INVESTIGATION OF *MAL* GENE REGULATION IN *SACCHAROMYCES*

by

Xin Wang

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy,

The City University of New York

2003

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

4/2/03
Date

Corinne A. Michels
Chair of Examining Committee
Dr. Corinne A. Michels, Queens College/CUNY

4/9/03
Date

Richard L. Chappell
Executive Officer
Dr. Richard L. Chappell

Dr. Susan Rotenberg, Queens College/CUNY

Cathy Savage-Dunn
Dr. Cathy Savage-Dunn, Queens College/CUNY

Dr. Dan Eshel, Brooklyn College/CUNY

Dr. Li Zhang, New York University

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

ABSTRACT

Investigation of *MAL* gene regulation in *Saccharomyces*

by

Xin Wang

Advisor: Corinne A. Michels

Maltose induction requires the maltose permease and the *MAL*-activator. But maltose permease does not appear to be a maltose sensor because we demonstrated that intracellular maltose is sufficient to induce *MAL* gene expression. *PmSUC2* encodes a sucrose transporter from the dicot plant *Plantago major* that exhibits no significant sequence homology to maltose permease and is capable of transporting maltose when expressed in *Saccharomyces*. Introduction of *PmSUC2* restores maltose inducible *MAL* gene expression to a maltose permease null mutant and this induction requires the *MAL*-activator. Furthermore, constitutive overexpression of either *MAL61* maltose permease or *PmSUC2* suppresses the noninducible phenotype of a defective *mal13* *MAL*-activator allele, suggesting that this suppression is solely a function of maltose transport activity and is not specific to the sequence of the permease.

A genetic selection for constitutive *MAL* mutants was carried out to identify additional regulators. 29 recessive mutants were identified and fall into at least two complementation groups: *rgr1* (10 alleles) and *sin4* (6 alleles). Both groups of mutants are pleiotropic and cause slow growth, flocculation and insensitiveness to glucose repression. Moreover, *RGR1* and *SIN4* repress the *MAL* gene expression in a common pathway.

Further genetic analysis was done to determine the roles of the Mediator complex in *MAL* gene regulation. *RGR1* and *SIN4* repress *MAL* gene expression independent of maltose signal pathway and do not act via Mig1/2 repressors. Each component of the Sin4 module plays a distinct role in regulating *MAL* gene expression. Gal11p is involved in both basal expression and induction. Pgd1p, and Med2p are required for maltose induction only. Additionally, the Swi/Snf complex is required for the full induction suggesting a role for chromatin remodeling in the activation of the *MAL* genes.

In summary, our results indicate that intracellular maltose is sufficient to induce *MAL* gene expression and maltose permease appears not to function as a maltose sensor. The Mediator complex is involved in controlling both basal and maltose-inducible *MAL* gene expression and each component of Sin4 module plays a distinct role in *MAL* gene regulation. The Swi/Snf complex is involved in maltose induction.

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my mentor, Dr. Corinne A. Michels, for her support, guidance, and encouragement. Without her excellent training and mentoring, it would not be possible to complete this project.

I am also indebted to all members of my dissertation committee, Dr. Susan Rotenberg, Dr. Cathy Savage-Dunn, Dr. Dan Eshel, and Dr. Li Zhang, for their valuable advice and support.

My great appreciation also goes to all my colleagues in Dr. Michels's laboratory, Igor Medintz, Bin Zhang, Mehtap Bali, Nidhi Gadura, Saima Cheema, and Marcelo Taborga, for their emotional support and helpful discussions.

I would also like to thank my parents, Fengyou Wang and Ruilian Huang, who inspired and nurtured my interest in science from a very young age.

I dedicate this dissertation to my wife Qiaoran Xi, whose love and support helped me make it through the difficult times. This dissertation could not have been possible without her love and support.

TABLE OF CONTENTS

LIST OF TABLES

Chapter 1

Chapter 2

Chapter 3

LIST OF FIGURES

Introduction

Chapter 1

Chapter 2

Chapter 3

Conclusion

INTRODUCTION

The transport of sugar into a cell is the first, and perhaps most important step of sugar metabolism. Regulation of sugar entry is a common strategy for a cell to adjust sugar metabolism and cell activities. In maltose-fermenting *Saccharomyces* strains, maltose transport is performed by maltose permease. Upon the availability of maltose, maltose permease expression is induced and maltose is transported into cells. How is maltose availability sensed and signaled to bring about the induced expression of maltose permease and maltase? Are regulators other than the *MAL*-activator and maltose permease involved in *MAL* gene regulation? The goals of my project are to determine the maltose sensing and signaling pathway in *Saccharomyces* and to identify more regulators of *MAL* gene expression and explore their mechanisms of action.

Part I. *MAL* gene regulation in *Saccharomyces*

The *MAL* system

Maltose fermentation in *Saccharomyces* yeast requires the presence of at least one of five unlinked *MAL* loci: *MAL 1, 2, 3, 4* or *6*. Each *MAL* locus consists of a cluster of three genes essential for maltose fermentation. Gene 1 encodes the maltose permease; Gene 2 encodes maltase that hydrolyzes maltose into two molecules of glucose; Gene 3 encodes the *MAL* transcription activator (*MAL*-activator) protein (Charron *et al.* 1989). The genetic nomenclature designates both locus position and the specific gene. At the *MAL6* locus, the three genes are referred to as *MAL61, MAL62,* and *MAL63*, respectively. The organization of a *MAL* locus is shown in Figure 1. Expression of maltose permease and maltase are induced by maltose and repressed by glucose at the transcriptional level

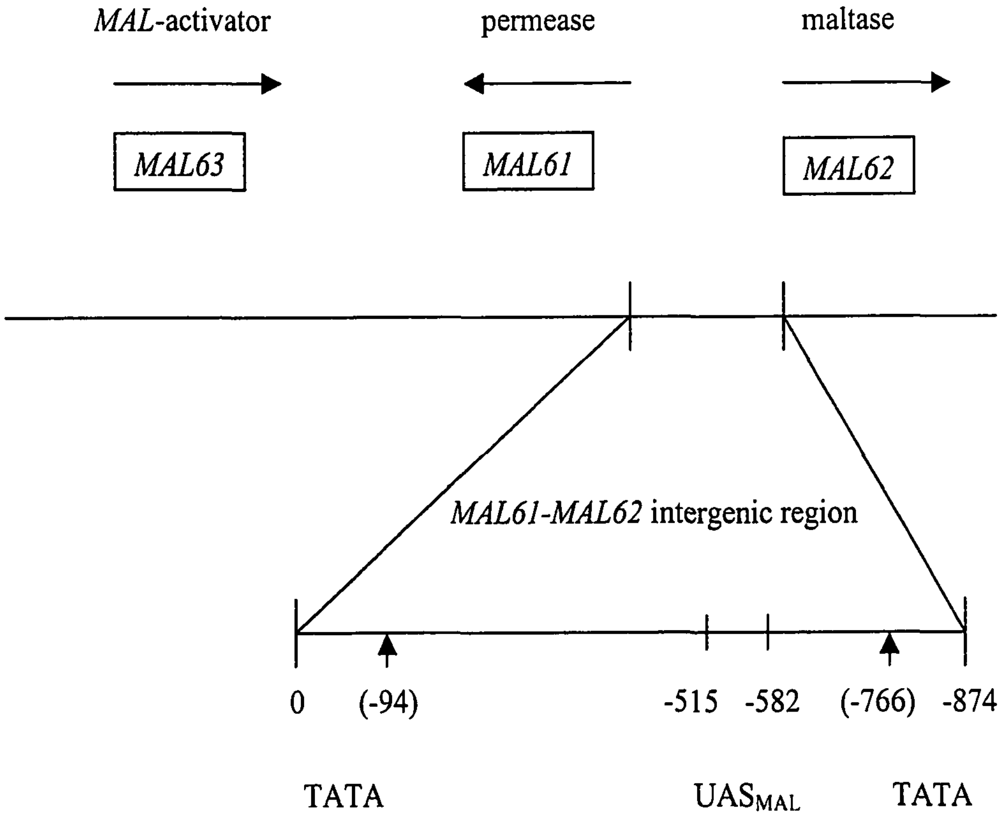


Figure 1. Organization of *MAL6* locus

(Hu *et al.* 1995; Lagunas 1993; Needleman *et al.* 1984; Needleman and Michels 1983). Maltose induction requires a functional *MAL*-activator.

Maltose permease and maltose transport

Transport by maltose permease is an active process that is coupled to the proton gradient and is independent of intracellular ATP levels (Serrano 1977). Kinetic analysis of maltose permease in induced cultures revealed both high-affinity (K_m of 1-4mM) and low-affinity transport (K_m of 70mM) (Busturia and Lagunas 1986; Cheng and Michels 1991). Maltose permease is responsible for the high-affinity maltose transport. Evidence has shown that the low-affinity component of maltose transport is an artifact of nonspecific binding of the maltose to the cell wall and plasma membrane (Benito and Lagunas 1992).

Maltose permease, encoded by Gene 1 at each *MAL* locus, is a high-affinity maltose/proton symporter (Cheng and Michels 1991). *MAL61* encodes a 614-residue protein with 12 putative hydrophobic transmembrane domains (Cheng and Michels 1989). Its sequence and structure exhibit significant homology to members of the sugar transport superfamily including sugar transporters from yeast, *E. coli*, and human. The topology of GLUT1 protein was determined in detail by immunological, protease accessibility, and chemical studies (Davies *et al.* 1990; Davies *et al.* 1987). Based on homology between *MAL61* and *GLUT1*, it is proposed that N- and C-terminal hydrophilic regions are situated on the cytoplasmic side of plasma membrane (Cheng and Michels 1989). Another high-affinity α-glucoside transporter, called Agt1p, has been identified and has 57% amino acid identity to the Mal61p (Han *et al.* 1995).

The addition of glucose to maltose fermenting yeast cells results in the rapid loss of maltose transport activity. This results both from repression of the maltose permease gene transcription and from the post-translational inactivation of maltose permease through a process called glucose induced inactivation. The inactivation consists of two separable processes, a rapid inhibition of maltose transport activity and a slower degradation of maltose permease protein (Medintz *et al.* 1996; Medintz *et al.* 1998). Degradation is dependent on endocytosis, vesicular sorting and vacuolar proteolysis and requires ubiquitin and ubiquitin-conjugating system (Medintz *et al.* 1996; Medintz *et al.* 1998). By mutational analysis, a putative PEST sequence in the N-terminal cytoplasmic domain of maltose permease was shown to be required for rapid glucose induced inhibition and degradation of maltose permease (Medintz *et al.* 2000).

MAL-activator and gene regulation

MAL63 encodes a positive regulatory protein, referred to as the *MAL*-activator, which is required for the maltose-inducible expression of the *MAL* structural genes encoding maltose permease and maltase (reviewed by Needleman 1991). Mal63p is a predicted 470-residue protein with a cysteine-rich amino-terminal region (residues 8-34) homologous to the cysteine-rich, zinc-cluster DNA-binding domains characterized in Gal4p and other yeast transcription activators (Kim and Michels 1988; Marmorstein *et al.* 1992; Schjerling and Holmberg 1996; Sollitti and Marmur 1988). Deletion analysis of the *MAL61-MAL62* intergenic promoter region identified a 68-basepair region necessary and sufficient for maltose induction of structural genes. This sequence is referred to as the $\mathrm{UAS_{MAL}}$ (Figure 1, Levine *et al.* 1992). Three putative Mal63p-binding sites were identified in *MAL61-MAL62* intergenic promoter region by DNA footprint analysis, two of which coincide with the UAS_{MAL} (Ni and Needleman 1990; Sirenko *et al.* 1995). The transcription activation domain of the *MAL*-activator is located within residues 60-283 (Hu *et al.* 1999). Residues 60-283 fused to full length LexA protein (residue 1-202) are sufficient to activate reporter gene expression. Several clusters of bulky hydrophobic residues including phenylalanine found in this region are likely to represent multiple transactivation subdomains similar to those found in Gcn4 protein. Residues 283-470 contain the maltose-response regulatory domain that negatively controls activation by the *MAL*-activator in the absence of maltose (Hu *et al.* 1999). How the presence of maltose in the medium activates the *MAL*-activator and which function(s) such as DNA-binding or transactivation are regulated is poorly understood.

Recently, the *MAL*-activator has been shown to be a client protein of the Hsp90 molecular chaperon complex (M Bali, B. Zhang and CA Michels, personal communication). Chaperon mutations, temperature sensitive *hsc82Δ hsp82-ts* and *hsc82Δ cpr7Δ*, are defective for maltose induction and the turnover rate of Mal63 activator protein is greater in these mutant strains compared to wild-type strains. Depletion of Hsp82p causes the degradation of Mal63 activator. Most significantly, Mal63 activator protein specifically co-purified with Hsp82 from the total cell extract. These findings indicate that the Hsp90 chaperon complex stabilizes the *MAL*-activator by protecting it from degradation and may also aid in folding of *MAL*-activator into a transcriptional active conformation.

Part II. Sugar sensing in *Saccharomyces*

Saccharomyces maintains a variety of nutrient sensing mechanisms that enable it to respond to different nutrients and monitor nutrient levels. These include sensing mechanisms for carbon sources, particularly glucose but also other fermentable carbon sources (reviewed in Ozcan and Johnston 1999); nitrogen sources, including ammonia, urea, and amino acids in general (reviewed in Forsberg and Ljungdahl 2001; ter Schure *et al.* 2000); and other requirements such as phosphate (Wykoff and O'Shea 2001). At least three sensing mechanisms are utilized to monitor glucose levels alone: the Snf1 protein kinase pathway, the Rgt2/Snf3 receptor pathway, and the Gpr1/Gpa2 signaling pathway (reviewed in Johnston 1999; Thevelein and de Winde 1999; Versele *et al.* 2001). Systems for sensing presence of specific sugars, such as galactose or maltose, or specific amino acids, such as histidine or proline, also are present.

Until recently, it was thought that yeast cells, in contrast to mammalian cells, primarily sense and monitor nutrient levels through the activity of intracellular sensors. Recent advances in our understanding in nutrient sensing indicate that yeast cells also possess several plasma membrane nutrient sensors that function similar to the traditional ligand-dependent receptors. I will describe examples of both types of signal pathways found in *Saccharomyces.*

Snf3p and Rgt2p: glucose sensors

Glucose, the preferred carbon and energy source for most eukaryotic cells, has significant and varied effects on cell function. Almost 20 glucose transporters and related proteins have been identified in *Saccharomyces*, including Snf3p and Rgt2p. Over the past few years, a great deal of evidence presented indicates that Snf3p and Rgt2p are not true glucose transporters but rather glucose sensors or receptors, which sense the levels of extracellular glucose, transduce this signal and trigger the induction of several *HXT* gene expression.

Snf3p and Rgt2p are members of 12 transmembrane sugar transporter superfamily. *SNF3* was first identified by isolating mutants defective in growth on sucrose or raffinose (Neigeborn and Carlson 1984). Mutations in *SNF3* result in a severe defect in high-affinity glucose transport and render cells unable to grow fermentively on low levels of glucose. *RGT2* was originally characterized as a dominant suppressor of *snf3* mutation (Marshall-Carlson *et al.* 1990). With extensive studies on both genes in the past decade, more and more evidence has accumulated indicating that Snf3p and Rgt2p play regulatory roles in glucose pathway rather than metabolic roles in transporting glucose (Bisson *et al.* 1993; Coons *et al.* 1995; Coons *et al.* 1997; Ko *et al.* 1993; Liang and Gaber 1996; Marshall-Carlson *et al.* 1990; Marshall-Carlson *et al.* 1991; Ozcan and Johnston 1995). Snf3p and Rgt2p are not able to transport sufficient amounts of glucose to overcome the growth defect of a multiple *hxt* null mutant strain and *HXT* genes can not restore the glucose signaling defect of *SNF3* and *RGT2* mutants, suggesting that Snf3p and Rgt2p act as glucose sensors rather than glucose transporters. The strongest evidence of this regulatory role is the isolation of a constitutive mutation of *RGT2* (Arg231Lys) able to induce *HXT1* gene expression even in the absence of glucose. The same mutation in *SNF3* also causes constitutive expression of *HXT* genes in the absence of glucose (Marshall-Carlson *et al.* 1990). Thus it is proposed that Rgt2p and Snf3p are glucose receptors that bind extracellular glucose and generate a signal to induce expression of certain *HXT* genes. Snf3p is a high-affinity glucose sensor and responds to low extracellular glucose concentrations while Rgt2p is a low-affinity glucose sensor and responds to high extracellular glucose concentrations (Ozcan *et al.* 1996a).

Snf3p and Rgt2p have a unique structural feature not found in other *HXT* proteins. They both have very long C-terminal tails: 303 aa in Snf3p and 218 aa in Rgt2p, which are predicted to be on the cytoplasmic side of plasma membrane. The sequences of the Snf3p and Rgt2p tails are dissimilar, except for a stretch of 25 amino acids, 16 of which are identical among the repeats. This amino acid sequence occurs twice in Snf3p and once in Rgt2p and contains putative target sites for casein kinase II as well as a nucleotide-binding site (Ozcan *et al.* 1996a). Overexpression of the Snf3 C-terminal cytoplasmic domain is able to complement the growth defect of a *snf3* null mutant independent of attachment to the membrane-spanning domains, but not the glucose-signaling defect of *snf3* or *rgt2* mutations. Attachment of Snf3p C-terminal tail to Hxt1p and Hxt2p converts these glucose transporters into glucose sensors, which, however, do not signal as well as Snf3p. In addition, removal of the C-terminal tails of the constitutive *SNF3-1* and *RGR2-1* alleles diminishes signaling. Taken together, these results suggest that the C-terminal tails are necessary but not sufficient for signaling and need other residues in the transmembrane domain region to signal optimally (Ozcan *et al.* 1998).

It is proposed that downstream signaling protein(s) may interact with C-terminal domain of Snf3p or Rgt2p. Recently, two-hybrid analysis has shown that Std1p and Mth1p physically interact with the C-terminal tails of Snf3p and Rgt2p (Lafuente *et al.* 2000; Schmidt *et al.* 1999). Mutations in *STD1* and *MTH1* increase the expression of glucose transporters indicating that Std1p and Mth1p have a repressive effect on the expression of *HXT*s. Std1p has been shown by two-hybrid analysis to be able to interact with Snf1p protein kinase and this indicates that Std1p may affect the Snf1p activity in the repression of *HXT*s (Hubbard *et al.* 1994; Schmidt *et al.* 1999). Additionally, the expression patterns of *STD1* and *MTH1* resemble those of *SNF3* and *RGT2* respectively in that *STD1* is repressed by glucose and *MTH1* is constitutively expressed. These results suggest that Std1p and Mth1p function in transmission of the glucose signal downstream of Snf3p and Rgt2p (Figure 2).

Schmidt *et al.* (1999) proposed a model in which *STD1* may act downstream of *RGT2* in modulating transcription by activating Snf1 kinase under no or low concentrations of glucose, while *MTH1* modulates the transcription of downstream targets of *SNF3* under non-signaling conditions. Std1p has been localized to both the cell periphery and the nucleus suggesting that Std1 may transmit the glucose signal from the cell surface to the site of transcription. *STD1* is a high copy suppressor of mutations in the TATA binding protein (TBP) and Std1p is able to physically interact with TBP suggesting that Std1p may directly bind to TBP and modulate transcription (Tillman *et al.* 1995).

Two additional proteins, Grr1p and Rgt1p, also play central roles in this signaling pathway. Grr1p is a SCF-F box protein and has leucine rich repeats that mediate protein-protein interaction (Bai *et al.* 1996). SCF is a family of protein complexes, each of which is comprised of Skp1, Cdc35/cullin, Rbx1/Roc1, and a different F-box protein. Each SCF complex interacts with a distinct group of protein targets for ubiquitination and subsequent degradation (Kamura *et al.* 1999; Kitagawa *et al.* 1999; Seol *et al.* 1999; Skowyra *et al.* 1999). $\mathrm{SCF}^{\mathrm{Grr1}}$ is one of the SCF complexes identified in yeast and has been found to target G1 cyclins Cln1p and Cln2p and the putative Cdc42 effector Gic2p

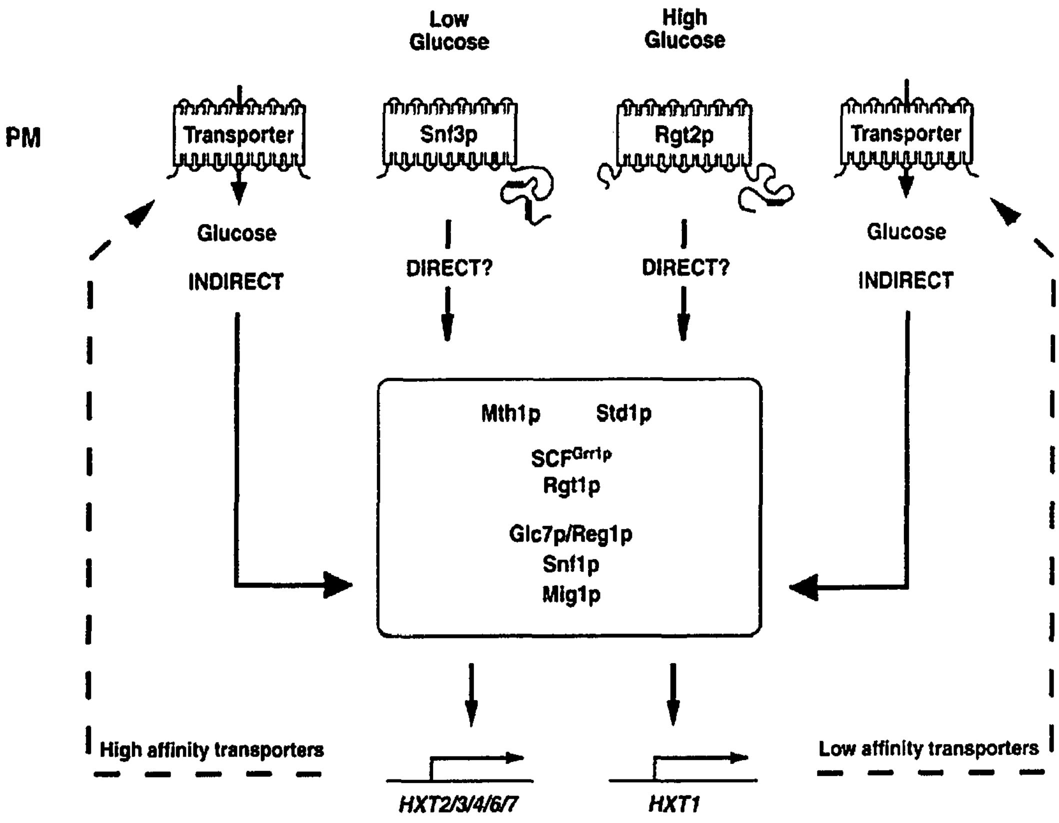


Figure 2. Schematic model of Snf3p/Rgt2p-dependent glucose-induced signaling. Snf3p and Rgt2p are localized to the plasma membrane (*PM*). *Solid arrows* represent signal transduction pathways and glucose uptake through hexose transporters. *Dashed arrows* reflect the post-transcriptional events leading to the functional expression of high- and low-affinity hexose transporter in the PM. *DIRECT* and *INDIRECT* refer to whether signals influencing the activity of downstream components are derived from the Snf3p and Rgt2p sensors, or from internalized glucose, respectively. Taken from Forsberg and Ljungdahl (2001).

for ubiquitination (Barral *et al.* 1995; Jaquenoud *et al.* 1998; Patton *et al.* 1998). Grr1p is required for glucose signaling pathway to induce *HXT* gene expression, since *grr1* mutants are defective in glucose induction of *HXT* genes (Ozcan and Johnston 1995). *RGT1* encodes a bi-functional transcriptional factor that regulates the *HXT* genes, and serves as both an activator and a repressor of *HXT* gene transcription in response to glucose levels (Ozcan *et al.* 1996b). In the absence of glucose, Rgt1p represses *HXT* gene expression while at high concentrations of glucose, Rgt1p is required for optimal activation of *HXT1* gene expression (Ozcan and Johnston 1995; Ozcan *et al.* 1996b). Repression of transcription by Rgt1p requires the general corepressor complex, Ssn6p and Tup1p. Activation of transcription by Rgt1p depends on signals generated by two glucose sensors, Snf3p and Rgt2p, and transduced by Grr1p (Ozcan *et al.* 1996b). In addition, mutations in *RGT1* suppress the defect in *HXT* gene expression caused by *grr1* mutations (Ozcan and Johnston 1995). These findings suggest that Grr1p inactivates Rgt1p in response to glucose possibly by ubiquitin-mediated proteolysis. It has been postulated that in response to glucose, signals generated by Snf3 and Rgt2 sensors facilitate the recruitment of Rgt1p to the SCF$^{\mathrm{Grr1}}$ complex, leading to degradation of Rgt1p and thereby activating *HXT* gene expression. But, ubiquitination of Rgt1p has not yet been demonstrated. It is also possible that SCF$^{\mathrm{Grr1}}$ acts on another regulator to indirectly stimulate inactivation of the repressor function of Rgt1p (Johnston 1999). Recently, Mosley *et al.* (2003) showed that glucose-mediated phosphorylation plays an essential role in Rgt1 function by converting Rgt1p from a transcriptional repressor to an activator. Rgt1p is hyperphosphorylated in response to glucose and this phosphorylation is required for Rgt1p to activate *HXT* gene transcription. Furthermore, in *snf3 rgt2* and *grr1* mutants, Rgt1p lacks the glucose-induced phosphorylation and behaves as a constitutive repressor independent of carbon sources. But, how Rgt1p phosphorylation is related to $\mathrm{SCF}^{\mathrm{Grr1}}$ action on Rgt1p remains to be answered.

Gpr1-Gpa2: sensor of fermentable carbon sources

The GTP-binding protein-coupled receptors (GPCRs) are conserved from yeast to human and are known to mediate cellular responses to ligands, including the neurotransmitters, hormones, and photons (Peroutka 1994). GPCRs are situated in the plasma membrane and characteristically contain seven transmembrane domains. Upon ligand binding, the GPCRs transduce signals via interaction with trimeric GTP-binding proteins, comprised of an α subunit (G_α), a β subunit (G_β), and a γ subunit (G_γ) (Gilman 1987; Lefkowitz 2000).

In yeast, three GPCRs have been characterized: Ste2p, Ste3p and Gpr1p. Ste2p and Ste3p are pheromone-binding receptors and are responsible for inducing gene expression of mating specific genes in response to a or α mating factor. Gpa1p and Gpa2p are the only two G-protein α subunits encoded by the yeast genome. Gpa1p is the Gα subunit that associates with the Ste2 and Ste3 receptors. The *GPA2* gene was cloned based on its homology to the mammalian heterotrimeric G_α protein and is believed to encode a GTP-binding protein α subunit in yeast (Nakafuku *et al.* 1988). Gpa2p was proposed to function in the regulation of cyclic AMP (cAMP) levels, based on the finding that overexpression of Gpa2p causes a 2-fold increase in the level of cAMP in response to glucose (Nakafuku *et al.* 1988). Gpr1p was isolated in several laboratories by the yeast two-hybrid system using Gpa2p as bait (Kraakman *et al.* 1999; Xue *et al.* 1998; Yun *et al.* 1997).

Addition of a high concentration of glucose to cells grown on a non-fermentable carbon source, or to cells in stationary phase causes a transient spike of intracellular cAMP levels. This transient rise in cAMP levels triggers a signaling cascade through the cAMP-PKA pathway, which has effects on a number of physiological events, including trehalose and glycogen metabolism, stress resistance, gluconeogenesis, glycolysis, and growth and proliferation (Thevelein and de Winde 1999). Two glucose sensors Snf3p and Rgt2p (described earlier) are not involved in this glucose-stimulated cAMP increase (Rolland 2000). Studies with the *gpr1* and *gpa2* mutants have indicated that both Gpr1p and Gpa2p are required for this response to glucose (Kraakman *et al.* 1999; Lorenz *et al.* 2000; Rolland *et al.* 2000; Yun *et al.* 1998). Epistasis analysis indicates that Gpr1p acts upstream of Gpa2p. Gpr1p is localized to the cell surface suggesting that it recognizes an extracellular ligand (Xue *et al.* 1998). However, the direct evidence that Gpr1p binds to glucose has not yet been obtained, but a requirement for Gpr1p in sensing of the extracellular glucose has been demonstrated (Rolland *et al.* 2000). These findings indicate that Gpr1p and Gpa2p constitute a GPCR system that is able to sense extracellular glucose and trigger a rapid increase in cAMP levels.

Gpr1p and Gpa2p have been demonstrated to be required for haploid invasive growth and diploid pseudohyphal differentiation (Kubler *et al.* 1997; Lorenz and Heitman 1997; Tamaki *et al.* 2000). A constitutive allele of *GPA2* suppresses the filamentous growth defect conferred by a *gpr1Δ* mutation, which supports the proposal that Gpr1p couples to Gpa2p and initiates signaling. The fact that a *RAS2* dominant active allele and addition of cAMP restore the filamentation in the *gpr1Δ* mutant strain suggests that Gpr1-Gpa2 regulates the filamentous growth through cAMP-PKA signaling pathway

(Lorenz *et al.* 2000). Pseudohyphal differentiation in response to nitrogen limitation requires the presence of a high level of a fermentable carbon source. The expression of Gpr1p is elevated under conditions that favor the pseudohyphal growth (Xue *et al.* 1998). Therefore, Gpr1 has been proposed to function as a receptor for fermentable sugars to stimulate the pseudohyphal growth (Lorenz *et al.* 2000).

