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Mutations in *SIN4* and *RGR1* Cause Constitutive Expression of *MAL* Structural Genes in *Saccharomyces cerevisiae*

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ABSTRACT

Transcription of the Saccharomyces *MAL* structural genes is induced 40-fold by maltose and requires the *MAL*-activator and maltose permease. To identify additional players involved in regulating *MAL* gene expression, we carried out a genetic selection for *MAL* constitutive mutants. Strain CMY4000 containing *MAL1* and integrated copies of *MAL61promoter-HIS3* and *MAL61promoter-lacZ* reporter genes was used to select constitutive mutants. The 29 recessive mutants fall into at least three complementation groups. Group 1 and group 2 mutants exhibit pleiotropic phenotypes and represent alleles of Mediator component genes *RGR1* and *SIN4*, respectively. The *rgr1* and *sin4* constitutive phenotype does not require either the *MAL*-activator or maltose permease, indicating that Mediator represses *MAL* basal expression. Further genetic analysis demonstrates that *RGR1* and *SIN4* work in a common pathway and each component of the Mediator Sin4 module plays a distinct role in regulating *MAL* gene expression. 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. A *sin4*Δ mutation is unable to suppress the defects in *MAL* gene expression resulting from loss of the Swi/Snf complex component Snf2p. The role of the Mediator in *MAL* gene regulation is discussed.

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 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.

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, demonstrate that maltose induction of *MAL* gene expression depends on the *MAL*-activator and maltose permease (Charron *et al.* 1986, 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). Our previous work reported that the role of maltose permease in induction is the accumulation of intracellular maltose but the means of sensing the presence of intracellular maltose remain undetermined (Wang *et al.* 2002). It is possible that the *MAL*-activator itself is the maltose-binding sensor. Alternatively, other positive or negative regulators may be involved but may not have 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 using a *MAL61promoter-HIS3* reporter. This approach should allow us to identify dominant constitutive alterations in positive regulators or recessive constitutive alterations in previously unidentified negative regulators. Here we report the identification of two genes, *SIN4* and *RGR1*, in which recessive mutations cause constitutive *MAL* gene expression. Our results indicate that Rgr1p and Sin4p are negative regulators of basal, but not induced, expression of the *MAL* structural genes. Because Sin4p and Rgr1p are both components of the Sin4 module of the yeast Mediator complex, we compared the roles of the other components of the Sin4 module in *MAL* gene regulation and found that each

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TABLE 1

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

Strain	Genotype	Reference and source
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** *mal11Δ::MAL61/HA MAL12 MAL13 GAL ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-200 leu2::MAL61pro-LacZ::MAL61pro-HIS3*	This study
CMY4001	*MATα mal11Δ::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 mal11Δ::MAL61/HA MAL12 MAL13 GAL leu2 ura3-52:* YIp355 *lys2-801 ade2-101 trp1-Δ63 his3-200 leu2:MAL61pro-LacZ:MAL61pro-HIS3*	This study
CM-31	*rgr1-31* (isogenic to CMY4000)	This study
CM-33	*sin4-33* (isogenic to CMY4000)	This study
CMY5003	*mal13Δ::G418* (isogenic to CMY4000)	This study
CMY5004	*mal11Δ::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 mal11Δ::G418* (isogenic to CMY4000)	This study
CMY5008	*sin4-33 mal11Δ::G418* (isogenic to CMY4000)	This study
CMY5009	*sin4Δ::G418* (isogenic to CMY4000)	This study
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	*snf2Δ::HygB* (isogenic to CMY4000)	This study
CMY5023	*sin4Δ::G418 snf2Δ::HygB* (isogenic to CMY4000)	This study
CMY5030	*rgr1-31 sin4Δ::G418* (isogenic to CMY4000)	This study
BLY1	*MATα lys2-801 his3-Δ200 ura3-52*	Brehon C. Laurent
BLY3	*MATα snf5-Δ2 his3-Δ200 ura3-52 ade2-101*	Brehon C. Laurent
BLY4	*MAT***a** *snf2-141oc his3-Δ200 ura3-52 suc2*	Brehon C. Laurent
BLY5	*MATα snf6-Δ2 his3-Δ200 ura3-52 suc2*	Brehon C. Laurent
BLY13	*MAT***a** *swi1Δ::LEU2 his3-Δ200 lys2-801 ura3-52 ade2-101 trp1Δ1 leu2-Δ1*	Brehon C. Laurent
BLY14	*MAT***a** *swi3Δ::TRP1 his3-Δ200 lys2-801 ura3-52 ade2-101 trp1Δ1 leu2-Δ1*	Brehon C. Laurent
BLY16	*MATα snf2Δ1::HIS3 his3-Δ200 lys2-801 ura3-52*	Brehon C. Laurent

plays a distinct role in basal and induced expression. The interplay between the Mediator and the Swi/Snf complex as it relates to *MAL* gene regulation is explored.

