Genomic Footprinting of Mig1p in the MAL62 Promoter

BINDING IS DEPENDENT UPON CARBON SOURCE AND COMPETITIVE WITH THE Mal63p ACTIVATOR*

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Mig1p inhibits gene expression in glucose by binding the Cyc8p (Ssn6p)-Tup1p repressor to the promoter of glucose-repressible genes. While the binding properties of Mig1p have been studied in vitro and the ability of Mig1p-Cyc8p (Ssn6p)-Tup1p to repress has been studied in vivo, no experiments have measured the effect of a carbon source on the in vivo binding of Mig1p or the effect of bound Mig1p on activator occupancy of the upstream activation sequence (UAS). To obtain this information, we used genomic footprinting to investigate glucose repression of MAL62, a gene that is also regulated by the Mal63p activator. These experiments show that two interrelated mechanisms are involved in the glucose repression of MAL62: 1) competition between the Mal63p activator and Mig1p for DNA binding and 2) modulation of Mig1p binding by the carbon source. Mig1p affects basal MAL62 expression in the absence of Mal63p by binding to a site in the MAL62 promoter and affects Mal63p-dependent synthesis by also inhibiting the access of Mal63p to site 1 in the UASMAL. The binding of Mig1p is increased in glucose and decreased in non-repressing sugars, but the increased binding in glucose is not due to an increase in the levels of Mig1p.

Glucose regulates carbon utilization in Saccharomyces cerevisiae by inhibiting the transcription of numerous genes, by affecting mRNA stability, and by inactivating preexisting proteins. The enzymes affected include those involved in gluconeogenesis, in the Krebs cycle, in respiration, and in the early steps in the utilization of galactose and other sugars (1). Together these mechanisms, collectively called glucose repression, function to partition carbon metabolism between glycolysis and gluconeogenesis and to ensure the utilization of glucose in preference to other carbon sources.

Although the mechanism of glucose repression is not understood, several of the genes involved have been identified. Central among these is MIG1, originally isolated as a multicopy inhibitor of GAL1 (2, 3). MIG1 encodes a Cys2His2 zinc finger protein that binds to several glucose-regulated promoters (e.g. GAL1–10, SUC2) and is in complexes with two other proteins, Tup1p and Cyc8p (Ssn6p), represses transcription during glucose growth. Deletion of MIG1 relieves glucose repression, and genetic experiments suggest that the ability of Mig1p to repress is regulated by a kinase encoded by SNF1 (4).

The genes required for maltose utilization are also subject to glucose repression. Maltose fermentation requires three proteins: the Mal63p activator, the Mal61p permease, and the Mal62p maltase. Maltose induces all three proteins; glucose inactivates maltose permease and represses transcription of MAL63, MAL61, and MAL62 (5–7). We have been studying the role of Mig1p in the MAL system and have recently shown that the deletion of a Mig1p binding site in the promoter of a constitutive allele of MAL63 (MAL63–D8) relieves glucose repression (8). This promoter site overlaps the transcriptional start site of MAL63 (7), implying that its removal may lead to an increased synthesis of Mal63p–D8p. If so, it remains to be explained how an increase in the synthesis of Mal63p can release glucose repression.

A clue is provided by the arrangement of binding sites for Mal63p and Mig1p in the MAL61-MAL62 divergent promoter. This promoter contains two Mig1p binding sites, with one, which we call site A, adjacent to the MAL63p binding site, site 1 (Fig. 1; Refs. 1 and 9). A simple explanation for the effect of increased Mal63p concentrations on glucose repression would be that the binding of Mig1p to site A decreases Mal63–D8p binding to site 1, reducing MAL62 expression. Increases in the synthesis of Mal63–D8p (e.g. by the removal of a Mig1p site in the MAL63 promoter) would lead to increased site 1 occupancy and to increased MAL62 expression in glucose. To explain derepression of MAL62 expression on sugars such as galactose, it is necessary to have a mechanism in which the binding to site A and/or the activity of the Mig1p-Cyc8p (Ssn6p)-Tup1p complex is reduced. In its simplest form, a satisfactory model would be that the affinity of Mig1p for site A is modulated by “upstream” proteins that respond to an unknown glucose repression signal; proteins like Snf1p, for example, could function by decreasing Mig1p binding.

However, there is no direct evidence for binding competition between Mig1p and Mal63p, and similar configurations of activator and repressor sites exist in yeast where mechanisms other than competitive binding are involved (10). Similarly, no information is available on the possible carbon source modulation of Mig1p binding in vivo.

In this report we present experiments that measure the binding of Mal63p and Mig1p in vivo to their sites in the MAL62 promoter and correlate this binding with MAL62 expression. The results support a model in which Mig1p affects basal MAL62 expression in the absence of Mal63p by binding to site A and affects Mal63p-dependent synthesis by also inhibiting the access of Mal63p to site 1 in the MAL62 promoter. Competition between Mig1p and Mal63p requires a functional site 1, showing that it is a direct competition for DNA binding.
and not an indirect mechanism like squechuling. Most importantly, the binding ability of Mig1p-Cys8p (Sn6p)-Tup1p is increased in glucose and decreased in nonrespiring sugars, indicating that regulation of Mig1p binding is involved in the glucose repression of MAL62 and probably other yeast genes.

