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Zhen Hu · Jan Olof Nehlin · Hans Ronne
Corinne A. Michels

MIG1-dependent and *MIG1*-independent glucose regulation of *MAL* gene expression in *Saccharomyces cerevisiae*

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Abstract Glucose repression is a global regulatory system in *Saccharomyces cerevisiae* controlling carbon-source utilization, mitochondrial biogenesis, gluconeogenesis and other metabolic pathways. Mig1p, a zinc-finger class of DNA-binding protein, is a transcriptional repressor regulating *GAL* and *SUC* gene expression in response to glucose. This report demonstrates that Mig1 protein represses transcription of the *MAL61* and *MAL62* structural genes and also the *MAL63* gene, which encodes the Mal-activator. Mig1p DNA-binding sites were identified upstream of all three *MAL* genes. Both of the Mig1p-binding sites found in the bidirectional *MAL61-MAL62* promoter were shown to function in the Mig1p-dependent glucose repression. Studies using constitutive Mal-activator alleles suggest that glucose regulation of inducer availability is a second major contributing factor in glucose repression of *MAL* gene expression and is even stronger than the Mig1p-dependent component of repression. Moreover, our results also suggest the contribution of other minor mechanisms in glucose regulation of *MAL* gene expression.

Key words Maltose fermentation · Glucose repression · *MIG1* · *MAL* genes

Introduction

MIG1 encodes a DNA-binding *Saccharomyces cerevisiae* transcriptional repressor with structural homology to mammalian Egr (early growth response) and Wilms' tumor proteins (Nehlin and Ronne 1990). In yeast, Mig1p is a component of the multi-faceted negative regulatory response to glucose, wherein the expression of a large number of enzymes, required for mitochondrial biogenesis, carbohydrate metabolism and gluconeogenesis, are repressed by glucose. Studies on glucose repression of the *SUC2* and *GAL* genes identified Mig1p as a downstream factor in the Snf1 protein kinase signal-transduction pathway which mediates repression of these genes (reviewed in Johnston and Carlson 1992; Trumbly 1992; Ronne 1995). It has been proposed that Mig1p may recruit a complex containing the Tup1p and Ssn6p (Cyc8p) proteins to its target promoters (Keleher et al. 1992). Tup1p is believed to be the effector subunit which mediates repression of the target gene (Tzamarias and Struhl 1994).

Maltose fermentation in *Saccharomyces* requires two enzyme functions, maltose permease (gene 1) and maltase (gene 2), and expression of these enzymes is induced by maltose and repressed by glucose (reviewed in Needleman 1991). Maltose induction is mediated by a transcription activator referred to as the Mal-activator (gene 3). The genes encoding these three functions map to a three-gene complex referred to as a *MAL* locus. Five *MAL* loci have been identified, each located at a telomere-associated site: *MAL1* (chromosome VII), *MAL2* (chromosome III), *MAL3* (chromosome II), *MAL4* (chromosome XI), and *MAL6* (chromosome VIII).

Several lines of evidence suggest strongly that Mig1p also functions as a repressor of *MAL* gene expression in response to glucose. First, a high-copy plasmid carrying *MIG1* inhibits growth on maltose (Nehlin and Ronne 1990). Second, deletion anaylsis of the shared, bidirectional promoter region of *MAL61* (maltose permease gene at *MAL6*) and *MAL62* (maltase gene at *MAL6*) identified an upstream glucose-responsive element with sequence homology both to the Mig1p DNA-binding consensus sequence and to the URS_G element of the *GAL1* promoter (Flick and Johnston 1992; Levine et al. 1992; Lundin et al. 1994). Finally, *snf1* mutants affect the utilization of maltose (Carlson et al. 1981). We undertook the present study

Z. Hu · C.A. Michels (✉)
Queens College and the Graduate School of CUNY,
Departments of Biology and Biochemistry,
65-30 Kissena Boulevard, Flushing, NY 11367, USA

J. O. Nehlin[1] · H. Ronne
Ludwig Institute for Cancer Research, Uppsala Branch,
Uppsala Biomedical Center, Box 595, S-751 24 Uppsala, Sweden

[1] *Present address:* Department of Cancer Biology, Lawrence Berkeley Laboratory, Building 70A, Room 1105, 1 Cyclotron Road, Berkeley, CA 94720, USA

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to characterize the relative significance of the role(s) of Mig1p in the glucose regulation of maltose fermentation and to identify other possible pathways which contribute to regulation.

Materials and methods

Yeast strains and media. The yeast strains used in the study were described previously (Needleman et al. 1984; Dubin et al. 1989). The genotype of strain 332-5A is *MAL61 MAL62 MAL63 mal64 mal11 MAL12 mal13 ura3-52 leu2-3,112 trp1 his.* It has a fully functional *MAL6* locus and a partially functional allele of the *MAL1* locus containing only an active *MAL12* gene encoding maltase. The constitutive strain R10 is isogenic to 332-5A except that it contains the constitutive Mal-activator allele *MAL64-R10* plus a deletion of *MAL63* constructed by removal of most of the ORF and insertion of the *URA3* gene. Strain R10u is a *ura3* derivative of R10 isolated on 5-fluoroorotic acid-containing medium (Boeke et al. 1984). *MIG1* and *SKO1* were disrupted in strain 332-5A using plasmids pJN22 and pHR97, respectively, as described previously (Nehlin and Ronne 1990; Nehlin et al. 1992). Yeast strains were grown at 30 °C on either rich media (1% yeast extract, 2% peptone) or minimal medium (0.67% yeast nitrogen base and appropriate amino acids minus uracil or leucine) plus various amounts of the specified carbon sources.

