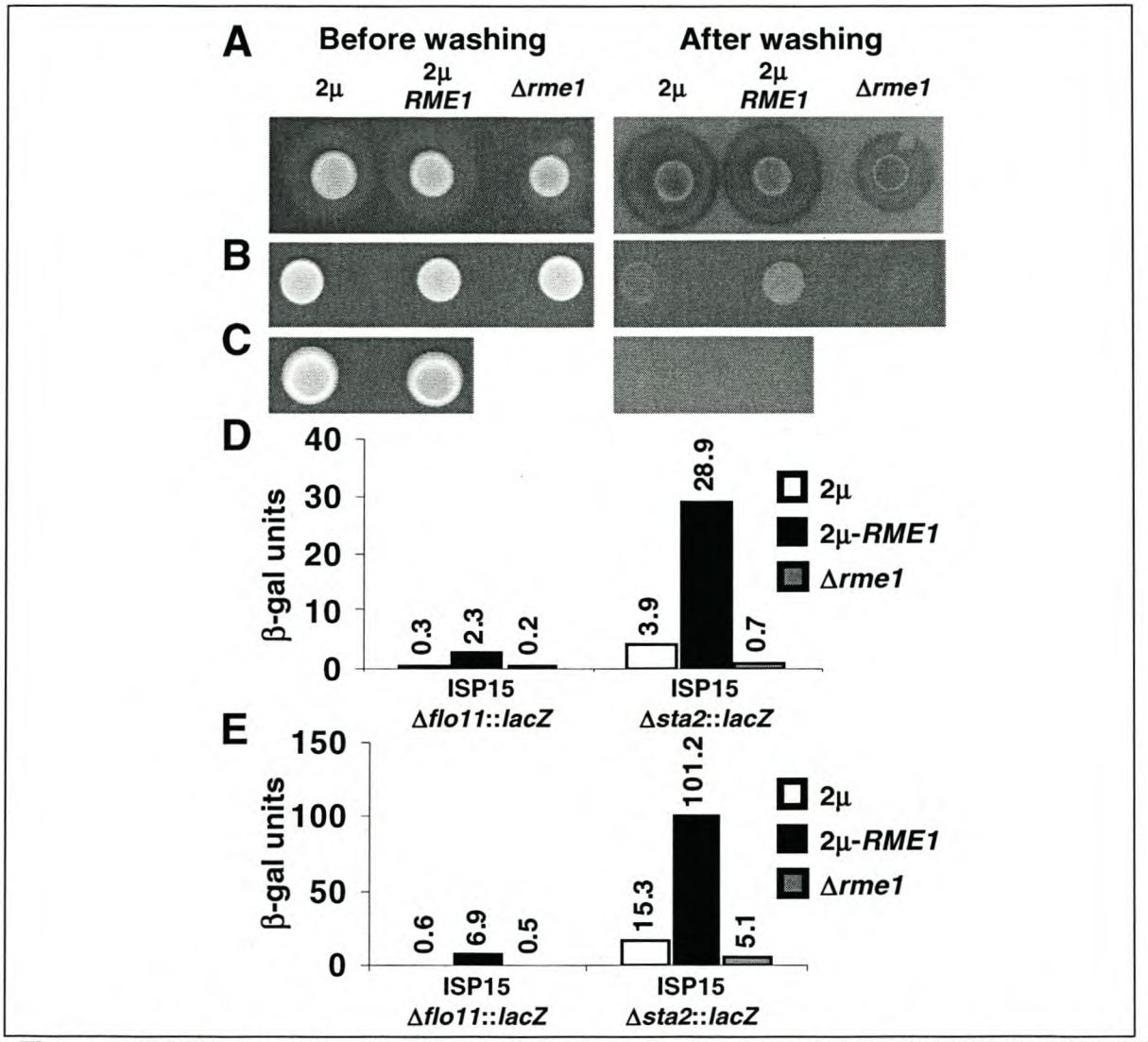
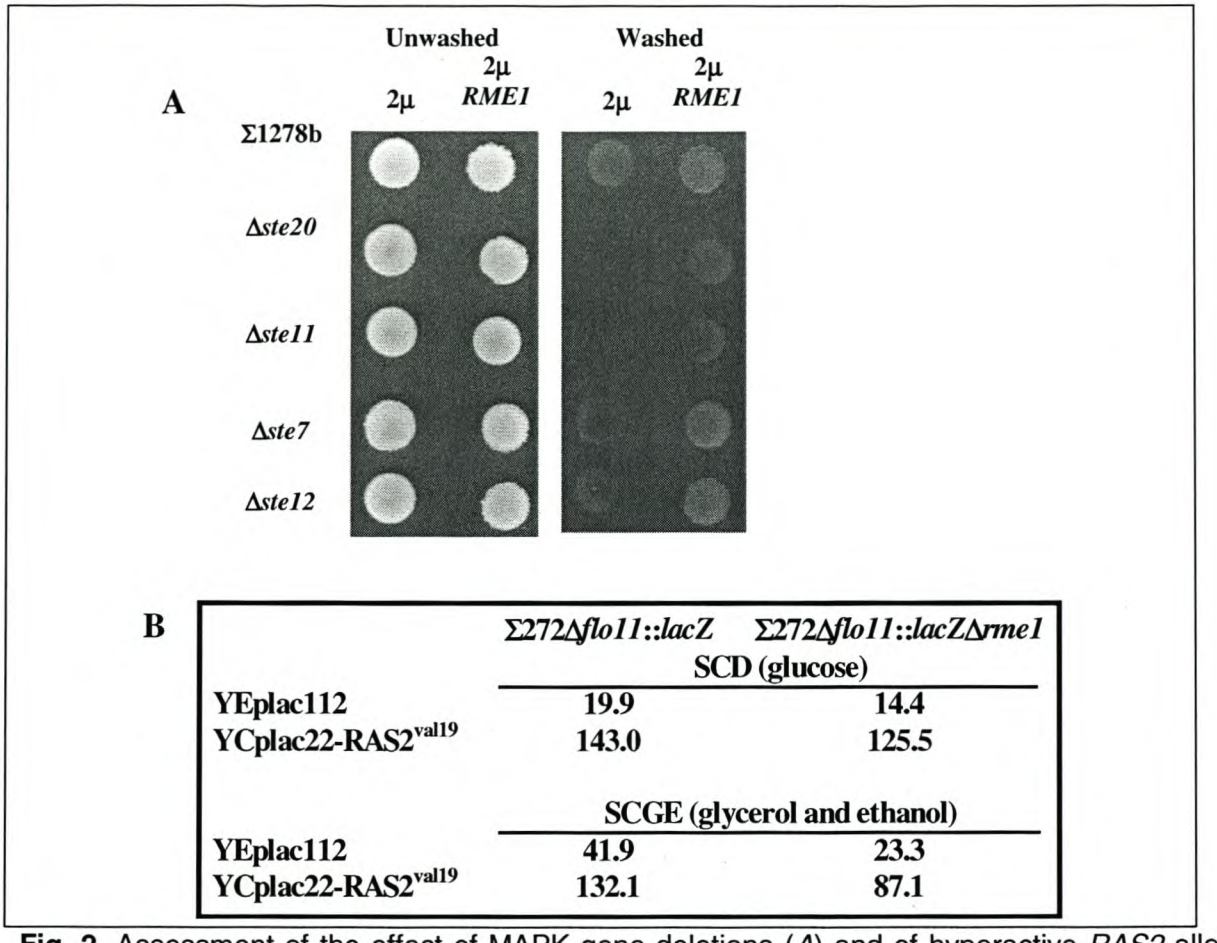