Recently, more components of the Gpr1-Gpa2 signaling pathway have been identified. Rgs2p, a membrane-bound protein, functions as a negative regulator of the Gpr1-Gpa2 signaling pathway (Versele *et al.* 1999). Rgs2p physically interacts with Gpa2p and stimulates the intrinsic GTPase activity of Gpa2p *in vitro* suggesting that Rgs2p acts as a GTPase-activating protein. *KRH1* and *KRH2* were identified by two-hybrid screen using as bait the constitutively active *GPA2* allele and were found to be involved in Gpr1-Gpa2 signaling pathway (Batlle *et al.* 2003). Cells containing *krh1Δ krh2Δ* mutations display increased invasive growth, decreased survival following heat shock, and decreased efficiency of sporulation, all of which are also seen in cells containing a constitutively active *GPA2* allele. This indicates that Krh1p and Krh2p play negative regulatory roles in Gpr1-Gpa2 pathway. The double *krh1Δ krh2Δ* mutation is able to suppress a *gpa2Δ* mutation indicating that Krh1p and Krh2p act downstream to Gpa2p. Another important discovery supporting Gpr1-Gpa2 signaling pathway is identification of Gβ and Gγ subunits that function specifically with Gpa2p. Gpb1 and Gpb2 bind to Gpa2p in two-hybrid assay. Overexpression of Gpb1p or Gpb2p inhibits filamentous growth. Strains lacking Gpb1p or Gpb2p or both exhibit enhanced filamentation indicating Gpb1, 2 proteins play an inhibitory role. Gpb1p and Gpb2p preferentially associate with the GDP-bound form of Gpa2p suggesting that Gpb1, 2p function as Gβ subunits (Harashima and Heitman 2002). Another Gpa2-interacting protein, Gpg1p was found to interact with Gpa2p indirectly and this interaction is mediated via Gpb1, 2p. The *gpg1* mutant exhibits a partial defect in *FLO11* expression and is modestly decreased for invasive growth. Gpg1p has been suggested to be a Gγ-like subunit with atypical features since its size and its predicted secondary structure are very similar to those of known Gγ subunits (Harashima and Heitman 2002).

Snf1 protein kinase signal transduction pathway

The Snf1 protein kinase pathway is the major signal pathway regulating glucose repression. Most components of this pathway have been identified and the regulatory mechanisms of glucose repressive genes by this pathway have been fairly well characterized. However, the glucose-sensing mechanism for this pathway and the identity of the intracellular small molecule signal regulating Snf1 kinase activity remains poorly understood.

The *SNF1* gene is essential for the derepression of genes repressed by glucose including *SUC2*, *GAL,* and *MAL* genes (Carlson *et al.* 1984; Celenza and Carlson 1986; Hu *et al.* 2000). *SNF1* encodes a serine/threonine protein kinase. The Snf1p is found associated with other proteins: Snf4p, Sip1p, Sip2p, and Gal83p (Celenza and Carlson 1989; Celenza *et al.* 1989; Lesage *et al.* 1996; Yang *et al.* 1992). The activating subunit Snf4p is required for Snf1 activity. Sip1p, Sip2p, and Gal83p constitute a family of related proteins that interact with Snf1p and Snf4p and serve as a scaffolding function in the kinase complex (Jiang and Carlson 1997). Snf1p has two domains: an amino-terminal catalytic domain and a carboxyl-terminal regulatory domain. At high concentrations of glucose, the Snf1 regulatory domain binds to the catalytic domain and inhibits the kinase activity. At low concentrations of glucose, Snf4p binds to the regulatory domain thereby activating the kinase activity (Jiang and Carlson 1996).

What is the glucose signal that regulates the Snfl activity? One suggested candidate is the AMP:ATP ratio (Johnston 1999). This comes from the realization that the components of Snfl kinase complex are similar to the subunits of the AMP-activated protein kinase (AMPK) of mammalian cells and the observation that changes in AMP:ATP ratio in response to glucose limitation correlate with the activity of Snflp (Wilson *et al.* 1996). When grown with abundant glucose, cells generate ATP by utilizing AMP leading to a low AMP:ATP ratio, which inactivates the Snfl kinase. When starved for glucose, cells produce AMP leading to a high AMP:ATP ration, which activates Snfl kinase. Another highly speculative possibility is that hexokianse 2 (Hxk2) senses the intracellular glucose levels. Hxk2p both phosphorylates glucose and is a key regulator of glucose repression (De Winde *et al.* 1996; Gancedo 1998; Ma *et al.* 1989b; Rose 1995). Recent data suggest that Hxk2p might play a more direct role in sensing glucose availability to the glucose repression. Hxk2p was found to reside in both the nucleus and the cytoplasm suggesting it functions in signaling in addition to catalysis (Herrero *et al.* 1998). Hexokianse 2 participation in DNA-protein complexes with cis-acting regulatory elements of *SUC2* promoter may suggest that it transduces the glucose signal by interacting directly with the transcriptional factors, such as Mig1p, that controls the expression of glucose-repressed genes (Herrero *et al.* 1998; Rolland *et al.* 2001). This proposed regulatory role is not supported by earlier studies by Ma and Botstein (Ma *et al.* 1989a; Ma *et al.* 1989c) that the degree of glucose repression correlates with the catalytic activity of a variety of *hxk2* mutations.

Mutations in *MIG1*, *SSN6* and *TUP1* suppress defects caused by the loss of *SNF1* (Vallier and Carlson 1994; Williams and Trumbly 1990). *MIG1* encodes a zinc finger protein that binds to a GC-box motif (Nehlin *et al.* 1991; Nehlin and Ronne 1990). The Mig1p binding sites have been identified in the promoters of many glucose repressed genes including *GAL1*, *GAL4*, *SUC2*, *CAT8*, and *MAL6* genes (Carlson 1997). Extensive research indicates that Mig1p exerts its repressive effects by recruiting the Ssn6-Tup1 corepressor complex that represses the transcription. Mutations in Tup1p and Ssn6p affect catabolite repression but are also highly pleiotropic (Gancedo 1992; Williams *et al.* 1991). The *SSN6* and *TUP1* gene products contain long stretches of polyglutamine that are found in many transcriptional regulatory proteins (Schultz *et al.* 1990; Williams and Trumbly 1990). Ssn6 and Tup1 proteins are found physically associated in a large general repressor complex (Williams *et al.* 1991). In addition, Mig1p and Ssn6p proteins interact in the two-hybrid system (Treitel and Carlson 1995). The subcellular localization of Mig1p appears to be regulated by the Snf1 protein kinase, because Mig1p remains in the nucleus in a *snf1* mutant (De Vit *et al.* 1997). Mig1p is differentially phosphorylated in response to glucose availability (Treitel and Carlson 1995). Its phosphorylation requires Snf1p, and Mig1p and Snf1p exhibit a two-hybrid interaction in vivo (Ostling and Ronne 1998; Treitel *et al.* 1998). These findings strongly suggest that phosphorylation by Snf1p regulates the localization of Mig1p.

Gal3: intracellular galactose sensor

To utilize galactose, yeast requires a specific galactose transporter, encoded by *GAL2*, and the Lelior pathway enzymes that convert galactose into glucose-1-phosphate encoded by *GAL1* (kinase), *GAL7* (transferase) and *GAL10* (epimerase) (review in

Johnston 1987). Expression of all of these genes occurs coordinately and is activated rapidly by several thousand-fold in presence of galactose as the sole carbon source.

Extensive studies have been done to decipher the mechanisms of the *GAL* gene regulation. *GAL* gene activation is dependent on Gal4p, the transcriptional activator (Hashimoto *et al.* 1983). Gal4p is constitutively bound to DNA upstream of its target genes and, in the absence of galactose, its transcriptional activity is repressed by Gal80p, a negative regulator, which interacts directly with the activation domain of Gal4p to inhibit its activity (Lue *et al.* 1987; Wu *et al.* 1996). But how is the inhibition released by the presence of galactose in the media? *GAL3* is required for rapid induction of the *GAL* genes. Overexpression of either Gal1p or Gal3p gives rise to the constitutive activation of *GAL* genes (Bhat and Hopper 1991; Bhat and Hopper 1992). Surprisingly, Gal3p protein shares high sequence homology to galactokinase (Gal1p), but does not itself have galactokinase activity (Bhat *et al.* 1990). *In vitro* studies have shown that Gal80p and Gal3p interact with each other and this interaction depends on galactose and ATP, and the Gal3p constitutive mutants interact with Gal80p in the absence of galactose (Blank *et al.* 1997; Sadowski *et al.* 1996; Suzuki-Fujimoto *et al.* 1996; Yano and Fukasawa 1997; Zenke *et al.* 1996). Based on studies on the formation of a Gal4p-Gal80p-Gal3p complex *in vitro*, a model of galactose induction was proposed (Platt and Reece 1998). In the absence of galactose, Gal80p interacts with Gal4p and inhibits the transcriptional activity of Gal4p. In the presence of galactose in the media, galactose is transported into the yeast cells and accumulates to reach a threshold level sufficient to saturate the available Gal3p. The binding of galactose as well as ATP promotes Gal3p to associate with Gal80p. Gal80p then undergoes a conformational change to release the activation domain of

Gal4p such that Gal4p can interact with its targets and activate the transcription of the *GAL* genes.

Recent evidence indicates that the subcellular distribution dynamics of Gal3p and Gal80p is involved in controlling Gal4p activity. Immunofluorescence studies show that Gal3p is located in the cytoplasm and is not detected in the nucleus. However, Gal80p is located in both the cytoplasm and nucleus and exhibits nucleocytoplasmic shuttling (Peng and Hopper 2000). When Gal3p is artificially sequestered in the cytoplasm by being anchored to the plasma membrane or the outer membrane of mitochondria, Gal3p retains its induction function suggesting that it is not necessary to enter the nucleus and act directly on the Gal80p-Gal4p complex to effect *GAL* gene expression. Moreover, Gal4p is responsive to the reduced levels of Gal80p in the nucleus by substituting Gal3p with a surrogate cytoplasmic Gal80p-binding factor (Peng and Hopper 2002). Based on this new evidence that galactose induction is achieved by modulating Gal3p-Gal80p interaction in two different cellular compartments, Peng and Hopper (2002) proposed a second model in which galactose triggers Gal3p-Gal80p complex formation in the cytoplasm to reduce the level of interaction of Gal80p with Gal4p in the nucleus (Figure 3).

The major difference between these two models is where Gal3p interacts with Gal80p and how this interaction causes the release of repression on Gal4p. But, in both models, Gal3p acts as a sensor of intracellular galactose.

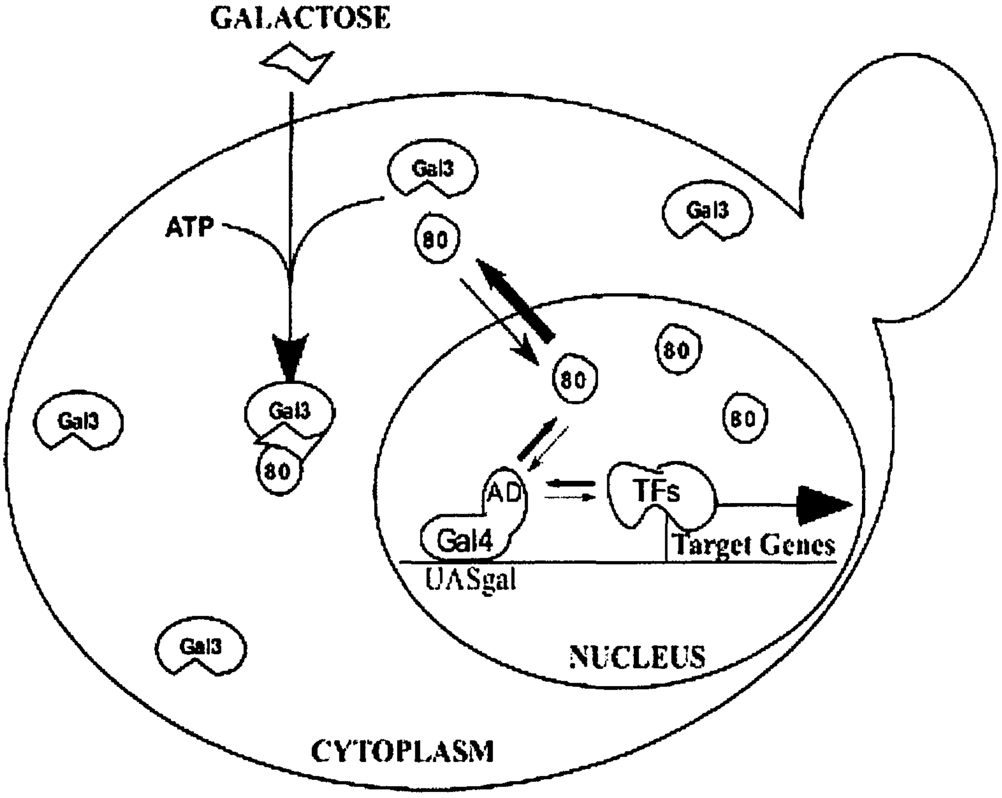


Figure 3. Model for transcriptional regulation of *GAL* genes through modulation of protein-protein interactions in the nucleus and in the cytoplasm. Gal3p (Gal3) is located in the cytoplasm, whereas Gal80p (80) is located in both the cytoplasm and the nucleus and undergoes rapid nuclear-cytoplasmic shuttling. In the nucleus, a number of transcription factors (TFs) compete with Gal80p to bind to the activation domain (AD) of Gal4p. In the absence of galactose, binding of Gal80p to Gal4p effectively limits active transcription of the *GAL* genes. Cellular uptake of galactose triggers Gal3p-Gal80p interaction in the cytoplasm and results in redistribution of Gal80p from the nucleus to the cytoplasm. These events lead to a reduced probability of binding between Gal80p and Gal4p. Taken from Peng and Hopper (2002).

Part III. An overview of the yeast Mediator complex

Discovery of the Mediator

Transcription by *Saccharomyces* RNA polymerase II (RNAPII) is a complex and highly regulated process that requires a large number of factors including the general transcriptional factors (TFIIA, -B, -C, -D, -E, -F, -G and -H), and TAFs (TBP-associated factors). Transcription studies in a reconstituted system indicate that additional factors are needed to respond to the activators. This led to the discovery of the Mediator initially as an activity in a yeast crude fraction able to relieve interference between different activators (Gill and Ptashne 1988). Subsequent biochemical studies identified 20 subunits in the Mediator (Flanagan *et al.* 1991; Kelleher *et al.* 1990; Kim *et al.* 1994). Demonstration of a physical interaction between the Mediator and the C-terminal domain (CTD) of the RNAPII largest subunit (Rpb1) functionally links the Mediator complex to RNAPII transcription (Thompson *et al.* 1993). Functional analysis of purified Mediator in an *in vitro* reconstituted system indicates that Mediator is required for both basal and regulated transcription, and regulates the TFIIH CTD kinase activity suggesting that the Mediator can function through the CTD (Kim *et al.* 1994; Myers *et al.* 1998). Mediator is now viewed as a modular and dynamic interface that connects diverse gene-specific regulatory proteins to the basal RNAPII transcriptional initiation apparatus by acting as signal sensor, integrator, and processor (Boube *et al.* 2002; Kang *et al.* 2001).

Structural organization of the Mediator

Yeast Mediator complex is comprised of at least 20 subunits including Srb proteins, Med proteins, and several other proteins originally discovered in a variety of genetic screens for transcriptional factors (Carlson 1997; Myers and Kornberg 2000).

Several Mediator complexes have been purified to homogeneity from yeast, human, and mouse cells. Recently, three-dimensional images of Mediator complexes have been determined by electron microscopy (Asturias *et al.* 1999; Dotson *et al.* 2000). As shown in Figure 4, electron microscopy image of the purified yeast Mediator/RNAP II holoenzyme complex revealed three major Mediator domains that wrap around the globular RNA polymerase II, termed head (h), middle (m), and tail (t) domains.

Genetic, biochemical analyses have revealed three functional substructures termed the Sin4 module, the Med9/Med10 module, and the Srb4 module (Figure 4). In a *sin4* mutant, the tail domain of the Mediator complex is missing, but the head and middle domains remain intact. Based on this information and physical interactions among the Mediator subunits, the Sin4, Med9/Med10, and Srb4 modules have been assigned to tail, middle and head domains, respectively (Dotson *et al.* 2000). The Sin4 module consists of Sin4, Rgr1, Med2, Pgd1, and Gal11 proteins and has been found to be involved in both transcriptional repression and activation. It is proposed that the Sin4 module acts as an integral part of Mediator directly sensing the repressing or activating signals from gene-specific regulators (Bhoite *et al.* 2001; Han *et al.* 1999; Myers *et al.* 1999). Park *et al.* (2000) have shown direct interaction between Gal11p and the activators Gal4, Gcn4, VP16, and Swi5 as well as an interaction between Pgd1p and Gcn4p. Pgd1p has been demonstrated to associate directly with the Cyc8-Tup1 corepressor complex (Papamichos-Chronakis *et al.* 2000). The Med9/Med10 module is composed of two stable submodules, the Med9 submodule containing Med1, -4 and -9 proteins, and the Med10 submodule containing Srb7, Med7, Med10, and Nut1 proteins. Biochemical analysis indicates that the Med9/Med10 module interacts physically with the CTD of

RNAPII, which is in agreement with the electron microscopy image (Kang *et al.* 2001). Genetic analysis suggests that the Med9 submodule is involved in transcriptional repression and the Med10 submodule is required to relay activating signals to the RNAPII initiation machinery (Han *et al.* 2001; Kang *et al.* 2001). The Srb4 module includes Srb2, -4, -5, -6, Rox3, Med6, -8, and -11 proteins and interacts directly with the RNAPII CTD, along with TBP and TFIIB (Kang *et al.* 2001). This is consistent with the observation that head domain physically interacts with RNAPII in electron microscopy images (Figure 4). Therefore, it is thought to directly modulate RNAPII activity.

In addition to Sin4, Med9/10, and Sin4 modules, some forms of the Mediator complex contain an accessory module, the Srb10 module. The Srb10 module is composed of four Srb proteins, Srb8-11 (Carlson 1997; Lee and Young 2000). Srb10p is a cyclin-dependent kinase and Srb11p is a Srb10-associated cyclin. Biochemical analysis indicates that Srb10 module interacts *in vitro* with the Med9 submodule (Kang *et al.* 2001). Srb10 kinase phosphorylates the CTD of RNAPII, thereby inhibiting transcription (Hengartner *et al.* 1998; Holstege *et al.* 1998). Genetic analysis suggests that the Srb10 module is implicated in transcriptional repression of genes that are regulated by glucose repression, meiotic development, mating type, and heat shock (Carlson 1997). Studies of *GAL* gene regulation, however, indicate that Srb10p also plays a positive role in *GAL* induction (Balciunas and Ronne 1995; Hirst *et al.* 1999; Kuchin *et al.* 1995; Liao *et al.* 1995; Vincent *et al.* 2001). Srb10p phosphorylates Gal4 on Ser699, and this phosphorylation is required for full induction of transcription (Hirst *et al.* 1999; Rohde *et al.* 2000; Sadowski *et al.* 1996). Recently, Srb10p was found to interact physically with Sip4p, a transcriptional activator of gluconeogenic genes and stimulate activation of a *LacZ* reporter by Sip4p. These findings suggest that the Srb10 kinase not only plays negative roles in transcriptional control but also has broad positive roles during growth in carbon sources other than glucose (Vincent *et al.* 2001).

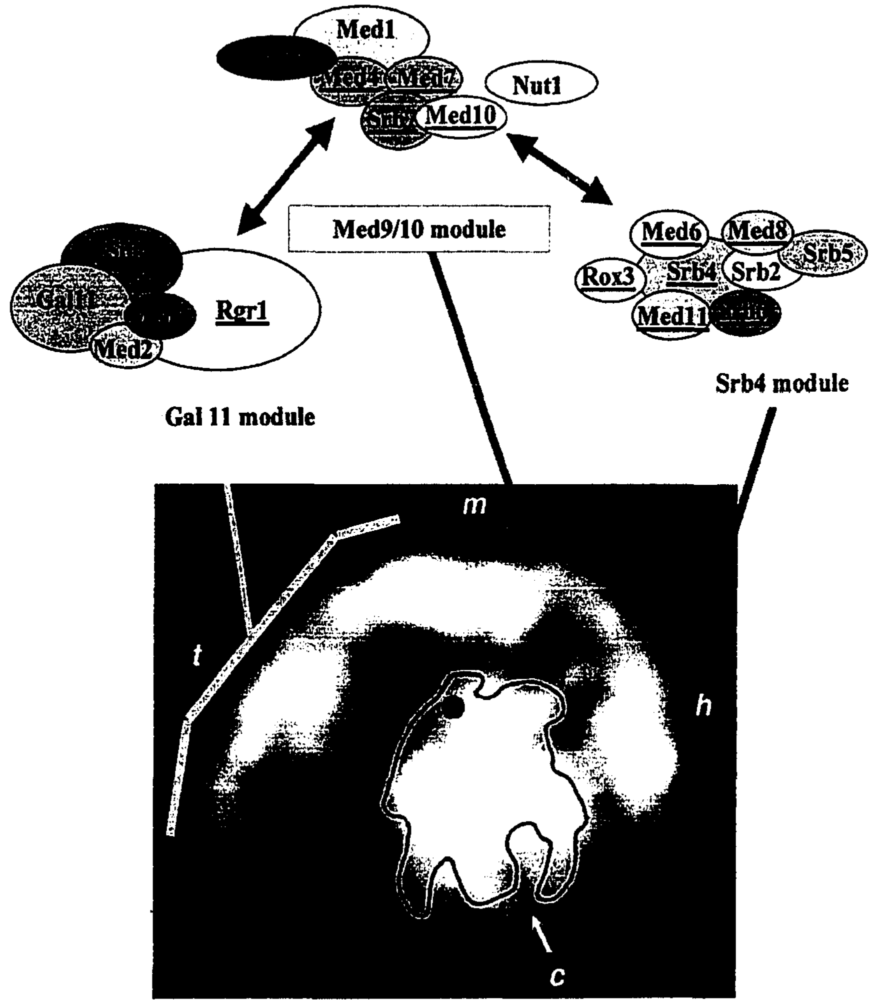


Figure 4. Structural organization of the *S. cerevisiae* Mediator associated with RNA Polymerase II. The upper part shows the proposed locations of Mediator modules and a model for subunit organization based on genetic, biochemical, and two-hybrid interactions. Underlined subunits are essential for viability. The lower part is a projection of the holoenzyme formed by Mediator and RNAP II as revealed by electron microscopy and image processing of purified particles. Mediator is in an extended conformation with head (h), middle (m), and tail (t) domains clearly distinguishable. RNAP II is delimited by a dark line, with the point of attachment of the CTD (dark circle) and the location of the DNA binding channel (c) indicated. Taken from Boube *et al.* (2000).

OVERVIEW

This thesis is organized into 3 main chapters. In Chapter 1, several lines of evidence are presented that intracellular maltose is sufficient to induce *MAL* gene expression and serves as a signaling molecule. Chapter 2 describes a genetic selection for Mal^c mutants in which *rgr1* and *sin4* mutations are identified. Chapter 3 describes a more detailed analysis on role of the Mediator in *MAL* gene regulation.

Chapter 1 is entitled "Intracellular maltose is sufficient to induce *MAL* gene expression in *Saccharomyces cerevisiae*". This was published as a full paper that was co-authored by myself, M. Bali, I. Medintz and C.A. Michels, and appeared in Eukaryotic Cell (2002) 1:696-703. In this study, we showed that introduction of *PmSUC2* restores maltose inducible *MAL* gene expression to a maltose permease null mutant and this induction requires the *MAL*-activator. These data indicate that intracellular maltose is sufficient to induce *MAL* gene expression independent of the mechanism of maltose transport. Further, we demonstrated that a rather low concentration of intracellular maltose is needed to trigger *MAL* gene expression. Additionally, we showed that suppression of the noninducible phenotype of a defective *mal13* *MAL*-activator allele by permease overexpression is solely a function of maltose transport activity. My contribution to this work includes the construction of most mutant strains and carrying out most of the maltose transport assay, maltase assay, and Western analysis. Mehtap Bali assisted in the construction of some strains and the results presented in Table 5. Igor Medintz assisted in the construction of various *MAL61* alleles and some of the maltose transport assay reported in Table 4.

Chapter 2 is entitled "A genetic study of *MAL* gene expression in *Saccharomyces*". This chapter describes a genetic selection by UV mutagenesis for *MAL* constitutive mutants. Two genes, *RGR1* and *SIN4*, are identified. Mutations in either gene cause multiple phenotypes, including the slow growth, flocculation, and reduced sensitivity to glucose repression. We also demonstrated that *RGR1* and *SIN4* act in a common pathway to repress *MAL* gene expression. I am solely responsible for the work reported in Chapter 2.

Chapter 3 is entitled "Role of the Mediator in *MAL* gene regulation". This chapter, together with Chapter 2, is currently being prepared for submission as a manuscript and will be coauthored by myself and C.A. Michels. In this report, we carried out a more detailed analysis on the possible roles that the Mediator plays in controlling *MAL* gene expression. We found that the Mediator regulates both the basal and maltose-inducible expression of *MAL* genes. We showed that *MAL* gene repression by Rgr1p and Sin4p is independent of maltose signaling pathway and that Rgr1p and Sin4p do not exert repressive effects via Mig1, 2p. Each component of the Sin4 module was found to play distinct roles in *MAL* gene regulation. Additionally, we report that the Swi/Snf complex is required for the full induction of *MAL* genes. The studies of Swi/Snf reported in Figure 4 were carried out by Bin Zhang.

CHAPTER 1

Intracellular Maltose Is Sufficient To Induce *MAL* Gene Expression In *Saccharomyces cerevisiae*

ABSTRACT

The presence of maltose induces *MAL* gene expression in *Saccharomyces* cells, but little is known about how maltose is sensed. Strains deleted for all maltose permease genes are unable to induce *MAL* gene expression. In this report, we studied the role of maltose permease in maltose sensing by substituting a heterologous transporter for the native maltose permease. *PmSUC2* encodes a sucrose transporter from the dicot plant *Plantago major* that exhibits no significant sequence homology to maltose permease. When expressed in *Saccharomyces* PmSUC2 is capable of transporting maltose, albeit at reduced rate. We showed that introduction of *PmSUC2* restores maltose inducible *MAL* gene expression to a maltose permease null mutant and this induction requires the *MAL*-activator. These data indicate that intracellular maltose is sufficient to induce *MAL* gene expression independent of the mechanism of maltose transport. Using strains expressing defective *mal61* mutant alleles, we demonstrate a correlation between the rate of maltose transport and the level of the induction, which is particularly evident in media containing very limiting concentrations of maltose. Moreover, our results indicate that a rather low concentration of intracellular maltose is needed to trigger *MAL* gene expression. We also showed that constitutive overexpression of either *MAL61* maltose permease or *PmSUC2* suppresses the noninducible phenotype of a defective *mal13 MAL*-activator allele, suggesting that this suppression is solely a function of maltose transport activity and is not specific to the sequence of the permease. Our studies indicate that maltose permease does not function as the maltose sensor in *Saccharomyces*.

INTRODUCTION

Saccharomyces responds to the presence of a wide variety of environmental nutrients via sensing and signaling pathways capable of identifying the nutrients, determining their approximate concentrations, and integrating the information from these several signals to regulate gene expression and cell growth, proliferation, and morphology. Few nutrient or metabolite sensors have been identified despite extensive efforts in a variety of regulated systems. For the most part, those nutrient sensors identified to date fall into two categories: integral membrane receptor-like proteins and cytoplasmic nutrient/metabolite binding proteins. Reports suggesting a dual role for certain nutrient transporters as both transporters and sensors have been presented, but conclusive evidence for such, in the form of constitutive alleles of the transporter genes, is lacking (Lorenz and Heitman 1998).

We are interested in identifying the *Saccharomyces* maltose sensor. Previous studies demonstrated that maltose permease plays an essential role in maltose induction of *MAL* gene expression (Charron *et al.* 1986). Moreover, the ability of an α-glucoside sugar to serve as an inducer of *MAL* gene expression appears to be dependent on the substrate specificity of the transporter (Han *et al.* 1995). These results suggested the possibility that the *Saccharomyces* maltose permease could also play the role of the maltose sensor.