Plasmids. Plasmids pHR81 and pMIG1 have been described previously (Nehlin and Ronne 1990). Plasmid YCpMAL43-C was constructed by inserting the *Bam*HI-*Sal*I fragment containing *MAL43-C* into the plasmid YCp50 (Charron and Michels 1987). The ARSH4/CEN6 cassette was obtained by PCR from plasmid pRS315 (Stratagene) and then inserted into the *Eco*RI site of plasmid YIp355, resulting in plasmid YCp355 (Myers et al. 1986). The ability of YCp355 to function as a stable, episomal plasmid was confirmed by yeast transformation followed by plasmid segregation. Plasmids pE2 and pD5 have been described previously (Dubin et al. 1989). Plasmid pHR81/MAL63 was constructed by inserting a 1.7-kb *Bam*HI fragment containing *MAL63* into pHR81.

Oligonucleotides. Double-stranded oligonucleotides were prepared as previously described (Nehlin et al. 1992). The oligonucleotides were end-labelled with ^{32}P-dCTP using Klenow polymerase and purified on Biospin columns (BioRad) prior to use.

Gel-shift assay. In vitro-translated Mig1 and Sko1 proteins were prepared as described previously (Nehlin and Ronne 1990). The Mig1p was incubated with ^{32}P-labelled oligonucleotides for 1 h at 20 °C in Mig1p-binding buffer (Nehlin and Ronne 1990). The reactions contained 1 µl of Mig1p lysate, 10–30 pg (3000 cpm) of labelled DNA, and 0.5 mg of poly(dI:dC) carrier DNA (Pharmacia LKB) in a total volume of 10 µl. Protein-DNA complexes were separated on 6% polyacrylamide gels, which were then dried and autoradiographed (Nehlin et al. 1992). Agarose-gel shift assays with end-labelled restriction fragments were performed as described previously (Nehlin and Ronne 1990; Nehlin et al. 1991).

Northern analysis. Yeast cells were harvested in mid-log phase. Total RNA was isolated as described in Ausubel et al. (1994) and separated on 1.2% agarose-formaldehyde gels. After transfer to Hybond-N nylon membranes (Amersham, IL.), the RNA and UV crosslinked and hybridized as described previously (Nehlin and Ronne 1990). Probes for the *MAL61*, *MAL62/12,* and *MAL63* genes were derived from plasmids pE2, pD5, and pHR81/MAL63, respectively. The amount of the hybridizing RNA was quantified in a Molecular Dynamics 400s Phosphorimager as described previously (Nehlin et al. 1992).

Mutagenesis and the construction of LacZ fusions. A 0.9-kb *Bam*HI fragment containing the entire intergenic region of *MAL61-62* (Levine et al. 1992), and including a few codons at the beginning of both structural genes, was created by PCR using plasmid pY6 as a template (Needleman et al. 1984). The *Bam*HI sites at both ends of this fragment were designed so that each could be fused, in-frame, to the *LacZ* gene in YCp355. This fragment was then subcloned into the *Bam*HI site of M13mp19 and the entire sequence was confirmed. Site-directed mutagenesis was carried out using the Muta-Gene Kit (BioRad) according to the manufacturer's instructions. Internal deletions of the two Mig1p-binding sites were created with oligonucleotides which exactly looped out the 6-bp GC box (Lundin et al. 1994) of the corresponding Mig1p-binding site and inserted no additional nucleotides. Sequences were confirmed using a DNA Sequencing Kit (USB) according to the manufacturer's instructions. The *Bam*HI fragment containing the entire intergenic region was inserted in both orientations into the *Bam*HI site of the reporter plasmid YCp355 to create in-frame fusions to the *LacZ* gene. All fusion plasmids were finally transformed into yeast strains 332-5A and R10u, with or without disruptions of *MIG1*.

Enzyme assays. Yeast cells were harvested in mid-log phase. Maltase activities were determined as described (Dubin et al. 1989). Maltase activity is expressed as nmoles of *p*-nitrophenol-α-glucoside (PNPG) hydrolyzed per min per mg of protein. The assay of β-galactosidase activity was carried out in permeabilized cells and the activity was normalized to the cell density (Guarente 1983; Ausubel et al. 1994). Assay values are the average of results obtained from two or more transformants. Standard errors were less than 10% for maltase assays and 20% for β-galactosidase assays.

Results

Effect of *MIG1* product on maltase gene expression

The *MAL6* strain 332-5A was transformed with plasmid pMIG1, which carries the *MIG1* gene in the *LEU2-d* defective plasmid pHR81. In leucine-deficient media, this plasmid amplifies to approximately 200–400 copies/cell leading to abundant expression of the *MIG1* gene (Nehlin and Ronne 1990). Strain 332-5A, carrying pMIG1 and grown in the absence of leucine, exhibits dramatically reduced levels of maltase in both induced (2% maltose, 3% glycerol and 2% lactate) and uninduced (3% glycerol and 2% lactate) growth conditions (Table 1). These results are comparable to those obtained by Nehlin and Ronne (1990) who found that this amplified plasmid severely inhibited growth on galactose and raffinose but not glucose, and strongly suggest that *MIG1* is involved in the regulation of maltose fermentation as well as in the utilization of galactose and sucrose. Under repressed conditions (2% glucose, 3% glycerol and 2% lactate) and under induced/repressed conditions (2% maltose, 2% glucose, 3% glycerol and 2% lactate), we see a reproducible two-fold increase in maltase expression in strains which over-express Mig1p. The basis of this increase is not clear.