Fig. 1. *RME1* regulates invasive growth and starch degradation. (*A*) YEplac112-RME1 increases starch degradation and invasive growth on starch-containing SCS media, while Δ*rme1* shows a reduction in both phenotypes. The halos surrounding the colonies reflect Sta2p glucoamylase activity. (*B*) Invasive growth phenotypes on nitrogen-limited SLAD medium. (*C*) Induction of invasive growth by YEplac112-RME1 is blocked in Δ*flo11* strain (ISP15Δ*flo11*::*lacZ*) on SLAD. (*D*) and (*E*) *RME1* regulates *FLO11* and *STA2* expression in SCD (*D*) and SCGE (*E*) liquid cultures. The genomic ORFs of *FLO11* and *STA2* were replaced with *lacZ* in both wild type and Δ*rme1* strains. β-galactosidase activity in Miller units (28).



B

	Σ272Δflo11::lacZ	Σ272Δflo11::lacZΔrme1
	SCD (glucose)	
YEplac112	19.9	14.4
YCplac22-RAS2^{val19}	143.0	125.5
	SCGE (glycerol and ethanol)	
YEplac112	41.9	23.3
YCplac22-RAS2^{val19}	132.1	87.1

Fig. 2. Assessment of the effect of MAPK gene deletions (*A*) and of hyperactive *RAS2* allele (*B*). (*A*) L5366h (Σ1278b), L5624h (Δ*ste20*), L5625h (Δ*ste11*), L5626h (Δ*ste7*) and L5627h (Δ*ste12*) were transformed with YEplac195 and YEplac195-RME1 and grown on SLAD for five days at 30 °C before washing. (*B*) β-galactosidase units for Σ272Δ*flo11*::*lacZ* and Σ272Δ*flo11*::*lacZ*Δ*rme1* bearing YEplac112 and Ycplac22-RAS2^{val19} in liquid SCD and SCGE media.

Table 4. P_{FLO11}-*lacz* expression and induction ratios conferred by 2μ-*RME1* in Σ1278b mutants in SCD and SCGE liquid media.

Reporter strain	SCD 2μ	SCD 2μ-*RME1*	SCD Ratio	SCGE 2μ	SCGE 2μ-*RME1*	SCGE Ratio
Σ1278b	**14.9**	**46.2**	**3.1**	**34.8**	**60.8**	**1.7**
Δ*gpa2*	29.7	63.0	2.1	32.3	49	1.5
Δ*ras2*	50.2	161.5	3.2	20.2	37.1	1.8
Δ*flo8*	1.6	5.7	3.6	1.3	6.6	5.1
Δ*msn1*	2.3	12.7	5.6	7.7	24.4	3.2
Δ*mss11*	1.6	4.8	3.0	1.1	6.8	6.1
Δ*phd1*	21.9	42.9	2.0	38.6	56.4	1.5
Δ*ste12*	3.6	12.4	3.5	22.4	48.7	2.2
Δ*tec1*	3.7	13.4	3.6	26.7	39.7	1.5

3.5.2 RME1P ACTS INDEPENDENTLY OF SIGNALING MOLECULES THAT REGULATE INVASIVE GROWTH

We assessed whether the regulation of *FLO11* by *RME1* would be affected by the absence of signaling modules that regulate invasive growth. For this purpose, the 2μ-*RME1* plasmid was transformed into strains with deletions or mutations in genes that affect cAMP-dependent signaling (Δ*ras2*, Δ*gpa2*, *RAS2*^{val19}) or the nutrient-regulated MAP kinase cascade. The experiments were conducted in the haploid Σ1278b genetic background. The data presented in Table 4 show that 2μ-*RME1* had a similar effect on *FLO11* transcription in the Σ1278b background as in the ISP15 strain, with *FLO11* induced approximately three-fold. Fig. 2*A* shows that this induction correlated well with the increased invasiveness of the corresponding strain. Table 4 shows that the deletion of either *RAS2* or *GPA2* did not influence the effect of multiple copies of *RME1* on the transcription of *FLO11*. Indeed, while both deletions resulted in an increase in basal reporter gene activity in SCD and appeared not to affect (Δ*gpa2*) or to reduce (Δ*ras2*) expression in SCGE, the fold-induction conferred by the 2μ-*RME1* plasmid was always similar to the one observed in the wild type. The same was true in the reverse situation, when the effects of the hyperactive *RAS2*^{val19} mutation were assessed in both wild type and Δ*rme1* genetic backgrounds. The increase in transcription was identical in both strains (Fig. 2*B*).