Five nearly identical *MAL* loci have been identified in *Saccharomyces cerevisiae*, each located at a telomere-associated site: *MAL1* (chromosome *VII*), *MAL2* (chromosome *III*), *MAL3* (chromosome *II*), *MAL4* (chromosome *XI*), and *MAL6* (chromosome *VIII*) (Charron *et al.* 1989). Different maltose fermenting strains carry at least one of these fully functional alleles but often two or more loci are present in a strain (Naumov *et al.* 1994). A typical *MAL* locus is a cluster of three genes all required for maltose fermentation. Gene 1 encodes maltose permease, a member of the 12-transmembrane domain family of sugar transporters; Gene 2 encodes maltase, an α-glucoside hydrolase; and Gene 3 encodes the *MAL*-activator, a DNA-binding transcription activator of the *MAL* genes (Charron *et al.* 1989). Genetic nomenclature uses both the locus number and the gene number. For example, *MAL61* encodes maltose permease at the *MAL6* locus. Natural variants of *MAL1* and *MAL3* containing nonfunctional alleles of Gene 1 or Gene 3 have been identified in strains from the wild and in common laboratory strains (Charron and Michels 1988; Michels *et al.* 1992). Induction of the *MAL* structural genes requires inducer, usually maltose, but certain other α-glucosides will also act as inducers in strains encoding an appropriate transport protein, such as maltose permease, and the *MAL*-activator (Charron *et al.* 1986; Han *et al.* 1995). Does maltose permease function simply for the accumulation of intracellular maltose whose presence is then monitored by some other mechanisms? Or, is maltose permease itself a maltose sensor capable of responding to extracellular maltose by initiating an intracellular signal?

Integral membrane proteins, particularly transporter-like homologues, are known to be utilized as sensors (Van Belle and Andre 2001). Well-studied examples are Snf3p and Rgt2p, integral membrane receptors that sense, respectively, low and high extracellular concentrations of glucose (Johnston 1999). Snf3 and Rgt2 proteins are structural and sequence homologues of the Hxt glucose transporters but are distinguished from the Hxt proteins by the presence of a long C-terminal cytoplasmic domain. Dominant gain-of-function mutations of *SNF3* and *RGT2* cause constitutive expression of the *HXT* genes. Moreover, the *HXT* genes cannot restore the signaling defect of *SNF3* or *RGT2* null mutations, and neither Snf3p nor Rgt2p are able to function as glucose transport proteins. Thus, although transporter-like, Snf3p and Rgt2p appear to serve solely as glucose sensors (Johnston 1999).

Additional members of the sugar transporter superfamily with unique structural features similar to Snf3p and Rgt2p have been identified as putative sugar sensors in other eukaryotes. Rco3 protein, a regulator of conidiation in *Neurospora crassa* (Madi *et al.* 1997), and Mst1 protein from *Amita muscaria* (Nehls *et al.* 1998) appear to act as glucose sensors. *SUT2* from *Arabidopsis* is proposed to encode a sucrose sensor in sieve element cells (Barker *et al.* 2000).

Utilization of transporter-like proteins as nutrient sensors is not unique to sugars. Ssy1 protein of *Saccharomyces* is a member of the large superfamily of amino acid permeases but is distinguishable from these permeases by its elongated N-terminal cytoplasmic domain (Iraqui *et al.* 1999). This cytoplasmic extension is reminiscent of the C-terminal domain of Snf3p and Rgt2p but no shared sequence homology has been identified. Results suggest that Ssy1 protein functions as a sensor of extracellular amino acids including leucine, isoleucine, and tryptophan. Similar to Snf3p and Rgt2p, Ssy1p requires the F-box protein Grr1p as a downstream effector in the amino acid signaling pathway.

Recent reports suggest that members of the G-protein coupled receptor class of proteins also function as glucose receptors in fungi. *Saccharomyces GPR1* encodes a member of the 7-transmembrane domain family of G protein coupled receptors that includes Ste2p and Ste3p, the mating-type pheromone receptors (Colombo *et al.* 1998;

Donzeau and Bandlow 1999; Kraakman *et al.* 1999; Lorenz *et al.* 2000; Xue *et al.* 1998). In conjunction with its G protein alpha subunit Gpa2p, Gpr1p regulates pseudohyphal differentiation in response to glucose. The *git3* gene of *Schizosaccharomyces pombe* encodes another member of the G protein coupled receptor family responsible for monitoring extracellular glucose (Welton and Hoffman 2000). Interestingly, both the *GPR1*-dependent pathway of *Saccharomyces* and the *git3*-dependent pathway of *Schizosaccharomyces pombe* regulate the activity of cAMP-dependent protein kinase (PKA), in particular the *Saccharomyces* Tpk2p isoform (Lorenz *et al.* 2000; Welton and Hoffman 2000; Yun *et al.* 1998).

Saccharomyces also utilizes intracellular nutrient sensors. The Snf1 protein kinase signaling pathway responds to high rates of glucose metabolism, possibly by monitoring changes in the ATP:AMP ratio produced by rapid glycolysis (Carlson 1999). Snf1 protein kinase is the catalytic subunit of a large protein complex that exhibits homology to mammalian AMP-activated protein kinases. It has been proposed that this kinase complex is the metabolite sensor, but this remains to be demonstrated in *Saccharomyces*. *Saccharomyces* Gal3 protein, a homologue of galactokinase (Gal1p), is the galactose sensor. Binding of galactose to Gal3p promotes Gal3p-Gal80p interaction thereby releasing the Gal4p transcription activation domain from Gal80p repression (Sil *et al.* 1999). Recent reports indicate that the Gal3p-Gal80p interaction occurs in the cytoplasm exclusively and effectively shifts the subcellular localization of Gal80p from the nucleus to the cytoplasm in the presence of galactose (Peng and Hopper 2000).

In the context of these studies on nutrient sensing in *Saccharomyces* and other fungi, we proposed to explore the role of the maltose transport protein, maltose permease, in the regulation of *MAL* gene expression. Our approach to resolving questions regarding the role of maltose permease in maltose sensing is based on the assumption that, if maltose permease were also to serve as a maltose sensor, this function would require specialized sequence features of maltose permease protein that could not be replaced by a heterologous protein capable of maltose transport. For this study we chose to use the high-affinity sucrose transporter from *Plantago major* encoded by *PmSUC2* (Gahrtz *et al.* 1994). PmSUC2 protein is a member of the 12-transmembrane domain superfamily of sugar transporters, and, although it exhibits little sequence homology with the *Saccharomyces* maltose permease, it is capable of transporting maltose albeit at lower affinity. We report here that expression of PmSUC2 in *Saccharomyces* restores maltose inducible *MAL* gene expression to maltose permease null mutants. Moreover, by other parameters explored in this study, we show that PmSUC2 transporter is able to fully replace maltose permease as a regulator of *MAL* gene expression.

MATERIALS AND METHODS

Yeast strains. The strains used in this study are listed in Table 1. CMY1001 is described in Medintz *et al.* (1996). It contains a single *MAL1* locus at which the *MAL11* maltose permease gene is replaced by the HA-tagged *MAL61*. No other *MAL* genes are present in this strain. Strain CMY1050 is a *MAL61/HA* null derivative of CMY1001 and was constructed by PCR-based gene disruption described in Medintz *et al.* (1998). Strain CMY1061 is a *MAL13* deletion disruption of CMY1050 and was constructed as follows. The appropriate upstream (5'-CCATGTAATCGCTGCATTCAGCGCAATTTGAACTGCACTCAGCTGAAGCTTCG TACGC) and downstream (5'-CGGTGCAAACAATAGTATGTCATGATTCGAA ATATGTCGGCATAGGCCACTAGTGGATCTG) primers were used to amplify the G418 resistance marker gene using pFA2-kanMX2 as template (Wach *et al.* 1994). Bases homologous to the template are underlined. The resulting PCR product, which has homology to *MAL13* sequence at both the 5' and 3' ends of the ORF, was then used for one-step replacement of *MAL13*. Candidate disruptants were confirmed by PCR analysis.

A similar PCR-based process was used to construct CMY1071 (*mal13Δ::G418*), CMY1072 (*mal33Δ::HygB*), and CMY1073 (*mal13Δ::G418 mal33Δ::HygB*) in strain YPH500. Strain YPH500 is isogenic to S288C and primers for the deletion of *mal13* and *mal33* were determined based on the sequence of S288C available at the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces/). The following primers 5'-ACTTTAACTAAGCAAACATGCGCCAAGCAGGCATGCGACTGCTG TCGATCAGCTGAAGCTTCGTACGC and 5'-ATCAAGGGTCTATGTCTTCA TTATCCTTGGGATAACCATCCAATTGTAAA GCATAGGCCACTAGTGGAT were

TABLE 1

***Saccharomyces cerevisiae* strains used in this study**

Strain	Genotype	Reference
CMY1001	*MATa MAL61/HA MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-200*	Medintz *et al.* (1996)
CMY1050	*mal61Δ::HIS3* (isogenic to CMY1001)	Medintz *et al.* (1998)
CMY1061	*mal61Δ::HIS3 mal13Δ::G418* (isogenic to CMY1001)	This study
YPH500	*MATα leu2-Δ1 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 ura3-52 AGT1 MAL12 mal13 MAL31 MAL32 mal33*	Sikorski and Hieter (1989)
CMY1071	*mal13Δ::G418* (isogenic to YPH500)	This study
CMY1072	*mal33Δ::HygB* (isogenic to YPH500)	This study
CMY1073	*mal13Δ::G418 mal33Δ::HygB* (isogenic to YPH500)	This study

used to amplify the $G418^R$ for *mal13* dispurtion. Primers 5'-ACTTTAGTCAAGTATGCATGCGACTATTGTCGTGTCCGTCGAGTAAAGTGCAGCTGAAGCTTCGTACGC and 5'-AGGAATTATGTCGTCTTCATCTTTGGAATCATCATTTAGGCGCAGTGGTCGCATAGGCCACTAGTGGAT were used to amplify Hyg^R for *mal33* disruption.

Plasmid construction and mutagenesis. *MAL61/HA* was constructed from *MAL61* by inserting a 12-codon sequence containing a single of copy of the HA epitope tag at the 5' end of the ORF (Medintz *et al.* 1996). *MAL61/HA* was subcloned into pUN30, yielding pMAL61/HA. The native *MAL61* promoter in pMAL61/HA was removed and replaced with the *ADH1* promoter at –12 basepair position of *MAL61*, yielding pADH1-MAL61/HA described in Medintz *et al.* (1998). Plasmids pMAL61/HA and pADH1-MAL61/HA were used as templates for *in vitro* mutagenesis using BioRad Mutagene Kit (Bio-Rad, Richland CA) to construct pMAL61/HA(Δ61-90) (Medintz *et al.* 2000), pMAL61/HA(Δ571-580), pADH1-MAL61/HA(Δ61-90), pADH1-MAL61/HA(Δ571-580) and a series of C-terminal nonsense mutations (pMAL61/HA-581NS, pMAL61/HA-575NS, pMAL61/HA-570NS and pMAL61/HA-560NS).

Plasmid pPTE18 was obtained from Norbert Sauer, University of Regensburg, Germany. *PmSUC2* cDNA encoding a *Plantago major* sucrose transporter was ligated into the *Eco*RI site of vector NEV-E yielding pPTE18 (Gahrtz *et al.* 1994). Expression of *PmSUC2* is controlled by the *S. cerevisiae PMA1* promoter.

Maltose transport assay. Maltose transport was measured as the rate of uptake of 1mM [^{14}C] maltose as described in Cheng and Michels (1989) and Medintz *et al.* (1996). Assays were done in duplicate on two or three transformants. V_{max} was determined by Lineweaver-Burk analysis as described in Medintz *et al.* (1996). Maltose transport activity and V_{max} are expressed as nanomoles of maltose transported per mg of cells per minute.

Maltase assay. Maltase activity was determined in total cell extracts as described by Dubin *et al.* (1985). Activity is expressed as nanomoles of PNPG (ρ-nitrophenyl β-D-glucopyranoside) hydrolyzed per mg of total protein per minute. The values reported are the average of duplicate assays obtained with extracts from at least two separate cultures.

Western analysis. Western blot analysis was carried out as described previously (Medintz *et al.* 1996). Mal61/HA protein was detected by using anti-HA specific antibody and the Vistra-ECF kit (Amersham) and the Storm 860 image analyzer (Molecular Dynamics). The relative protein levels were determined using the Storm 860 image capture software. Western blot analysis was done in duplicate on extracts prepared from duplicate experiments carried out with at least two independent transformants.

RESULTS

***PmSUC2* suppresses the noninducible phenotype of a maltose permease deletion**. CMY1001 is a maltose fermenting strain containing *MAL1* as the sole *MAL* locus (Medintz *et al.* 1996). The *MAL1* locus of CMY1001 encodes maltose permease (*MAL61/HA*), maltase (*MAL12*), and the *MAL*-activator (*MAL13*). Charron *et al.* (1986) showed that loss of either maltose permease or the *MAL*-activator blocks maltose induction of *MAL* gene expression but loss of maltase has no obvious effect on induction. *MAL61/HA* was deleted from strain CMY1001 to create CMY1050 (*mal61Δ::HIS3*) and maltose induction of maltase expression was determined (see Table 2). Under uninduced conditions (3% glycerol/2% lactate), CMY1001 expresses a low but significant level of maltose transport activity (0.17μM/min/mg dry wt cells). Deletion of the maltose permease gene reduces this rate to a background rate of 0.09 μM/min/mg that probably represents the nonspecific low-affinity binding activity described by Benito and Lagunas (Benito and Lagunas 1992) and not true transport. Table 2 also confirms that maltose induction is dependent on maltose permease. A 6-hour induction period is sufficient for full induction of maltase expression in CMY1001 while the permease deletion strain CMY1050 exhibits no induction during the same period.

PmSUC2 encodes a sucrose transporter from *Plantago major* that is a member of twelve transmembrane domain family of sugar transporters (Gahrtz *et al.* 1994). Blast analysis comparing the amino acid sequence of PmSUC2 to that of Mal61p indicates that these transporters do not share significant sequence homology (only 10% identity largely in transmembrane domains). When expressed in *Saccharomyces*, PmSUC2 was found to be capable of transporting sucrose and this sucrose transport was inhibited by maltose,

TABLE 2

Effect of *PmSUC2* on the expression of *MAL* genes

Relevant *MAL* genotype (Strain)	Plasmid	Maltose transport activity		Maltase activity	
		G/L	G/L + M	0h	6h
MAL61 MAL12 MAL13 (CMY1001)	Vector	0.17	5.61	48	576
mal61Δ MAL12 MAL13 (CMY1050)	Vector	0.09	0.11	21	20
	pPTE18 (*PmSUC2*)	0.68	ND	27	706
	pADH1-MAL61/HA	5.98	ND	26	643
mal61Δ MAL12 mal13Δ (CMY1061)	Vector	0.11	ND	27	21
	pPTE18 (*PmSUC2*)	0.56	ND	12	25
	pADH1-MAL61/HA	6.10	ND	12	18

ND: not determined.

Strain CMY1050 (*MAL61Δ::HIS3*) and CMY1061 (*MAL611Δ::HIS3 MAL13Δ::G418*) were transformed with plasmids pUN70, pPTE18 (*PmSUC2*), or pADH1-MAL61. Maltose transport activity was determined in early mid-log phase cells grown in selective medium containing 3% glycerol and 2% lactate (G/L) or G/L plus 2% maltose (G/L + M). For maltase activity, cells were grown to early mid-log phase in selective synthetic medium containing 3% glycerol and 2% lactate (G/L), and then transferred to G/L medium containing 2% maltose (G/L + M). Maltase activity was determined at the time of transfer to maltose (0 hr) and 6 hr after the transfer. Maltase activity and transport activity were assayed as described in Materials and Methods. Experimental variation for maltose transport activity is <20% and for maltase activity is <30%.

indicating that PmSUC2 could also be capable of transporting maltose (Gahrtz *et al.* 1994). Plasmid pPTE18, which carries the *PmSUC2* gene under the control of the constitutive *Saccharomyces PMA1* promoter, was introduced into the maltose permease deletion strain CMY1050 and the ability to support maltose induction tested. As shown in Table 2, growth in glycerol/lactate (G/L) allows expression of *PmSUC2* and produces a low but significant level of maltose transport activity. The maltose transport rates in cells expressing *PMA1promoter-PmSUC2* are approximately 10-fold lower than cells carrying *ADH1-MAL61*, but this reduced transport rate is nevertheless sufficient to cause wild-type levels of maltase induction. CMY1050 carrying the constitutively expressed *ADH1promoter-MAL61* is presented as a control.

To test whether PmSUC2-mediated induction is dependent on the *MAL*-activator, *MAL13* was deleted from CMY1050 creating CMY1061 (*mal61Δ mal13Δ*) and maltose induction of maltase was assayed in transformants constitutively expressing either Mal61p or PmSUC2. The results in Table 2 demonstrate that loss of the *MAL13* activator gene blocks maltose-induced maltase expression by both the PmSUC2 transporter and Mal61/HA permease. Thus, *PmSUC2*-dependent maltose induction requires the *MAL*-activator. Taken together, theses results indicate that intracellular maltose is sufficient to induce *MAL* gene expression independent of the mechanism of maltose transport and that induction by intracellular maltose is dependent on the *MAL*-activator.

The concentration of intracellular maltose correlates with the level of the induction. Medintz *et al.* (1998) constructed a series of deletions of N-terminal cytoplasmic domain of Mal61/HA permease as part of a study to localize the target site of glucose-induced endocytosis and proteolysis. One interesting mutation, a deletion of residues 61-90, exhibited little or no transport activity, but nonetheless was able to induce *MAL* gene expression. Further analysis of this mutant is shown in Table 3. Maltose transport activity of strains expressing *mal61/HA(Δ61-90)* from the native promoter when measured at a substrate concentration of 1mM maltose (standard assay condition) does not differ significantly from the vector control. Measurement of V_{max} using Lineweaver-Burk analysis found an approximately 3-fold increase in V_{max} compared to the vector control and suggests that the low level of maltose induction exhibited by this strain could be a function of this very low rate of maltose transport (Table 3). The K_m of the Mal61/HA(Δ61-90) permease is unaffected (approximately 1.1mM for both alleles). Expression of the *mal61/HA(Δ61-90)* from the constitutive *ADH1* promoter allowed for a modest increase in maltose transport activity and a slight increase in maltase induction levels. These activities were still too low to allow rapid maltose fermentation. As a result, the strain grows slowly on maltose as the sole carbon source and growth is not associated with carbon dioxide bubble formation.

An additional series of *MAL61/HA* mutants containing deletions of the C-terminal cytoplasmic domain were constructed by creation of a translation stop site at codon 560, 570, 575, or 581 and by removal of codons 571 to 580 as described in Materials and Methods. Plasmid-borne copies of these mutant alleles were transformed into strain CMY1050 and the transformants were tested for their ability to induce maltase activity. The results are shown in Table 4. Also shown are the levels of maltose transport activity and permease protein expressed by the transformants. A clear correlation can be observed between the levels of maltose transport activity and maltase induction. Additionally, residues 571-580 appear to play an important role in maltose transport activity but not an

TABLE 3

Maltose induction of *MAL* gene expression in strains carrying *MAL61/HA (Δ61-90)*

MAL61-HA allele	Maltose fermentation	V_{max}	Maltose transport activity	Maltase activity
MAL61/HA	+	7.14	5.96	1652
mal61/HA(Δ61-90)	-	0.21	0.07	320
ADH1-mal61/HA(Δ61-90)	-	1.42	0.21	463
Vector control	-	0.08	0.06	35

Strain CMY1050 was transformed with plasmid carrying the indicated *MAL61/HA* allele in vector pUN30 (Elledge and Davis 1988). Cells were grown in selective induced medium with 3% glycerol, 2% lactate and 2% maltose to mid-log phase. Maltase activity and maltose transport activity were determined as described in Materials and Methods. Fermentation was determined as growth in YP+Maltose medium plus the production of gas within 7 days. V_{max} was calculated by Lineweaver-Burk plot of maltose transport activity over a range of substrate concentrations as described in Medintz *et al.* (1996).

essential role in induction. Truncation of Mal61p to residue 580 has only a modest effect on transport activity while truncation to residue 574 or 559 or deletion of residues 571 to 580 severely reduces or eliminates transport (Table 4). Deletion of residues 571 to 580 reduces transport activity almost 20-fold but maltase induction is reduced to about one-third of wild-type levels (Table 4). The results reported in Tables 3 and 4 suggest that extremely low levels of intracellular maltose are able to trigger *MAL* gene induction and that, at these limiting intracellular maltose concentrations, the level of induction correlates with the presumed level of intracellular maltose.

Low rates of maltose transport activity cause a delay in induction by very low extracellular concentrations of maltose. The finding that at very low levels of maltose transport activity induction correlates with transport rates suggests that the threshold level of intracellular maltose needed to induce the *MAL* genes is very low. If a strain expressing *mal61/HA(Δ61-90)* from its native promoter were grown in medium containing a very low concentration of maltose, we would expect a delay in induction because greater time would be needed to accumulate sufficient intracellular maltose to reach this threshold level. We wished to test this prediction. Strains expressing either *mal61/HA(Δ61-90)* or *MAL61/HA* were grown to early log phase in uninduced conditions and transferred to media containing either 0.5% or 0.01% maltose. The time courses of maltase induction at these two concentrations are compared in Figure 1. When transferred to media containing 0.5% maltose, the strain expressing wild-type maltose permease initiates induction at approximately 30 minutes while induction is delayed to about 1-1.5 hours in the strain expressing mutant permease. Moreover, the rate of increase in maltase activity is far more rapid in cells expressing wild-type permease. In contrast, when the

TABLE 4

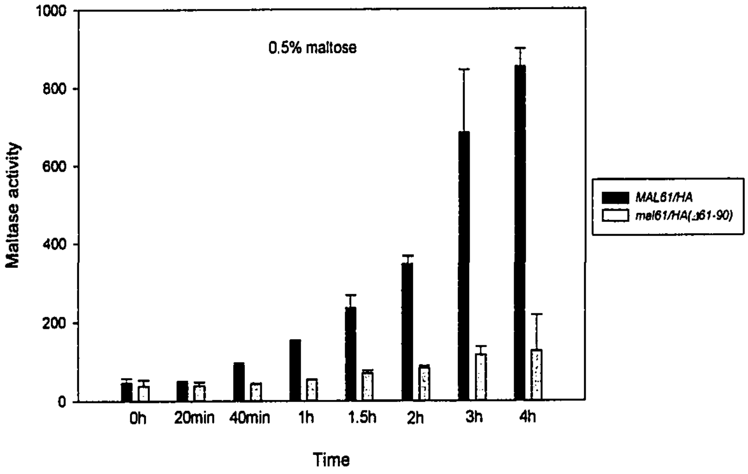
Maltose induction of *MAL* gene expression in strains carrying mutations in the C-terminal cytoplasmic domain of Mal61/HA maltose permease

MAL61/HA allele	Maltose fermentation	Maltase activity	Maltose transport activity	Relative level of Mal61/HA protein
MAL61/HA	+	2231	5.45	100
MAL61/HA-581NS	+	2117	2.68	79
MAL61/HA-575NS	+	1582	0.63	41
MAL61/HA-570NS	-	69	0.06	<1
MAL61/HA-560NS	-	77	0.06	<1
MAL61/HA(Δ571-580)	-	702	0.31	32
Vector control	-	42	0.06	<1

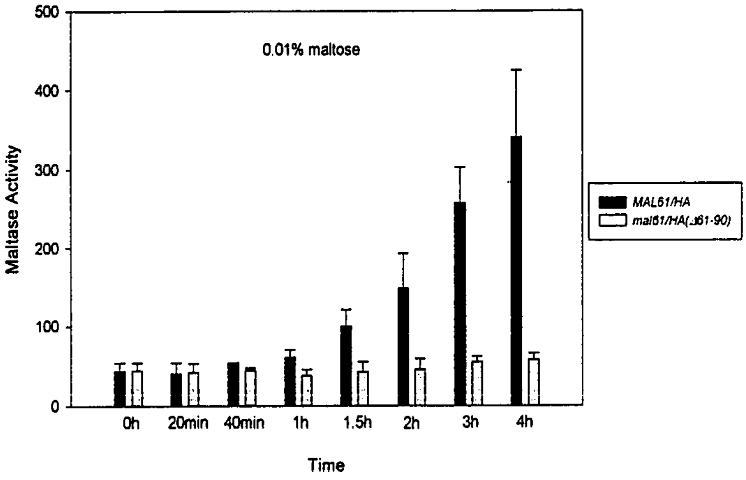
Strain CMY1050 was transformed with plasmid carrying the indicated *MAL61/HA* allele in vector pUN30. Cells were grown in selective induced medium with 3% glycerol, 2% lactate and 2% maltose to mid-log phase. Fermentation was determined as described in Table 3. Maltase activity, and maltose transport activity were determined as described in Materials and Methods. The relative protein levels of Mal61/HA permease were determined by comparison of Western blots using total cell extracts from cells grown under maltose-induced conditions.

Figure 1. Time course of maltase induction in strains expressing either *MAL61/HA* or *mal61/HA(Δ61-90)* maltose permease. CMY1050 strains expressing either *mal61/HA(Δ61-90)* or *MAL61/HA* were grown to early log phase in uninduced conditions (3% glycerol and 2% lactate) and transferred to inducing media containing 3% glycerol, 2% lactate plus either 0.5% (13.9mM) (A) or 0.01% (0.3mM) (B) maltose. Cells were collected at the indicated time points and maltase activity assayed as described in Materials and Methods. The data represent the averages and standard deviations of results obtained in at least two independent assays for cultures grown in both conditions.

A.


0.5% maltose
Maltase activity
1000
800
600
400
200
0
0h
20min
40min
1h
1.5h
2h
3h
4h
Time
MAL61/HA
mal61/HA(Δ61-90)

B.


0.01% maltose
Maltase Activity
500
400
300
200
100
0
0h
20min
40min
1h
1.5h
2h
3h
4h
Time
MAL61/HA
mal61/HA(Δ61-90)

time course of induction is followed in media containing 0.01% maltose, induction in the wild-type strain still initiates at about 30 minutes, although the rate of increase in maltase activity is slowed, but no significant induction is observed in the strain expressing mutant maltose permease even after 4 hours. These results are consistent with the proposal that a threshold level of intracellular maltose is needed in order to trigger *MAL* gene induction and that this threshold level is rather low.

Overexpression of Mal61 maltose permease rescues the defect in *MAL* induction caused by the *mal13*, but not *mal33* mutant activator. Strain YPH500, which is essentially isogenic to S288C, contains two *MAL* loci mapping to the right telomeres of chromosomes *VII* (*MAL1*) and *II* (*MAL3*), but is unable to ferment maltose and is defective for maltose induction (Table 5, line 3). This induction defect is complemented by a plasmid-borne copy of *MAL63* encoding the *MAL*-activator from *MAL6* (Table 5, line 1) indicating that the noninducible phenotype of YPH500 results from a lack of a functional *MAL*-activator. The amino acid sequence of the defective mal13p and mal33p *MAL*-activators encoded by YPH500 is 70.7% and 71.2% identical to Mal63p, respectively.

As part of a separate study of the *MAL*-activator, we introduced a plasmid carrying the *ADH1promoter-MAL61* gene into YPH500. Much to our surprise, we found that constitutive high-level expression of Mal61 permease is able to restore maltose inducibility of maltase expression to wild-type levels in strain YPH500 despite its defective *MAL*-activators (Table 5, lines 1, 2 and 4).

To determine whether rescue by *ADH1pro-MAL61/HA* is dependent on *mal13*, *mal33* or both, the single and double deletions of both genes were constructed in YPH500

TABLE 5

Constitutive *MAL61* expression suppresses the defective *mal13* *MAL*-activator but not *mal33* of strain YPH500

	MAL-activator alleles	Plasmid	Maltase activity	
			G/L	G/L + Maltose
1	*mal13 mal33* [*MAL63*]	Vector	40	1177
2	*mal13 mal33* [*MAL63*]	pADH1-MAL61/HA	37	1522
3	*mal13 mal33*	Vector	50	129
4	*mal13 mal33*	pADH1-MAL61/HA	16	1180
5	*mal13Δ mal33*	Vector	30	38
6	*mal13Δ mal33*	pADH1-MAL61/HA	56	97
7	*mal13 mal33Δ*	Vector	30	53
8	*mal13 mal33Δ*	pADH1-MAL61/HA	42	1240
9	*mal13Δ mal33Δ*	Vector	38	32
10	*mal13Δ mal33Δ*	pADH1-MAL61/HA	12	134

Strain YPH500 and isogenic disruptions of the defective *mal13* (CMY1071), *mal33* (CMY1072), and both *mal13* and *mal33* (CMY1073) were transformed with plasmid pADH1-MAL61/HA or the vector control pUN30. As a control, YPH500 was also transformed with the functional *MAL*-activator *MAL63* carried in vector pUN70 (lines 1 and 2). Cells were grown in selective uninduced (3% glycerol and 2% lactate) and induced media (3% glycerol, 2% lactate and 2% maltose) to mid-log phase. Maltase activity was assayed as described in Materials and Methods.

strain yielding CMY1071, CMY1072, and CMY1073, and maltose induction of maltase assayed. Loss of *mal13* blocked the suppressing effect of constitutive Mal61 permease (Table 5, line 6 and 10). The strain lacking only *mal33* was still rescued by overexpressed Mal61 permease as well as YPH500 strain (Table 5, line 8). These results clearly indicate that constitutive, high-level Mal61 permease expression rescues the induction defect caused by *mal13*, but not by *mal33*.