Deletion of *MIG1* in strain 332-5A led to slightly increased maltase levels in induced conditions but no effect was seen in cells grown in uninduced conditions. The *mig1* disruption relieved glucose-repression and increased maltase expression in repressed and induced/repressed cells 40–60-fold (Table 2). These results confirm the involvement of *MIG1* in the glucose repression of maltase expression. Moreover, they demonstrate a six-fold induction of

Table 1 Effect of *MIG1* over-expression on maltase expression. Strain 332-5A was transformed with plasmid pMIG1 or the vector control plasmid, pHR81, and maltase levels determined as described in Dubin et al. 1989. Cells were grown in minimal media lacking leucine plus 3% glycerol and 2% lactate (v/v) and 2% (w/v) of the indicated sugar(s)

	Maltase activity			
Sugar added	–	Maltose	Glucose	Mal + Glu
Plasmid				
pHR81	7.8	441	0.8	2.0
pMIG1	1.4	26.4	1.7	4.2

Table 2 Effect of a *MIG1* deletion on maltase expression. A deletion/disruption of the *MIG1* gene was constructed in strain 332-5A as described in Materials and methods. Strains were grown in rich media plus 3% glycerol and 2% lactate and 2% of the indicated sugar. Maltase was assayed as described for Table 1

	Maltase activity			
Sugar added	–	Maltose	Glucose	Mal + Glu
Genotype				
MIG1	4.5	350	0.5	0.8
mig1Δ	4.7	570	31.2	32.0

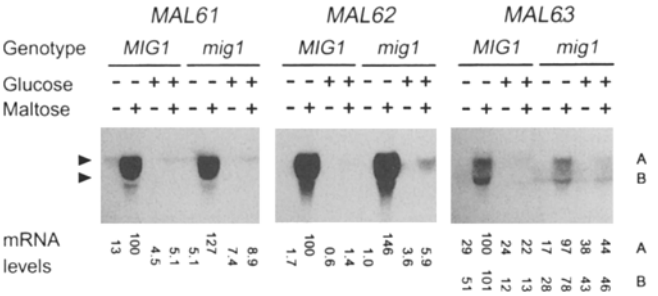


Fig. 1 Effect of *mig1* gene disruption on the expression of the *MAL61*, *MAL62* and *MAL63* mRNAs. Strain 332-5A (relevant genotype *MAL61 MAL62 MAL63 mal11 MAL12 mal13*) and 332-5A *mig1Δ* were grown as described in Table 2. Total mRNA was isolated, size-separated using a horizontal agarose gel, and transferred to a nylon membrane which was then hybridized to probes derived from *MAL61*, *MAL62*, and *MAL63* (Needelman et al. 1984) as described in Materials and methods. The probe used is indicated above each panel. It should be noted that, because of the high level of homology, the *MAL62*-derived probe also detects the *MAL12* mRNA. mRNAs levels were quantified in a phosphorimager, and normalized using hybridization to an *ACT1* probe. For each mRNA, the level in wild-type cells grown on maltose was set at 100%. The 2.0-kb mRNA was set at 100% in the case of *MAL63*. The *arrowheads* indicate the positions of the two *MAL61*-homologous mRNAs. The letters *A* and *B* distinguish the two *MAL63*-homologous mRNAs

maltase activity by glucose in the *mig1Δ* strain compared to the level of expression in uninduced cells. A transient glucose-induced expression of *SUC2* was seen in a *mig1Δ* strain (Nehlin et al. 1992).

Effect of *MIG1* on the expression of the *MAL61*, *MAL62*, and *MAL63* mRNAs

The expression of the *MAL* genes was further investigated by Northern-blot analysis of total RNA isolated from strain 332-5A and an isogenic *mig1Δ* strain. The probes utilized were specific for the *MAL61*, *MAL62/12* and *MAL63* mRNAs expressed by this strain. Yeast cells were grown in glycerol/lactate media with or without added maltose and/or glucose. The results are shown in Fig. 1. The mRNA levels reported were quantified by a phosphorimager and normalized to the levels of actin mRNAs (*ACT1* mRNAs are not shown in Fig. 1).

The *MAL62/12* transcript is induced by maltose and repressed by glucose. Disruption of *MIG1* increased the levels of the *MAL62/12* transcript 4–6-fold both in the repressed and induced/repressed media. Additionally, *MAL62/12* mRNA levels are higher in the repressed and induced/repressed conditions than in the uninduced condition in the *mig1* disruption strain. This confirms our finding of a glucose induction of maltase activity in the absence of *MIG1* (Table 2). There also remains a 25-fold glucose repression which is independent of Mig1p. Interestingly, this repression is seen only in the presence of maltose. Thus, we conclude that glucose repression of *MAL62/12* can be separated into a least two mechanisms. One mechanism requires Mig1p and operates both in the presence and in the absence of maltose. The second mechanism is independent of Mig1p and is detected only in the presence of maltose, suggesting that it involves inhibition of the induction process.