Similarly, multiple copies of *RME1* were able to activate invasive growth in the absence of elements of the invasive growth-regulating MAP kinase cascade (Fig. 2*A*). As reported previously (16, 25), deletion of the different *STE* genes resulted in reduced invasive growth, with the strains Δ*ste20* and Δ*ste11* showing the severest phenotypes. Multiple copies of *RME1* were able to restore the invasive growth phenotype in all the mutants.

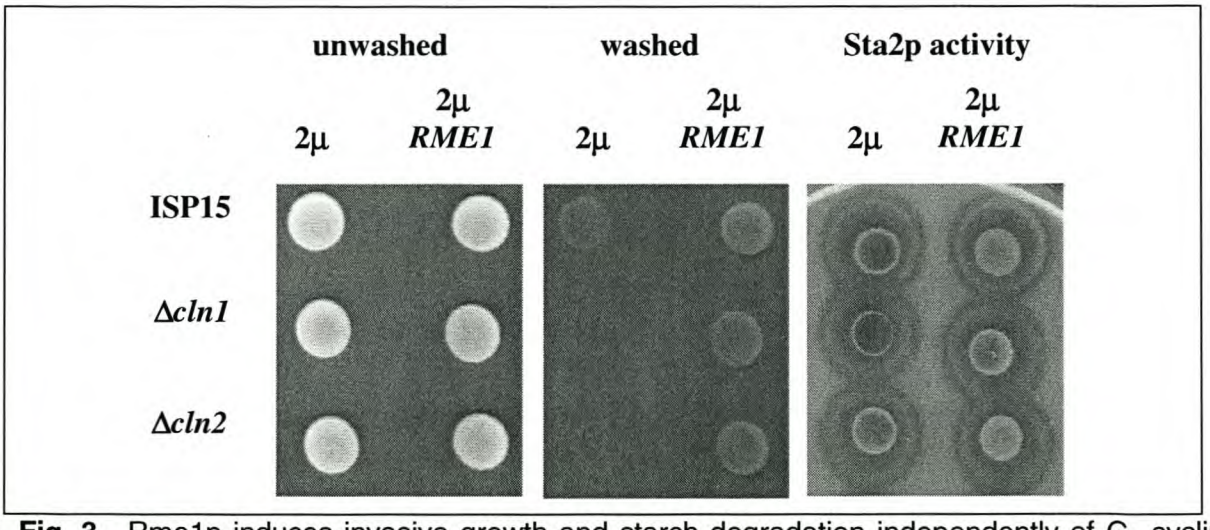


Fig. 3. Rme1p induces invasive growth and starch degradation independently of G_1 cyclins. ISP15 wild type, Δ*cln1* and Δ*cln2* were transformed with YEplac112 and YEplac112-RME1 and grown on SLAD and SCS (Sta2p activity).

3.5.3 RME1P INDUCES INVASIVE GROWTH AND STARCH DEGRADATION INDEPENDENTLY OF CLN1P AND CLN2P

Since Rme1p is known to control *CLN2* expression, and since G_1 cyclins regulate invasive growth, we investigated whether the effect of Rme1p on *FLO11* was dependent on the presence of Cln1p or Cln2p. For this purpose, we generated strains deleted for *CLN1*, *CLN2* or both. However, the Δ*cln1*/Δ*cln2* double mutant showed clear growth defects and was excluded from the analysis. In accordance with the results of Loeb *et al.* (22), the Δ*cln1* strain showed the severest defect, while the Δ*cln2* strain also displayed a clear reduction in invasive growth (Fig. 3). The presence of 2μ-*RME1* in both the Δ*cln1* and Δ*cln2* strains strongly enhanced the level of invasion.