Rescue of defective mal13 *MAL*-activator is not specifically dependent on Mal61 maltose permease. The results shown in Table 5 may be interpreted in two ways. First, if maltose permease protein itself were to play a direct role in induction, simple restoration of maltose transport activity by an unrelated transport protein such as PmSUC2 permease should not be able to rescue the defective *mal13 MAL*-activator. An example of such is the direct binding of the *E. coli* MalT transcription activator to the maltose transport complex ($MalEFGK_2$) (Panagiotidis *et al.* 1998). Alternatively, if rapid delivery of an initial very high concentration of intracellular maltose were capable of triggering the *MAL* gene induction by the defective *mal13 MAL*-activator, different permeases with varied transport activities should vary in their abilities to rescue *mal13*. Thus, even the PmSUC2 encoded transporter should be capable of rescuing *mal13* despite its lack of sequence homology to Mal61 permease. To test this, plasmids constitutively expressing different versions of maltose permease genes (*MAL61/HA*, *mal61/HA(Δ61-90)*, and *PmSUC2*) were introduced to YPH500 and maltase induction assayed. The results in Table 6 clearly indicate that PmSUC2 permease is able to rescue the defective *mal13 MAL*-activator. Moreover, the significantly reduced level of maltose transport by PmSUC2 has a relatively modest impact on *mal13* suppression suggesting

TABLE 6

Constitutive *PmSUC2* suppresses the defective *MAL*-activator genes of strain YPH500

Plasmid	Maltose transport activity	Maltase activity	
		G/L	G/L + Maltose
Vector	0.08	50	129
pADH1-MAL61/HA	1.98	16	1180
pADH1-mal61/HA(Δ61-90)	0.34	16	290
pPTE18 (*PmSUC2*)	0.50	38	525

Strain YPH500 was transformed with the indicated plasmids that constitutively express *MAL61*, *mal61/HA(Δ61-90)*, or *PmSUC2*. Transformants were grown in selective uninduced (3% glycerol and 2% lactate) and induced (3% glycerol, 2% lactate and 2% maltose) media to mid-log phase. Maltase and maltose transport activity were assayed as described in Materials and Methods. Maltose transport activity is reported for cells grown in induced conditions.

that the suppression is solely a function of the maltose transport activity of PmSUC2 permease and is unrelated to any specific sequence feature of this protein.

DISCUSSION

Intracellular maltose is sufficient to induce *MAL* gene expression. We provide several lines of evidence demonstrating that intracellular maltose is sufficient to induce *MAL* gene expression in *Saccharomyces*. PmSUC2, a sucrose transporter from *Plantago major*, was used as a surrogate for the *Saccharomyces* maltose permease to transport maltose into a strain lacking the native maltose permease. Blast analysis shows that PmSUC2 shares no significant sequence homology to the Mal61 and Agt1 maltose permeases. Sequence identity among these transporters is about 10% and is largely confined to the transmembrane domains. Given the lack of significant sequence homology, we feel confident in concluding that PmSUC2 is only functioning as a maltose transporter in our *Saccharomyces* strains. The finding that PmSUC2 is able to replace the *Saccharomyces* maltose permease therefore provides strong evidence that accumulation of intracellular maltose concentrations is sufficient to stimulate *MAL* gene induction. Our results do not exclude the possibility that the true inducer is a metabolite of maltose and not maltose itself, but we believe this to be unlikely.

The level of intracellular maltose required to initiate induction and to maintain the induced state appears to be quite low. Strains expressing defective maltose permeases exhibit a significant reduction in their maximal induced levels of maltase activity (Tables 3 and 4) and induction correlates well with the reduced rate of maltose transport capacity of these strains. Moreover, very low levels of maltose transport cause a delay in maltase induction when extracellular maltose concentrations are limiting. Both findings suggest that accumulation of intracellular maltose to a threshold level is needed to initiate the induction and that the threshold level is rather low.

Maltose permease does not appear to function as a maltose sensor. Demonstrating that intracellular maltose is sufficient to stimulate *MAL* gene induction does not exclude the possibility that the *Saccharomyces* maltose permease also serves a sensor-like role in induction. Our finding that constitutive high-level expression of Mal61 maltose permease suppresses the defective *mal13*-encoded *MAL*-activator suggested a possible role in induction for maltose permease similar to that the *E. coli* maltose transport complex in the bacterial maltose utilization system. In the *E. coli* maltose-maltodextrin system, uptake is mediated by a periplasmic-binding-protein-dependent ABC transporter (the MalEFGK$_2$ complex). MalT, a transcriptional activator, is sequestered at the plasma membrane in the inactive monomeric form by binding to the idling transporter complex via MalK. Transport of maltose by the transporter complex releases MalT from MalK-MalT interaction enabling it to form active oligomeric MalT in the presence of the effectors ATP and maltotriose (Boos and Bohm 2000).

One possible mechanism by which constitutive Mal61 permease expression could suppress the defective *mal13* is by specifically binding the activator. In uninduced growth conditions the permease might sequester the *MAL*-activator at the plasma membrane and thereby prevent the *MAL*-activator from entering the nucleus and activating *MAL* genes transcription. Conformational changes in maltose permease induced by maltose transport might destabilize the putative interaction between maltose permease and the *MAL*-activator resulting in release of *MAL*-activator, nuclear entry, and *MAL* gene activation. Binding of defective mal13 activator to the constitutive elevated levels of maltose permease might protect it from degradation or other forms of inactivation thereby suppressing this mutant form of the activator.

Our finding that constitutive expression of *PmSUC2* also suppresses the defective *mal13* *MAL*-activator argues against the specific-binding model and supports a second model. In this model the large bolus of maltose transported into the cell via the abundant maltose permease suppresses the defect of mal13p, suggesting that the defect of the mutant activator protein is in its ability to respond to the maltose signal.

Based on these results, we propose that maltose permease serves solely as a maltose transporter. It accumulates intracellular concentrations of maltose to levels sufficient to both induce *MAL* gene expression and provide an energy source for growth. In cells grown in uninduced conditions, the basal low level of maltose permease expression is sufficient to transport enough maltose into the cell to allow for the accumulation of intracellular maltose to levels that are adequate to promote the activation of the *MAL*-activator and induce further structural gene expression. Wykoff and O'Shea (Wykoff and O'Shea 2001) similarly conclude that the phosphate transporter Pho84p, which is essential during phosphate starvation, does not serve as the phosphate sensor and suggest that intracellular phosphate concentration regulates *PHO* gene expression.

So, what is the maltose sensor? One likely possibility is that the *MAL*-activator itself binds to maltose directly. In work to be reported elsewhere we have shown that maltase induction is dependent on the Hsp90 molecular chaperone complex (B. Zhang, M. Bali, K. Morano and C.A. Michels; personal communication). Several other transcriptional activators are known to associate with Hsp90 (Helmbrecht *et al.* 2000). Members of the steroid receptor family of activators are released from the chaperone complex following direct binding of the cognate steroid (Richter and Buchner 2001). Similarly, Hap1 activator release from the Hsp90 chaperone complex is dependent on heme binding (Hach *et al.* 2000; Zhang *et al.* 1998). An alternate possibility is that a Gal3-like protein might serve as the maltose sensor. We do not favor this hypothesis because, despite extensive genetic analysis, specific maltose nonfermenting mutants have not been isolated in genes unlinked to the *MAL* loci. Currently, we are undertaking genetic approaches to identify the maltose-binding sensor.

CHAPTER 2

A Genetic Study Of *MAL* Gene Expression In *Saccharomyces*

ABSTRACT

In *Saccharomyces*, transcription of the *MAL* structural genes is induced 40-fold by maltose and induction requires the *MAL*-activator and maltose permease (to accumulate intracellular inducer). To identify possible additional players involved in regulating *MAL* gene expression, we carried out a genetic selection for *MAL* constitutive (Mal^c) mutants. Strain CMY4000 was constructed containing *MAL1* and integrated copies of *MAL61pro-HIS3* and *MAL61pro-LacZ* reporter genes. CMY4000 was UV mutagenized and mutants capable of growth on galactose medium lacking histidine were selected. Of 33 His+ mutants, 31 were constitutive for maltase and *LacZ* reporter expression (10-70% of fully induced levels). The 29 recessive mutants fall into at least two complementation groups: Group 1 (10 alleles) and Group 2 (6 alleles). Both groups of mutants are pleiotropic and cause slow growth, flocculation and insensitiveness to glucose repression. The genes represented by Group 1 and Group 2 mutations are demonstrated to be *RGR1* and *SIN4* respectively. Moreover, we show that S*IN4* and *RGR1* regulate *MAL* gene expression in a common pathway.

INTRODUCTION

Saccharomyces maintains a variety of nutrient sensing mechanisms that enable it to respond to different nutrients and monitor nutrient levels. These include sensing mechanisms for carbon sources, particularly glucose but also other fermentable carbon sources (reviewed Ozcan and Johnston 1999); nitrogen sources, including ammonia, urea, and amino acids in general (reviewed in Forsberg and Ljungdahl 2001; ter Schure *et al.* 2000); and other requirements such as phosphate (Wykoff and O'Shea 2001). At least three sensing mechanisms are utilized to monitor glucose levels alone: the Snfl protein kinase pathway, the Rgt2/Snf3 receptor pathway, and the Gprl/Gpa2 signaling pathway (reviewed in Johnston 1999; Thevelein and de Winde 1999; Versele *et al.* 2001). Systems for sensing specific sugars, such as galactose or maltose, or specific amino acids, such as histidine or proline, also are present. Both the specific systems and the more global regulatory systems are integrated via multiple mechanisms.

The work of many laboratories has contributed to our understanding of the mechanism of galactose sensing. Gal3 protein is central to galactose sensing (Bhat and Murthy 2001). Gal3p exhibits homology to Gal1 kinase, the first enzyme in the galactose utilization pathway. Recent studies from the Hopper laboratory suggest that internalized galactose binds to Gal3p thereby activating the ability of Gal3p to bind Gal80 repressor (Peng and Hopper 2000; Peng and Hopper 2002). Nuclear-localized Gal80 repressor binds to the activation domain of Gal4 transcription activator blocking its ability to activate the *GAL* genes. Gal80 repressor shuttles between the nucleus and the cytoplasm. But, when Gal80p is sequestered in the cytoplasm by interaction with galactose-bound Gal3p, Gal4p is free to activate *GAL* gene expression. Thus, Gal3p is the primary galactose sensor in *Saccharomyces* and galactose-induced *GAL* gene expression is delayed 2-3 days in *gal3* mutants.

A major interest of our laboratory is the sensing mechanism for maltose and other α-glucosides. Studies of maltose fermentation undertaken during the last 50 years, including work from our laboratory, demonstrated that maltose induction of *MAL* gene expression depends on the *MAL*-activator and maltose permease (Charron *et al.* 1986; Charron *et al.* 1989). Deletion of the gene encoding maltase causes a nonfermentable phenotype but maltose induction of maltose permease is unaffected indicating that this enzyme is not required for induction but only for utilization of maltose (Charron *et al.* 1986). In Chapter 1 of this thesis, we report that the role of maltose permease in induction is the accumulation of intracellular maltose but the means of sensing the presence of intracellular maltose remains undetermined. It is possible that the *MAL*-activator itself is the maltose-binding sensor. Alternately, other positive or negative regulators may be involved but may have not been identified as yet because they are encoded by repeated or essential genes. To identify possible additional players involved in regulating *MAL* gene expression, we designed a sensitive genetic selection for *MAL* constitutive (Mal^c) mutants. This approach should allow us to identify dominant constitutive alterations in positive regulators or recessive alterations in previously unidentified negative regulators.

MATERIALS AND METHODS

Yeast strains, media, and genetic methods. The strains used in this study are listed in Table 1. CMY1001 is described in Medintz *et al.* (1996). It contains a single *MAL1* locus at which the *MAL11* maltose permease gene is replaced by the HA-tagged *MAL61*. No other *MAL* genes are present. Other strains listed in Table 1 are derived from CMY1001. Strain CMY4000 was constructed by inserting two YIp355-based plasmids carrying *MAL61pro-LacZ* and *MAL61pro-HIS3* reporter genes into the *leu2* gene of CMY1001 by targeted integration. Strain CMY4001 was created by changing mating type of CMY4000 from **a** to α using plasmid pGHOT obtained from R. Rothstein (Columbia University). A *URA3* plasmid (YIp355) was integrated into the *ura3-52* gene of CMY4001 to create a strain CMY4002.

Yeast standard media are as described in Ausubel *et al.* 2002. All yeast strains were grown in rich media or selective minimal media with 2% glucose (SD), 2% maltose (SMal) or 2% galactose, 3% glycerol and 2% lactic acid (SGalG/L). Yeast transformations were done via the lithium acetate / polyethylene glycol protocol of Schiestl *et al.* (Schiestl *et al.* 1993). Mating, sporulation and tetrad analysis were done by standard *Saccharomyces* genetic methods (Ausubel 1988; Guthrie and Fink 1991).

Mutagenesis and isolation of *MAL* constitutive mutants. Strain CMY4000 was grown in YPD to mid-log phase. Cells were collected by centrifugation, washed, and resuspended in sterile water. The cell suspension was mutagenized by exposure to UV light of wavelength 254 nm for sufficient time so as to allow for approximately 15% survival. The mutagenized cells were immediately plated onto SGalG/L media lacking histidine, and the plates were incubated in the dark for 5 days at 25°C. Thirty-three

TABLE 1

***Saccharomyces cerevisiae* strains used in this study**

Strain	Genotype	Reference
CMY1001	*MAT***a** *MAL61/HA MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-200*	Medintz *et al.* (1996)
CMY4000	*MAT***a** *MAL61/HA MAL12 MAL13 GAL ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-200 leu2::MAL61pro-LacZ::MAL61pro-HIS3*	This study
CMY4001	*MATα MAL61/HA MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-200 leu2::MAL61pro-LacZ::MAL61pro-HIS3*	This study
CMY4002	*MATα MAL61/HA MAL12 MAL13 GAL leu2 ura3-52:* YIp355 *lys2-801 ade2-101 trp1-Δ63 his3-200 leu2:MAL61pro-LacZ:MAL61pro-HIS3*	This study
CMY5009	*sin4Δ::G418* (isogenic to CMY4000)	This study
CMY5030	*rgr1-31 sin4Δ::G418* (isogenic to CMY4000)	This study

independent potential *MAL* constitutive (Mal^c) mutants formed colonies. Their ability to grow on the SGalG/L-His media was confirmed. Each mutant was screened for expression of the *MAL61pro-LacZ* reporter and *MAL12* expression by β-galactosidase plate assay and maltase assay, respectively, using galactose-grown cells.

Plasmid construction. Plasmid pUN30-MAL61pro-ADE2 carrying the *ADE2* open reading frame under the control of the *MAL61* promoter was constructed as follows. Plasmid YIp365-I61 (Danzi *et al.* 2000) carrying the *MAL61* promoter was digested with *Eco*RI and *Sal*I to liberate a 0.9kb fragment containing basepairs –874 to –1 of the *MAL61* promoter. This was subcloned into vector pUN30 (Elledge and Davis 1988) forming pUN30-MAL61pro. The *ADE2* open reading frame (approximately 1.7kb) was amplified by PCR from plasmid pRS402 (ATCC87477) with primers 5'-GGGGGTCGACATGGATTCTAGAACAGTTGG-3' and 5'-GGGGGCATGCAGATCTTATGTATGAAATTC-3'. This amplified PCR product was digested with *Sal*I and *Sph*I and inserted downstream of the *MAL61* promoter in pUN30-MAL61pro to create pUN30-MAL61pro-ADE2.

Cloning of wild-type alleles of mutant gene in strain CM-31 and CM-33. The constitutive mutants CM-31 and CM-33 were chosen as representative strains from complementation group 1 and group 2, respectively. CM-31 and CM-33 are pink on SM media because of the presence of the *ade2-101* mutant allele. Each strain was transformed with pUN30-MAL61pro-ADE2 and the resulting strain forms white colonies on SGalG/L media because of the constitutive expression of the *MAL61pro-ADE2* reporter. These strains were then transformed with a centromere-based YCp50 genomic library containing *URA3* prepared from strain S288C (a gift from Lucy Robinson). Pink

Ura+ transformants on SGalG/L media were isolated. These potentially carry the dominant wild-type allele of the mutation present in CM-31 or CM-33. Dependence of pink color on the presence of the library plasmid was determined and complementation of constitutive maltase expression phenotype was confirmed for each transformant. The library plasmid was isolated from each transformant and retransformed into the CM-31 or CM-33 [pUN30-MAL61pro-ADE2] mutant strains to confirm the complementation. The yeast insert in each library plasmid was identified by sequencing the YCp50-insert junction.

Sequencing of *rgr1* mutant alleles. The genomic copy of each of the *rgr1* mutant alleles was amplified by PCR using primers 5'-GTAGAGGTCTGTTGTAAAGATCATC-3' (53-77 bases before the start codon) and 5'-TTCAGGAGAGGGGTTACAATCTCC-3' (complementary to sequence 36-59 bases after the stop codon) and High Fidelity Platinum *Taq* DNA Polymerase (Invitrogen) to insure the fidelity of amplification product. Seven sequencing primers were designed every about 500 base pairs along the ORF based on the *RGR1* sequence in S288C obtained at the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces/). The site of the alteration was sequenced in independent amplifications to insure that it was not a PCR error.

Maltase assay. Maltase activity was determined in total cell extracts as described by Dubin *et al.* (1985). Activity is expressed as nanomoles of PNPG (ρ-nitrophenyl β-D-glucopyranoside) hydrolyzed per mg of total protein per minute. The values reported are the average of duplicate assays obtained with extracts from at least two separate cultures. The values from different cultures varied approximately 15%.

β-Galactosidase plate assay. Cells were patched onto a plate containing the appropriate selective medium and grown for two days. A substrate-agarose mixture was prepared by mixing melted agarose with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal) solution and maintained as a liquid at 55-60°C. The final mixture contains 0.5% agarose, 0.5M Na_2HPO_4-NaH_2PO_4 pH7 buffer, 0.1% SDS, 2% dimethylformaride, and 0.05% X-Gal. Approximately 10ml of this mixture was poured over the surface of the culture plate and photographs were taken following 6-12 hours of blue color development.

Flocculation assay. Cells were grown overnight in 5ml liquid minimal medium to approximately mid log phase. The culture was vortexed briefly to separate and suspend the cells as best as possible in the medium, and the culture tubes were photographed immediately, and after 15 and 30 minutes of standing in a test tube rack.

RESULTS

Isolation of mutants that constitutively express *MAL* genes. To select for *MAL* constitutive mutants (Mal^c) we constructed strain CMY4000, which carries the complete *MAL1* locus encoding maltose permease (*MAL61/HA*), maltase (*MAL12*), and the *MAL*-activator (*MAL13*) as well as integrated copies of both *MAL61pro-HIS* and *MAL61pro-LacZ* reporter genes (see Material and Methods) as diagrammed in Figure 1. The ability of CMY4000 to grow in the absence of histidine is dependent on the presence of maltose in the growth medium. Mutations causing constitutive *MAL* gene expression should provide the ability to grow in the absence of histidine even under uninduced growth conditions (galactose or glycerol/lactate medium) and should allow for the constitutive expression of β-galactosidase, maltose permease, and maltase.

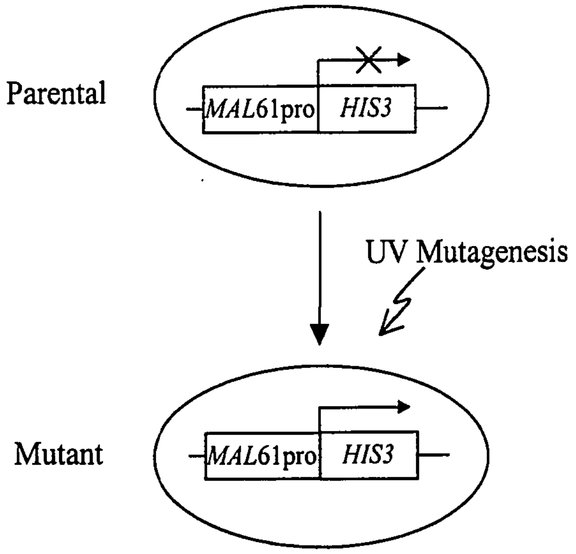
To select for Mal^c mutants, approximately 10^7 cells were mutagenized with UV-light and plated directly onto minimal medium containing galactose plus glycerol/lactate and lacking histidine. A total of 33 potential Mal^c mutant colonies were isolated and the phenotype of each is listed in Table 2. Of these, 31 also exhibited constitutive expression of the *MAL61pro-lacZ* reporter gene based on plate assay (data not shown) and constitutive maltase expression ranging from 10% to 70% of fully induced levels (Table 2). Thus, the 31 Mal^c mutants carry alterations in transacting regulators of *MAL* gene expression. Mutants CM-22 and CM-26 exhibit only constitutive expression of the *MAL61pro-HIS3* reporter and were not considered further. It is noted that mutant CM-15 showed a significant defect in maltase induction.

To determine whether the constitutive mutations are dominant or recessive, each Mal^c mutant was mated to CMY4002, a *MAT*α strain isogenic to CMY4000. Growth on

Figure 1. Selection strategy for Mal^c mutants. Strain CMY4000 (*MAL1 his3Δ-200 MAL61pro-HIS3 MAL61pro-LacZ)* is a histidine auxotroph in media lacking maltose. CMY4000 cells were UV mutagenized as described in the Materials and Methods and histidine prototrophs were selected in galactose media lacking histidine. Potential Mal^c mutants were confirmed by measuring *LacZ* expression by plate assay and maltase activity levels in galactose-grown cells.

Starting strain: CMY4000 (*his3 MAL61pro-HIS3*)

Expected Phenotype



MAL61pro off on Galactose
His$^-$ on Galactose

MAL61pro constitutively on
His$^+$ on Galactose (Mal^c)

TABLE 2

MAL gene expression in Mal^c mutants

Strain	Growth on media lacking histidine		Maltase activity	
	2% Maltose	2% Galactose	2% Maltose	2% Galactose
CMY4000	+++	-	1728	41
CM-1	+++	+++	2059	302
CM-2	+++	+	1128	162
CM-3	+++	++	2134	527
CM-4	+++	+++	2261	278
CM-5	++	++	2346	348
CM-6	++	++	1806	269
CM-7	++	++	2174	456
CM-8	+++	++	1701	212
CM-9	++	++	1574	452
CM-10	+++	++	1249	117
CM-11	+++	+	1020	167
CM-12	+	+	1452	234
CM-13	+++	+++	1312	315
CM-14	+++	+	1143	180
CM-15	++	++	556	281
CM-16	++	++	2290	214
CM-17	++	++	1771	794
CM-18	+	+	928	305
CM-19	++	+	1160	308
CM-20	+++	+++	1592	359
CM-21	+++	+	2158	396
CM-22	++	+++	351	39
CM-23	+++	+	1177	173
CM-24	++	++	2944	1236
CM-25	+++	+	1248	180
CM-26	+++	+++	1763	36
CM-27	+++	+	1158	158
CM-28	+++	+++	1557	435
CM-29	+++	++	1574	524
CM-30	+++	++	1420	347
CM-31	+++	++	1218	239
CM-32	+++	++	1932	876
CM-33	++	++	1779	465

CMY4000 and the Mal^c mutants were streaked for single colonies on minimal media lacking histidine with either 2% maltose or 2% galactose and growth rates monitored for 3 days. Maltase activity was assayed in cells grown in minimal media with either 2% maltose or 2% galactose as described in Materials and Methods.

minimal galactose medium lacking histidine and maltase expression in galactose-grown cells was determined in the heterozygous diploids. The results in Table 3 clearly show that two mutants, CM-24 and CM-32, contain dominant Mal^c mutations. The alteration in mutant CM-24 is fully dominant and the heterozygous diploid exhibits nearly fully induced levels of maltase. CM-32 carries a partially dominant alteration. The alterations in the remaining 29 mutants are recessive.

The diploids obtained from the recessive Mal^c mutants were sporulated for tetrad analysis. Unfortunately, sporulation levels were very poor for all of the diploids. Most asci contained only 2 ascospores and germination rates were low making genetic analysis extremely difficult. Only for mutants CM-31 and CM33 were we able to obtain sufficient 3- or 4-spored tetrads to follow segregation of the Mal^c phenotype. Totally 11 4-spored and 2, 3-spored tetrads were obtained from the mating of CM-31 to CMY4002; and 12, 4-spored tetrads were obtained from the mating of CM-33 to CMY4002. In both cases, the Mal^c phenotype segregated 2:2 ratio, that is, in a 4-spored tetrad 2 segregants grew on galactose medium lacking histidine (the Mal^c phenotype) and 2 segregates did not (data not shown). Thus, both mutant strains CM-31 and CM-33 contain a single mutant gene causing the Mal^c phenotype.

Complementation analysis of the remaining Mal^c mutants was carried out as follows. Haploid *MATα* segregants carrying the Mal^c mutation obtained from the cross of CM-31 with CMY4002 or CM-33 with CMY4002 were mated to each of the 29 Mal^c mutants and the phenotype of the diploid determined. Lack of complementation should give constitutive diploids capable of growth on minimal medium lacking histidine in the absence of maltose. Complementation should restore the maltose-inducible phenotype

TABLE 3

Determination of dominance of Mal^c mutants

Diploid strain	Growth in media lacking histidine		Maltase activity
	2% maltose	2% Galactose	2% Galactose
CMY4000 x CMY4002	+++	-	36
CM-1 x CMY4002	++	-	34
CM-2 x CMY4002	+++	-	17
CM-3 x CMY4002	++	-	24
CM-4 x CMY4002	++	-	15
CM-5 x CMY4002	+++	-	22
CM-6 x CMY4002	+++	-	15
CM-7 x CMY4002	+++	-	16
CM-8 x CMY4002	+++	-	48
CM-9 x CMY4002	+++	-	30
CM-10 x CMY4002	++	-	50
CM-11 x CMY4002	++++	-	31
CM-12 x CMY4002	++	-	32
CM-13 x CMY4002	+++	-	30
CM-14 x CMY4002	+++	-	31
CM-15 x CMY4002	+++	-	30
CM-16 x CMY4002	+++	-	38
CM-17 x CMY4002	+++	-	35
CM-18 x CMY4002	++	-	43
CM-19 x CMY4002	+++	-	32
CM-20 x CMY4002	++	-	24
CM-21 x CMY4002	++	-	24
CM-23 x CMY4002	+++	-	33
CM-24 x CMY4002	+++	+	1665
CM-25 x CMY4002	+++	-	41
CM-27 x CMY4002	++	-	30
CM-28 x CMY4002	+++	-	27
CM-29 x CMY4002	++	-	21
CM-30 x CMY4002	++	-	21
CM-31 x CMY4002	+++	-	23
CM-32 x CMY4002	++	+	190
CM-33 x CMY4002	+++	-	28

CMY4000 and each Mal^c mutant were mated with CMY4002 and the resulting diploids assayed for maltase activity as described in Materials and Methods. Growth on minimal media lacking histidine with either 2% maltose or 2% galactose was determined as described for Table 2.

causing the diploid to be unable to grow on minimal medium lacking histidine in uninduced conditions. The results in Table 4 indicate that the 29 recessive Mal^c mutants fall into at least two complementation groups. Group 1 includes 10 Mal^c mutants: CM-7, -9, -10, -13, -17, -18, -19, -20, -30, and -31. Group 2 includes 5 Mal^c mutants: CM-5, -21, -28, -29, and -33. The remaining 14 recessive mutants lie in at least one additional complementation group but have not been studied further.

Group 1 and Group 2 mutations are pleiotropic. During the genetic analysis of the Mal^c mutants, we observed that some grew much more slowly than the parental strain and also that some were flocculent, that is, when grown in liquid media the cells quickly settled to the bottom of the culture tube. We compared growth rates on rich media (YPD) and flocculation rate of all of the mutants in both complementation groups. As shown in Figure 2, six out of ten Group 1 mutants, CM-7, -9, -10, -13, -18, and -31, exhibit a slow growth phenotype but the remaining four mutants, CM-17, -19, -20, and -30, grow as well as the parental strain indicating allelic variation. All Group 2 mutants grow normally.