MATERIALS AND METHODS

Yeast strains and plasmids: 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*, referred to as *mal11Δ::MAL61/HA*. No other *MAL* genes are present in this strain. Strain CMY4000 was constructed by inserting two YIp365-based plasmids (Myers *et al.* 1986) carrying *MAL61promoter-lacZ* and *MAL61promoter-HIS3* reporter genes into the *leu2* gene of CMY1001 by targeted integration. Strain CMY4001 was created by changing mating type of CMY4000 from mating type a to α, using plasmid pGHOT obtained from R. Rothstein (Columbia University). The *URA3* plasmid YIp355 was integrated into the *ura3-52* gene of CMY4001 to create a strain CMY4002. Strains CM-31 and CM-33 were isolated by UV mutagenesis as described below.

Gene disruptions were done by the PCR-based one-step gene replacement method in appropriate strains. The primer pairs used for each different gene disruption were determined on the basis of the sequence of S288C available at the Saccharomyces Genome Database (http://genome-www.stanford.edu/Saccharomyces/). 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). Candidate disruptants were confirmed by PCR analysis. Plasmid pLN1384 containing *SNF2* (obtained from Brehon Laurent) was introduced into strain CMY5022 (*snf2Δ::HygB*) prior to the disruption of the chromosomal copy of *SIN4* to avoid difficulties resulting from the very slow growth rate of *sin4 snf2* double-mutant strains. Following the successful disruption of *sin4*, the plasmid was cured from the strain to create CMY5023 (*sin4Δ::G418 snf2Δ::HygB*).

Plasmid pUN30-MAL61promoter-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 0.9-kb fragment containing base pairs −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 (∼1.7 kb) 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.

Mutagenesis and isolation of *MAL* constitutive mutants: Strain CMY4000 was grown in YPD to midlog 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 to ∼15% survival. The mutagenized cells were immediately plated onto minimal medium containing 2% galactose, 3% glycerol, and 2% lactate (SGalG/L) lacking histidine, and the plates were incubated in the dark for 5 days at 25° until His+ colonies appeared. The potential Mal^c mutants were screened by assaying expression of the *MAL61promoter-lacZ* reporter using the standard β-galactosidase plate assay and *MAL12* expression by assaying maltase activity levels in galactose-grown cells.

Cloning of wild-type alleles of a mutant gene in strains CM-31 and CM-33: Constitutive mutants CM-31 and CM-33 were chosen as representatives of complementation groups 1 and 2, respectively. CM-31 and CM-33 are pink because of the presence of the *ade2-101* mutant allele. Each strain was transformed with pUN30-MAL61promoter-ADE2. The resulting transformants form white colonies on SGalG/L media because of the constitutive expression of the *MAL61promoter-ADE2* reporter. These were then transformed with a centromere-based YCp50 genomic library prepared from strain S288C. Pink Ura$^+$ transformants on SGalG/L media were isolated as potential carriers of 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 and complementation of the constitutive maltase expression phenotype was determined for each transformant. The library plasmid was isolated from each transformant and reintroduced into the CM-31 [pUN30-MAL61promoter-ADE2] or CM-33 [pUN30-MAL61promoter-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′-TTCAGGAGAGGGGT TACAATCTCC-3′ (complementary to sequence 36–59 bases after the stop codon) and high-fidelity platinum *Taq* DNA polymerase (Invitrogen, San Diego) to ensure the fidelity of amplification product. Seven sequencing primers were designed, each annealing to sites about every 500 bp along the *RGR1* ORF, on the basis of sequence of this gene in S288C (http://genome-www.stanford.edu/Saccharomyces/). The site of the alteration was sequenced using DNA from independent amplifications to ensure that the detected alteration does not result from 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 ρ-nitrophenyl β-d-glucopyranoside (PNPG) hydrolyzed per milligram of total protein per minute. The values reported are the average of duplicate assays obtained with extracts from at least duplicate cultures of the same strain. The values from different cultures varied ∼15%.

β-Galactosidase plate assay: Cells were patched onto a plate containing the appropriate selective medium and grown for 2 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°. The final mixture contains 0.5% agarose, 0.5 m Na_2HPO_4-NaH_2PO_4 pH 7 buffer, 0.1% SDS, 2% dimethylformamide, and 0.05% X-Gal. Approximately 10 ml of this mixture was poured over the surface of the culture plate and photographs were taken following 6–12 hr of blue color development.