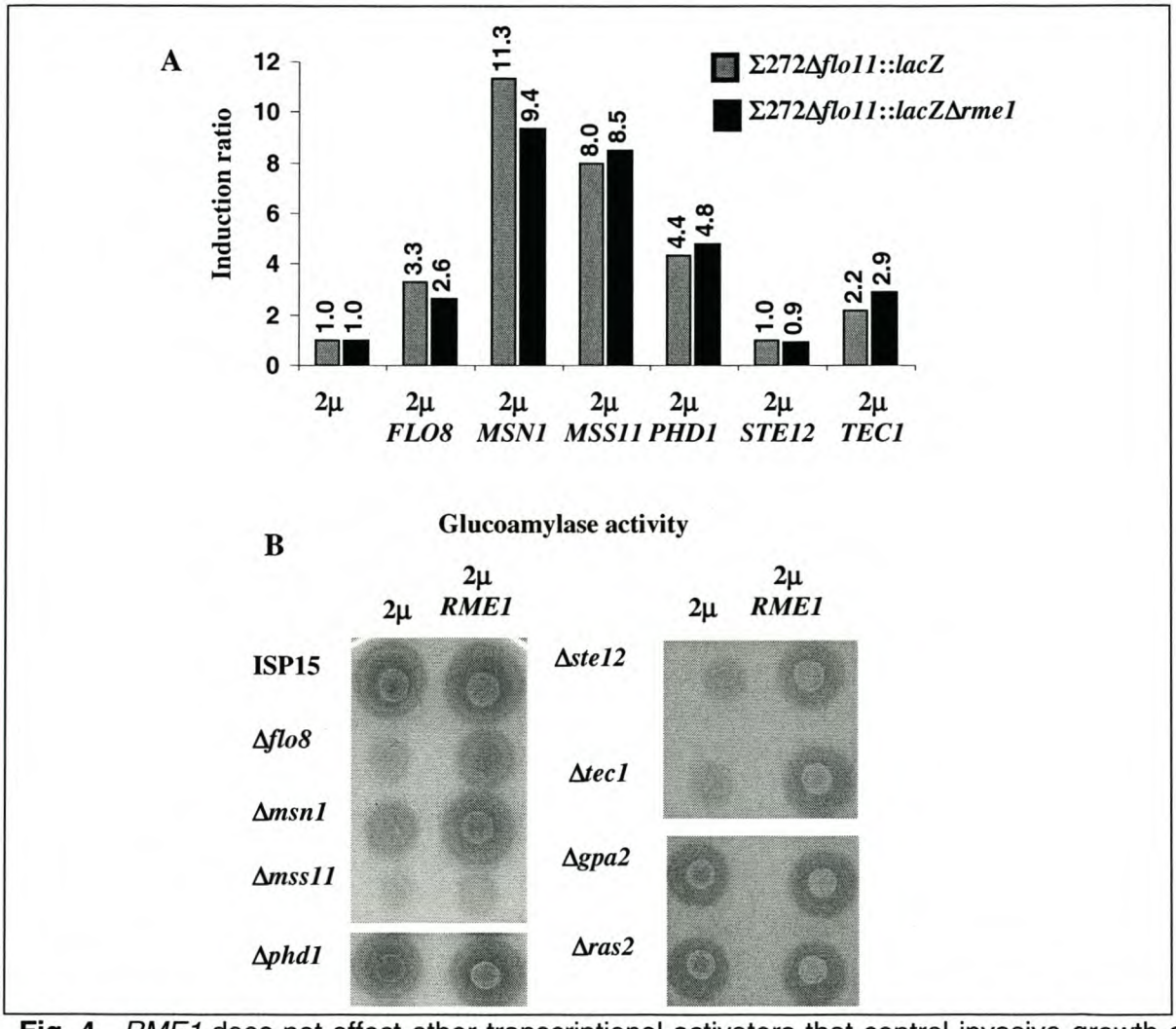


Fig. 4. *RME1* does not affect other transcriptional activators that control invasive growth and starch utilization. (*A*) Histogram of the induction ratios for Σ272Δ*flo11*::*lacZ* and Σ272Δ*flo11*::*lacZ*Δ*rme1* transformed with YEplac112 without insert, or with *FLO8*, *MSN1*, *MSS11*, *PHD1*, *STE12* and *TEC1*. The absolute SCD β-gal values were normalized to the YEplac112 control to obtain the induction ratios for every construct in each strain. (*B*) YEPlac112 and YEplac112-RME1 were introduced into wild-type ISP15 and isogenic strains with deleted copies of *FLO8*, *MSN1*, *MSS11*, *PHD1*, *STE12*, *TEC1*, *GPA2* and *RAS2*. Glucoamylase activity was tested on starch-containing SCS media.

When tested on starch-containing SCS plates, the deletion of the cyclin genes did not lead to changes in starch degradation, and the presence of 2µ-*RME1* resulted in similar increases in the wild type and the two cyclin-mutated strains.

3.5.4 RME1P DOES NOT REQUIRE OTHER TRANSCRIPTIONAL ACTIVATORS

Several transcription factors have been shown to activate *FLO11* expression (26, 27). To assess whether Rme1p would require the presence of these factors, we transformed 2µ-*RME1* into the Σ272Δ*flo11*::*lacZ* strain and the isogenic mutants Δ*flo8*, Δ*msn1*, Δ*mss11*, Δ*phd1*, Δ*ste12* and Δ*tec1* (Table 4). Basal levels of *lacZ* activity in the wild type grown in SCD were severely affected by deletions of *FLO8*, *MSN1*, *MSS11*, *STE12* and *TEC1*. Δ*phd1* did not appear to affect *FLO11* expression significantly, and even resulted in a 1.4-fold increase in reporter gene activity. In SCGE, however, only deletions of *FLO8*, *MSN1* and *MSS11* caused a significant decrease in *lacZ* expression, suggesting that *STE12* and *TEC1* may not be required to the same extent in glucose-derepressed conditions. However, under both conditions and in all the mutants, 2µ-*RME1* increased reporter gene activity significantly. As a general rule, the fold induction conferred by the plasmid was higher for strains with low basal transcriptional activity, and lower for strains with high initial activity. In the reverse situation, all 2µ plasmids carrying the genes of the different factors were able to activate transcription by the same induction factor in the wild type and Δ*rme1* strain (Fig. 4*A*). In all cases, the expression data also correlated well with invasive growth (data not shown).

We furthermore assessed whether starch degradation in ISP15 was similarly affected (Fig. 4*B*) by the deletions. When comparing the data in Table 4 with the starch degradation phenotypes, a good correlation between the two data sets, reflecting two different genetic backgrounds, can be observed.

Promoters with Rme1p Response Elements	Sequences
P_{FLO11} (-1 427 to -1 417)	GTACCACAAAA
P_{STA2} (-1 314 to -1 304)	GTACCACAAAA
P_{IME1} (-2 040 to -2 030)	GTACCTCAAGA
P_{IME1} (-1 959 to -1 949)	GTACCTCAAAA
P_{CLN2} (-683 to -673)	GAACCTCAGTA
P_{CLN2} (-563 to -553)	GAACCTCAAAA
RRE consensus	GWACCWCARDA
Mutated P_{FLO11} RRE	ATATTATAAAA

Fig. 5. Alignment of RREs in the promoters of *FLO11*, *STA2*, *IME1* and *CLN2* and consensus sequence. W = A or T; R = A or G; D = A or G or T.

3.5.5 RME1P INDUCES *FLO11* EXPRESSION VIA AN RME1P RESPONSE ELEMENT

Sequence analysis of P_{FLO11} and P_{STA2} revealed the presence of a putative RRE, GTACCACAAAA, at positions -1 427 and -1 314 respectively (Fig. 5). The only difference between this sequence and the previously identified RREs is a T to A substitution in position 6 of the consensus sequence in P_{FLO11} and $P_{STA\ 2}$. To assess the role of this sequence, we mutated the GTACCACAAAA to ATATTATAAAA, since the guanine and cytosine nucleotides had been shown to be required for Rme1p-DNA interaction (13, 32). Fig. 6*A* shows that 2µ-*RME1* was no longer able to properly activate the P_{FLO11} with the mutated RRE. In strain ISP15Δ*flo11*::*lacZ*, the 2µ-*RME1* plasmid resulted in the production of *β*-galactosidase, as indicated by the blue color of the colony in X-Gal-containing medium, whereas the strain with the RRE mutation exhibited very little activity.