Flocculation is easily observed in liquid medium. CMY4000 cells separate soon after cytokinesis and cultures take hours to clarify because the cells settle-out slowly. Flocculent strains, in which the daughter cells adhere tightly and form large clusters, sediment much more quickly. To measure the extent of flocculation, each mutant strain was grown overnight in liquid minimal medium, vortexed briefly to separate and suspend the cells in the culture medium, and the culture tubes photographed immediately and after 15 and 30 minutes. As shown in Figure 3, Group 1 mutants are all modestly flocculent to varying degrees compared to CMY4000, in which only a small clear region can be seen

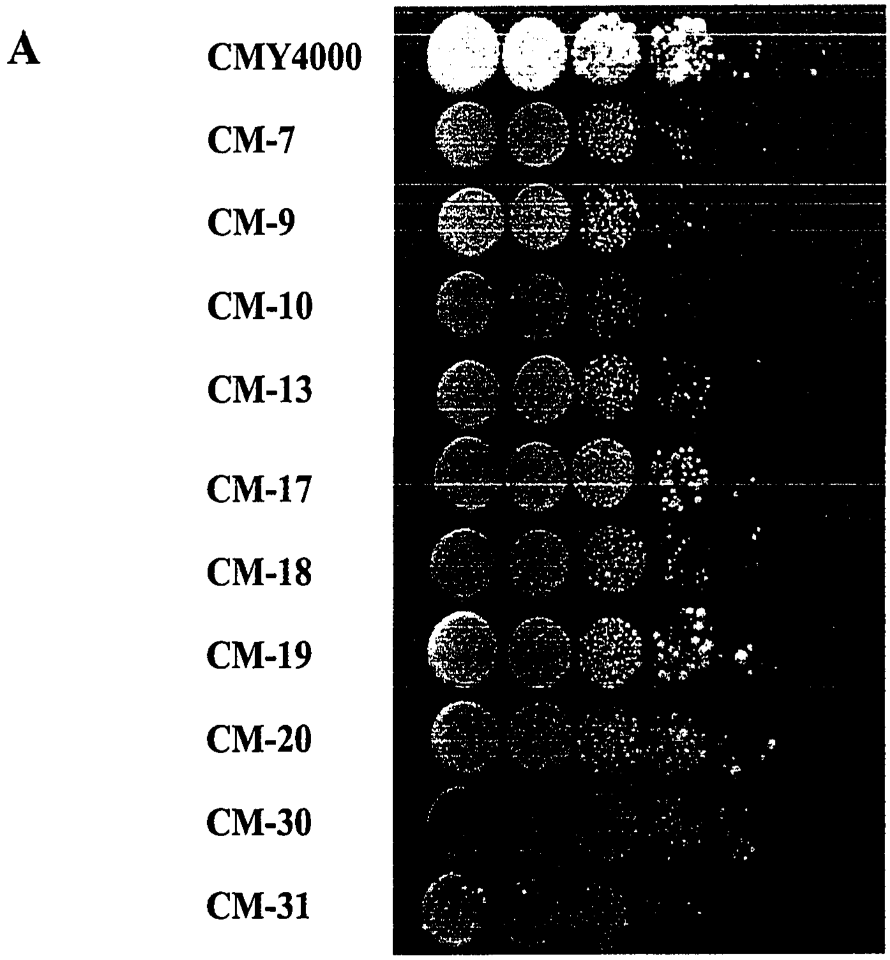
TABLE 4

Complementation analysis of recessive Mal^c mutants

Parental strain	Growth of diploid on 2% galactose media lacking histidine	
	Group 1 tester strain (from CM-31)	Group 2 tester strain (from CM-33)
CM-1	-	-
CM-2	-	-
CM-3	-	-
CM-4	-	-
CM-5	-	+
CM-6	-	-
CM-7	+	-
CM-8	-	-
CM-9	+	-
CM-10	+	-
CM-11	-	-
CM-12	-	-
CM-13	+	-
CM-14	-	-
CM-15	-	-
CM-16	-	-
CM-17	+	-
CM-18	+	-
CM-19	+	-
CM-20	+	-
CM-21	-	+
CM-23	-	-
CM-25	-	-
CM-27	-	-
CM-28	-	+
CM-29	-	+
CM-30	+	-
CM-31	+	-
CM-33	-	+

Haploid *MATα* segregants derived from the mating of CM-31 or CM-33 with CMY4002 were mated to each of the 29 recessive Mal^c mutants. The ability of the resulting diploids to grow on minimal media lacking histidine with 2% galactose was monitored as described for Table 2. No growth (-) indicates complementation. Growth (+) indicates that the Mal^c mutations do not complement.

Figure 2. Growth rates of group 1 mutants (A) and group 2 mutants (B). Cells of CMY4000 and each of the group 1 mutants (A) and group 2 mutants (B) were grown in YPD overnight to an OD600 of 0.1. A series of 10-fold dilutions were performed and 3μl of each dilution was spotted onto YPD media. The plates were incubated for two days and photographed.


A
CMY4000
CM-7
CM-9
CM-10
CM-13
CM-17
CM-18
CM-19
CM-20
CM-30
CM-31

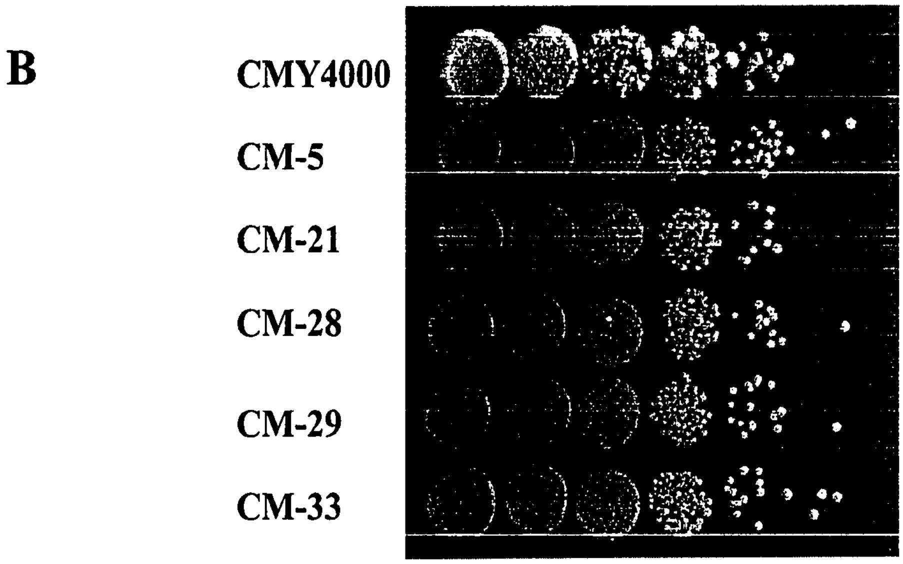
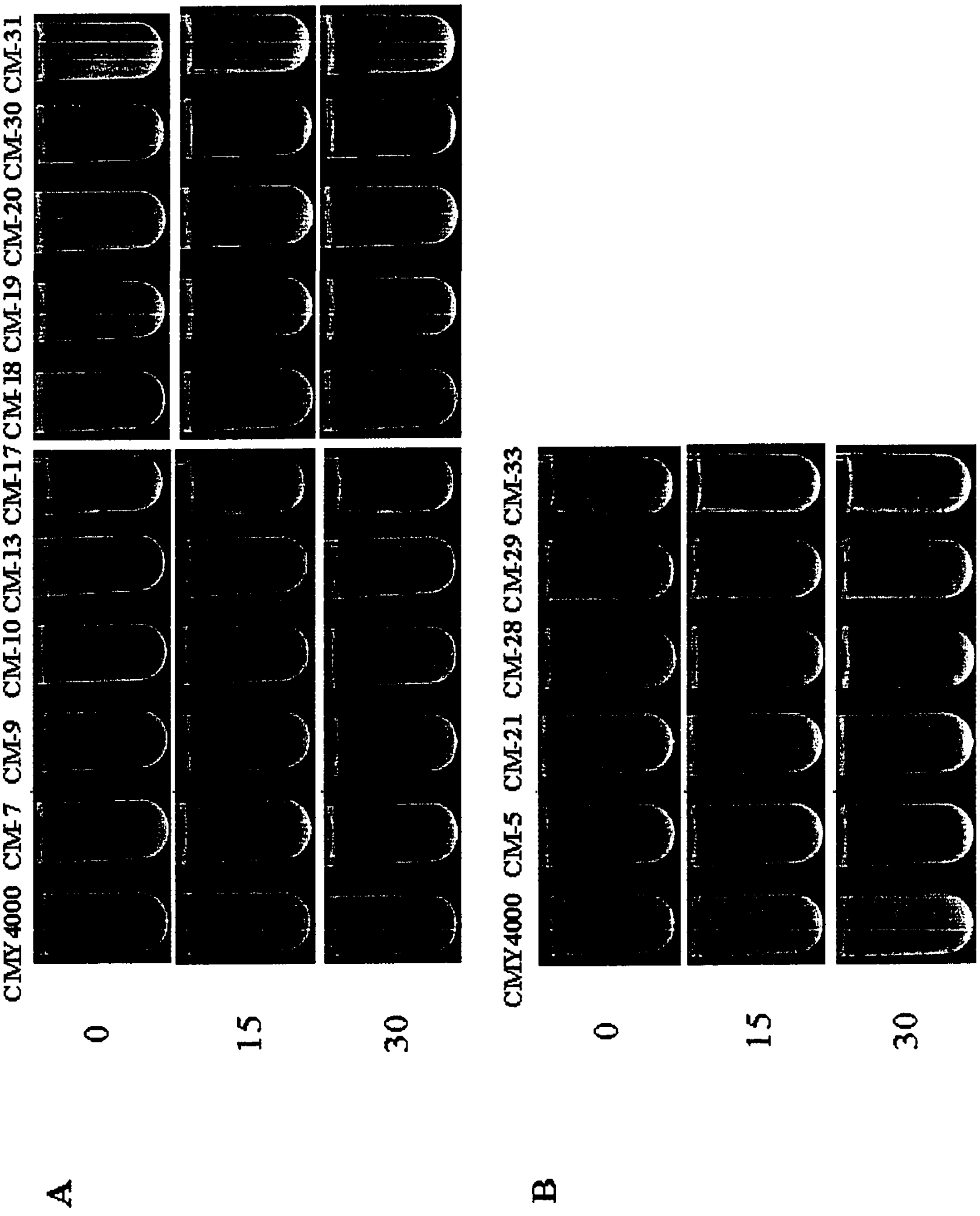

B
CMY4000
CM-5
CM-21
CM-28
CM-29
CM-33

Figure 3. Flocculation phenotype of group 1 mutants (A) and group 2 mutants (B). Cultures of CMY4000 and each group 1 mutant (A) and group 2 mutant (B) were grown overnight in liquid minimal medium, vortexed briefly to separate and suspend the cells in the culture medium, and allowed to stand without further agitation. The culture tubes were photographed immediately and after 15 and 30 minutes.


A
CMY4000
CM-7
CM-9
CM-10
CM-13
CM-17
CM-18
CM-19
CM-20
CM-30
CM-31
0
15
30
B
CMY4000
CM-5
CM-21
CM-28
CM-29
CM-33
0
15
30

at the top of the tube. All of the Group 2 mutants are so flocculent that the cells grew in clumps, can not be adequately resuspended even after vigorous vortexing, and largely remain at the bottom of the tube.

Glucose as the preferred carbon source inhibits transcription of *MAL* genes, a phenomenon referred to as glucose repression. To investigate whether Group 1 or Group 2 mutants affect glucose repression, maltase expression in strains carrying the mutant allele from CM-31 and CM-33 were assayed following growth under maltose-induced and uninduced growth conditions. The results in Table 5 clearly demonstrate that mutations in both genes partially relieve glucose repression.

Taken together, these results indicate that the Group 1 and Group 2 mutations are pleiotropic suggesting that the genes encode global regulators controlling the expression of diverse genes, not specifically the *MAL* genes.

Group 1 and Group 2 Mal^c mutations represent alleles of *RGR1* and *SIN4*, respectively. The wild-type alleles of the Group 1 and Group 2 Mal^c mutations were cloned by complementation from a low-copy yeast genomic library (YCp50-based) using the following strategy. A reporter plasmid carrying the *ADE2* gene under the control of the *MAL61* promoter was introduced into the Mal^c mutant strain, either CM-31 or CM-33, which also carry the *ade2-101* mutation. As a result of the constitutive expression of the plasmid-borne *MAL61promoter-ADE2* gene the transformant strains form white colonies on galactose-containing medium. Library plasmids carrying the dominant wild-type allele should restore the maltose inducible phenotype thereby blocking expression of the *MAL61promoter-ADE2* reporter and producing pink colonies on galactose-containing medium. Transformant colonies carrying a library plasmid were selected on minimal

TABLE 5

Group 1 and group 2 mutations relieve glucose repression

Strain	Maltase activity		
	2% Galactose 3% Gly + 2% Lac	2% Maltose	2% Glucose
CMY4000	46	1728	2
CM-31	254	1218	116
CM-33	432	1779	108

CMY4000, CM-31 (a group 1 mutant) and CM-33 (a group 2 mutant) were grown in minimal media under uninduced (2% galactose, 3% glycerol and 2 % lactate), induced (2% maltose) and repressed (2% glucose) conditions and maltase activity assayed as described in Materials and Methods.

medium lacking uracil with 2% galactose and screened for pink colonies. Dependence on the library plasmid was confirmed by plasmid loss. The transformants were screen to identify those that also exhibit low uninduced levels of maltase expression and normal flocculation rates. The library plasmid was recovered from each independent transformant and the sequence of the ends of the yeast insert determined.

Two plasmids were isolated that restored inducible expression of the *MAL61promoter-ADE2* reporter in CM-31, pM31-2 and pM31-10. The yeast inserts are diagramed in Figure 4. The overlapping region contains six intact ORFs on both strands. Two plasmids, pUN70/RGR1 and YCp50-Half, were constructed from pM31-10 and pUN70/RGR1 containing only *RGR1* was able to partially complement the Mal^c phenotype of CM-31 and fully complement the flocculation phenotype. YCp50-Half containing the other five ORFs did not complement either phenotype.

The partial complementation of the Mal^c phenotype concerned us so the complete open reading of all 10 presumed *rgr1* mutant alleles obtained by our selection scheme was determined to confirm the presence of a mutation. The results are summarized in Table 6. All *rgr1* alleles contain a single alteration, either a nonsense or a frame-shift mutation located in the region of codons 710-910 of this 1082-codon ORF. Thus, Group 1 mutants are alleles of *RGR1*, an essential gene encoding a scaffold-like component in the middle and tail region of the RNAPII mediator complex (Asturias *et al.* 1999; Dotson *et al.* 2000). The lack of full complementation of the *rgr1-31* allele by plasmid-borne *RGR1* is unexplained but could indicate decreased plasmid stability.

Six plasmids, pM33-1, 3, 5, 6, 11, and 12, were isolated from the CEN genomic library that restored inducible expression of the *MAL61promoter-ADE2* reporter in CM-

Figure 4. Cloning of the gene that complements group 1 mutations. Strains CMY4000 (wild-type) and CM-31 (a group 1 mutant) carrying the indicated plasmid were tested for Mal^c phenotypes. The yeast genomic inserts were depicted based on the sequence information available at the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces/) and the insert-vector boundary delimited by dotted lines. Transformants were grown in minimal media lacking uracil with 2% galactose, 3% glycerol and 2% lactate and maltase activity assayed as described in Materials and Methods. Flocculation was tested as described in Materials and Methods.

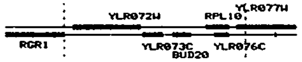
Host strain	Plasmid	Insert	Maltase Activity	Flocculation
CMY4000	YCp50	None	68	-
CM-31	YCp50	None	260	+
	pM31-2		84	-
	pM31-10		79	-
	YCp50-Half		289	+
	pUN70-RGR1		126	-

TABLE 6

Mutation type and position of *rgr1* alleles

Allele	Mutation type	Codon
rgr1-7	nonsense	897
rgr1-9	nonsense	710
rgr1-10	nonsense	897
rgr1-13	nonsense	892
rgr1-17	frame-shift	848
rgr1-18	nonsense	731
rgr1-19	nonsense	892
rgr1-20	frame-shift	854
rgr1-30	nonsense	893
rgr1-31	frame-shift	910

33. These plasmids complemented both the Mal^c and the flocculation phenotypes (Figure 5). All six plasmids carry a genomic insert fragment from the same chromosomal region. Plasmids pM33-1 and pM31-3 contain the same insert fragment. The overlap of these insert fragments contains two intact ORFs, *SIN4* and *YNL235C*. Subsequent cloning of *SIN4* in pUN70 done using primers that exclude the promoter region of *YNL235C* (forming plasmid pUN70-SIN4) enabled us to demonstrate that the complementing region is the *SIN4* gene. Thus, the Group 2 mutations are alleles of *SIN4*.

Sin4p is a component in the tail region of RNAP II mediator complex (Asturias *et al.* 1999; Dotson *et al.* 2000; Myers *et al.* 1999). *SIN4* is not an essential gene in *Saccharomyces*. Therefore we were able to delete the entire *SIN4* ORF in strain CMY4000 to create CMY5009. Table 7 compares the level of constitutive expression of *sin4Δ* to the *sin4-33* allele present in CM-33. The phenotypes are similar suggesting that the *sin4* mutations isolated here are null alleles.

***SIN4* and *RGR1* regulate *MAL* gene expression in a common pathway.** Although Sin4p and Rgr1p are both components of the mediator complex, it is possible that each could transmit a different signal to the RNA polymerase II to regulate *MAL* gene expression. To test this, *SIN4* was deleted in CMY4000 and the isogenic *rgr1-31* strain (CMY5001) to create CMY5009 and CMY5030, respectively. Maltase activity was assayed in cells grown in uninduced conditions. As shown in Figure 6, the double mutant *rgr1-31 sin4Δ* displays a similar level of constitutive maltase expression compared to the single mutant *sin4Δ* indicating that *RGR1* and *SIN4* function in a common pathway to regulate *MAL* gene expression. This conclusion is not in conflict with the fact that lower

Figure 5. Cloning of the gene that complements group 2 mutations. Strains CMY4000 and CM-33 (a group 2 mutant) carrying the indicated plasmid were tested for Mal^c phenotypes. The yeast genomic inserts were depicted based on the sequence information available at the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces/) and the insert-vector boundary delimited by dotted lines. Transformants were grown in minimal media lacking uracil with 2% galactose, 3% glycerol and 2% lactate and maltase activity assayed as described in Materials and Methods. Flocculation was tested as described in Materials and Methods.

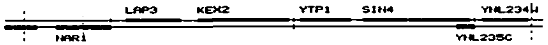
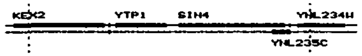
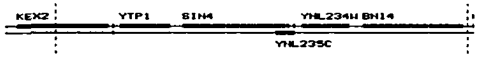
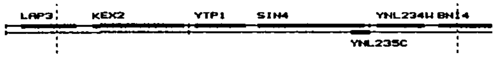
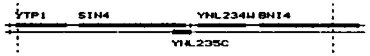
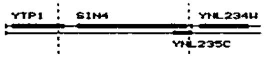
Host strain	Plasmid	Insert	Maltase Activity	Flocculation
CMY4000	YCp50	None	68	-
CM-33	YCp50	None	432	+
	pM33-1/3		75	-
	pM33-5		111	-
	pM33-6		84	-
	pM33-11		86	-
	pM33-12		81	-
	pUN70-SIN4		83	-

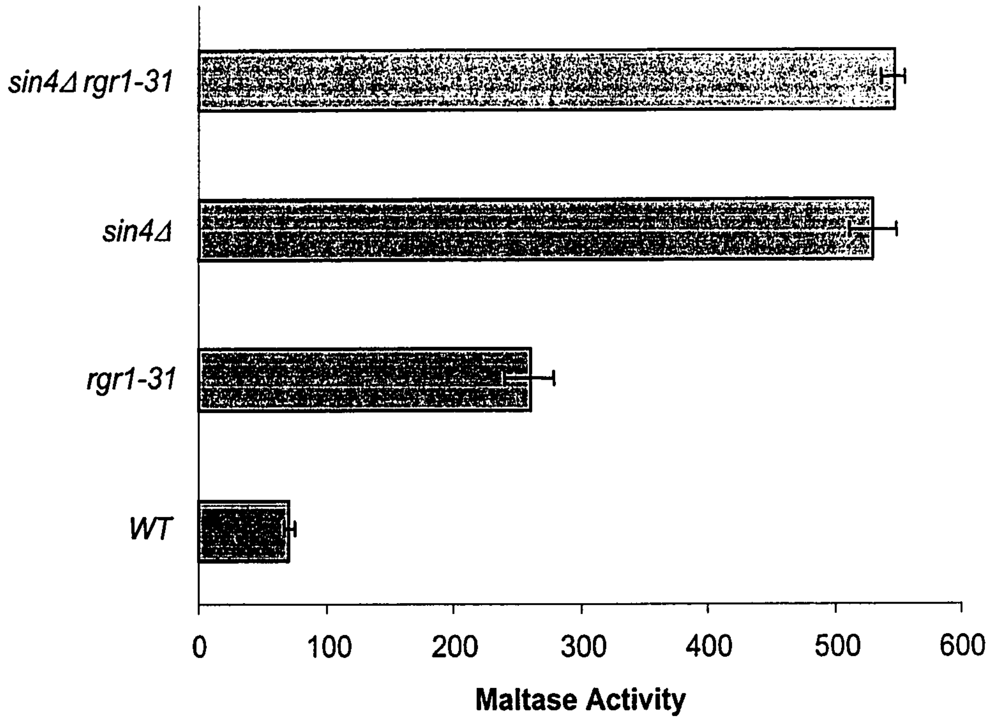
TABLE 7

Mal^c mutaions in *sin4* are null alleles

Strain	Relevant genotype	Maltase activity	
		2% Maltose	2% Galactose 3% Gly + 2% Lac
CMY4000	*SIN4*	1728	46
CM-33	*sin4-33*	1779	432
CMY5009	*sin4Δ*	1899	457

CMY4000 (wild-type), CM-33 (*sin4-33*) and CMY5009 (*sin4Δ::G418*) were grown in minimal media containing 2% maltose or 2% galactose, 3% glycerol and 2 % lactate and maltase activity assayed as described in Materials and Methods.

Figure 6. *RGR1* and *SIN4* are in a common pathway. CMY4000 (wild-type), CM-31 (*rgr1-31*), CMY5009 (*sin4Δ::G418*) and CMY5030 (*rgr1-31 sin4Δ::G418*) were grown in minimal media with 2% galactose, 3% glycerol and 2% lactate. Maltase activity was assayed as described in Materials and Methods.


sin4Δ rgr1-31
sin4Δ
rgr1-31
WT
0
100
200
300
400
500
600
Maltase Activity

levels of maltase activity were observed in *rgr1-31* (CM-31) than in *sin4Δ* (CMY5009) because *RGR1* is an essential gene and *rgr1-31* is only a partial loss-of-function allele.

DISCUSSION

We set out to isolate *MAL* constitutive mutations in an effort to identify novel regulatory factors involved in the maltose sensing pathway of *Saccharomyces*. One class of mutants that one might have expected to isolate was constitutive alterations in the *MAL*-activator. None were obtained but in retrospect this is not surprising. The strain used in this study, CMY4000, carries *MAL1*. *MAL1*, unlike *MAL6*, lacks the linked nonfunctional *mal64* gene at which a simple nonsense mutation is capable of producing a dominant constitutive mutant (Gibson *et al.* 1997). Other previously isolated constitutive mutations mapping to *MAL*-activator genes were obtained by X-ray mutagenesis or appeared to result from rare genomic conversion events (Khan *et al.* 1973; Wang and Needleman 1996). The two dominant constitutive mutations isolated in this study have not been extensively characterized but preliminary work does not suggest that these alterations map to the *MAL13* gene. Interestingly, both strains CM-24 and CM-32 tend to lose their integrated reporter genes during growth, probably by recombination between tandem repeats of the inserted copies, although CM-24 is more stable. This suggests that the dominant constitutive mutations present in CM-24 and CM-32 are in global regulators and not in the *MAL*-activator.

RGR1 is essential for viability in *Sacchromyces* and has homologs in higher eukaryotes such as *C. elegans*, *Drosophila*, mouse, and human (Boube *et al.* 2002; Woychik and Hampsey 2002). Sequence comparison between yeast and *C. elegans* Rgr1 protein reveals that the three regions of greatest homology are found in the N-terminus, the essential portion of the protein (Myers and Kornberg 2000). The N-terminus of Rgr1p interacts with the Med9/Med10 sub-module of the middle region of the mediator complex

(Boube *et al.* 2002). The middle region reportedly makes contact with Srb4 module in the head region and provides a crucial function in RNAPII transcription initiation by relaying activating signal to the initiation machinery (Han *et al.* 2001; Kang *et al.* 2001). The C-terminal region of Rgr1p exhibits a great deal more variability, consistent with the suggestion that this region is the site of signal input. It has been demonstrated by biochemical studies that C-terminus of Rgr1p anchors the Sin4 module, comprised of Sin4p, Gal11p, Med2p and Pgd1p, to the mediator complex (Li *et al.* 1995; Myers *et al.* 1999). None of these are essential components in *Saccharomyces* but, as will be described in detail in Chapter 3, they play different roles in *MAL* gene expression.

Mutations in the Rgr1p C-terminus cause a variety of phenotypes, such as resistance to glucose repression, sporulation deficiency, and defects in the regulation of *HO* and *IME1* expression (Jiang *et al.* 1995; Jiang and Stillman 1992; Sakai *et al.* 1990). Our collection of *rgr1* alleles is the most extensive reported and may help determine the functional domains of Rgr1p. It is significant that only alterations causing C-terminal truncations of Rgr1 protein were obtained. This suggests that missense mutations in this region do not sufficiently disrupt the reported compact three-dimensional structure of this region to affect its function in the mediator complex. Based on the phenotypes of the *rgr1* nonsense and frame-shift mutations investigated in this study, we observed no clear correlation between the severity of the phenotype and the map position of the mutation. It is premature to draw conclusions regarding the role of the C-terminal region of Rgr1 protein in the absence of information regarding the stability of each Rgr1 mutant protein. Nonetheless, a more complete analysis of these alleles comparing all of the pleiotropic phenotypes should provide useful information regarding a structure-function analysis of this multifunctional scaffold-like protein. Our finding that Sin4p and Rgr1p function in a common pathway for regulating *MAL* gene expression implies that the C-terminal region of Rgr1p is required for the interaction with Sin4p, which is consistent with the reported findings (Li *et al.* 1995; Myers *et al.* 1999). Its role in the interaction with other components of the tail complex of the *Saccharomyces* mediator and their affect on *MAL* gene expression will be explored in Chapter 3.

Genetic analysis suggests that mediator is involved in both transcriptional activation and repression depending on the specific promoter (Myers and Kornberg 2000). The results described here indicate that Rgr1p and Sin4p play negative roles in the regulation of *MAL* gene expression. The Sin4p module, consisting of Sin4p, Pdg1p/Med2p, Med3p, and Gal11p, has been proposed to play a dedicated role in direct sensing of signals from gene-specific regulators. Gal11 and Pgd1 have been shown to bind specifically with transcriptional activator and repressor proteins including Gal4p, Gcn4p, Swi5p, and Tup1p via distinct and/or overlapping surfaces (Bhoite *et al.* 2001; Han *et al.* 1999; Myers *et al.* 1999). Previous work from our laboratory demonstrated that Mig1p and Mig2p are involved in the glucose repression of *MAL* gene expression but deletion of both *MIG1* and *MIG2*, while relieving glucose repression of *MAL* gene expression (Hu *et al.* 1995; Hu *et al.* 2000), does not lead to the levels of constitutive expression seen here for *sin4* and *rgr1* mutants. In Chapter 3 we explore the possibility that Mig1, 2 repressors and/or other unidentified repressors recruit the yeast Mediator to the *MAL* structural gene promoter via interaction with the Sin4 module and dissect the role of each component of the Sin4 module in *MAL* gene regulation.

CHAPTER 3

The Role of the Mediator in *MAL* Gene Regulation

ABSTRACT

The Mediator complex of *Saccharomyces cerevisiae* is required for diverse regulatory functions controlling transcription by RNA polymerase II. The Sin4 module has been proposed as an integral part of the Mediator utilized for sensing repressing or activating signals from gene-specific regulators. Previously we demonstrated that Rgr1p and Sin4p act to repress the basal expression of *MAL* genes in a common pathway. In this report we show that Rgr1p and Sin4p do not act via Mig1, 2 repressors. The derepression caused by mutations in *RGR1* and *SIN4* does not involve either the *MAL*-activator or the maltose permease and thus is independent of the maltose sensing/signaling pathway. We also demonstrate that each component of the Sin4 module plays a distinct role in regulating *MAL* gene expression. Gal11p is involved in both basal expression and induction. Pgd1p, and Med2p are required for maltose induction only. We show that Swi/Snf complex is required for the full induction of the *MAL* genes suggesting a role for chromatin remodeling in the activation of the *MAL* genes.

INTRODUCTION

Transcription by *Saccharomyces* RNA polymerase II (RNAPII) is a complex and highly regulated process that involves a large number of factors including the general transcriptional factors (TFIIA, -B, -C, -D, -E, -F, -G and -H), TAFs (TBP-associated factors), and the Mediator complex. The Mediator was first discovered as an activity in a yeast crude fraction able to relieve interference between different activators (Gill and Ptashne 1988). The Mediator is functionally linked to the RNAPII through the physical interaction with the C-terminal domain of the largest subunit (Rbp1) of RNAPII (Thompson *et al.* 1993). Functional analysis of purified Mediator in an *in vitro* reconstituted system indicates that Mediator is required for both basal and regulated transcription, as well as to stimulate the phosphorylation of the C-terminal domain (CTD) of the largest subunit of RNA polymerase II Rpb1 by the TFIIH-associated kinase (Kim *et al.* 1994; Myers *et al.* 1998). More recently, a number of the Mediator-like multiprotein complexes have been isolated from mammalian cells, including TRAP/SMCC, ARC/DRIP, mMED, hSUR2, NAT, CRSP and PC2 complexes (Malik and Roeder 2000; Naar *et al.* 2001; Rachez and Freedman 2001).