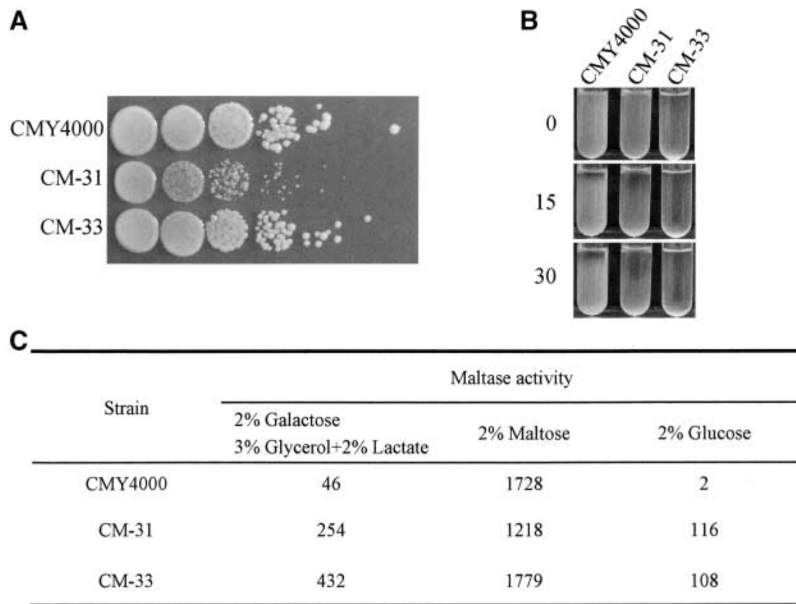
Flocculation assay: Cells were grown overnight in 5 ml liquid minimal medium to approximately midlog phase. The culture was vortexed briefly to separate and suspend the cells in the medium as best as possible, and the culture tubes were photographed immediately and after 15 and 30 min of standing in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

RESULTS

Isolation and genetic analysis of *MAL* constitutive mutants: To select for Mal^c mutants we constructed strain CMY4000, which carries the complete *MAL1* locus encoding maltose permease (*mal11Δ::MAL61/HA*), maltase (*MAL12*), and the *MAL*-activator (*MAL13*) as well as integrated copies of both *MAL61promoter-HIS3* and *MAL61promoter-lacZ* reporter genes (see materials and methods). 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 carry out the selection for Mal^c mutants, ∼10^7 cells of CMY4000 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. Of these, 31 also exhibited constitutive expression of the *MAL61-promoter-lacZ* reporter gene on the basis of plate assay and constitutive maltase expression ranging from 10 to 70% of fully induced levels (data not shown).

The 31 Mal^c mutant strains were mated to strain CMY4002 and the phenotype of the diploids was determined. Two mutants, CM-24 and CM-32, were found to be dominant. Complementation analysis was performed on the remaining 29 recessive mutants. Due to very poor sporulation and spore viability, we were able to obtain sufficient four-spore tetrads to follow segregation of the Mal^c phenotype only for mutants CM-31 and CM-33. For the crosses involving CM-31 and CM-33, the Mal^c phenotype segregated 2:2, indicating that both carried a single-mutant alteration. Haploid *MAT*α 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 recessive Mal^c mutants. The results indicate that these 29 Mal^c mutants fall into at least three complementation groups. Group 1 includes 10 Mal^c mutants, CM-7, -9, -10, -13, -17, -18, -19, -20, -30, and -31; and group 2 includes 5 Mal^c mutants, CM-5, -21,



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

Figure 1.—Phenotypic consequences of mutations in CM-31 (group 1) and CM-33 (group 2). (A) Growth rates of CM-31, CM-33, and CMY4000 on YPD plates. (B) Flocculation phenotype. Cultures of CMY4000, CM-31, and CM-33 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 min. (C) Effects of mutations on glucose repression. CMY4000, CM-31, and CM-33 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 was assayed as described in materials and methods.

-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 significantly 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 rates of all mutant strains. Figure 1 shows the results obtained with mutants CM-31 and CM-33, representatives from group 1 and group 2, respectively, but in all cases members of the same complementation group exhibited similar phenotypes (data not shown).

CM-31 exhibits a slow growth phenotype but CM-33 grows normally, as can be seen from the dilution assay shown in Figure 1A. To measure the extent of flocculation, CM-31, CM-33, and CMY4000 were grown overnight in liquid minimal medium, vortexed briefly to separate and suspend the cells in the culture medium, and the culture tubes were photographed immediately and after 15 and 30 min. The results presented in Figure 1B demonstrate that CM-31 is modestly flocculent compared to nonflocculent CMY4000. In contrast, CM-33 is so flocculent that the cells grow in clumps, cannot 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 the *MAL* genes, a phenomenon referred to as glucose repression. We found that both group 1 and group 2 mutants relieve glucose repression. Maltase expression was assayed following growth under maltose-induced, glucose-repressed, and uninduced growth conditions. The results for strains carrying the mutant allele from CM-31 and CM-33 are presented in Figure 1C and clearly demonstrate that mutations in both genes partially relieve glucose repression. Similar results were found for other group 1 and group 2 mutants (data not shown).

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 carries 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 thus should produce pink colonies on galactose-containing medium. Transformant colonies carrying a library plasmid were selected on minimal medium lacking uracil with 2% galactose and screened for pink colonies. Dependence on the library plasmid of the pink colony color was confirmed by plasmid loss. The pink transformants were screened to identify those that also exhibited 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 fully restored induc-

TABLE 2

Mutant alterations of *rgr1* alleles

Allele	Mutation	Amino acid replacements
rgr1-7	C2689T	Q897*
rgr1-9	C2128T	Q710*
rgr1-10	C2689T	Q897*
rgr1-13	A2674T	K892*
rgr1-17	2547: insertion of A	N849KF*
rgr1-18	T2193A	Y731*
rgr1-19	A2674T	K892*
rgr1-20	2561: deletion of A	N854TSR*
rgr1-30	A2674T	K892*
rgr1-31	2730: insertion of T	F910FLR*

*, stop codon.