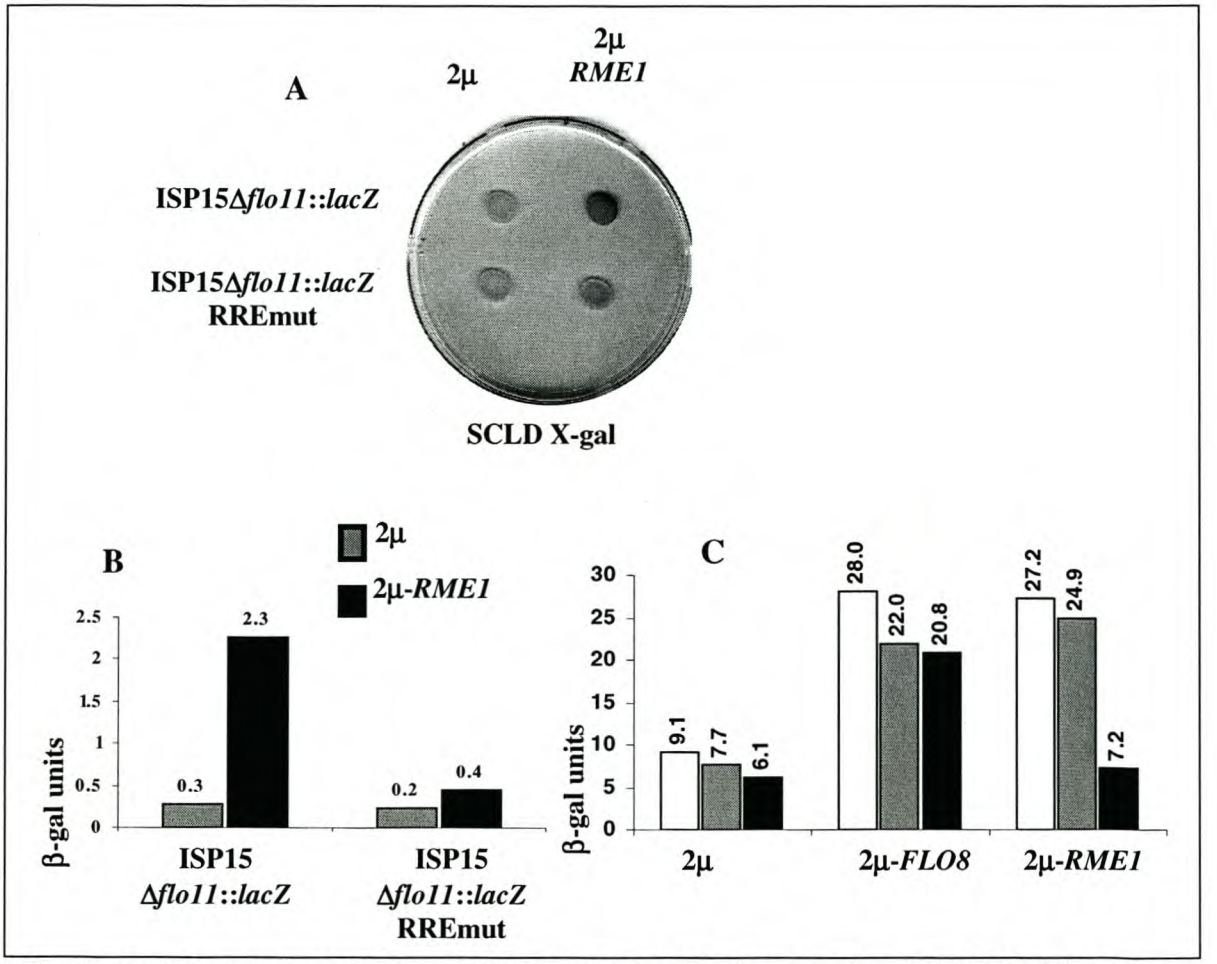


Fig. 6. Rme1p requires the P_{FLO11} RRE to induce *lacZ* expression. (*A*) ISP15Δ*flo11*::*lacZ* and ISP15Δ*flo11*::*lacZ*RREmut were transformed with YEplac195 and YEplac195-RME1 and grown on SCLD (0.1% glucose) supplemented with X-gal for 12 days. The dark coloring of the colony consisting of ISP15Δ*flo11*::*lacZ* with YEplac195-RME1 is indicative of *lacZ* expression. (*B*) *β*-galactosidase activity of the ISP15 strains used in (*A*) grown in SCD. (*C*) *lacZ* expression in Σ272Δ*flo11*::*lacZ* (white bar), Σ272Δ*flo11*::*lacZ*Δ*rme1* (gray bar) and Σ272Δ*flo11*::*lacZ*RREmut (black bar) transformed with YEplac181, YEplac181-FLO8 and YEplac181-RME1. Σ272 derives from the Σ1278b genetic background.

When β-galactosidase values were measured in liquid media, a 30% reduction of activity was observed for the RREmut-controlled reporter gene, similar to the reduction observed for the *RME1* deletion (Fig. 6*B*). As would be expected, a much more significant difference in reporter gene activity was observed in the presence of 2μ-*RME1*. However, transcriptional activation by Rme1p appeared not to be entirely abolished, since the 2μ-*RME1* plasmid still resulted in a two-fold increase in β-galactosidase activity, compared to the eight-fold increase observed in the wild type strain. To further verify whether RREmut specifically affected *RME1*-dependent activation, the same strains were transformed with a 2μ-*FLO8*. Fig. 6*C* shows that the mutated promoter was fully activated by Flo8p, both in terms of absolute β-galactosidase units and induction ratio. Reporter gene activity is multiplied by 3.1 and 3.4 in the presence of 2μ-*FLO8* in the wild type and the RRE-mutated strains respectively, while the corresponding values for 2μ-*RME1* are 3 and 1.2.