Yeast Mediator complex is comprised of at least 20 subunits including Srb proteins, Med proteins, and several other proteins originally discovered in a variety of genetic screens for transcriptional factors (Carlson 1997; Myers and Kornberg 2000). Genetic, biochemical, and three-dimensional imaging analyses have revealed three functional substructures termed the Sin4 module, the Med9/Med10 module, and the Srb4 module corresponding to the tail, middle, and head domains observed in the electron microscopy image of the Mediator, respectively. The Srb4 module includes Srb2, -4, -5, -

6, Rox3, Med6, -8, and -11 proteins and interacts directly with the RNAPII CTD, along with TBP and TFIIB (Kang *et al.* 2001). Therefore, it is thought to directly modulate RNAPII activity. The Med9/Med10 module is composed of two stable submodules, the Med9 submodule containing Med1, -4 and -9 proteins, and the Med10 submodule containing Srb7, Med7, Med10, and Nut1 proteins. Genetic analysis suggests that the Med9 submodule is involved in transcriptional repression and the Med10 submodule is required to relay activating signals to the RNAPII initiation machinery (Han *et al.* 2001; Kang *et al.* 2001). The Sin4 module consists of Sin4, Rgr1, Med2, Pgd1, and Gal11 proteins and has been found to be involved in both transcriptional repression and activation. It is proposed that the Sin4 module acts as an integral part of Mediator directly sensing the repressing or activating signals from gene-specific regulators (Bhoite *et al.* 2001; Han *et al.* 1999; Myers *et al.* 1999). Park *et al.* (2000) have shown direct interaction between Gal11p and the activators Gal4, Gcn4, VP16, and Swi5 as well as an interaction between Pgd1p and Gcn4p. Pgd1p has been demonstrated to associate directly with the Cyc8-Tup1 corepressor complex (Papamichos-Chronakis *et al.* 2000).

We isolated recessive mutations in *SIN4* and *RGR1* in a genetic selection for *MAL* constitutive (Mal^c) mutants and demonstrated that Sin4p and Rgr1p act in a common pathway to repress *MAL* gene expression (Chapter 2). In this chapter, we show that the Mediator controls both basal and maltose-inducible *MAL* gene expression. We examine different *MAL* gene-specific regulators in an effort to identify those responsible for the repressing signal mediated by Sin4 and Rgr1 proteins. The roles of other components of the Sin4 module were compared. Gal11p acts to activate both basal-level and maltose-induced *MAL* gene expression. Pgd1p and Med2p are required only for maltose induction. The role of Swi/Snf complex in *MAL* gene activation is also explored.

MATERIALS AND METHODS

Yeast strains, media, and genetic methods. The strains used in this study are listed in Table 1. CMY1001 is described in Medintz *et al.* (1996). It contains a single *MAL1* locus at which the *MAL11* maltose permease gene is replaced by the HA-tagged *MAL61*. No other *MAL* genes are present in this strain. Strain CMY4000 was constructed by integrating plasmids carrying *MAL61pro-LacZ* and *MAL61pro-HIS3* reporter genes into CMY1001 as described in Chapter 2. Strains CMY5001 and CMY5002 were isolated by UV mutagenesis as described in Chapter 2. The remaining strains in Table 1 from CMY5003 to CMY5024 were constructed by disrupting the corresponding gene(s) with PCR-based one-step gene replacement method in CMY4000, CMY5001 or CMY5002. Primer pairs used in each different gene disruption were determined based on the sequence of S288C available at the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces/) and are listed in Table 2. The appropriate upstream and downstream primers were used to amplify the G418 resistance marker gene using pFA2-kanMX2 as template or the hygromycin resistance marker gene using pAG32 as template (Wach *et al.* 1994). Sequences homologous to template pFA2-kanMX2 or pAG32 are underlined. Candidate disruptants were confirmed by PCR analysis.

Yeast standard media are as described (Ausubel 1988; Guthrie and Fink 1991). All yeast strains were grown in selective complete synthetic media with 2% glucose (SD), 2% maltose (SMal) or 2% galactose, 3% glycerol, and 2% lactic acid (SGalG/L). Yeast transformations were done via lithium acetate / polyethylene glycol protocol of Schiestl *et al.* (1993).

TABLE 1

Saccharomyces cerevisiae strains used in this study

Strain	Genotype	Reference
CMY1001	*MAT*a *MAL61/HA MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-200*	Medintz *et al.* (1996)
CMY4000	*MAT*a *MAL61/HA MAL12 MAL13 GAL ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-200 leu2::MAL61pro-LacZ::MAL61pro-HIS3*	Chapter 2
CMY5001	*rgr1-31* (isogenic to CMY4000)	Chapter 2
CMY5002	*sin4-33* (isogenic to CMY4000)	Chapter 2
CMY5003	*mal13Δ::G418* (isogenic to CMY4000)	This Study
CMY5004	*mal61Δ::G418* (isogenic to CMY4000)	This study
CMY5005	*rgr1-31 mal13Δ::G418* (isogenic to CMY4000)	This study
CMY5006	*sin4-33 mal13Δ::G418* (isogenic to CMY4000)	This study
CMY5007	*rgr1-31 mal61Δ::G418* (isogenic to CMY4000)	This study
CMY5008	*sin4-33 mal61Δ::G418* (isogenic to CMY4000)	This study
CMY5009	*sin4Δ::G418* (isogenic to CMY4000)	Chapter 2
CMY5010	*pgd1Δ::G418* (isogenic to CMY4000)	This Study
CMY5011	*med2Δ::G418* (isogenic to CMY4000)	This Study
CMY5012	*gal11Δ::G418* (isogenic to CMY4000)	This Study
CMY5013	*mig1Δ::G418* (isogenic to CMY4000)	This Study
CMY5014	*mig2Δ::HygB* (isogenic to CMY4000)	This Study
CMY5015	*mig1Δ::G418 mig2Δ::HygB* (isogenic to CMY4000)	This Study
CMY5016	*rgr1-31 mig1Δ::G418* (isogenic to CMY4000)	This Study
CMY5017	*rgr1-31 mig2Δ::HygB* (isogenic to CMY4000)	This Study
CMY5018	*rgr1-31 mig1Δ::G418 mig2Δ::HygB* (isogenic to CMY4000)	This Study
CMY5019	*sin4-33 mig1Δ::G418* (isogenic to CMY4000)	This Study
CMY5020	*sin4-33 mig2Δ::HygB* (isogenic to CMY4000)	This Study
CMY5021	*sin4-33 mig1Δ::G418 mig2Δ::HygB* (isogenic to CMY4000)	This Study
CMY5022	*srb10Δ::G418* (isogenic to CMY4000)	This Study
CMY5023	*rgr1-31 srb10Δ::G418* (isogenic to CMY4000)	This Study
CMY5024	*sin4Δ::HygB srb10Δ::G418* (isogenic to CMY4000)	This Study
BLY1	*MATα lys2-801 his3-Δ200 ura3-52*	Gifts from Brehon C. Laurent
BLY3	*MATα snf5-Δ2 his3-Δ200 ura3-52 ade2-101*	
BLY5	*MATα snf6-Δ2 his3-Δ200 ura3-52 suc2*	
BLY13	*MAT*a *swi1Δ::LEU2 his3-Δ200 lys2-801 ura3-52 ade2-101 trp1Δ1 leu2-Δ1*	
BLY14	*MAT*a *swi3Δ::TRP1 his3-Δ200 lys2-801 ura3-52 ade2-101 trp1Δ1 leu2-Δ1*	
BLY16	*MATα snf2Δ1::HIS3 his3-Δ200 lys2-801 ura3-52*	

TABLE 2
Primers used in this study

Gene disruption	Primer pair
MAL13	5'- CAATGAGAAGAACTGTACTGTTCGGGGGAATATTTAGAAGGACAGCTGAAGCTTCGTACGC 5'- ATTGTGATCATACTTATTTACGACATCTACCAATGCATTGGCGCATAGGCCACTAGTGGATCTG
MAL61	5'- AAGGGATTATCCTCATTAATAAACAGAAAAAAAGACAGGCAGCTGAAGCTTCGTACGC 5'- TTTGTTCACAACAGATGGGGTGCTTCGCCCTTCATCTACGCATAGGCCACTAGTGGATCTG
SIN4	5'- AAAGAAACTAGCAGACCTGACCTTCTGTTGGTAAATATTAGTATGCAGCTGAAGCTTCGTACGC 5'- TTTAAAACAATTCTATACAAAACTATGCTATAGTACTAATAATCAGCATAGGCCACTAGTGGATCTG
PGD1	5'- AATATAATATATAACACTAACACTTTCCTGAGGATTTTCGGTATGCAGCTGAAGCTTCGTACGC 5'- ATTATACAGATAATTACTATCTTGGATACATAGATGCACCAGTCAGCATAGGCCACTAGTGGATCTG
MED2	5'- TTGAAGGCGGATCCTCCCAAATAAACTGCCCGTCTGAAAGTAATGCAGCTGAAGCTTCGTACGC 5'- AACACGGTTTACAAGTCAATAGTTAACAATAGGAAGACCAAGCTAGCATAGGCCACTAGTGGATCTG
GAL11	5'- TCAAAGATCAAGGATTAAAACGCTATTTCTTTTAAATCTGCTATGCAGCTGAAGCTTCGTACGC 5'- AAGTAACTTCAAAAGTATCAAAAGTATGGAAACTTCAAATGTTCAGCATAGGCCACTAGTGGATCTG
MIG1	5'- CACGAGAGTTGAGTATAGTGGAGACGACATACTACCATAGCCATGCAGCTGAAGCTTCGTACGC 5'- TGTCTTTTGATTTATCTGCACCGCCAAAAACTTGTCAGCGTATCAGCATAGGCCACTAGTGGATCTG
MIG2	5'- TATTGACCTCGAGAACAAACAAAATAAAAATAAAAAAAGAGAATGCAGCTGAAGCTTCGTACGC 5'- TCGTTAGAGGAAAAATGGTGAGATAAAAAGGGGCCGTAAAGGTTAGCATAGGCCACTAGTGGATCTG
SRB10	5'- AATTGAATTAAGGCCGCCTAGTTTTGACGGGAGGAGAGAGAAATGCAGCTGAAGCTTCGTACGC 5'- GCTGTGGAATGAAAAATTCCAAATATATATAAAAATAGAAGCCTAGCATAGGCCACTAGTGGATCTG

Note: Sequences underlined are homologous to either 5' or 3' end of the gene to be disrupted.

Maltase assay. Maltase activity was determined in total cell extracts as described by Dubin *et al.* (1985). Activity is expressed as nanomoles of PNPG (ρ-nitrophenyl β-D-glucopyranoside) hydrolyzed per mg of total protein per minute. The values reported are the average of duplicate assays obtained with extracts from at least two separate cultures.

RESULTS

The Sin4 module of the Mediator is thought to sense signals from gene-specific activators and repressors. Two gene-specific regulators of the *MAL* structural genes, the *MAL*-activator and Mig1/2 repressor, have been identified and are possible candidates for interaction with the Mediator.

The constitutive phenotype of *rgr1* and *sin4* is not dependent on either the *MAL*-activator or maltose permease. The *MAL*-activator gene (*MAL13*) or the maltose permease gene (*MAL61)* were deleted from CMY4000 (*RGR1 SIN4*), CMY5001 (*rgr1-31 SIN4*), and CMY5002 (*RGR1 sin4-33*), all of which carry an integrated reporter *MAL61pro-HIS3* gene. The resulting strains were tested for their ability to grow on maltose medium lacking histidine and galactose medium lacking histidine, and maltase expression levels were assayed in galactose-grown cells. As was found previously (Charron *et al.* 1986; Charron *et al.* 1989), strains lacking *MAL*-activator (*mal13*Δ) or maltose permease (*mal61*Δ) are not maltose-inducible (Table 3). The double mutant strains, *rgr1-31 mal13*Δ and *rgr1-31 mal61*Δ, are able to grow in galactose medium lacking histidine and express the similar levels of maltase as the strains carrying the *rgr1-31* mutation alone. Similarly, loss of the *MAL*-activator or maltose permease has no effect on *MAL* gene expression in the *sin4-33* mutant strain (Table 3). These results indicate that constitutive expression of the *MAL* genes in *rgr1* or *sin4* mutant strains is not dependent on either the *MAL*-activator or maltose permease. It should be noted that the constitutive level of *MAL* gene expression in the *rgr1* or *sin4* mutant strains lacking a *MAL*-activator is not sufficient to allow for growth on maltose medium lacking histidine.

TABLE 3

The constitutive phenotype of *rgr1* and *sin4* is not dependent on either *MAL*-activator or maltose peamease.

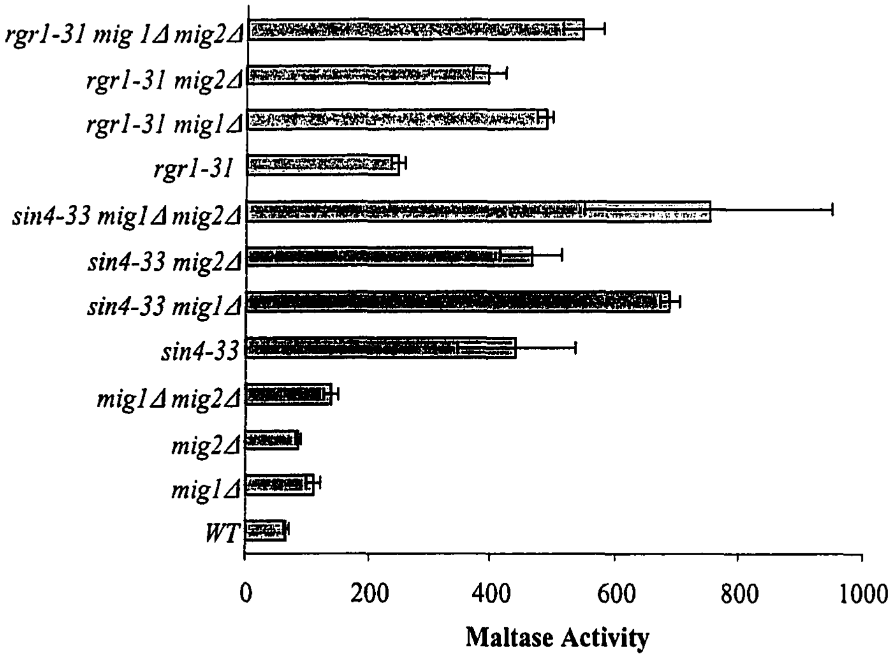
Strain	Relative genotype	Growth		Maltase activity
		Mal -His	Gal -His	(SGalG/L)
CMY4000	*RGR1 SIN4 MAL61 MAL13*	+	-	46
CMY5004	*RGR1 SIN4 mal61Δ MAL13*	-	-	27
CMY5003	*RGR1 SIN4 MAL61 mal13Δ*	-	-	21
CMY5001	*rgr1-31 SIN4 MAL61 MAL13*	+	+	254
CMY5007	*rgr1-31 SIN4 mal61Δ MAL13*	-	+	280
CMY5005	*rgr1-31 SIN4 MAL61 mal13Δ*	-	+	285
CMY5002	*RGR1 sin4-33 MAL61 MAL13*	+	+	432
CMY5008	*RGR1 sin4-33 mal61Δ MAL13*	-	+	326
CMY5006	*RGR1 sin4-33 MAL61 mal13Δ*	-	+	347

The *MAL*-activator gene (*MAL13*) or the maltose permease gene (*MAL61)* were disrupted from CMY4000 (*RGR1 SIN4 MAL61 MAL13*), CMY5001 (*rgr1-31 SIN4 MAL61 MAL13*), and CMY5002 (*RGR1 sin4-33 MAL61 MAL13*). The resulting strains were streaked for single colonies on minimal media lacking histidine with either 2% maltose or 2% galactose and growth monitored for 3 days. Maltase activity was assayed in cells grown in minimal media with either 2% galactose, 3% glycerol and 2% lactate (SGalG/L) as described in Materials and Methods.

***SIN4* and *RGR1* act synergistically with *MIG1* and *MIG2* in the repression of *MAL* gene expression.** We tested the possibility that Mig1p and/or Mig2p repress the *MAL* gene expression through direct or indirect interaction with the Sin4 module. *MIG1*, *MIG2,* and both were disrupted in strain CMY4000, the *rgr1-31* mutant (CMY5001), and the *sin4-33* mutant (CMY5002). Maltase expression levels were determined in uninduced growth conditions (SGalG/L) (Figure 1). Consistent to the previous studies (Hu *et al.* 2000), deletion of *MIG1*, *MIG2* or both modestly increases the basal level of maltase expression to levels about 4 times lower than the maltase levels observed in *sin4* and *rgr1* mutants, indicating that *MIG1* and *MIG2* only play minor roles in repressing basal level *MAL* gene expression. Disruption of either *MIG1* or *MIG2* in either *rgr1-31* or *sin4-33* mutant strains causes further increases in maltase expression levels and disruption of both *MIG1* and *MIG2* in the *rgr1-31* or *sin4-33* strains leads to even higher levels of maltase activity than either strain containing the single *mig1Δ* and *mig2Δ* alone. These findings indicate that Mig1p and Mig2p repress the expression of *MAL* genes independently of the Mediator Sin4 module and act to repress *MAL* gene expression via a separate pathway.

Srb10 acts in the same pathway with Sin4/Rgr1 to repress *MAL* gene expression. The Srb10 module is thought to be an accessory module of the core Mediator complex because it is absent from some classes of purified Mediator complexes. It is composed of four subunits, Srb8-11 proteins (reviewed by Carlson 1997; Lee and Young 2000). Among these subunits, Srb10p is a cyclin-dependent kinase interacting *in vitro* with the Med9 module of Mediator complex (Kang *et al.* 2001) and Srb11p is the Srb10-associated cyclin. The Srb10 module is thought to act primarily in transcriptional

Figure 1. ***SIN4*** **and** ***RGR1*** **act synergistically with** ***MIG1*** **and** ***MIG2*** **in the repression of** ***MAL*** **gene expression.** *MIG1*, *MIG2* and both were deleted from strain CMY4000, the *rgr1-31* mutant strain CMY5001, and the *sin4-33* mutant strain CMY5002. Maltase activity was assayed in cells grown in minimal media with 2% galactose, 3% glycerol and 2% lactate (SGalG/L) as described in Materials and Methods.

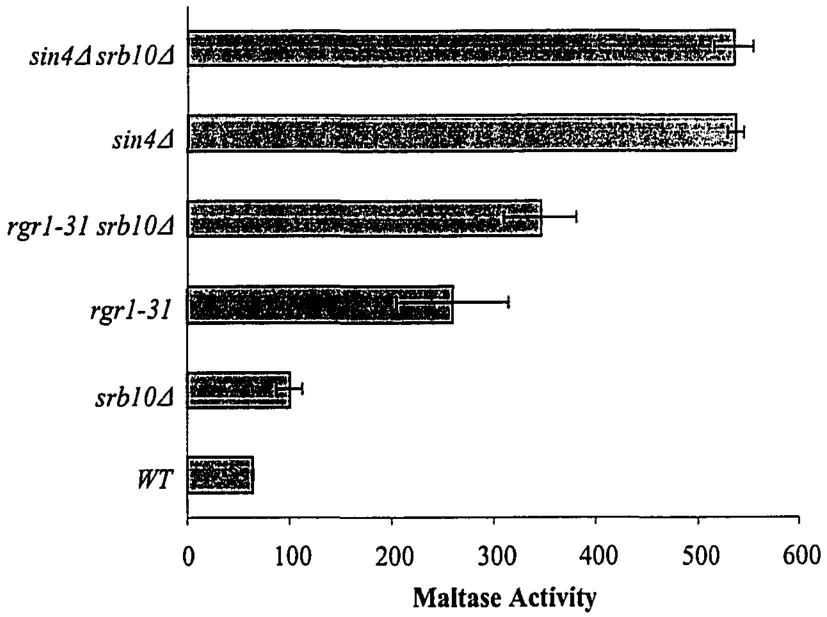

rgr1-31 mig 1Δ mig2Δ
rgr1-31 mig2Δ
rgr1-31 mig1Δ
rgr1-31
sin4-33 mig1Δ mig2Δ
sin4-33 mig2Δ
sin4-33 mig1Δ
sin4-33
mig1Δ mig2Δ
mig2Δ
mig1Δ
WT
0
200
400
600
800
1000
Maltase Activity

repression (Hengartner *et al.* 1998; Malik *et al.* 2000; Mittler *et al.* 2001; Wang *et al.* 2001). We asked whether it is also involved in repression of *MAL* genes and, if so, is it in the same regulatory pathway as Rgr1/Sin4 proteins.

The *SRB10* gene was disrupted in parental strain (CMY4000), the *rgr1-31* strain (CMY5001), and the *sin4*Δ strain (CMY5009) and maltase expression levels determined in cells grown under uninduced conditions. As shown in Figure 2, maltase levels are slightly increased in the *srb10*Δ strain compared to the isogenic *SRB10* strain indicating that Srb10p contributes to the repression of basal expression of *MAL* genes. The double mutant *sin4*Δ *srb10*Δ strain displays a similar level of maltase expression as the single mutant *sin4*Δ strain indicating that *SRB10* and *SIN4* function in a same pathway to repress *MAL* gene expression and suggesting that *SIN4* is downstream of *SRB10*. The double mutant *rgr1-31 srb10*Δ strain displays a slightly higher level of maltase expression compared to the single mutant *rgr1-31*, but this difference is barely significant and may only reflect the fact that Rgr1p is essential and that *rgr1-31* is a partial loss-of-function allele. Thus, we suggest that *SRB10* and *RGR1* function in a same pathway to repress *MAL* gene expression and that *RGR1* is downstream of *SRB10*.

Each component of the Sin4 module of the Mediator plays a distinct role in regulation *MAL* gene expression. Biochemical analysis indicates that the Sin4 module of the Mediator complex contains Sin4p, Gal11p, Med2p, and Pgd1p, and binds to Rgr1p which reportedly serves as a bridge connecting the Sin4 module to the Med9/Med10 module (Li *et al.* 1995; Myers *et al.* 1999). Genetic evidence reveals negative as well as positive regulatory roles of the Sin4 module and Rgr1p depending on the promoters (Myers and Kornberg 2000).

Figure 2. Srb10 acts in the same pathway with Sin4/Rgr1 to repress *MAL* gene expression. The *SRB10* gene was deleted from strain CMY4000, the *rgr1-31* strain CMY5001, and the *sin4*Δ strain CMY5009. Maltase activity was assayed in cells grown in minimal media with 2% galactose, 3% glycerol and 2% lactate (SGalG/L) as described in Materials and Methods.


sin4Δ srb10Δ
sin4Δ
rgr1-31 srb10Δ
rgr1-31
srb10Δ
WT
0
100
200
300
400
500
600
Maltase Activity

To elucidate the role of the various Sin4 module subunits in *MAL* gene regulation, the effects of deletion of the nonessential *SIN4*, *GAL11*, *MED2*, and *PGD1* genes on maltase expression were tested. Strains CMY5009 (*sin4*Δ), CMY5010 (*pgd1*Δ), CMY5011 (*med2*Δ), and CMY5012 (*gal11*Δ) were constructed by one-step gene replacement and maltase expression levels assayed in induced (SMal) and uninduced (SGalG/L) growth conditions. As shown in Figure 3, the *sin4*Δ strain displays a significant increase in basal maltase expression with no significant effect on induced expression levels. In contrast, the *med2*Δ and *pgd1*Δ strains exhibit no significant impact on basal expression but dramatically decrease induced expression of maltase. Deletion of *GAL11* significantly decreases both basal and induced expression of maltase. Basal expression of maltase is extremely low (11 units) in the *gal11*Δ strain but approximately 10-fold induction is observed. This is significant but lower than the 30-fold induction observed in strain CMY4000. Thus, the components of the Sin4 module have distinct effects on basal and induced *MAL* gene expression.

Swi/Snf complex is involved in *MAL* gene activation. Swi/Snf is a 2 MDa multisubunit complex that plays key roles in the regulation of eukaryotic gene expression (Peterson and Workman 2000). Swi/Snf is required for changes in chromatin structure that accompany transcriptional induction of *SUC2* and *PHO8* and other yeast promoters (Gregory *et al.* 1998; Gregory *et al.* 1999; Wu and Winston 1997). To examine whether the Swi/Snf is required for *MAL* gene activation, we measured maltase induction in strains containing mutations in *SWI1*, *SNF2*, *SWI3*, *SNF5* or *SNF6* encoding components of the Swi/Snf complex. As shown in Figure 4, loss of any one of these functions caused a dramatic decrease in induced maltase expression compared to the isogenic wild-type

Figure 3. Effects of deletion of the Sin4 module components in basal maltase expression and maltase induction. The *SIN4*, *GAL11*, *MED2,* or *PGD* gene was deleted from strain CMY4000. Maltase activity was assayed in cells grown in minimal media with 2% galactose, 3% glycerol and 2% lactate (SGalG/L, uninduced conditions) and with 2% maltose (SMal, induced conditions) as described in Materials and Methods.

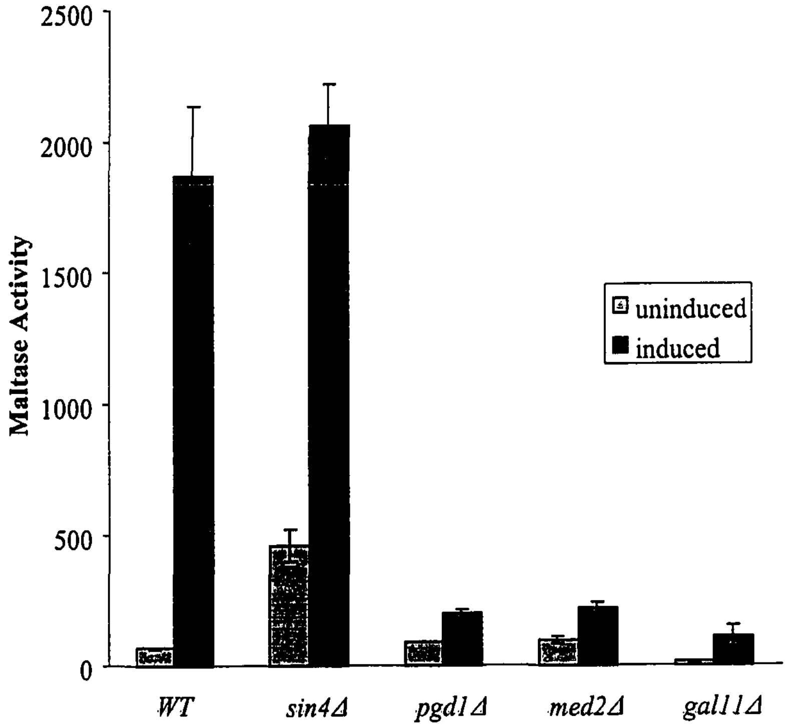
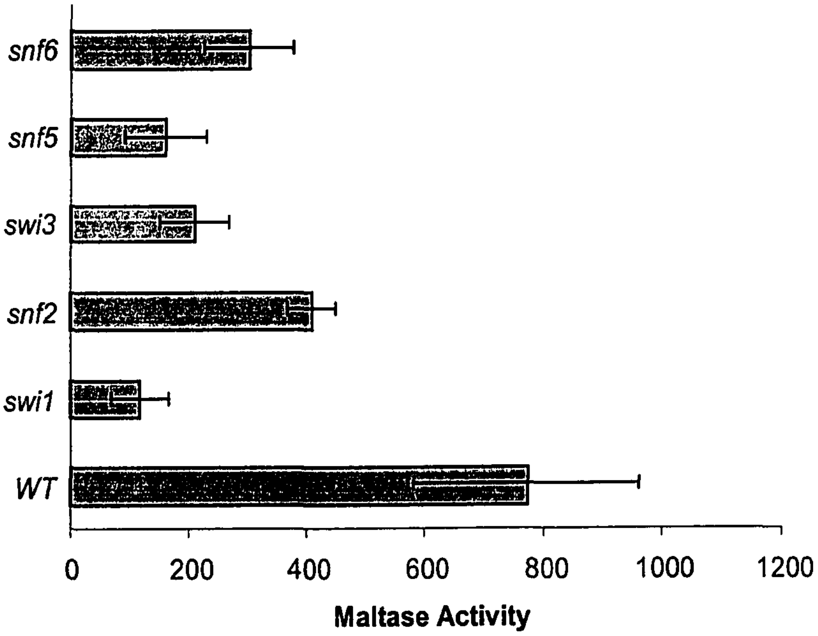

2500
2000
1500
1000
500
0
Maltase Activity
uninduced
induced
WT
sin4Δ
pgd1Δ
med2Δ
gal11Δ

Figure 4. The Swi/Snf complex is involved in maltose induction. Plasmid YCp50-MAL63 carrying the *MAL63 MAL*-activator gene (Danzi *et al.* 2000) was transformed into strain BLY1 and the isogenic strain series, BLY13 (*swi1Δ*), BLY16 (*snf2Δ*), BLY14 (*swi3Δ*), BLY3 (*snf5Δ*) and BLY5 (*snf6Δ*). Cells were grown in selective uninduced media containing 0.2% glucose to early-log phase. Cells were then collected and transferred to selective induced media containing 2% maltose. After six hours, maltase activity was assayed as described in Materials and Methods.


snf6
snf5
swi3
snf2
swi1
WT
0
200
400
600
800
1000
1200
Maltase Activity

strain. Thus, the Swi/Snf complex is required for the full induction of *MAL* gene expression.