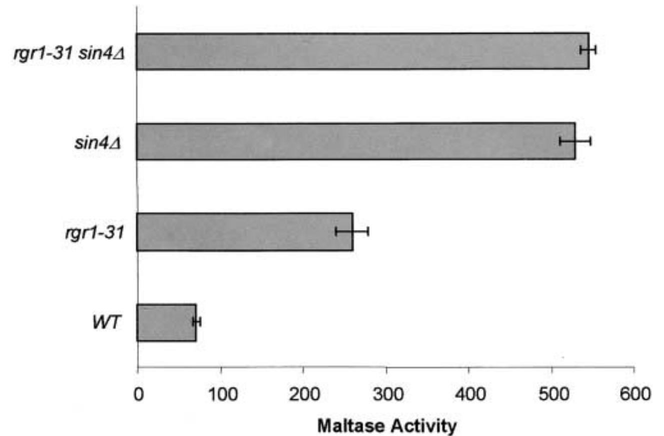


Figure 2.—*RGR1* and *SIN4* are in a common pathway for *MAL* gene regulation. CMY4000 (wild type), CM-31 (*rgr1-31*), CMY5009 (*sin4*Δ*::G418*), and CMY5030 (*rgr1-31 sin4*Δ*::G418*) were grown under uninduced conditions in minimal media with 2% galactose, 3% glycerol, and 2% lactate. Maltase activity was assayed as described in materials and methods.

ible expression of the *MAL61promoter-ADE2* reporter in CM-31. The overlapping region of the insert fragments is derived from the right arm of chromosome XII and contains six intact ORFs (*YLR071C–YLR076C*). Subcloning identified a *Bgl*II-*Eco*RI fragment capable of partially complementing the Mal^c phenotype of CM-31 and fully complementing the flocculation phenotype. This fragment contains only one complete ORF, *RGR1*. The basis of the partial complementation by this shorter insert fragment is unexplained but is observed with both multicopy and CEN vectors carrying only the *RGR1* gene. In heterozygous diploids all of the *rgr1* mutant alleles are purely recessive to *RGR1* for all phenotypes. Thus, the partial complementation is unlikely to be of functional significance. Nonetheless, it concerned us so the complete sequence of the open reading frame of all 10 presumed *rgr1* mutant alleles obtained by our selection scheme was determined to confirm the presence of a mutation. All 10 *rgr1* alleles contain a single alteration, either a nonsense or a frameshift mutation, located in the region of codons 710–910 of this 1082-codon ORF (listed in Table 2). Thus, group 1 mutants are alleles of *RGR1,* an essential gene encoding a scaffold-like component in the middle and tail regions of the RNA polymerase II (RNAPII) mediator complex (Asturias *et al.* 1999; Dotson *et al.* 2000). It is interesting to note that the *rgr1* alleles isolated here differed in the level of constitutive maltase expression and the severity of the flocculation phenotype but no clear correlation between their phenotype and the position of the mutant alteration is evident (data not shown).

Six plasmids were isolated from the CEN genomic library that restored inducible expression of the *MAL61-promoter-ADE2* reporter in CM-33. These plasmids complemented both the Mal^c and the flocculation phenotypes. The overlap of these insert fragments contains two intact ORFs, *SIN4* and *YNL235C. SIN4* was amplified by PCR from CMY4000, using primers that exclude the promoter region of *YNL235C.* Subsequent cloning of this fragment in pUN70 enabled us to demonstrate that the complementing region is the *SIN4* gene, suggesting that the group 2 mutations are alleles of *SIN4.* Sin4p is a component in the tail region of the RNAPII mediator complex (Asturias *et al.* 1999; Myers *et al.* 1999; Dotson *et al.* 2000). We deleted the nonessential *SIN4* in strain CMY4000 to create CMY5009 and observed similar levels of constitutive maltase expression in the *sin4*Δ null allele as in all five of the *sin4* mutant strains (data not shown). Thus, loss of Sin4p results in constitutive maltase expression.

***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 act independently to regulate *MAL* gene expression. To test this, *SIN4* was deleted in CMY4000 and the isogenic *rgr1-31* strain (CM-31) to create CMY5009 and CMY5030, respectively. Maltase activity was assayed in cells grown under uninduced conditions. As shown in Figure 2, the double mutant *rgr1-31 sin4*Δ displays a similar level of constitutive maltase expression compared to the single mutant *sin4*Δ, 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 levels of maltase activity were observed in *rgr1-31* (CM-31) than in *sin4*Δ (CMY5009) because *RGR1* is an essential gene and *rgr1-31* is only a partial loss-of-function allele.