In the case of *MAL61*, low levels of mRNA are detected in uninduced and repressed conditions due to the presence of a transcript which is somewhat larger than the major inducible mRNA (Fig. 1; Needleman et al. 1984). The smaller transcript is undetectable under these conditions. Both transcripts respond to maltose induction. The values in Fig. 1 are presented as a total of both the inducible and the partially constitutive transcripts. From these results, we are unable to demonstrate an effect of *MIG1* disruption on the expression patterns of either transcript.

MAL63 has two mRNAs of different sizes and both transcripts are induced by maltose (Fig. 1; Needleman et al. 1984). Expression levels of the larger transcript are similar under uninduced, repressed and induced/repressed conditions, and disruption of *MIG1* only slightly increased the expression in the presence of glucose. The smaller transcript is sensitive to glucose-repression, about four-fold, and *MIG1* disruption leads to an approximate four-fold increase in the levels of this transcript in repressed and induced/repressed conditions. As with *MAL62*, there is also a Mig1p-independent component of repression, which is only seen under induced/repressed conditions.

MIG1-dependent glucose repression in Mal-activator constitutive strains

As noted above, glucose repression of maltase (*MAL62/12*) mRNA appears to have at least two components: a 4–6-fold repression of maltase (*MAL62/12*) mRNA appears to have at least two components: a 4–6-fold repression mediated by Mig1p and a 25-fold Mig1p-independent component which we suggest involves inhibition of maltose induction. To confirm that inhibition of the induction process contributes to glucose repression of the *MAL* genes, we used two Mal-activator constitutive alleles. *MAL43-C* is a dominant, constitutive allele of the Mal-activator gene encoded by the *MAL4* locus (Charron and Michels 1987). This gene was introduced into both the *MAL6 332-5A* and isogenic *mig1Δ* strains on a CEN plasmid. *MAL64* is a linked homologue of *MAL63* which can be activated by mutation to encode a dominant, constitutive activator of *MAL* gene expression (Dubin et al. 1989). Strain R10 is isogenic to 332-5A except for the presence of the constitutive *MAL64-R10* allele and a deletion of *MAL63*. Maltase expression was determined in both the constitutive and isogenic *mig1Δ* deletion strains (Table 3).

Both strains are fully constitutive. Indeed, maltase levels are reproducibly higher in the *MAL64-R10* strain when grown in uninduced rather than induced conditions. This constitutive expression is only repressed approximately ten-fold by glucose, and deletion of *MIG1* causes a three-fold derepression. In the strain carrying the *MAL43-C* plasmid, maltase expression was only three-fold repressed by glucose, and disruption of *MIG1* caused a two-fold derepression (Table 3). These results confirm that inhibition of the induction process plays a significant role in glucose repression of the *MAL* genes.

Mig1p binds to the upstream regions of *MAL61*, *MAL62*, and *MAL63*

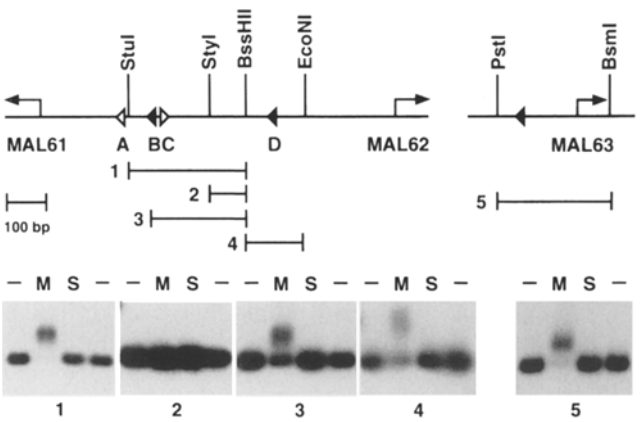
The results reported above suggest that *MIG1* is involved in the glucose regulation of maltose fermentation. The DNA-binding specificity of *MIG1* has been documented by extensive analysis demonstrating that the two zinc fingers of Mig1p recognize the degenerate GC-box (G/C)(C/T)GG(G/A)G (Nehlin and Ronne 1990; Nehlin et al. 1991; Lundin et al. 1994). Previous studies identified binding sites for Mig1p in the promoter regions of *GAL1*, *GAL4* and *SUC2* (Nehlin and Ronne 1990; Nehlin et al. 1991). The Mig1p-binding sites in the *GAL1* and *GAL4* promoters lie within regions defined as essential for the glucose-repression of these genes, referred to as URS_G for upstream repression sequences (Flick and Johnston 1990, 1992; Griggs and Johnston 1991). Deletion analysis of the *MAL61-MAL62* bidirectional promoter region identified a sequence adjacent to the UAS_{MAL} which appears to be involved in the glucose-repression of *MAL62* and which exhibits sequence homology to the Mig1p DNA-binding site consensus sequence (Levine et al. 1992; Lundin et al. 1994). In fact, perusal of the *MAL61-MAL62* intergenic region reveals four sites matching this sequence, which we will refer to as sites A (*MAL61* proximal) to D (*MAL62* proximal) (Fig. 2). The *MAL63* promoter contains a single possible Mig1p site approximately 120-bp upstream of the *MAL63* ORF (Fig. 2) (Kim and Michels 1988).