DISCUSSION

The Mediator complex plays an essential role in both activation and repression of RNA polymerase II-mediated transcription. Its involvement in *MAL* gene regulation was discovered during a genetic selection for Mal^c mutants, which identified mutations in *RGR1* and *SIN4* genes (Chapter 2). In this chapter we further explore the roles of components of the Mediator Sin4 module in *MAL* gene expression. We demonstrated that Sin4p, Gal11p, Pgd1p, and Med2p have distinct effects on basal and induced *MAL* gene expression.

Rgr1p and Sin4p repress basal level expression of *MAL* genes, but have little or no effect on maltose induction. Mutations in *RGR1* and *SIN4* increase maltase expression to about 20-30% of the fully induced levels under uninduced growth conditions and partially relieve the glucose repression. Here we demonstrate that this constitutive expression does not require the *MAL*-activator or the maltose-induction signal generated by maltose permease. Thus, Rgr1p and Sin4p repress basal expression of *MAL* genes and act independent of the *MAL*-activator mediated induction. Moreover, the results reported in Table 5 of Chapter 2 and Figure 4 of this chapter demonstrate that Sin4p is not required for maltose induction since both *sin4-33* and *sin4Δ* mutant strains exhibit wild-type levels of induced maltase expression.

Mechanism of Rgr1p and Sin4p repression of *MAL* gene expression. Studies in our laboratory identified two Mig1-binding sites in the bi-directional promoter between *MAL61* and *MAL62* (Hu *et al.* 1995). Mig1p exerts its repressive effects by recruiting the Ssn6-Tup1 corepressor complex (Treitel and Carlson 1995). Given reports that Mediator interacts with Ssn6-Tup1 corepressor (Papamichos-Chronakis *et al.* 2000), we tested the genetic interaction between *MIG1/2* and our Mal^c *rgr1* and *sin4* mutations. Deletion of *MIG1*, *MIG2,* or both in strains carrying *rgr1-31* or *sin4-33* mutations acts synergistically to further increase maltase expression (Figure 1). This indicates that Sin4p and Rgr1p are not the mediators of the Mig1, 2 repression signal but instead function in an independent repression pathway.

Two approaches were taken to identify other possible *MAL* gene-specific repressors. First, we searched the bi-directional promoter sequence of *MAL61* and *MAL62* for consensus DNA-binding sites using the TRANSFAC database (Krull *et al.* 2003; Matys *et al.* 2003). Potential binding sites for several DNA-binding activators were identified including Gcr1p and Abf1p but, other than the Mig1, 2p binding sites, no additional repressor binding sites were revealed. The genome sequences of *S. mikatae, S. kudriavzevii,* and *S. bayanus* all of species closely related to *S. cerevisiae*, are now available (M. Johnston, personal communication). We performed a multiple alignment analysis on *MAL* promoters from these four species using program the ClustalW to identify possible consensus DNA-binding sites. Again, no repressor binding sites other than that for Mig1, 2 repressor were identified. In fact, the only sequences common to all four promoters are the UAS$_{\mathrm{MAL}}$ and the Mig1, 2p binding sites.

So how do Sin4p and Rgr1p repress basal level expression of *MAL* genes? Previous studies have suggested that Sin4p and Rgr1p affect transcription by altering chromatin structure. Mutations in *SIN4* and *RGR1* increase the linking number of plasmid DNA possibly because of decreases in nucleosome density and stability (Jiang *et al.* 1995; Jiang and Stillman 1992). Loss of *SIN4* results in an increase in chromatin accessibility as measured by increased sensitivity to micrococcal nuclease digestion but does not appear to alter nucleosome positioning, histone expression, or histone modification (Macatee *et al.* 1997). Rgr1p is required for nucleosomal repression of transcription in a plasmid-chromosome transcriptional system (Moss and Laybourn 2000). Taken together, these results indicate that Sin4p is a negative regulator of transcription by acting to inhibit chromatin reorganization and/or maintain the inactive chromatin structure. In light of findings supporting a role for Sin4p as a transcriptional activator (Jiang *et al.* 1995; Jiang and Stillman 1992; Li *et al.* 1995), it has been suggested that Sin4p participates indirectly in chromatin reorganization, possibly by activating the expression of genes encoding chromatin modifying enzymes or nonhistone chromatin components that promote the inactive state. Loss of Sin4p would be expected to constitutively activate genes in which chromatin structure plays an important regulatory role in gene activity. Our inability to identify *MAL* gene specific negative regulator other than Mig1, 2 repressor leads us to propose that Sin4p and Rgr1p most likely regulate *MAL* gene basal transcription indirectly.

Relationship between the Mediator and the Swi/Snf complex. Several patterns of genetic interactions between the Mediator and the Swi/Snf complex have been described. Defects in components of both complexes are suppressed by similar mutations in the chromatin components, that is, mutations in histones and associated factors (so-called *sin* mutations), suggesting that both complexes are involved in chromatin modeling (Kruger *et al.* 1995; Prelich and Winston 1993). Several *swi/snf* mutations are partially suppressed by a *sin4* null mutation (Sternberg *et al.* 1987; Stillman *et al.* 1994). These findings suggest that the Mediator and Swi/Snf complexes may have antagonistic effects on chromatin structure. Swi/Snf and Mediator are both required for *MAL* gene induction.

A number of transcriptional activators, such as Gal4p, VP16, Gcn4p, and Swi5p, can interact with both complexes and recruit them to promoters (Bhoite *et al.* 2001; Lee *et al.* 1999; Natarajan *et al.* 1999; Neely *et al.* 1999; Park *et al.* 2000). Gal11p is the Mediator component that interacts directly with these transcriptional activators (Bhoite *et al.* 2001; Lee *et al.* 1999; Park *et al.* 2000). The results reported in Figure 4 indicate that the Swi/Snf complex is required for *MAL* gene expression implying that chromatin reorganization is an important step in *MAL* gene induction. In contrast, mutations in components of the SAGA complex, including Ada2p, Ada3p, and Gcn5p, have no significant effect on *MAL* gene expression, suggesting the acetylation of the chromatin template is not required for maltose induction (B. Zhang unpublished results). Given that Gal11p, Med2p, and Pgd1p are required for maltose induction, they may play a role in recruiting the Mediator to *MAL* gene promoter via their interaction with the *MAL*-activator. Our finding that the Swi/Snf complex is involved in maltose induction may suggest that the Swi/Snf complex is also recruited to the *MAL* promoter, again possibly by the *MAL*-activator. Both proposals are under investigation.

Structural organization of the Sin4 module at the *MAL* promoter. The Sin4 module of the Mediator complex contains Sin4p, Gal11p, Med2p, and Pgd1p (Myers *et al.* 1999). Structural organization of the Sin4 module has been revealed by biochemical and electron microscopy imaging analyses. It has been reported that Sin4p anchors all of the module components to the Mediator complex through its interaction with C-terminus of Rgr1p. Mediator complexes purified from *sin4Δ* and *rgr1-Δ2* strains lack all of the components of the Sin4 module (Asturias *et al.* 1999; Dotson *et al.* 2000; Jiang *et al.* 1995; Li *et al.* 1995; Myers *et al.* 1999). Pgd1p, Med2p, and Gal11p are dependent on each other for their stable association within the Mediator and their loss has little effect on Sin4p association with Rgr1p (Lee *et al.* 1999; Myers *et al.* 1999). Our results reported in Figure 3 speak against this proposed organization. If binding of these components to the Mediator occurred via Sin4p, loss of Sin4p should destabilize interaction with Rgr1p resulting in loss of the entire Sin4 module thereby blocking the *MAL* induction, because Gal11p, Pgd1p, and Med2p are required for *MAL* induction. Our experimental results dispute this assumption because maltose induction is not affected in the *sin4Δ* strain (Figure 3). While this does not exclude Pgd2p, Med2p, and Gal11p binding to Sin4p, it is not likely that this binding is necessary for their interaction with the Mediator, at least at the *MAL* promoter.

Based on previous findings and our results, we propose a model in which Mediator plays a negative role in the basal level expression, and a positive role in induction of *MAL* genes. The Mediator, primarily Sin4p and Rgr1p, activates the expression of proteins that promote the inactive state of chromatin, thereby it indirectly blocks the RNAP II from accessing the *MAL* promoter. This results in the repression of basal *MAL* gene transcription. Under the induced conditions, the *MAL*-activator binds to the *MAL* promoter, and recruits the Mediator and the Swi/Snf complex. The Swi/Snf complex then promotes chromatin reorganization thereby overcoming its repressive effects. With the help of Gal11p, Pdg1p, and Med2p, the *MAL*-activator activates the *MAL* gene transcription through the Mediator complex modulation of the RNAP II activity.

CONCLUSION

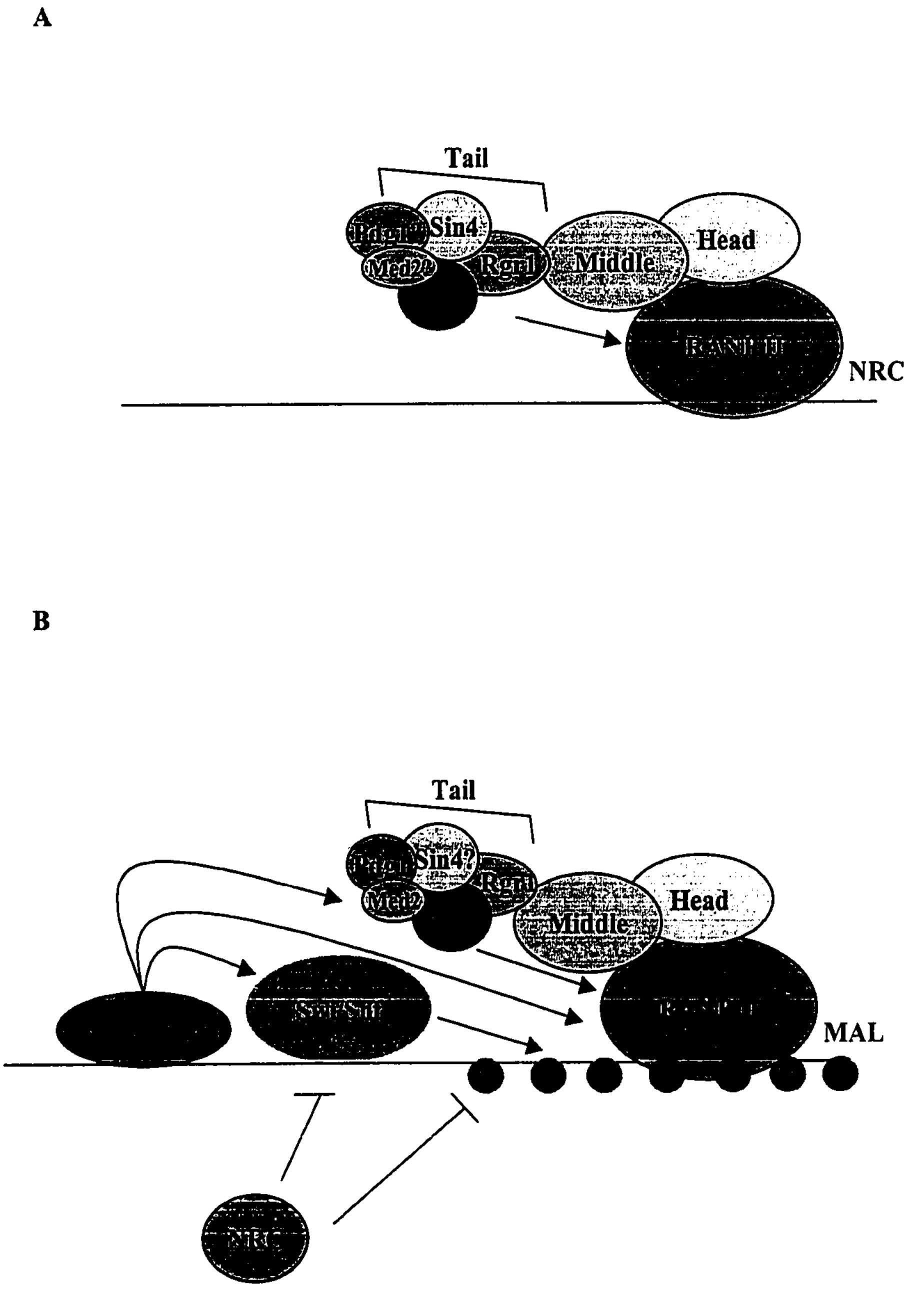
A hypothetical model consistent with the results of my research is presented here that describes the roles of the Mediator complex and Swi/Snf complex in *MAL* gene regulation (see Figure 1). Chapter 2 and 3 demonstrate that Rgr1p and Sin4p, components of the Mediator Sin4 module, are involved in repression of *MAL* basal gene expression. Further genetic analysis indicates that each component of the Sin4 module plays a distinct role in regulating *MAL* gene expression. Gal11p is involved in both basal expression and induction. Pgd1p and Med2p are required for maltose induction only. Additionally, the Swi/Snf chromatin remodeling complex is required for full induction suggesting a role for chromatin remodeling in the regulation of *MAL* gene expression.

We propose that Sin4p and Rgr1p participate **indirectly** to repress *MAL* gene basal transcription. The model suggests that Sin4p and Rgr1p activate the expression of genes encoding protein(s) that promote the inactive state of chromatin, possibly inhibitors of chromatin remodeling, chromatin modifying enzymes, or nonhistone chromatin components. This is diagrammed in Figure 1A. Deletion/mutation of *SIN4* would block expression of the negative regulator of chromatin (NRC) leading to a more active state for chromatin and thereby allowing elevated constitutive basal expression of all genes that require chromatin reorganization, including *MAL* genes. Figure 1B illustrates the events of *MAL* gene induction. Under induced conditions, the *MAL*-activator binds to *MAL* promoters and recruits Mediator and Swi/Snf complex. The Swi/Snf complex then causes chromatin reorganization and promotes transcription initiation. Gal11p, Pdg1p, Med2p, and Rgr1p in the Mediator tail complex are required for *MAL* induction. Loss of Sin4p does not impair *MAL* induction. Thus, Sin4p is not an essential component of

Mediator at a *MAL* promoter. Perhaps the subunit composition of the Mediator tail complex is heterogeneous and differs at different promoters.

The results reported in this thesis do not allow us to distinguish the function of the *MAL*-activator. It may activate RNAP II directly or indirectly via the Mediator and/or by recruiting Swi/Snf directly or indirectly via the Mediator. These are illustrated in Figure 1B. Work is in progress to explore these possibilities.

Figure 1. Proposed model of roles of the Mediator complex in basal level expression and maltose induction. Under uninduced conditions, Sin4p and Rgr1p activate expression of genes encoding negative regulators of chromatin (NRC), possibly chromatin modifying enzymes or nonhistone chromatin components, thereby promoting the inactive state of chromatin. Under induced conditions, *MAL*-activator binds to the *MAL* promoter and recruits the Mediator and the Swi/Snf complexes. Gal11p, Pdg1p, and Med2p positively modulate RNAP II activity. The role of Sin4p in *MAL* gene activation is unclear. The Swi/Snf complex remodels the chromatin structure by disrupting the nucleosomes (N).


A
Tail
Pdg1?
Sin4
Med2?
Rgr1
Middle
Head
RNAPII
NRC
B
Tail
Pdg1
Sin4?
Rgr1
Med2
Middle
Head
Swi/Snf
MAL
NRC

]

BIBLIOGRAPHY

Asturias, F. J., Y. W. Jiang, L. C. Myers, C. M. Gustafsson and R. D. Kornberg, 1999 Conserved structures of mediator and RNA polymerase II holoenzyme. Science **283:** 985-7.

Ausubel, F. M., 1988 *Current protocols in molecular biology*. Published by Greene Pub. Associates and Wiley- Interscience : J. Wiley, New York.

Bai, C., P. Sen, K. Hofmann, L. Ma, M. Goebl *et al.*, 1996 *SKP1* connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell **86:** 263-74.

Balciunas, D., and H. Ronne, 1995 Three subunits of the RNA polymerase II mediator complex are involved in glucose repression. Nucleic Acids Res **23:** 4421-5.

Barker, L., C. Kuhn, A. Weise, A. Schulz, C. Gebhardt *et al.*, 2000 *SUT2*, a putative sucrose sensor in sieve elements. Plant Cell **12:** 1153-64.

Barral, Y., S. Jentsch and C. Mann, 1995 G1 cyclin turnover and nutrient uptake are controlled by a common pathway in yeast. Genes Dev **9:** 399-409.

Batlle, M., A. Lu, D. A. Green, Y. Xue and J. P. Hirsch, 2003 Krh1p and Krh2p act downstream of the Gpa2p Gα subunit to negatively regulate haploid invasive growth. J Cell Sci **116:** 701-710.

Benito, B., and R. Lagunas, 1992 The low-affinity component of *Saccharomyces cerevisiae* maltose transport is an artifact. J Bacteriol **174:** 3065-9.

Bhat, P. J., and J. E. Hopper, 1991 The mechanism of inducer formation in *gal3* mutants of the yeast galactose system is independent of normal galactose metabolism and mitochondrial respiratory function. Genetics **128:** 233-9.

Bhat, P. J., and J. E. Hopper, 1992 Overproduction of the *GAL1* or *GAL3* protein causes galactose-independent activation of the *GAL4* protein: evidence for a new model of induction for the yeast *GAL/MEL* regulon. Mol Cell Biol **12:** 2701-7.

Bhat, P. J., and T. V. Murthy, 2001 Transcriptional control of the *GAL/MEL* regulon of yeast *Saccharomyces cerevisiae*: mechanism of galactose-mediated signal transduction. Mol Microbiol **40:** 1059-66.

Bhat, P. J., D. Oh and J. E. Hopper, 1990 Analysis of the *GAL3* signal transduction pathway activating *GAL4* protein-dependent transcription in *Saccharomyces cerevisiae*. Genetics **125:** 281-91.

Bhoite, L. T., Y. Yu and D. J. Stillman, 2001 The Swi5 activator recruits the Mediator complex to the *HO* promoter without RNA polymerase II. Genes Dev **15:** 2457-69.

Bisson, L. F., D. M. Coons, A. L. Kruckeberg and D. A. Lewis, 1993 Yeast sugar transporters. Crit Rev Biochem Mol Biol **28:** 259-308.

Blank, T. E., M. P. Woods, C. M. Lebo, P. Xin and J. E. Hopper, 1997 Novel Gal3 proteins showing altered Gal80p binding cause constitutive transcription of Gal4p-activated genes in *Saccharomyces cerevisiae.* Mol Cell Biol **17:** 2566-75.

Boos, W., and A. Bohm, 2000 Learning new tricks from an old dog: MalT of the *Escherichia coli* maltose system is part of a complex regulatory network. Trends Genet **16:** 404-9.

Boube, M., L. Joulia, D. L. Cribbs and H. M. Bourbon, 2002 Evidence for a mediator of RNA polymerase II transcriptional regulation conserved from yeast to man. Cell **110:** 143-51.

Busturia, A., and R. Lagunas, 1986 Catabolite inactivation of the glucose transport system in *Saccharomyces cerevisiae.* J Gen Microbiol **132:** 379-85.

Carlson, M., 1997 Genetics of transcriptional regulation in yeast: connections to the RNA polymerase II CTD. Annu Rev Cell Dev Biol **13:** 1-23.

Carlson, M., 1999 Glucose repression in yeast. Curr Opin Microbiol **2:** 202-7.

Carlson, M., B. C. Osmond, L. Neigeborn and D. Botstein, 1984 A suppressor of *SNF1* mutations causes constitutive high-level invertase synthesis in yeast. Genetics **107:** 19-32.

Celenza, J. L., and M. Carlson, 1986 A yeast gene that is essential for release from glucose repression encodes a protein kinase. Science **233:** 1175-80.

Celenza, J. L., and M. Carlson, 1989 Mutational analysis of the *Saccharomyces cerevisiae SNF1* protein kinase and evidence for functional interaction with the *SNF4* protein. Mol Cell Biol **9:** 5034-44.

Celenza, J. L., F. J. Eng and M. Carlson, 1989 Molecular analysis of the *SNF4* gene of *Saccharomyces cerevisiae*: evidence for physical association of the *SNF4* protein with the *SNF1* protein kinase. Mol Cell Biol **9:** 5045-54.

Charron, M. J., R. A. Dubin and C. A. Michels, 1986 Structural and functional analysis of the *MAL1* locus of *Saccharomyces cerevisiae.* Mol Cell Biol **6:** 3891-9.

Charron, M. J., and C. A. Michels, 1988 The naturally occurring alleles of *MAL1* in *Saccharomyces* species evolved by various mutagenic processes including chromosomal rearrangement. Genetics **120:** 83-93.

Charron, M. J., E. Read, S. R. Haut and C. A. Michels, 1989 Molecular evolution of the telomere-associated *MAL* loci of *Saccharomyces*. Genetics **122:** 307-16.

Cheng, Q., and C. A. Michels, 1989 The maltose permease encoded by the *MAL61* gene of *Saccharomyces cerevisiae* exhibits both sequence and structural homology to other sugar transporters. Genetics **123:** 477-84.

Cheng, Q., and C. A. Michels, 1991 *MAL11* and *MAL61* encode the inducible high-affinity maltose transporter of *Saccharomyces cerevisiae.* J Bacteriol **173:** 1817-20.

Colombo, S., P. Ma, L. Cauwenberg, J. Winderickx, M. Crauwels *et al.*, 1998 Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signaling in the yeast *Saccharomyces cerevisiae.* EMBO J **17:** 3326-41.

Coons, D. M., R. B. Boulton and L. F. Bisson, 1995 Computer-assisted nonlinear regression analysis of the multicomponent glucose uptake kinetics of *Saccharomyces cerevisiae.* J Bacteriol **177:** 3251-8.

Coons, D. M., P. Vagnoli and L. F. Bisson, 1997 The C-terminal domain of Snf3p is sufficient to complement the growth defect of *snf3* null mutations in *Saccharomyces cerevisiae*: *SNF3* functions in glucose recognition. Yeast **13:** 9-20.

Danzi, S. E., B. Zhang and C. A. Michels, 2000 Alterations in the *Saccharomyces MAL*-activator cause constitutivity but can be suppressed by intragenic mutations. Curr Genet **38:** 233-40.

Davies, A., T. L. Ciardelli, G. E. Lienhard, J. M. Boyle, A. D. Whetton *et al.*, 1990 Site-specific antibodies as probes of the topology and function of the human erythrocyte glucose transporter. Biochem J **266:** 799-808.

Davies, A., K. Meeran, M. T. Cairns and S. A. Baldwin, 1987 Peptide-specific antibodies as probes of the orientation of the glucose transporter in the human erythrocyte membrane. J Biol Chem **262:** 9347-52.

De Vit, M. J., J. A. Waddle and M. Johnston, 1997 Regulated nuclear translocation of the Mig1 glucose repressor. Mol Biol Cell **8:** 1603-18.

De Winde, J. H., M. Crauwels, S. Hohmann, J. M. Thevelein and J. Winderickx, 1996 Differential requirement of the yeast sugar kinases for sugar sensing in establishing the catabolite-repressed state. Eur J Biochem **241:** 633-43.

Donzeau, M., and W. Bandlow, 1999The yeast trimeric guanine nucleotide-binding protein alpha subunit, Gpa2p, controls the meiosis-specific kinase Ime2p activity in response to nutrients. Mol Cell Biol **19:** 6110-9.

Dotson, M. R., C. X. Yuan, R. G. Roeder, L. C. Myers, C. M. Gustafsson *et al.*, 2000 Structural organization of yeast and mammalian mediator complexes. Proc Natl Acad Sci U S A **97:** 14307-10.

Dubin, R. A., R. B. Needleman, D. Gossett and C. A. Michels, 1985 Identification of the structural gene encoding maltase within the *MAL6* locus of *Saccharomyces carlsbergensis*. J Bacteriol **164:** 605-10.

Elledge, S. J., and R. W. Davis, 1988 A family of versatile centromeric vectors designed for use in the sectoring-shuffle mutagenesis assay in *Saccharomyces cerevisiae.* Gene **70:** 303-12.

Flanagan, P. M., R. J. Kelleher, 3rd, M. H. Sayre, H. Tschochner and R. D. Kornberg, 1991 A mediator required for activation of RNA polymerase II transcription in vitro. Nature **350:** 436-8.

Forsberg, H., and P. O. Ljungdahl, 2001 Sensors of extracellular nutrients in *Saccharomyces cerevisiae.* Curr Genet **40:** 91-109.

Gahrtz, M., J. Stolz and N. Sauer, 1994 A phloem-specific sucrose-H+ symporter from *Plantago major* L. supports the model of apoplastic phloem loading. Plant J **6:** 697-706.

Gancedo, J. M., 1992 Carbon catabolite repression in yeast. Eur J Biochem **206:** 297-313.

Gancedo, J. M., 1998 Yeast carbon catabolite repression. Microbiol Mol Biol Rev **62:** 334-61.

Gibson, A. W., L. A. Wojciechowicz, S. E. Danzi, B. Zhang, J. H. Kim *et al.*, 1997 Constitutive mutations of the *Saccharomyces cerevisiae MAL*-activator genes *MAL23*, *MAL43*, *MAL63*, and *mal64*. Genetics **146:** 1287-98.

Gill, G., and M. Ptashne, 1988 Negative effect of the transcriptional activator *GAL4*. Nature **334:** 721-4.

Gilman, A. G., 1987 G proteins: transducers of receptor-generated signals. Annu Rev Biochem **56:** 615-49.

Gregory, P. D., A. Schmid, M. Zavari, L. Lui, S. L. Berger *et al.*, 1998 Absence of Gcn5 HAT activity defines a novel state in the opening of chromatin at the *PHO5* promoter in yeast. Mol Cell **1:** 495-505.

Gregory, P. D., A. Schmid, M. Zavari, M. Munsterkotter and W. Horz, 1999 Chromatin remodelling at the *PHO8* promoter requires SWI-SNF and SAGA at a step subsequent to activator binding. EMBO J **18:** 6407-14.

Guthrie, C., and G. R. Fink, 1991 *Guide to yeast genetics and molecular biology.* Academic Press, San Diego.

Hach, A., T. Hon and L. Zhang, 2000 The coiled coil dimerization element of the yeast transcriptional activator Hap1, a Gal4 family member, is dispensable for DNA binding but differentially affects transcriptional activation. J Biol Chem **275:** 248-54.

Han, E. K., F. Cotty, C. Sottas, H. Jiang and C. A. Michels, 1995 Characterization of *AGT1* encoding a general alpha-glucoside transporter from *Saccharomyces.* Mol Microbiol **17:** 1093-107.

Han, S. J., J. S. Lee, J. S. Kang and Y. J. Kim, 2001 Med9/Cse2 and Gal11 modules are required for transcriptional repression of distinct group of genes. J Biol Chem **276:** 37020-6.

Han, S. J., Y. C. Lee, B. S. Gim, G. H. Ryu, S. J. Park *et al.*, 1999 Activator-specific requirement of yeast mediator proteins for RNA polymerase II transcriptional activation. Mol Cell Biol **19:** 979-88.

Harashima, T., and J. Heitman, 2002 The Gα protein Gpa2 controls yeast differentiation by interacting with kelch repeat proteins that mimic Gβ subunits. Mol Cell **10:** 163-73.

Hashimoto, H., Y. Kikuchi, Y. Nogi and T. Fukasawa, 1983 Regulation of expression of the galactose gene cluster in *Saccharomyces cerevisiae.* Isolation and characterization of the regulatory gene *GAL4.* Mol Gen Genet **191:** 31-8.

Helmbrecht, K., E. Zeise and L. Rensing, 2000 Chaperones in cell cycle regulation and mitogenic signal transduction: a review. Cell Prolif **33:** 341-65.

Hengartner, C. J., V. E. Myer, S. M. Liao, C. J. Wilson, S. S. Koh *et al.*, 1998 Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases. Mol Cell **2:** 43-53.

Herrero, P., C. Martinez-Campa and F. Moreno, 1998 The hexokinase 2 protein participates in regulatory DNA-protein complexes necessary for glucose repression of the *SUC2* gene in *Saccharomyces cerevisiae.* FEBS Lett **434:** 71-6.

Hirst, M., M. S. Kobor, N. Kuriakose, J. Greenblatt and I. Sadowski, 1999 *GAL4* is regulated by the RNA polymerase II holoenzyme-associated cyclin-dependent protein kinase *SRB10/CDK8.* Mol Cell **3:** 673-8.

Holstege, F. C., E. G. Jennings, J. J. Wyrick, T. I. Lee, C. J. Hengartner *et al.*, 1998 Dissecting the regulatory circuitry of a eukaryotic genome. Cell **95:** 717-28.

Hu, Z., A. W. Gibson, J. H. Kim, L. A. Wojciechowicz, B. Zhang *et al.*, 1999 Functional domain analysis of the *Saccharomyces MAL*-activator. Curr Genet **36:** 1-12.

Hu, Z., J. O. Nehlin, H. Ronne and C. A. Michels, 1995 *MIG1*-dependent and *MIG1*-independent glucose regulation of *MAL* gene expression in *Saccharomyces cerevisiae.* Curr Genet **28:** 258-66.

Hu, Z., Y. Yue, H. Jiang, B. Zhang, P. W. Sherwood *et al.*, 2000 Analysis of the mechanism by which glucose inhibits maltose induction of *MAL* gene expression in *Saccharomyces.* Genetics **154:** 121-32.