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 (*mal11*Δ*::MAL61*) were deleted from CMY4000 (*RGR1 SIN4*), CM-31 (*rgr1-31 SIN4*), and CM-33 (*RGR1 sin4-33*), all of which carry an integrated reporter *MAL61promoter-HIS3* gene. The resulting strains

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 (SGalG/L)
		Mal − His	Gal − His	
CMY4000	*RGR1 SIN4 mal11Δ::MAL61 MAL13*	+	−	46
CMY5004	*RGR1 SIN4 mal11Δ MAL13*	−	−	27
CMY5003	*RGR1 SIN4 mal11Δ::MAL61 mal13Δ*	−	−	21
CMY5001	*rgr1-31 SIN4 mal11Δ::MAL61 MAL13*	+	+	254
CMY5007	*rgr1-31 SIN4 mal11Δ MAL13*	−	+	280
CMY5005	*rgr1-31 SIN4 mal11Δ::MAL61 mal13Δ*	−	+	285
CMY5002	*RGR1 sin4-33 mal11Δ::MAL61 MAL13*	+	+	432
CMY5008	*RGR1 sin4-33 mal11Δ MAL13*	−	+	326
CMY5006	*RGR1 sin4-33 mal11Δ::MAL61 mal13Δ*	−	+	347

The *MAL*-activator gene (*MAL13*) or the maltose permease gene (*mal11Δ::MAL61*) was disrupted from CMY4000 (*RGR1 SIN4 mal11Δ::MAL61 MAL13*), CMY5001 (*rgr1-31 SIN4 mal11Δ::MAL61 MAL13*), and CMY5002 (*RGR1 sin4-33 mal11Δ::MAL61 MAL13*). The resulting strains were streaked for single colonies on minimal media lacking histidine with either 2% maltose or 2% galactose and growth was monitored for 3 days. 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.

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, 1989), strains lacking the *MAL*-activator (*mal13Δ*) or maltose permease (*mal11Δ*) are not maltose inducible (Table 3). The double-mutant strains, *rgr1-31 mal13Δ* and *rgr1-31 mal11Δ*, are able to grow in galactose medium lacking histidine and express 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.

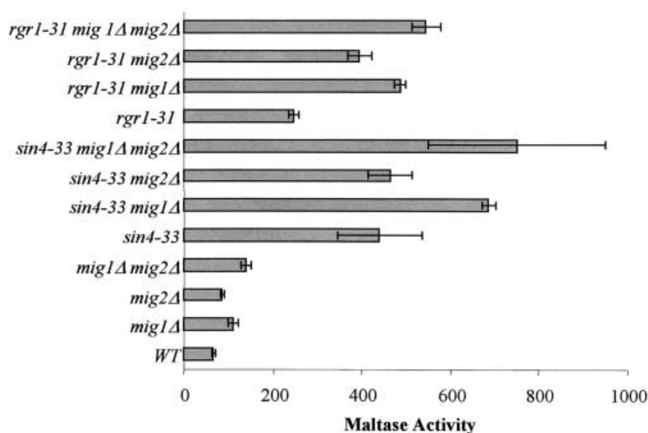


Figure 3.—*SIN4* and *RGR1* act synergistically with *MIG1* and *MIG2* in the repression of *MAL* gene expression. *MIG1*, *MIG2*, and both were deleted from strain CMY4000, the *rgr1-31* mutant strain CMY5001, and the *sin4-33* mutant strain CMY5002. Maltase activity was assayed in cells grown under uninduced conditions in minimal media with 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:** The Sin4 module of the Saccharomyces Mediator is thought to sense signals from gene-specific activators and repressors. Other than the *MAL*-activator the only gene-specific regulators of the *MAL* structural genes identified are the Mig1,2 repressors and thus these are possible candidates for interaction with the Mediator. 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 (CM-31), and the *sin4-33* mutant (CM-33). Maltase expression levels were determined under uninduced growth conditions (SGalG/L; Figure 3). Consistent with previous studies (Hu *et al.* 2000), deletion of *MIG1*, *MIG2*, or both modestly increases the basal level of maltase expression to levels about four times lower than the maltase levels observed in *sin4* and *rgr1* mutants, indicating that *MIG1* and *MIG2* play only 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 in either strain containing the single *mig1Δ* or *mig2Δ* alone. These findings indicate that Mig1p and Mig2p repress the expression of *MAL* genes

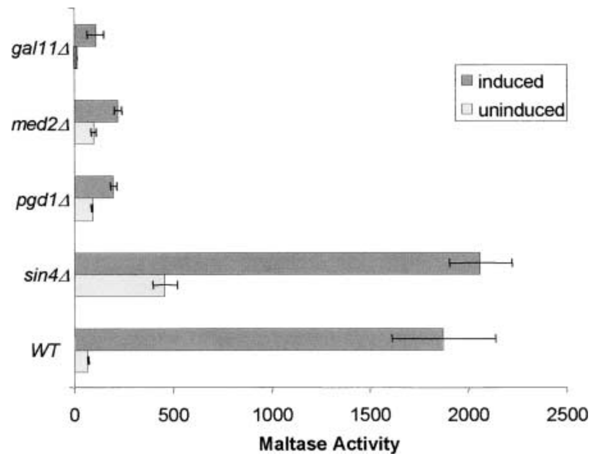


FIGURE 4.—Effects of deletion of the Sin4 module components on basal maltase expression and maltase induction. The *SIN4*, *GAL11*, *MED2*, or *PGD* gene was deleted from strain CMY4000. Maltase activity was assayed in cells grown in minimal media with 2% galactose, 3% glycerol, and 2% lactate (SGalG/L, uninduced conditions) and with 2% maltose (SMal, induced conditions) as described in MATERIALS AND METHODS.