Agarose-gel shifts with end-labelled restriction fragments were used to scan the promoter regions of *MAL61*, *MAL62*, and *MAL63*. The *MAL61-MAL62* intergenic region contains at least two independent Mig1p-binding sites, as evidenced from the fact that the non-overlapping fragments 1 and 4 are both retarded in the presence of Mig1

Table 3 Effect of a *MIG1* disruption in Mal-activator constitutive strains. The *MAL64-R10* strain, strain R10, is isogenic to strain 332-5A but carries the constitutive *MAL64-R10* allele and a *mal63Δ::URA3* deletion. This strain and the isogenic *mig1Δ* strain were grown as described in Table 2. To construct the *MAL6* [pMAL43-C], strain 332-5A was transformed with a CEN plasmid carrying the *MAL43-C* constitutive Mal-activator allele in vector Ycp50. This strain and the isogenic *mig1Δ* were grown in minimal media lacking uracil plus 3% glycerol and 2% lactate and 2% of the indicated sugar. Maltase activities were assayed as described in Table 1

	Maltase activity			
Sugar added	–	Maltose	Glucose	Mal + Glu
MAL64-R10 strain				
MIG1	1027	752	77.5	69.2
mig1Δ	1211	1233	211	174
MAL6 [pMAL43-C] strain				
MIG1	506	561	185	150
mig1Δ	505	732	377	332

Fig. 2 Agarose-gel shift assay for Mig1p-binding to restriction fragments from the *MAL* promoters. Restriction maps of the *MAL61-MAL62* and *MAL63* promoters are shown at the top, with the fragments tested for binding displayed below the maps. Possible *Mig1*p-binding sites are shown as *arrows*, the direction of which indicates the orientation of the GC-box. *Filled arrows* represent sites to which *Mig1*p binds strongly. The agarose-gel shifts are shown at the bottom. Abbreviations: *M Mig1* protein; *S* Sko1 protein; –, no added protein



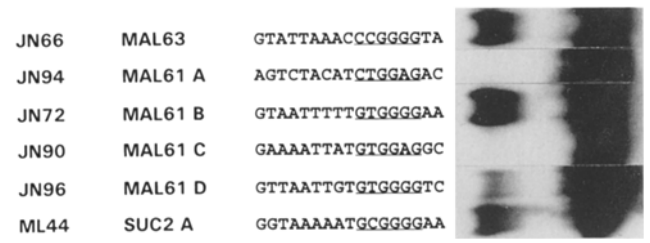


Fig. 3 Gel-shift assay for *Mig1*p-binding to oligonucleotides derived from potential binding sequences found in the *MAL* gene promoters. The top strand of each oligonucleotide is shown to the left, with the GC box *underlined*. The gel shift is oriented with *Mig1*-DNA complexes to the left and free DNA to the right

protein (Fig. 2). Fragment 4 contains bases from –217 to –371 with respect to the ATG of *MAL62*. The Mig1p-binding region in fragment 1 was further localized using fragments 2 and 3 to a site between base pairs –214 and –417 with respect to the ATG of *MAL61* (Fig. 2). For the *MAL63* gene, we found that a fragment which contains DNA from base –196 to +78 is retarded in the presence of Mig1 protein.

We also considered the possibility that the glucose induction of maltase expression in the *mig1Δ* strain could involve the *SKO1* gene which functions as a transcriptional repressor of *SUC2* (Nehlin et al. 1992). The transient glucose induction of *SUC2* in the *mig1Δ* strain seems to be associated with the loss of Sko1p-dependent repression (Nehlin et al. 1992). Therefore, the restriction fragments were also tested for binding to the Sko1 protein. In no case did we detect any retardation (Fig. 2). This is consistent with the fact that a deletion of *SKO1* had little effect on *MAL* gene expression (data not shown).

The results in Fig. 2 suggest that Mig1p binds to the *MAL63* promoter and to at least two distinct sites in the *MAL61-MAL62* promoter. We used gel-shift analysis with short double-standard oligonucleotides corresponding to the various potential Mig1p-binding sites identified in the promoter regions of these genes to determine which are capable of binding Mig1p in vitro. The results are shown in Fig. 3. As a positive control, we also included an oligonucleotide corresponding to the Mig1p site A in the *SUC2* promoter identified previously (Nehlin and Ronne 1990). We found that the different sites vary considerably in their ability to bind Mig1p. The site in the *MAL63* promoter bound Mig1p strongly, as did site B from the *MAL61-MAL62* region. The *MAL62* proximal site D also bound Mig1p, though less efficiently than site B. Site C bound Mig1p very weakly, which was evident only after prolonged exposure of the gel. The remaining site A did not bind Mig1p at all. These results are consistent with the above results using restriction fragments (Fig. 2).

The sequences of the Mig1p-binding sites identified in the *MAL* promoter regions are in excellent agreement with previously determined rules for Mig1p binding (Lundin et al. 1994). High-affinity binding to Mig1p requires a GC-box, (G/C)(C/T)GG(G/A)G, with a G being preferred in position 5. In addition, an AT-rich region 5′ to this GC-box is also important for binding. The three oligonucleotides that clearly bound Mig1p (JN66, JN72, and JN96) all have a GC-box with a G in position 5, and a well conserved AT-box (Fig. 3). JN90, which bound Mig1p very weakly, has both a GC-box and an AT-box, but the GC-box has an A in position 5. In contrast, the oligonucleotide JN94 that failed to bind Mig1p lacks the AT-box. Moreover, it has an A in position 5 of the GC-box.