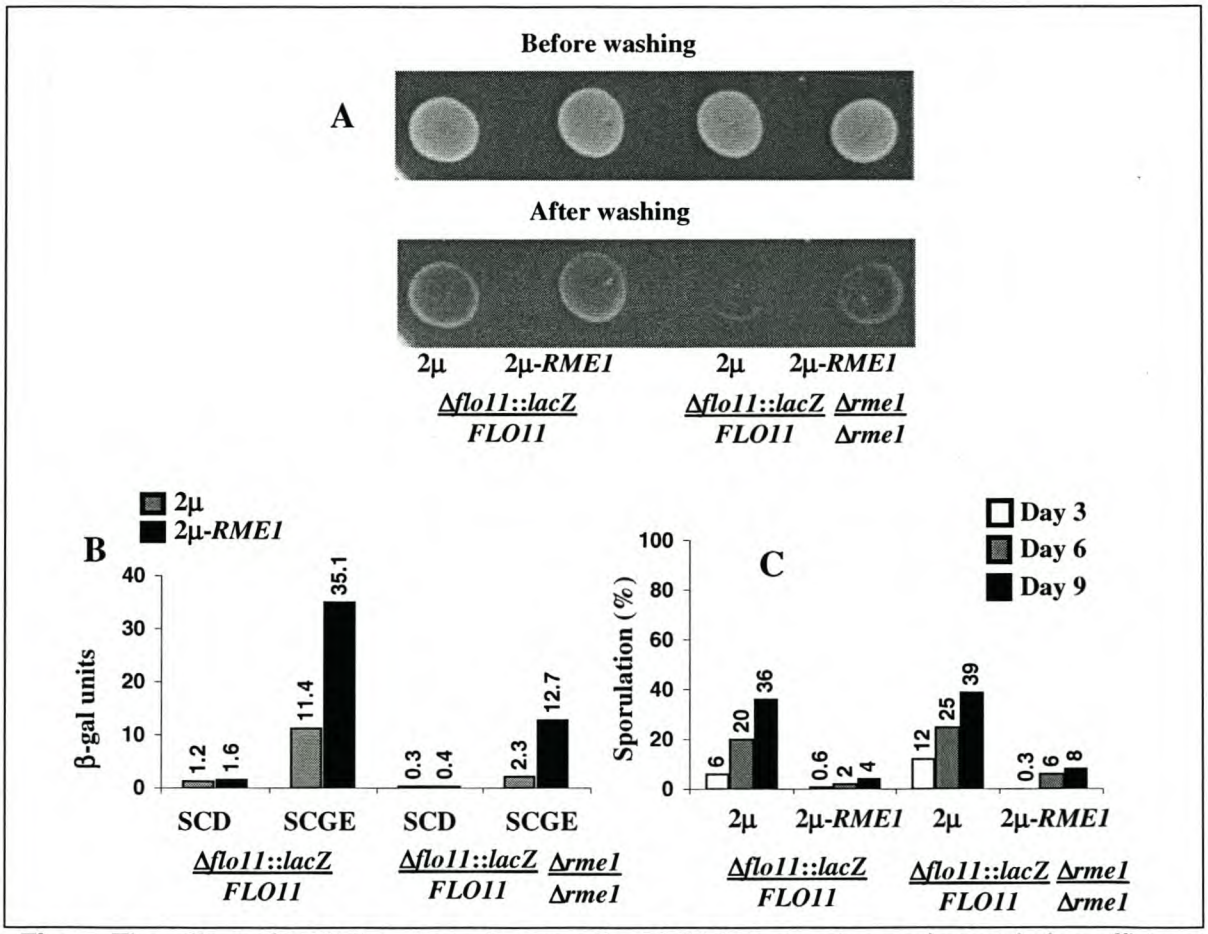


Fig. 7. The effect of *RME1* on invasive growth, *FLO11* expression and sporulation efficiency in Σ1278 diploid strains. (*A*) Strains 2NΣ272Δ*flo11*::*lacZ* and 2NΣ272Δ*flo11*::*lacZ*Δ*rme1*, both carrying one functional copy of *FLO11*, were transformed with YEplac181 and YEplac181-RME1 and subsequently spotted onto SLAD media and allowed to grow for five days at 30 °C before washing. (*B*) β-galactosidase activity of the transformants used in (A) in liquid SCD and SCGE media. (*C*) Sporulation efficiency of the transformants used in (*A*) and (*B*). The histogram depicts the ratio between sporulating and non-sporulating cells.

3.5.6 RME1P REGULATES *FLO11* EXPRESSION AND SPORULATION IN DIPLOIDS

In order to assess whether Rme1p acts as a genetic switch between invasive growth and sporulation, *FLO11* expression and sporulation efficiency were tested in diploid strains. Strains 2NΣΔ*flo11*::*lacZ* and 2NΣΔ*flo11*::*lacZ*Δ*rme1*/Δ*rme1*, both still bearing one functional copy of *FLO11*, were transformed with 2μ-*RME1*. Fig. 7*A* shows that, in the presence of 2μ-*RME1*, both strains presented increased invasive growth and the Δ*rme1*/Δ*rme1* strain exhibited significantly reduced invasive growth when compared to the wild type. We also assessed whether *RME1* controls the formation of pseudohyphae in the diploid strains. The only significant difference was an earlier onset of hyphae formation in the 2μ-*RME1* strain in comparison to the wild type and the Δ*rme1* strain (data not shown). The Δ*rme1* strain formed hyphae with similar efficiency than the wild type.

FLO11 expression levels were four- to five-fold lower in the Δ*rme1* strain in both SCD and SCGE (Fig. 7*B*). Contrary to the haploid Σ1278b strain (Table 4), the 2μ-*RME1*-transformed diploids showed only limited (30%) *lacZ* induction when grown in SCD. On the other hand, when grown in SCGE medium, a more than three-fold induction above the wild type level was observed in the 2μ-*RME1* transformed strain. The level of induction was even more significant in the Δ*rme1* strain, in which a five-fold increase was observed.

The sporulation efficiency of the same strains correlated inversely with the *FLO11* expression data. The Δ*rme1*/Δ*rme1* strain sporulated more efficiently than the wild type (Fig. 7*C*), with 12% of cells of this strain having formed spores after three days on sporulation media, compared to 6% of wild type cells and 0.6% of cells of the 2μ-*RME1* strain. The difference between the wild type strain and the Δ*rme1*/Δ*rme1* strain became less obvious after several more days, indicating inactivation of Rme1p in the wild type and, consequently, efficient sporulation. However, multiple copies of *RME1* exerted a strong repressive effect on sporulation, even after nine days.