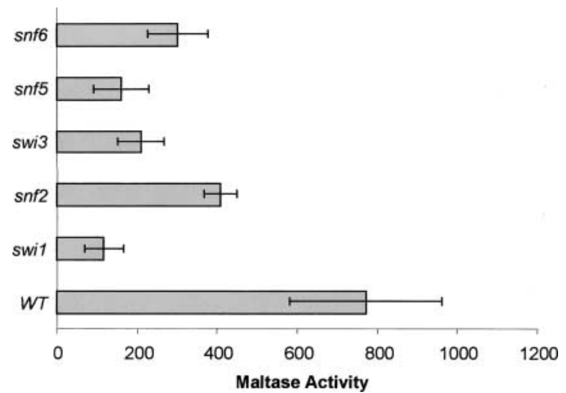


FIGURE 5.—The Swi/Snf complex is involved in maltose induction. Plasmid YCp50-MAL63 carrying the *MAL63 MAL*-activator gene (DANZI *et al.* 2000) was transformed into strain BLY1 and the isogenic strain series, BLY13 (*swi1*Δ), BLY16 (*snf2*Δ), BLY14 (*swi3*Δ), BLY3 (*snf5*Δ), and BLY5 (*snf6*Δ). Cells were grown under uninduced conditions in selective media containing 0.2% glucose to early-log phase. Cells were then collected and transferred to selective induced media containing 2% maltose. After 6 hr, maltase activity was assayed as described in MATERIALS AND METHODS.

independently of the Mediator Sin4 module and act to repress *MAL* gene expression via a separate pathway.

Each component of the Sin4 module of the Mediator plays a distinct role in regulation of *MAL* gene expression: Biochemical analysis indicates that the Saccharomyces Sin4 module of the Mediator complex contains Sin4p, Gal11p, Med2p, and Pgd1p. These bind to Rgr1p that 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).

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 CMY-5012 (*gal11*Δ) were constructed by one-step gene replacement and maltase expression levels assayed under induced (SMal) and uninduced (SGalG/L) growth conditions. As shown in Figure 4, 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 maltose-induced expression of maltase is dramatically decreased. 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 ~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. Moreover, Sin4p is a negative regulator of basal expression of the *MAL* structural genes, Med2p and Pgd1p are positive regulators of induced expression, and Gal11p is required for both basal and induced expression.

***MAL* gene induction is defective in strains carrying Swi/Snf complex mutations:** Swi/Snf is a 2-MD 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 (WU and WINSTON 1997; GREGORY *et al.* 1998, 1999). To examine whether the Swi/Snf complex is required for *MAL* gene induction, we measured maltase induction in an isogenic series of strains containing mutations in *SWI1*, *SNF2*, *SWI3*, *SNF5*, or *SNF6* encoding components of the Swi/Snf complex. As shown in Figure 5, loss of any one of these functions causes a dramatic decrease in induced maltase expression compared to that of the isogenic wild-type strain. Thus, the Swi/Snf complex is required for the full induction of *MAL* gene expression. 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 by Gcn5p is not required for maltose induction (B. ZHANG and C. A. MICHELS, unpublished results).

Constitutive *MAL* gene expression caused by loss of *SIN4* is dependent on the Swi/Snf complex: Previous studies have suggested that Sin4p negatively regulates gene transcription by acting to inhibit chromatin reorganization and/or maintain the inactive chromatin structure (JIANG and STILLMAN 1992; JIANG *et al.* 1995;

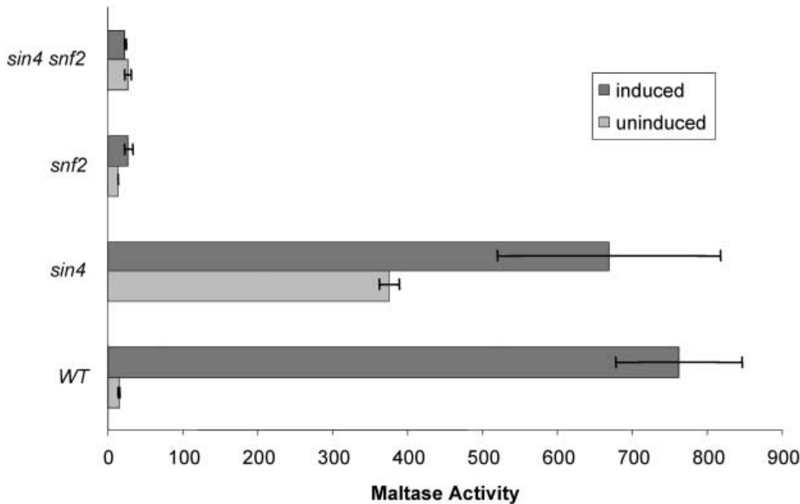


Figure 6.—Effects of *sin4*Δ and *snf2*Δ on *MAL* gene expression. Maltase expression was determined in the isogenic strain series CMY4000 (*SIN4 SNF2*), CMY5009 (*sin4*Δ *SNF2*), CMY5022 (*SIN4 snf2*Δ), and CMY5023 (*sin4*Δ *snf2*Δ). Strains were grown to midlog in synthetic medium containing 0.1% glucose plus either 2% maltose (induced) or 2% galactose (uninduced) and harvested, and maltase activity was assayed as described in materials and methods. The low concentration of glucose is not repressing but is required because of the very slow growth rate of CMY5023, in particular.