Deletion analysis of the Mig1p-binding sites in the *MAL61-MAL62* promoter

To determine whether the Mig1p-binding sites in the *MAL61-MAL62* identified in vitro also function in vivo and whether Mig1p functions to repress *MAL61*, we monitored the effects of deleting one or both sites on *MAL61* and *MAL62* expression. A 0.9-kp fragment containing the full 874-bp *MAL61-MAL62* intergenic promoter sequence and a few codons from the 5′-end of both the *MAL61* and *MAL62* open reading frames was fused, in-frame, to the *LacZ* gene of plasmid YCp355, a CEN derivative of YIp355 (Myers et al. 1986; Levine et al. 1992). Deletions in which the 6-bp GC-box was exactly removed from site B (–275 to –280), site D (–580 to –585), and from both sites B and D, were constructed. The wild-type promoter and the three deletions were fused to the *LacZ* reporter in both the *MAL61* and *MAL62* orientations and these plasmids introduced into the *MAL6* strain 332-5A and an isogenic *mig1Δ* strain. Reporter gene expression was determined in uninduced, induced and repressed conditions (see Table 4).

Expression of *LacZ* from the wild-type *MAL62* promoter in the wild-type *MAL6 MIG1* host strain was induced only 2–3-fold, well below the 60–80-fold induction seen in measurements of induction based on *MAL62/12* transcript levels (Fig. 1) and maltase enzyme levels (Table 2). These results are consistent with our previous findings which used a single, integrated copy of the *LacZ* fusion plasmid (Levine et al. 1992). The basis for this low induction ratio is unclear. Nevertheless, it is important to note that the sensitivity to glucose repression exhibited here appears to the comparable to that in the other assay systems.

Expression from the wild-type promoter in the *MAL62-LacZ* orientation was repressed 30–80-fold by glucose, and deletion of *MIG1* leads to a ten-fold derepression (Table 4). Deletion of the *MAL62*-proximal Mig1p-binding site, site D, relieves glucose repression of *MAL62-LacZ* about threefold. Deletion of the *MAL61*-proximal site B alone has no effect on *MAL62-LacZ* expression, but shows a modest effect when site D also is deleted. Deletion of *MIG1* caused only a minor increase in *MAL62-LacZ* expression in the presence of glucose when both Mig1p sites had been deleted (Table 4). This demonstrates that the effect of Mig1p on *MAL62* expression is mediated almost entirely by the Mig1p-binding sites.

Interestingly, uninduced expression from the *MAL62* promoter was significantly reduced as a result of the dele-

Table 4 Effect of deletion of the two Mig1p-binding sites in the *MAL61-MAL62* promoter on *LacZ* reporter gene expression. Mutations in the *MAL61-MAL62* 874-bp bidirectional promoter sequence, deleting one or both of the G/C-boxes of the two Mig1p-binding sites, were constructed and fused, in-frame, to the *LacZ* gene of plasmid YCp355 (see Materials and methods) in both the *MAL61* and *MAL62* promoter orientations. These were transformed into strain 332-5A and an isogenic *mig1Δ*, and β-galactosidase activity assayed in cells grown in minimal media lacking uracil plus 3% glycerol and 2% lactate (v/v) and 2% (w/v) of the indicated sugar

	β-Galactosidase activity							
	MAL6 MIG1 strain				*MAL6 mig1Δ* strain			
Sugar added	–	Mal	Glu	Mal+Glu	–	Mal	Glu	Mal+Glu
Promoter sequence:								
MAL61-LacZ Orientation								
Wild-type	2	18	<1	<1	2	35	<1	1
ΔB	2	17	<1	<1	2	19	1	2
ΔD	1	30	<1	<1	2	40	1	2
ΔB, ΔD	<1	16	<1	<1	<1	24	1	2
MAL62-LacZ Orientation								
Wild-type	69	170	2	2	60	200	20	22
ΔB	50	169	2	3	48	212	20	27
ΔD	16	101	6	6	14	124	19	21
ΔB, ΔD	14	122	9	9	16	155	19	21

Table 5 Effect of deletion of the Mig1p-binding sites in the *MAL61-MAL62* promoter on *LacZ* reporter gene expression in a *MAL64-R10* constitutive strain. The *MAL61-LacZ* and *MAL62-LacZ* promoter deletion constructs described in Table 4 were introduced into strain R10u, a uracil-requiring spontaneous mutant isolated from strain R10 using 5-FOA selection. The cells were grown and β-galactosidase levels assayed as described in Table 4

	β-Galactosidase activity			
Sugar added	–	Maltose	Glucose	Mal+Glu
Promoter sequence:				
MAL61-LacZ Orientation				
Wild-type	110	40	8	8
ΔB	123	49	16	16
ΔD	189	53	13	15
ΔB, ΔD	90	34	21	21
MAL62-LacZ Orientation				
Wild-type	387	224	85	88
ΔB	369	229	105	108
ΔD	244	193	122	121
ΔB, ΔD	388	201	154	151

tion of site D. Loss of Mig1p had no effect on this uninduced expression. This suggests that an activator protein may also bind to site D (see Discussion).