3.6 DISCUSSION

3.6.1 RME1P CONTROLS INVASIVE GROWTH

Our data suggest that Rme1p acts as a genetic switch between two nutrient-controlled cellular differentiation pathways, namely invasive growth and meisosis. Previously, the ability of Rme1p to activate *CLN2* expression, coupled with the cell cycle-dependent expression of *RME1*, has been taken as evidence for the involvement of Rme1p in the regulation of mitosis (9). Taken together with our data, it appears likely that Rme1p plays a general role as a transcriptional regulator of genes that are central to the

control of three nutrient-dependent cellular growth forms, i.e. unicellular mitotic growth, invasive and pseudohyphal growth and spore formation.

Previous attempts to link Rme1p to invasive growth have failed in at least one reported attempt (10). The failure may have been due to the specific conditions or the genetic background of the strain employed by this group. We found that the effect of *RME1* deletion in the haploid Σ1278b genetic background was less obvious than in the ISP15 genetic background or in the Σ1278 diploid strain.

Our initial identification of *RME1* was based on the ability of the ISP15 *S. cerevisiae* strain to use starch as the sole carbon source. This screen allows the identification of genes that may be overlooked by other procedures, since starch degradation is a self-amplifying phenotype. Higher starch degradation results in faster growth, which will lead, in turn, to increased starch degradation. We confirmed our data in strains from two independent genetic backgrounds, as well as for haploid and diploid strains.

3.6.2 RME1P REGULATES *FLO11* TRANSCRIPTION VIA AN RRE

Rme1p acts directly via an RRE sequence in the promoter of the *FLO11* gene. As in the case of the RREs in P_{IME1} and P_{CLN2}, the *FLO11* and *STA2* RREs are situated far upstream of the ATG translation start codons, in positions –1427 and –1314 respectively. Mutations within the *FLO11* RRE significantly reduce, but do not completely eliminate, the ability of multiple copies of *RME1* to activate transcription. This might suggest the presence of a second RRE in the promoter of *FLO11*, which would be similar to the situation in P_{CLN2} and P_{IME1}. However, careful scanning did not reveal the presence of a second consensus sequence in the 3.5 kb of P_{FLO11} and P_{STA2}. Alternatively, Rme1p might play a role independent of its DNA-binding activity.

The RRE is situated in an area that was pinpointed by several groups as being essential for the regulation of *FLO11* (26, 27, 33), and Pan and Heitman (33) showed that Flo8p acts in close proximity to the RRE. It therefore is significant that the mutations in the RRE did not affect the ability of Flo8p to activate *FLO11*.

3.6.3 RME1P ACTS INDEPENDENTLY OF OTHER REGULATORS OF INVASIVE GROWTH

Rme1p acts independently of the invasive growth-regulating signaling pathways, the cAMP/PKA pathway and the invasive growth-modulating MAPK pathway. It also does not require the G_1 cyclins. In fact, the deletion of *CLN1* or *CLN2* has no consequences on the ability of Rme1p to induce invasive growth.

The data also show that other transcriptional activators of *FLO11* and *STA2* are not affected by Rme1p. Indeed, all factors investigated are still able to confer similar levels of induction in a Δ*rme1* and in a wild type strain when present on multiple copy

plasmids, albeit from a lower basal level. Similarly, 2µ-*RME1* leads to increased *FLO11* expression in strains deleted for these factors.

3.6.4 POSSIBLE MECHANISM OF RME1P-DEPENDENT REGULATION OF *FLO11*

It is unclear how Rme1p interacts with other elements that regulate invasive and pseudohyphal growth, and which signal is responsible for this regulation. A possible link between *RME1* and invasive growth may be established through the further investigation of factors that regulate *RME1* transcription. For example, Swi5p has recently been shown to regulate *RME1* expression and has also been implicated in the regulation of *FLO11* (34).

It has been suggested that Rme1p acts by excluding other factors from promoters (8, 12). Since this exclusion can occur at sites that are situated at significant distances from the RRE, it has been hypothesized that this effect may be chromatin dependent (8). The activation of *FLO11* transcription by Rme1p therefore may be due to the exclusion of one or several transcriptional repressors. We are currently investigating whether the effect of *RME1* is dependent on the exclusion of specific or general repressor proteins that regulate *FLO11* transcription, including Sok2p (34), Sfl1p (33), Nrg1 or Nrg2 (35, 36), as well as on the Tup1p-Ssn6p general repressor complex (37). The *SOK2* gene is repressed by glucose and multiple copies of *RME1* were unable to activate *FLO11* transcription in the diploid strain when grown on glucose, suggesting a potential link between the two factors. However, in the haploid strains, *RME1* activated *FLO11* similarly in both glucose and glycerol/ethanol containing media, and it therefore is unlikely that the effect of Rme1p is due to Sok2p exclusion alone. A similar argument can be made for Nrg1, which was shown to interact with the P_{STA2} homologue P_{STA1} and mediate glucose repression (36). Sfl1p, on the other hand, has been suggested to antagonize the effect of Flo8p on *FLO11* expression (33). If this hypothesis is true, our data suggest that it is unlikely that Rme1p acts by excluding Sfl1p from the *FLO11* or *STA2* promoters, since the induction observed in the presence of 2µ-*FLO8* is of similar efficiency in both the wild type and in the Δ*rme1* strain. A role for Rme1p in lifting general repression therefore appears the most likely, and would also best fit the other regulatory roles of the protein.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

GENERAL DISCUSSION AND CONCLUSION

4.1 CONCLUDING REMARKS AND PERSPECTIVES

Changes in the growth environment of single-cell organisms require that cells adapt to their surroundings. The cell has a multitude of receptors that inform it of changes in its immediate growth environment. Changes in the nutritional composition, the presence of an opposite mating factor and changes in the water potential or salt concentration are examples of the physiological factors that a single-cell organism has to contend with. Each stress condition elicits a different response from the yeast. The external stress factors are detected by receptors located in the cell wall. These signals are then transmitted to the nucleus via signal transduction networks. The respective signals can be amplified or overridden by other signals transmitted by the network. For example, abundant nutritional signals will be overridden by a mating signal in a haploid yeast, but no response will be given to the mating signal in a diploid yeast and mitosis will continue unabated.