Macatee *et al.* 1997; Moss and Laybourn 2000). To investigate the relationship between these two complexes in regulation of *MAL* gene transcription *SIN4* and *SNF2* were individually and together deleted in strain CMY1001. These strains were grown under uninduced and induced conditions and maltase activity was determined. As shown in Figure 6, and consistent with the findings shown above in Figure 5, the *snf2*Δ mutation causes a severe defect in maltase expression under induced conditions that is not suppressed by *sin4*Δ. In contrast, no effect is observed on uninduced maltase expression levels in *snf2*Δ but there is an approximately twofold increase in maltase activity in the *sin4*Δ *snf2*Δ double mutant (Figure 6). It is interesting to note that the effects of *snf2*Δ are significantly greater in this strain series compared to those in the strains shown in Figure 5 and probably are a function of differences in the strain backgrounds and/or the *MAL* loci of these strains. The results in Figure 6 indicate that the constitutive expression of *MAL* genes caused by loss of Sin4p is largely dependent on the Swi/Snf complex, and that Sin4p acts upstream of the Swi/Snf in controlling basal *MAL* gene expression.

DISCUSSION

The Mediator complex plays an essential role in both activation and repression of RNA polymerase II-mediated transcription and the Sin4 module to act in regulatory signal transduction. Our results suggest that the components of the Sin4p module of the yeast Mediator function differentially and in distinct combinations to modulate *MAL* gene transcription and regulate basal expression via as yet unidentified *MAL* promoter-binding repressor(s) and activator(s).

Sin4p and the C-terminal region of Rgr1p repress basal level expression of *MAL* genes, but have little or no effect on maltose induction: Mutations in *RGR1* that truncate up to 270 residues of the C-terminal region and *SIN4* null mutations increase maltase expression to ~20–30% of the fully induced levels under uninduced growth conditions and partially relieve glucose repression independent of the *MAL*-activator and maltose permease. Thus, the C terminus of Rgr1p and Sin4p repress basal expression of the *MAL* genes.

Mig1,2 repressor regulates glucose repression of *MAL* gene transcription (Hu *et al.* 1995) but actions of this repressor are distinct from the Sin4p effects observed here. Mig1p exerts its repressive effects by recruiting the Ssn6-Tup1 corepressor complex, which reportedly interacts with the Mediator (Treitel and Carlson 1995; Papamichos-Chronakis *et al.* 2000). Nonetheless, deletion of *MIG1*, *MIG2*, or both in strains carrying *rgr1-31* or *sin4-33* mutations shows that Mig1,2 repressor further increases maltase expression and thus acts synergistically with the *sin4* and *rgr1* mutations (Figure 3), indicating that Sin4p and the C terminus of Rgr1p are not involved in transmission of the Mig1,2p repression signal but instead function via an independent repression pathway.

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). Biochemical analyses and electron microscopy imaging propose that Sin4p anchors the other module components to the Mediator complex through its interaction with the C terminus of Rgr1p. Mediator complexes purified from *sin4*Δ and *rgr1-Δ2* strains lack all of the components of the Sin4 module (Jiang *et al.* 1995; Li *et al.* 1995; Asturias *et al.* 1999; Myers *et al.* 1999; Dotson *et al.* 2000). Pgd1p, Med2p, and Gal11p are dependent on each other in regard to their ability to form a stable association within the Mediator but their loss has little effect on the association of Sin4p with Rgr1p (Lee *et al.* 1999; Myers *et al.* 1999).

Our results conflict with this proposed anchoring function of Sin4p. Given the essential requirement of

Gal11p, Pgd1p, and Med2p in *MAL* gene induction demonstrated here, loss of Sin4p should block *MAL* gene induction. This is clearly not the observed result (Table 3 and Figure 4). Both *sin4-33* and *sin4*Δ mutant strains exhibit wild-type levels of induced maltase expression. Thus, Pgd2p, Med2p, and Gal11p binding to Sin4p could not be required for their interaction with the Mediator. We suggest instead that Sin4p interacts with the C-terminal 270 residues of Rgr1p and that Gal11p, Pgd1p, and Med2p bind elsewhere on Rgr1p. Loss of Sin4p may destabilize the complex *in vitro* but not *in vivo*, at least not enough to alter induced expression at the *MAL* promoter.