Unfortunately, *LacZ* expression from the *MAL61* promoter is surprisingly low even under induced conditions and maltose induction of the *MAL61-LacZ* fusion is only about ten-fold (Table 4). In an effect to examine the effect of *MIG1* on *MAL61*, we decided to test the promoter deletions in the *MAL64-R10* constitutive strain. All eight *LacZ* fusion constructions were introduced into strain R10u which is isogenic to strain R10 described above except for a *ura3* mutation selected for the *mal63::URA3* disruption using 5-FOA. Expression of *MAL61-LacZ* is now adequate to allow us to evaluate the effects of deletions of sites B and D. As has been noted previously in constitutive strains, the uninduced levels of expression are higher than those under induced conditions (Table 3; Dubin et al. 1989). Table 5 shows a 14-fold glucose-repression of *MAL61-LacZ* expression for the wild-type promoter sequence. Deletion of site B relieves glucose repression of *MAL61-LacZ* ex-

Table 6 Effect of a *MIG1* disruption on *LacZ* reporter gene expression in a *MAL64-R10* constitutive strain. A *mig1Δ* null mutation was introduced into strain R10u as described in Table 4 above. Plasmids carrying the *MAL61-LacZ* and *MAL62-LacZ* fusions with the wild-type promoter sequence were introduced into strain R10u and the isogenic *mig1Δ* strain, and β-galactosidase activities determined as described in Table 4

	β-Galactosidase activity							
	MAL64-R10 MIG1 strain				*MAL64-R10 mig1Δ* strain			
Sugar added	–	Mal	Glu	Mal+Glu	–	Mal	Glu	Mal+Glu
Promoter orientation:								
MAL61-LacZ	110	40	8	8	97	109	48	41
MAL62-LacZ	387	224	85	88	372	413	212	198

pression two-fold. Deletion of site D slightly increases expression under all conditions but the 14-fold glucose-sensitivity remains. The double deletion results in a three-fold increase in glucose-repressed expression. Thus, glucose repression of *MAL61* is mediated by site B, and site D does not contribute significantly to the glucose repression of *MAL61*. The effects of glucose on the expression of the *MAL62-LacZ* fusions in the constitutive strain are consistent with those seen in the wild-type strain (Table 4).

The wild-type *LacZ* fusions were also introduced into a *mig1Δ* of strain R10u and *LacZ* expression determined (Table 6). Induced expression of both *MAL61* and *MAL62* was increased to levels comparable to the uninduced conditions. We found that glucose repression of *MAL61* and *MAL62* was relieved five-fold and 2.5-fold, respectively. An approximately two-fold glucose repression remains for both genes. These results are consistent with those reported in Table 3 where maltase levels were determined in a *MAL64-R10* constitutive strain and confirm that Mig1p is involved in the glucose repression of *MAL61* as well as *MAL62*.

Discussion

Mig1p is a glucose-responsive repressor of the *MAL* genes

The results described here allow us to define several components contributing to the glucose repression of maltose fermentation. Of these, Mig1p-dependent transcription repression is a significant factor regulating all three genes of the *MAL6* gene cluster. Mig1p-binding sites were identified in the shared, bidirectional promoter sequence of *MAL61* and *MAL62* at base pairs –273 to –288, called site B, and –578 to –592, called site D. The nucleotide numbers indicate the distance from the *MAL61* ORF. Site D begins 282 base pairs upstream of the *MAL62* ORF and is immediately adjacent to the UAS_{MAL}, a Mal63p-binding site (Ni and Needleman 1990; Levine et al. 1992; Yao et al. 1994). Our results indicate that site D (proximal to *MAL62*) contributes significantly to the glucose repression of *MAL62*, while site B (proximal to *MAL61*) is primarily responsible for the repression of *MAL61*. In the absence of the proximal binding site, the distal site also seems to have a modest effect on Mig1p-dependent repression as evidenced by the slight relief of glucose repression in the double-site deletions compared to the single deletion of the proximal sites, implying some degree of bidirectionality (Tables 4 and 5). Stronger evidence for the bidirectional function of Mig1p-binding sites is found in the *GAL* system. A single Mig1p-binding site is found in the shared, bidirectional *GAL1-GAL10* promoter, referred to as element A of the upstream repressing sequence, URS_G (Nehlin et al. 1991; Flick and Johnston 1992). The URS_G A and C elements are located between the UAS_G and the ATG codon of *GAL1* and appear to function independently of orientation to a heterologous promoter (Flick and Johnston 1992). No URS_G was found between the UAS_G and the ATG codon of *GAL10* (Flick and Johnston 1992) and, while *GAL10* expression is clearly sensitive to glucose repression, the role of the URS_G A element in Mig1p-dependent glucose repression of *GAL10* was not tested directly.

Deletion of site D reduced the *MAL62-LacZ* expression in uninduced and induced conditions, and this effect was independent of Mig1p. This result suggests that site D may also bind a transcriptional activator. Potential candidates are the products of the *MSN2* and/or *MSN4* genes which were isolated as multicopy suppressors of a *snf1-ts* mutation (Estruch and Carlson 1993). Msn2p and Msn4p are closely related zinc-finger proteins whose DNA-binding domains are somewhat similar to the Mig1p DNA-binding domain. However, Msn2p and Msn4p differ from Mig1p at position 18 of the second finger which determines DNA-binding specificity (Ronne 1995). This suggests that their binding specificity probably differs from that of Mig1p, but does not rule out the possibility that the three proteins bind to partially overlapping sites.

Dual level control of the *MAL* genes by Mig1p

A Mig1p-binding site was identified in the sequence –116 to –131 base pairs upstream of the *MAL63* ORF. Deletion of *MIG1* increases the expression of both *MAL63* mRNAs under repressed conditions but the effect on the smaller 1.6-kb transcript is greater (Fig. 1). It has not been determined if both transcripts are functional, but in studies of a strain carrying the *MAL1* locus and no others, only the 1.6-kb transcript was detected suggesting that the smaller *MAL63* transcript is functional, if not both (Charron et al. 1986). Thus, Mig1p regulates the transcription of the *MAL* structural genes not only directly but indirectly by regulating the transcription of the Mal-activator.