Meiosis is a highly specialised process that is available only to diploid organisms. During meiosis, the chromosome number is halved, while four haploid cells are created, thus facilitating sexual reproduction. These haploid cells are able to mate with another haploid of the opposite mating type and create a diploid organism with the same chromosome number as the parental cells. The genetic regulation of meiosis is very complex, with the signals of several signal transduction networks culminating to regulate meiosis. About 500 genes products are required during meiosis in *S. cerevisiae* and these control everything from the regulation of the initiation of meiosis to the completion of sporulation (Vershon and Pierce, 2000).

The two main factors determining the initiation of meiosis in yeast are i) the nutritional status of the cell and its environment and ii) the *MAT* status of the cell – the requirement for an **a**/α diploid. The master regulator of meiosis, *IME1*, has an extremely large promoter on which both the nutritional and *MAT* signals converge (Kassir *et al.*, 1988; Smith *et al.*, 1990). Sagee *et al.* (1998) demonstrated that the *IME1* promoter contains many regulatory regions. Most have shown regulation only on a genetic level, with only a few proteins identified to bind to the *IME1* promoter directly. The literature review focuses on the initiation of meiosis, with the expression of *IME1* commanding the greater part of the literature study.

Rme1p is a zinc finger-containing DNA-binding protein that was first identified as a repressor of meiosis. It binds to Rme1p Response Elements (RREs) in the *IME1* promoter and prevents its expression (Kassir *et al.*, 1988; Mitchell and Herskowitz, 1986; Sagee *et al.*, 1998). Additionally, Rme1p plays a positive role during mitosis and induces G_1 cyclin expression. The *CLN2* promoter contains RREs to which Rme1p binds to induce expression (Toone *et al.*, 1995). Both mitosis and meiosis have many

other regulatory proteins that regulate their respective processes. Furthermore, as both processes require a large amount of metabolic energy, it is vital that mitosis and meiosis never occur simultaneously. Co-occurrence would be fatal to the cell. To this end, several proteins, among which Rme1p, can induce mitosis while repressing meiosis, thus preventing co-occurrence.

In this study we show that *RME1* has additional functions. Rme1p enhances the growth of yeast on a starch medium and, secondly, it functions in the invasive growth pathway. We attempted to place Rme1p with respect to known nutrient signalling molecules involved in invasive growth. We determined that Rme1p functions independently of the MAP kinase cascade. Also, the cell cyclins Cln1p and Cln2p are not required by Rme1p to induce invasive growth. *FLO11/MUC1* encodes a cell wall protein that is required for invasive growth (Lambrechts *et al.*, 1996; Lo and Dranginis, 1996). *FLO11* has a large promoter and several factors that induce invasive growth have been shown to regulate *FLO11* expression. Data obtained show that Rme1p requires *FLO11* to induce invasive growth. We have identified the presence of an RRE in the promoter of *FLO11* to which Rme1p binds to induce expression. Mutation of the RRE prevents Rme1p from inducing invasive growth in SCD media. The expression data showed a minor increase of *FLO11* expression in response to *RME1* overexpression in SCGE medium. This would indicate that a second RRE could be present, as found in the promoters of *IME1* and *CLN2*, although sequence analysis did not reveal any further RREs in the *FLO11* promoter.

The epistasis analysis between Rme1p and other signalling molecules delivered some interesting results. Phenotypically, it appears that Rme1p requires *MSS11* and *FLO8* to induce invasion. However, data obtained from the β-galactosidase experiments revealed that *RME1* overexpression can induce *FLO11* expression, regardless of the deletion of *MSS11*, *MSN1*, *FLO8*, *TEC1*, *PHD1* or *TEC1*. Also, the overexpression of these factors can overcome *RME1* deletion. Thus, the positioning of Rme1p relative to other signalling modules is not yet established.

It appears that Rme1p has a role to play in any nutritional condition that the yeast cell may encounter and could function as a molecular switch. If the nutrients are abundant, Rme1p enhances mitotic cell division by binding to the *CLN2* promoter. If the nutrients are limited or not utilised efficiently, Rme1p can bind to the promoter of *FLO11* and enhance invasive growth. In both these nutritional conditions, Rme1p also represses meiosis by binding to the promoter of *IME1* and preventing expression. The expression of *RME1* is repressed in growth conditions that induce meiosis. In the absence of Rme1p, repression of *IME1* is alleviated and transcriptional activators can bind to the *IME1* promoter and induce its expression. Rme1p requires Rgr1p and Sin4p to repress *IME1* expression (Sakai *et al.*, 1990). Rgr1p and Sin4p are known to maintain high density chromatin and make the DNA inaccessible to activator proteins or the RNA polymerase II complex (Covitz *et al.*, 1994; Jiang *et al.*, 1995). Toone *et al.* (1995) suggested that Rme1p can function either as an activator or a repressor,

depending on the context of its binding site. In the *CLN2* promoter, the RRE is located in the UAS1 region, from where it induces *CLN2* expression. The UAS1 region also contains the SCB and MCB elements. However, Rme1p-dependent activation of *CLN2* is independent of these elements, and this was confirmed by the ability of Rme1p to bypass the requirement for Swi4p, Swi6p and Mbp1p.

Rme1p induces *FLO11* expression by direct binding to its promoter, but the exact molecular mechanism by which Rme1p induces *FLO11* expression and any co-regulators required by Rme1p remain to be elucidated. It is possible that Rme1p does not function in a hierarchical signalling module with respect to the above-mentioned factors, but rather in a more general system that enhances the ability of the transcriptional activators to exert their effect – the inverse of the repressive effect seen on the *IME1* promoter. However, there is no direct evidence to support this theory.

Invasive growth and pseudohyphal development give yeast a developmental option that provides a mechanism to penetrate its growth medium. In nature, this gives yeast a competitive advantage by enabling it to forage for nutrients. Pathogenic fungi, such as *Candida albicans* and *Ustilago maydis*, require the ability to form filaments and invade its host for their pathogenicity (for a review see Madhani and Fink, 1998). Loss of the ability to grow invasively reduces the virulence of these pathogens significantly. The study of the signal transduction networks governing invasive growth and pseudohyphal development in the model organism *S cerevisiae* has helped to unravel the regulation of the filamentous growth of pathogens such as *C. albicans* and *U. maydis*.

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