Swi/Snf chromatin reorganization is required for *MAL* gene expression. The results reported in Figures 5 and 6 indicate that the Swi/Snf chromatin-reorganizing complex is required for induced *MAL* gene expression as well as for the elevated basal rate of *MAL* gene expression exhibited in *sin4* mutants. We suggest that Sin4p negative regulation of basal *MAL* gene transcription is achieved by blocking Swi/Snf-dependent chromatin reorganization. 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 (Jiang and Stillman 1992; Jiang *et al.* 1995). 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). Moreover, defects in components of both the Mediator and the Swi/Snf complex are suppressed by similar mutations in chromatin components, the so-called *sin* mutations in histones and associated factors (Prelich and Winston 1993; Kruger *et al.* 1995).

We report little or no suppression of the *snf2*Δ defect in basal and induced maltase expression by *sin4*Δ (Figure 6). Several *swi/snf* mutations had been reported to be partially suppressed by a *sin4* null mutation (Sternberg *et al.* 1987; Stillman *et al.* 1994), which would suggest a role for Sin4p downstream of this chromatin-reorganizing complex. These finding may be an artifact since suppression is observed only with *LacZ* reporter genes and not with the genomic copy of the same genes (Yu *et al.* 2000). Thus, our results, similar to results for the *HO* gene, indicate that Sin4p acts upstream of the Swi/Snf complex to maintain the low basal level of *MAL* gene expression probably by blocking chromatin reorganization and/or maintaining an inactive chromatin structure.

Role of the Sin4 module in *MAL* gene regulation: Sin4p and Gal11p play opposing roles in regulating basal expression from the *MAL* promoter. Sin4p is a negative regulator of basal *MAL* gene expression. Gal11p is a positive regulator of both basal and *MAL*-activator-dependent maltose-induced *MAL* gene expression. We propose that Sin4p represses basal expression of *MAL* gene promoters by blocking Gal11p-mediated chromatin reorganization. Our findings are consistent with the reported roles of these Mediator components in yeast transcription regulation. Gal11p is considered a positive regulator of transcription, although modest negative effects have been reported (Yu and Fassler 1993; Nishizawa *et al.* 1994; Nishizawa 2001). Artificial tethering of a Gal11p fusion protein to the promoter region of a reporter construct produces strong activation of reporter gene expression (Jiang and Stillman 1992; Barberis *et al.* 1995). Alternately, with the exception of *CTS1*, *MAT*α*2*, *Ty1*, and *HIS4*, Sin4p is regarded as a negative regulator of transcription (Jiang and Stillman 1992; Jiang *et al.* 1995). We propose that Sin4p-bound Mediator complex interacts with a DNA-bound repressor and blocks the functions of Gal11p. In the absence of Sin4p, Gal11p interacts with a DNA-bound activator, recruits Mediator to the activator, and activates basal transcription in a Swi/Snf-dependent manner. It should be noted that interactions between DNA-bound Mig1,2 repressor and Mediator do not similarly interfere with this Gal11p-dependent activation of basal expression.

Sin4p is not involved in regulation of maltose-induced *MAL* gene expression (Figure 1). For maltose-induced expression, we propose that a Mediator complex containing Gal11p, Med2p, and Pgd1p interacts with the *MAL*-activator and activates transcription in a Swi/Snf-dependent manner. Thus, the Mediator plays different roles in *MAL* gene expression and acts as both an antagonist and protagonist of *MAL* gene expression under different growth conditions by utilizing different components of the Sin4p module. Whether these results suggest different structural conformations of the Mediator Sin4p module or that the composition the Mediator Sin4 module is heterogeneous and varies from promoter to promoter, as a function of the growth medium, or when bound to different DNA-binding transcription factors remains to be determined.

Studies support the proposal that the Mediator complex is recruited to promoters by means of interactions between the Sin4p module and DNA-bound transcription regulators (Lee *et al.* 1999; Bhoite *et al.* 2001). Evidence of direct interaction between Gal11p and several transcription activators including Gal4p, Gcn4p, and the VP16 activation domain has been reported by Park *et al.* (2000). Bhoite *et al.* (2001) show that Swi5 activator recruits Mediator to the *HO* promoter via direct interaction. Sin4p has been shown to immunoprecipitate with Sfl1p, a repressor of *SUC2*, *FLO11*, and *HSP26* (Conlan and Tzamarias 2001). What DNA-binding transcription factors are involved in regulating basal *MAL* gene expression? Sequence analysis of the bidirectional *MAL61* and *MAL62* promoter (Krull *et*

al. 2003; Matys *et al.* 2003) and multiple-alignment analysis of *MAL* promoters from *S. mikatae, S. kudriavzevii,* and *S. bayanus* were largely uninformative. Deletion analysis carried out by Levine *et al.* (1992) points to a possible repressor-binding site located in base pairs −348 to −514 upstream of the *MAL61* ORF, a region that exhibits sequence similarities to the proposed Sfl1p-binding site (Conlan and Tzamarias 2001). We are currently involved in studies to identify novel regulators of basal *MAL* gene transcription with particular attention to Sfl1 repressor.

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