Hubbard, E. J., R. Jiang and M. Carlson, 1994 Dosage-dependent modulation of glucose repression by *MSN3* (*STD1*) in *Saccharomyces cerevisiae.* Mol Cell Biol **14:** 1972-8.

Iraqui, I., S. Vissers, F. Bernard, J. O. de Craene, E. Boles *et al.*, 1999 Amino acid signaling in *Saccharomyces cerevisiae*: a permease-like sensor of external amino acids and F-Box protein Grr1p are required for transcriptional induction of the *AGP1* gene, which encodes a broad- specificity amino acid permease. Mol Cell Biol **19:** 989-1001.

Jaquenoud, M., M. P. Gulli, K. Peter and M. Peter, 1998 The Cdc42p effector Gic2p is targeted for ubiquitin-dependent degradation by the SCFGrr1 complex. EMBO J **17:** 5360-73.

Jiang, R., and M. Carlson, 1996 Glucose regulates protein interactions within the yeast *SNF1* protein kinase complex. Genes Dev **10:** 3105-15.

Jiang, R., and M. Carlson, 1997 The Snf1 protein kinase and its activating subunit, Snf4, interact with distinct domains of the Sip1/Sip2/Gal83 component in the kinase complex. Mol Cell Biol **17:** 2099-106.

Jiang, Y. W., P. R. Dohrmann and D. J. Stillman, 1995 Genetic and physical interactions between yeast *RGR1* and *SIN4* in chromatin organization and transcriptional regulation. Genetics **140:** 47-54.

Jiang, Y. W., and D. J. Stillman, 1992 Involvement of the *SIN4* global transcriptional regulator in the chromatin structure of *Saccharomyces cerevisiae.* Mol Cell Biol **12:** 4503-14.

Johnston, M., 1987 A model fungal gene regulatory mechanism: the *GAL* genes of *Saccharomyces cerevisiae.* Microbiol Rev **51:** 458-76.

Johnston, M., 1999 Feasting, fasting and fermenting. Glucose sensing in yeast and other cells. Trends Genet **15:** 29-33.

Kamura, T., D. M. Koepp, M. N. Conrad, D. Skowyra, R. J. Moreland *et al.*, 1999 Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. Science **284:** 657-61.

Kang, J. S., S. H. Kim, M. S. Hwang, S. J. Han, Y. C. Lee *et al.*, 2001 The structural and functional organization of the yeast mediator complex. J Biol Chem **276:** 42003-10.

Kelleher, R. J., 3rd, P. M. Flanagan and R. D. Kornberg, 1990 A novel mediator between activator proteins and the RNA polymerase II transcription apparatus. Cell **61:** 1209-15.

Khan, N. A., F. K. Zimmermann and N. R. Eaton, 1973 Genetic control of maltase formation in yeast. II. Evidence for a gene regulating the level of maltase production. Mol Gen Genet **124:** 365-7.

Kim, J., and C. A. Michels, 1988 The *MAL63* gene of *Saccharomyces* encodes a cysteine-zinc finger protein. Curr Genet **14:** 319-23.

Kim, Y. J., S. Bjorklund, Y. Li, M. H. Sayre and R. D. Kornberg, 1994 A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. Cell **77:** 599-608.

Kitagawa, K., D. Skowyra, S. J. Elledge, J. W. Harper and P. Hieter, 1999 *SGT1* encodes an essential component of the yeast kinetochore assembly pathway and a novel subunit of the SCF ubiquitin ligase complex. Mol Cell **4:** 21-33.

Ko, C. H., H. Liang and R. F. Gaber, 1993 Roles of multiple glucose transporters in *Saccharomyces cerevisiae.* Mol Cell Biol **13:** 638-48.

Kraakman, L., K. Lemaire, P. Ma, A. W. Teunissen, M. C. Donaton *et al.*, 1999 A *Saccharomyces cerevisiae* G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. Mol Microbiol **32:** 1002-12.

Kruger, W., C. L. Peterson, A. Sil, C. Coburn, G. Arents *et al.*, 1995 Amino acid substitutions in the structured domains of histones H3 and H4 partially relieve the requirement of the yeast SWI/SNF complex for transcription. Genes Dev **9:** 2770-9.

Krull, M., N. Voss, C. Choi, S. Pistor, A. Potapov *et al.*, 2003 TRANSPATH: an integrated database on signal transduction and a tool for array analysis. Nucleic Acids Res **31:** 97-100.

Kubler, E., H. U. Mosch, S. Rupp and M. P. Lisanti, 1997 Gpa2p, a G-protein alpha-subunit, regulates growth and pseudohyphal development in *Saccharomyces cerevisiae* via a cAMP-dependent mechanism. J Biol Chem **272:** 20321-3.

Kuchin, S., P. Yeghiayan and M. Carlson, 1995 Cyclin-dependent protein kinase and cyclin homologs *SSN3* and *SSN8* contribute to transcriptional control in yeast. Proc Natl Acad Sci U S A **92:** 4006-10.

Lafuente, M. J., C. Gancedo, J. C. Jauniaux and J. M. Gancedo, 2000 Mth1 receives the signal given by the glucose sensors Snf3 and Rgt2 in *Saccharomyces cerevisiae.* Mol Microbiol **35:** 161-72.

Lagunas, R., 1993 Sugar transport in *Saccharomyces cerevisiae.* FEMS Microbiol Rev **10:** 229-42.

Lee, T. I., and R. A. Young, 2000 Transcription of eukaryotic protein-coding genes. Annu Rev Genet **34:** 77-137.

Lee, Y. C., J. M. Park, S. Min, S. J. Han and Y. J. Kim, 1999 An activator binding module of yeast RNA polymerase II holoenzyme. Mol Cell Biol **19:** 2967-76.

Lefkowitz, R. J., 2000The superfamily of heptahelical receptors. Nat Cell Biol **2:** E133-6.

Lesage, P., X. Yang and M. Carlson, 1996 Yeast *SNF1* protein kinase interacts with *SIP4,* a C6 zinc cluster transcriptional activator: a new role for *SNF1* in the glucose response. Mol Cell Biol **16:** 1921-8.

Levine, J., L. Tanouye and C. A. Michels, 1992 The UAS$_{\mathrm{MAL}}$ is a bidirectional promotor element required for the expression of both the *MAL61* and *MAL62* genes of the *Saccharomyces MAL6* locus. Curr Genet **22:** 181-9.

Li, Y., S. Bjorklund, Y. W. Jiang, Y. J. Kim, W. S. Lane *et al.*, 1995 Yeast global transcriptional regulators Sin4 and Rgr1 are components of mediator complex/RNA polymerase II holoenzyme. Proc Natl Acad Sci U S A **92:** 10864-8.

Liang, H., and R. F. Gaber, 1996 A novel signal transduction pathway in *Saccharomyces cerevisiae* defined by Snf3-regulated expression of *HXT6*. Mol Biol Cell **7:** 1953-66.

Liao, S. M., J. Zhang, D. A. Jeffery, A. J. Koleske, C. M. Thompson *et al.*, 1995 A kinase-cyclin pair in the RNA polymerase II holoenzyme. Nature **374:** 193-6.

Lorenz, M. C., and J. Heitman, 1997 Yeast pseudohyphal growth is regulated by *GPA2*, a G protein alpha homolog. EMBO J **16:** 7008-18.

Lorenz, M. C., and J. Heitman, 1998 The *MEP2* ammonium permease regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. EMBO J **17:** 1236-47.

Lorenz, M. C., X. Pan, T. Harashima, M. E. Cardenas, Y. Xue *et al.*, 2000 The G protein-coupled receptor Gprl is a nutrient sensor that regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. Genetics **154:** 609-22.

Lue, N. F., D. I. Chasman, A. R. Buchman and R. D. Kornberg, 1987 Interaction of *GAL4* and *GAL80* gene regulatory proteins *in vitro*. Mol Cell Biol **7:** 3446-51.

Ma, H., L. M. Bloom, S. E. Dakin, C. T. Walsh and D. Botstein, 1989a The 15 N-terminal amino acids of hexokinase II are not required for in vivo function: analysis of a truncated form of hexokinase II in *Saccharomyces cerevisiae*. Proteins **5:** 218-23.

Ma, H., L. M. Bloom, C. T. Walsh and D. Botstein, 1989b The residual enzymatic phosphorylation activity of hexokinase II mutants is correlated with glucose repression in *Saccharomyces cerevisiae*. Mol Cell Biol **9:** 5643-9.

Ma, H., L. M. Bloom, Z. M. Zhu, C. T. Walsh and D. Botstein, 1989c Isolation and characterization of mutations in the *HXK2* gene of *Saccharomyces cerevisiae*. Mol Cell Biol **9:** 5630-42.

Macatee, T., Y. W. Jiang, D. J. Stillman and S. Y. Roth, 1997 Global alterations in chromatin accessibility associated with loss of *SIN4* function. Nucleic Acids Res **25:** 1240-7.

Madi, L., S. A. McBride, L. A. Bailey and D. J. Ebbole, 1997 *rco-3*, a gene involved in glucose transport and conidiation in *Neurospora crassa*. Genetics **146:** 499-508.

Malik, S., W. Gu, W. Wu, J. Qin and R. G. Roeder, 2000 The USA-derived transcriptional coactivator PC2 is a submodule of TRAP/SMCC and acts synergistically with other PCs. Mol Cell **5:** 753-60.

Malik, S., and R. G. Roeder, 2000 Transcriptional regulation through Mediator-like coactivators in yeast and metazoan cells. Trends Biochem Sci **25:** 277-83.

Marmorstein, R., M. Carey, M. Ptashne and S. C. Harrison, 1992 DNA recognition by *GAL4*: structure of a protein-DNA complex. Nature **356:** 408-14.

Marshall-Carlson, L., J. L. Celenza, B. C. Laurent and M. Carlson, 1990 Mutational analysis of the *SNF3* glucose transporter of *Saccharomyces cerevisiae.* Mol Cell Biol **10:** 1105-15.

Marshall-Carlson, L., L. Neigeborn, D. Coons, L. Bisson and M. Carlson, 1991 Dominant and recessive suppressors that restore glucose transport in a yeast *snf3* mutant. Genetics **128:** 505-12.

Matys, V., E. Fricke, R. Geffers, E. Gossling, M. Haubrock *et al.*, 2003 TRANSFAC: transcriptional regulation, from patterns to profiles. Nucleic Acids Res **31:** 374-8.

Medintz, I., H. Jiang, E. K. Han, W. Cui and C. A. Michels, 1996 Characterization of the glucose-induced inactivation of maltose permease in *Saccharomyces cerevisiae.* J Bacteriol **178:** 2245-54.

Medintz, I., H. Jiang and C. A. Michels, 1998 The role of ubiquitin conjugation in glucose-induced proteolysis of *Saccharomyces* maltose permease. J Biol Chem **273:** 34454-62.

Medintz, I., X. Wang, T. Hradek and C. A. Michels, 2000 A PEST-like sequence in the N-terminal cytoplasmic domain of *Saccharomyces* maltose permease is required for glucose-induced proteolysis and rapid inactivation of transport activity. Biochemistry **39:** 4518-26.

Michels, C. A., E. Read, K. Nat and M. J. Charron, 1992 The telomere-associated *MAL3* locus of *Saccharomyces* is a tandem array of repeated genes. Yeast **8:** 655-65.

Mittler, G., E. Kremmer, H. T. Timmers and M. Meisterernst, 2001 Novel critical role of a human Mediator complex for basal RNA polymerase II transcription. EMBO Rep **2:** 808-13.

Mosley, A. L., J. Lakshmanan, B. K. Aryal and S. Ozcan, 2003 Glucose-mediated phosphorylation converts the transcription factor Rgt1 from a repressor to an activator. J Biol Chem .

Moss, D. R., and P. J. Laybourn, 2000 Upstream nucleosomes and Rgr1p are required for nucleosomal repression of transcription. Mol Microbiol **36:** 1293-305.

Myers, L. C., C. M. Gustafsson, D. A. Bushnell, M. Lui, H. Erdjument-Bromage *et al.*, 1998 The Med proteins of yeast and their function through the RNA polymerase II carboxy-terminal domain. Genes Dev **12:** 45-54.

Myers, L. C., C. M. Gustafsson, K. C. Hayashibara, P. O. Brown and R. D. Kornberg, 1999 Mediator protein mutations that selectively abolish activated transcription. Proc Natl Acad Sci U S A **96:** 67-72.

Myers, L. C., and R. D. Kornberg, 2000 Mediator of transcriptional regulation. Annu Rev Biochem **69:** 729-49.

Naar, A. M., B. D. Lemon and R. Tjian, 2001 Transcriptional coactivator complexes. Annu Rev Biochem **70:** 475-501.

Nakafuku, M., T. Obara, K. Kaibuchi, I. Miyajima, A. Miyajima *et al.*, 1988 Isolation of a second yeast *Saccharomyces cerevisiae* gene (*GPA2*) coding for guanine nucleotide-binding regulatory protein: studies on its structure and possible functions. Proc Natl Acad Sci U S A **85:** 1374-8.

Natarajan, K., B. M. Jackson, H. Zhou, F. Winston and A. G. Hinnebusch, 1999 Transcriptional activation by Gcn4p involves independent interactions with the SWI/SNF complex and the SRB/mediator. Mol Cell **4:** 657-64.

Naumov, G. I., E. S. Naumova and C. A. Michels, 1994 Genetic variation of the repeated *MAL* loci in natural populations of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus*. Genetics **136:** 803-12.

Needleman, R., 1991 Control of maltase synthesis in yeast. Mol Microbiol **5:** 2079-84.

Needleman, R. B., D. B. Kaback, R. A. Dubin, E. L. Perkins, N. G. Rosenberg *et al.*, 1984 *MAL6* of *Saccharomyces*: a complex genetic locus containing three genes required for maltose fermentation. Proc Natl Acad Sci U S A **81:** 2811-5.

Needleman, R. B., and C. Michels, 1983 Repeated family of genes controlling maltose fermentation in *Saccharomyces carlsbergensis*. Mol Cell Biol **3:** 796-802.

Neely, K. E., A. H. Hassan, A. E. Wallberg, D. J. Steger, B. R. Cairns *et al.*, 1999 Activation domain-mediated targeting of the SWI/SNF complex to promoters stimulates transcription from nucleosome arrays. Mol Cell **4:** 649-55.

Nehlin, J. O., M. Carlberg and H. Ronne, 1991 Control of yeast *GAL* genes by *MIG1* repressor: a transcriptional cascade in the glucose response. EMBO J **10:** 3373-7.

Nehlin, J. O., and H. Ronne, 1990 Yeast *MIG1* repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. EMBO J **9:** 2891-8.

Nehls, U., J. Wiese, M. Guttenberger and R. Hampp, 1998 Carbon allocation in ectomycorrhizas: identification and expression analysis of an *Amanita muscaria* monosaccharide transporter. Mol Plant Microbe Interact **11:** 167-76.

Neigeborn, L., and M. Carlson, 1984 Genes affecting the regulation of *SUC2* gene expression by glucose repression in *Saccharomyces cerevisiae.* Genetics **108:** 845-58.

Ni, B. F., and R. B. Needleman, 1990 Identification of the upstream activating sequence of *MAL* and the binding sites for the *MAL63* activator of *Saccharomyces cerevisiae.* Mol Cell Biol **10:** 3797-800.

Ostling, J., and H. Ronne, 1998 Negative control of the Mig1p repressor by Snf1p-dependent phosphorylation in the absence of glucose. Eur J Biochem **252:** 162-8.

Ozcan, S., J. Dover and M. Johnston, 1998 Glucose sensing and signaling by two glucose receptors in the yeast *Saccharomyces cerevisiae.* EMBO J **17:** 2566-73.

Ozcan, S., J. Dover, A. G. Rosenwald, S. Wolfl and M. Johnston, 1996a Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. Proc Natl Acad Sci U S A **93:** 12428-32.

Ozcan, S., and M. Johnston, 1995 Three different regulatory mechanisms enable yeast hexose transporter (*HXT*) genes to be induced by different levels of glucose. Mol Cell Biol **15:** 1564-72.

Ozcan, S., and M. Johnston, 1999 Function and regulation of yeast hexose transporters. Microbiol Mol Biol Rev **63:** 554-69.

Ozcan, S., T. Leong and M. Johnston, 1996b Rgt1p of *Saccharomyces cerevisiae*, a key regulator of glucose-induced genes, is both an activator and a repressor of transcription. Mol Cell Biol **16:** 6419-26.

Panagiotidis, C. H., W. Boos and H. A. Shuman, 1998 The ATP-binding cassette subunit of the maltose transporter MalK antagonizes MalT, the activator of the *Escherichia coli mal* regulon. Mol Microbiol **30:** 535-46.

Papamichos-Chronakis, M., R. S. Conlan, N. Gounalaki, T. Copf and D. Tzamarias, 2000 Hrs1/Med3 is a Cyc8-Tup1 corepressor target in the RNA polymerase II holoenzyme. J Biol Chem **275:** 8397-403.

Park, J. M., H. S. Kim, S. J. Han, M. S. Hwang, Y. C. Lee *et al.*, 2000 *In vivo* requirement of activator-specific binding targets of mediator. Mol Cell Biol **20:** 8709-19.

Patton, E. E., A. R. Willems, D. Sa, L. Kuras, D. Thomas *et al.*, 1998 Cdc53 is a scaffold protein for multiple Cdc34/Skp1/F-box proteincomplexes that regulate cell division and methionine biosynthesis in yeast. Genes Dev **12:** 692-705.

Peng, G., and J. E. Hopper, 2000 Evidence for Gal3p's cytoplasmic location and Gal80p's dual cytoplasmic-nuclear location implicates new mechanisms for controlling Gal4p activity in *Saccharomyces cerevisiae.* Mol Cell Biol **20:** 5140-8.

Peng, G., and J. E. Hopper, 2002 Gene activation by interaction of an inhibitor with a cytoplasmic signaling protein. Proc Natl Acad Sci U S A **99:** 8548-53.

Peroutka, S. J., 1994 5-Hydroxytryptamine receptors in vertebrates and invertebrates: why are there so many? Neurochem Int **25:** 533-6.

Peterson, C. L., and J. L. Workman, 2000 Promoter targeting and chromatin remodeling by the SWI/SNF complex. Curr Opin Genet Dev **10:** 187-92.

Platt, A., and R. J. Reece, 1998 The yeast galactose genetic switch is mediated by the formation of a Gal4p-Gal80p-Gal3p complex. EMBO J **17:** 4086-91.

Prelich, G., and F. Winston, 1993 Mutations that suppress the deletion of an upstream activating sequence in yeast: involvement of a protein kinase and histone H3 in repressing transcription in vivo. Genetics **135:** 665-76.

Rachez, C., and L. P. Freedman, 2001 Mediator complexes and transcription. Curr Opin Cell Biol **13:** 274-80.

Richter, K., and J. Buchner, 2001 Hsp90: chaperoning signal transduction. J Cell Physiol **188:** 281-90.

Rohde, J. R., J. Trinh and I. Sadowski, 2000 Multiple signals regulate *GAL* transcription in yeast. Mol Cell Biol **20:** 3880-6.

Rolland, F., J. H. De Winde, K. Lemaire, E. Boles, J. M. Thevelein *et al.*, 2000 Glucose-induced cAMP signalling in yeast requires both a G-protein coupled receptor system for extracellular glucose detection and a separable hexose kinase-dependent sensing process. Mol Microbiol **38:** 348-58.

Rolland, F., J. Winderickx and J. M. Thevelein, 2001 Glucose-sensing mechanisms in eukaryotic cells. Trends Biochem Sci **26:** 310-7.

Rose, M., 1995 Molecular and biochemical characterization of the hexokinase from the starch-utilizing yeast *Schwanniomyces occidentalis.* Curr Genet **27:** 330-8.

Sadowski, I., C. Costa and R. Dhanawansa, 1996 Phosphorylation of Gal4p at a single C-terminal residue is necessary for galactose-inducible transcription. Mol Cell Biol **16:** 4879-87.

Sakai, A., Y. Shimizu, S. Kondou, T. Chibazakura and F. Hishinuma, 1990 Structure and molecular analysis of *RGR1*, a gene required for glucose repression of *Saccharomyces cerevisiae*. Mol Cell Biol **10:** 4130-8.

Schiestl, R. H., M. Dominska and T. D. Petes, 1993 Transformation of *Saccharomyces cerevisiae* with nonhomologous DNA: illegitimate integration of transforming DNA into yeast chromosomes and *in vivo* ligation of transforming DNA to mitochondrial DNA sequences. Mol Cell Biol **13:** 2697-705.

Schjerling, P., and S. Holmberg, 1996 Comparative amino acid sequence analysis of the C6 zinc cluster family of transcriptional regulators. Nucleic Acids Res **24:** 4599-607.

Schmidt, M. C., R. R. McCartney, X. Zhang, T. S. Tillman, H. Solimeo *et al.*, 1999 Std1 and Mth1 proteins interact with the glucose sensors to control glucose-regulated gene expression in *Saccharomyces cerevisiae*. Mol Cell Biol **19:** 4561-71.

Schultz, J., L. Marshall-Carlson and M. Carlson, 1990 The N-terminal TPR region is the functional domain of *SSN6*, a nuclear phosphoprotein of *Saccharomyces cerevisiae*. Mol Cell Biol **10:** 4744-56.

Seol, J. H., R. M. Feldman, W. Zachariae, A. Shevchenko, C. C. Correll *et al.*, 1999 Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. Genes Dev **13:** 1614-26.

Serrano, R., 1977 Energy requirements for maltose transport in yeast. Eur J Biochem **80:** 97-102.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19-27.

Sil, A. K., S. Alam, P. Xin, L. Ma, M. Morgan *et al.*, 1999 The Gal3p-Gal80p-Gal4p transcription switch of yeast: Gal3p destabilizes the Gal80p-Gal4p complex in response to galactose and ATP. Mol Cell Biol **19:** 7828-40.

Sirenko, O. I., B. Ni and R. B. Needleman, 1995 Purification and binding properties of the Mal63p activator of *Saccharomyces cerevisiae*. Curr Genet **27:** 509-16.

Skowyra, D., D. M. Koepp, T. Kamura, M. N. Conrad, R. C. Conaway *et al.*, 1999 Reconstitution of G1 cyclin ubiquitination with complexes containing SCFGrr1 and Rbx1. Science **284:** 662-5.

Sollitti, P., and J. Marmur, 1988 Primary structure of the regulatory gene from the *MAL6* locus of *Saccharomyces carlsbergensis*. Mol Gen Genet **213:** 56-62.

Sternberg, P. W., M. J. Stern, I. Clark and I. Herskowitz, 1987 Activation of the yeast *HO* gene by release from multiple negative controls. Cell **48:** 567-77.

Stillman, D. J., S. Dorland and Y. Yu, 1994 Epistasis analysis of suppressor mutations that allow *HO* expression in the absence of the yeast *SW15* transcriptional activator. Genetics **136:** 781-8.

Suzuki-Fujimoto, T., M. Fukuma, K. I. Yano, H. Sakurai, A. Vonika *et al.*, 1996 Analysis of the galactose signal transduction pathway in *Saccharomyces cerevisiae*: interaction between Gal3p and Gal80p. Mol Cell Biol **16:** 2504-8.

Tamaki, H., T. Miwa, M. Shinozaki, M. Saito, C. W. Yun *et al.*, 2000 GPR1 regulates filamentous growth through *FLO11* in yeast *Saccharomyces cerevisiae*. Biochem Biophys Res Commun **267:** 164-8.

ter Schure, E. G., N. A. van Riel and C. T. Verrips, 2000 The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. FEMS Microbiol Rev **24:** 67-83.

Thevelein, J. M., and J. H. de Winde, 1999 Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. Mol Microbiol **33:** 904-18.

Thompson, C. M., A. J. Koleske, D. M. Chao and R. A. Young, 1993 A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. Cell **73:** 1361-75.

Tillman, T. S., R. W. Ganster, R. Jiang, M. Carlson and M. C. Schmidt, 1995 *STD1 (MSN3)* interacts directly with the TATA-binding protein and modulates transcription of the *SUC2* gene of Saccharomyces cerevisiae. Nucleic Acids Res **23:** 3174-80.

Treitel, M. A., and M. Carlson, 1995 Repression by *SSN6-TUP1* is directed by *MIG1*, a repressor/activator protein. Proc Natl Acad Sci U S A **92:** 3132-6.

Treitel, M. A., S. Kuchin and M. Carlson, 1998 Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in *Saccharomyces cerevisiae*. Mol Cell Biol **18:** 6273-80.

Vallier, L. G., and M. Carlson, 1994 Synergistic release from glucose repression by *mig1* and *ssn* mutations in *Saccharomyces cerevisiae*. Genetics **137:** 49-54.

Van Belle, D., and B. Andre, 2001 A genomic view of yeast membrane transporters. Curr Opin Cell Biol **13:** 389-98.

Versele, M., J. H. de Winde and J. M. Thevelein, 1999 A novel regulator of G protein signalling in yeast, Rgs2, downregulates glucose-activation of the cAMP pathway through direct inhibition of Gpa2. EMBO J **18:** 5577-91.

Versele, M., K. Lemaire and J. M. Thevelein, 2001 Sex and sugar in yeast: two distinct GPCR systems. EMBO Rep **2:** 574-9.

Vincent, O., S. Kuchin, S. P. Hong, R. Townley, V. K. Vyas *et al.*, 2001 Interaction of the Srb10 kinase with Sip4, a transcriptional activator of gluconeogenic genes in *Saccharomyces cerevisiae*. Mol Cell Biol **21:** 5790-6.

Wach, A., A. Brachat, R. Pohlmann and P. Philippsen, 1994 New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast **10:** 1793-808.

Wang, G., G. T. Cantin, J. L. Stevens and A. J. Berk, 2001 Characterization of mediator complexes from HeLa cell nuclear extract. Mol Cell Biol **21:** 4604-13.

Wang, J., and R. Needleman, 1996 Removal of Mig1p binding site converts a *MAL63* constitutive mutant derived by interchromosomal gene conversion to glucose insensitivity. Genetics **142:** 51-63.

Welton, R. M., and C. S. Hoffman, 2000 Glucose monitoring in fission yeast via the Gpa2 Gα, the git5 Gβ and the git3 putative glucose receptor. Genetics **156:** 513-21.

Williams, F. E., and R. J. Trumbly, 1990 Characterization of *TUP1*, a mediator of glucose repression in *Saccharomyces cerevisiae*. Mol Cell Biol **10:** 6500-11.

Williams, F. E., U. Varanasi and R. J. Trumbly, 1991 The *CYC8* and *TUP1* proteins involved in glucose repression in *Saccharomyces cerevisiae* are associated in a protein complex. Mol Cell Biol **11:** 3307-16.

Wilson, W. A., S. A. Hawley and D. G. Hardie, 1996 Glucose repression/derepression in budding yeast: *SNF1* protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. Curr Biol **6:** 1426-34.

Woychik, N. A., and M. Hampsey, 2002 The RNA polymerase II machinery: structure illuminates function. Cell **108:** 453-63.

Wu, L., and F. Winston, 1997Evidence that Snf-Swi controls chromatin structure over both the TATA and UAS regions of the *SUC2* promoter in *Saccharomyces cerevisiae*. Nucleic Acids Res **25:** 4230-4.

Wu, Y., R. J. Reece and M. Ptashne, 1996 Quantitation of putative activator-target affinities predicts transcriptional activating potentials. EMBO J **15:** 3951-63.

Wykoff, D. D., and E. K. O'Shea, 2001 Phosphate transport and sensing in *Saccharomyces cerevisiae*. Genetics **159:** 1491-9.

Xue, Y., M. Batlle and J. P. Hirsch, 1998 *GPR1* encodes a putative G protein-coupled receptor that associates with the Gpa2p Gα subunit and functions in a Ras-independent pathway. EMBO J **17:** 1996-2007.

Yang, X., E. J. Hubbard and M. Carlson, 1992 A protein kinase substrate identified by the two-hybrid system. Science **257:** 680-2.

Yano, K., and T. Fukasawa, 1997 Galactose-dependent reversible interaction of Gal3p with Gal80p in the induction pathway of Gal4p-activated genes of *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A **94:** 1721-6.

Yun, C. W., H. Tamaki, R. Nakayama, K. Yamamoto and H. Kumagai, 1997 G-protein coupled receptor from yeast *Saccharomyces cerevisiae*. Biochem Biophys Res Commun **240:** 287-92.

Yun, C. W., H. Tamaki, R. Nakayama, K. Yamamoto and H. Kumagai, 1998 Gpr1p, a putative G-protein coupled receptor, regulates glucose-dependent cellular cAMP level in yeast *Saccharomyces cerevisiae*. Biochem Biophys Res Commun **252:** 29-33.

Zenke, F. T., R. Engles, V. Vollenbroich, J. Meyer, C. P. Hollenberg *et al.*, 1996 Activation of Gal4p by galactose-dependent interaction of galactokinase and Gal80p. Science **272:** 1662-5.

Zhang, L., A. Hach and C. Wang, 1998 Molecular mechanism governing heme signaling in yeast: a higher-order complex mediates heme regulation of the transcriptional activator *HAP1*. Mol Cell Biol **18:** 3819-28.