This dual level control of the *MAL6* genes by Mig1p is similar to its role in the repression of the *GAL* genes (Griggs and Johnston 1991; Nehlin et al. 1991), and also to the repression of the *alc* genes by CREA, the Mig1p homologue in *Aspergillus nidulans* (Mathieu and Felenbok 1994). It therefore seems to be a general finding that glucose repression acts both on specific activators and on their respective target genes. One cannot rule out that other genes such as *SUC2*, which are directly repressed by Mig1p, may also have specific activators whose expression is similarly repressed. The reason for this dual-level control is not clear. However, direct repression of the *GAL1* promoter is very fast, and it is therefore thought to be important during the early stages of repression (Johnston et al. 1994).

Glucose repression of *GAL4* is five-fold at the mRNA level, and is completely dependent on Mig1p (Nehlin et al. 1991). Our results suggest that repression of *MAL63* is of a similar magnitude (Fig. 1). However, the situation is complicated by the presence of two *MAL63* transcripts, and also by the fact that *MAL63*, unlike *GAL4*, is autoregulated. The system is therefore more similar to the *alc* genes in *Aspergillus*, where the specific activator gene *alcR* is reg-

ulated both by CREA and by its own gene product (Mathieu and Felenbok 1994). A Mig1p-independent glucose repression of *MAL63* is still seen in the presence of maltose (Fig. 1). Since *MAL63* is autoregulated, it seems likely that this repression simply reflects a glucose-dependent inhibition of the induction pathway.

Curiously, the Mig1p-binding site present in the *MAL63* promoter is not found at a comparable position upstream of *MAL23* and *MAL43-C* (Gibson and Michels, unpublished results). Approximately 200 bp upstream of both genes has been sequenced and the three promoters are 98% identical overall in this region. The sequence of the *MAL63* Mig1p-binding site differs by a single base pair from the comparable sites in the *MAL23* and *MAL43-C* promoters, respectively, which lack one G/C base pair in the critical GC-box. However, we cannot rule out that other Mig1p sites may exist further upstream in these promoters. Whether Mig1p-dependent regulation of *MAL63* transcription is common to all of the Mal-activator genes therefore remains to be established.

Down-regulation of the induction pathway contributes to glucose repression of the *MAL* genes

Throughout this investigation, *MAL* gene expression in glucose-repressed conditions was determined both with and without maltose present in the medium. Our results suggest that induction is inhibited 25-fold by glucose and that this inhibition is Mig1p-independent. Several possible mechanisms can be suggested, the most likely of which is inducer exclusion probably resulting from the glucose-induced inactivation of the maltose permease (reviewed in Johnston and Carlson 1992). As a result, intracellular levels of maltose would be inadequate for the induction of *MAL* gene expression. The *MAL64-R10* and *MAL43-C* Mal-activator mutant alleles are fully constitutive and have enabled us to examine the contribution of the inducer-dependent component of the glucose regulation of *MAL* gene expression in addition to the Mig1p-dependent component. In strains carrying these alleles, expression of the *MAL* genes shows less than ten-fold sensitivity to glucose repression as compared to the 700-fold repression seen in the isogenic inducible strain (Tables 2 and 3). Significantly, disruption of *MIG1* in the constitutive strains only relieves glucose repression of *MAL62* by 2–3-fold (Tables 3 and 6) and *MAL61* by five-fold (Table 6). These studies strongly support the proposal that the glucose-dependent inhibition of the induction signal is responsible in a large part for the Mig1p-independent glucose repression, but do not exclude the possibility that glucose affects the synthesis and/or function of the Mal-activator by a Mig1p-independent, induction-independent pathway.

Role of other factors in the regulation of the *MAL* genes

Based on the results of *MIG1* disruption in the inducible *MAL6* strain (Tables 2 and 4), it was expected that disruption of *MIG1* in the *MAL64-R10* constitutive strain would all but eliminate glucose-repression sensitivity. It did not (Tables 3 and 6). The persistence of a 2–6-fold glucose repression of either *MAL61-LacZ*, *MAL62-LacZ* or maltase expression in constitutive, *mig1Δ* strains indicates that other glucose-responsive regulatory factors are also involved. It has been shown that *MIG1* acts synergistically with *SSN2* through *SSN5*, *SSN7* and *SSN8* in mediating glucose repression of *SUC2* (Vallier and Carlson 1994). Conceivably, the latter genes could also be involved in glucose repression of the *MAL* genes.

In summary, we have uncovered several pathways involved in the glucose regulation of *MAL* gene expression as has been demonstrated for the *GAL* genes (Flick and Johnston 1990, 1992; Griggs and Johnston 1991; Nehlin et al. 1991; Lamphier and Ptashne 1992). One major pathway is the Mig1p-mediated repression of *MAL61*, *MAL62* and *MAL63* transcription. Another even more significant component of repression is seen only under induced/repressed conditions and most likely reflects the glucose regulation of inducer availiblity. These two components account for all but about 2–6-fold of the approximately 700-fold glucose-repression seen in the *MAL6* strain used here. The remaining repression is likely to result from the sum of several minor contributions at transcriptional and post-transcriptional levels.

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