**EXPERIMENTAL PROCEDURES**

**Strains**—The strains used and the full genotypes are shown in Table I. The wild type *S. cerevisiae* strain was 332-5B (MAL6 MAL12). Strain 332-F is isogenic to 332-5B except for the introduction of a mal63 frameshift mutation. This was constructed by first replacing by homologous recombination approximately 0.4 kb of *MAL63* (from the AesII site in the promoter at 278 base pair to position +130 base pair in the coding sequence) with *URA3* (11); the resulting strain, strain 332-Δ52, was a gift from Man Chang. Using primer TGGAGAAGAGAGTGCA-CAATCCATTAG, a 130 base pair fragment containing the *UAS* was inserted into pS1 (15); pJW30 contained the same *Sal*I-fragment of *MAL63*–D8 in the same vector. The single copy plasmids pJW4 and pJW5 containing *MAL63* or *MAL63*+, respectively, were described previously (8). Plasmid pJW14 is a derivative of pJW1 and contains a 1.5 kb XbaI fragment from the *mal63* coding region and more than 1.5 kb of the *MAL62* promoter; it was used to generate the *MAL63* antisense RNA used in the RNA protection assay. Plasmid pJW49 was constructed by inserting an EcoRI-SpeI fragment of *MIG1* into pS1.

Southern Transfer Analysis, PCR, Sequencing, and in Vitro Footprinting—The methods used were described previously (8, 9).

**DNA Protection Assay—**Rats for in vitro transcription and RNA protection (Ambion) were used for both antisense RNA preparation and for the RNA protection assay. A *Pst*I-*Bgl*II fragment of pOX1 was used as the template for the generation of *MAL62* antisense RNA, and a *Xba*I-*Taq*I fragment cloned into pS1 (pS1-45) was used to generate *MAL63* antisense RNA. The *Mlu*I-SpeI fragment from plasmid pJW49 was used as a template for *MIG1* antisense RNA. The actin control template is an EcoRI-BamHI fragment from the plasmid pPL9 (a gift from Man Chang). Direct counting of gels in an Ambis radiation analysis system was used to quantify mRNA levels.

**DNA Preparation for Genomic Footprinting Assay—**The DNA preparation for genomic footprinting was essentially the same as that used in Diffley et al. (18). After the cells were grown to log phase, they were centrifuged at 4°C and resuspended in 4–5 volumes (v/v) of lysis buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM phenylmethylsulfonyl fluoride, 5 mM 2-mercaptoethanol, 2 µg/ml leupeptin, and 1 µg/ml pepstatin A. Lysis was by vortexing with an equal volume of 5-mM fluorescein isothiocyanate (FITC)-labeled oligonucleotide probes of 33- to 50-mer length, each localized to a specific promoter region. The probes were labeled with FITC at the 5’ end and then hybridized to yeast DNA. The DNA was then precipitated with an equal volume of cold ethanol. The DNA pellet was dissolved in 30 mM sodium phosphate buffer (pH 7.2) and used for hybridization.

**Maltose to Glucose Shift Experiment—**Cells grown to log phase on YEPM, centrifuged, washed, and transferred to YPD medium containing 100 µg/ml cycloheximide. Footprinting was done after incubation for 30 minutes at 30°C.

**TABLE I Yeast strains**

| Strains | Genotypes |
|---------|-----------|
| 332-5B  | MAL6 MAL612 MIG1 ura3-52 leu2-3, 112 trp1 his1 |
| A9      | Isogenic to 332-5B except for a deletion-insertion that replaced part of the MAL62 coding sequence |
| 332-F   | Isogenic to 332-5B except mal63 (frameshift) |
| 332 ΔMIG1 | Isogenic to 332-5B except MIG1:URA3 |
| 332-F ΔMIG1 | Isogenic to 332-F except MIG1:URA3 |
| 332-J1  | Isogenic to 332-5B except MAL62:URA3 site 1 mutation |
| 332 ΔTUP1 | Isogenic to 332-5B except TUP1:URA3 |
| 332 ΔMIG1 ΔTUP1 | Isogenic to 332-5B except TUP1:URA3 |
| 332 ΔCYC8 | Isogenic to 332-5B except CYC8:URA3 |
| 332 ΔMIG1 ΔCYC8 | Isogenic to 332-5B except SSNS6 except MIG1:URA3 |

**Medium and Growth Conditions**—Yeast was grown in YPD medium (0.7% yeast nitrogen base, the required nutritional supplements, and a carbon source); better footprints were obtained on this medium. Minimal medium was SD (minimal plus glucose) and SM (minimal plus maltose). Complex media was YEP (1% yeast extract, 2% peptone). Carbon sources were used at 2% for glucose (YPD, SD) and maltose (YPM, SM). YEPGE contained 3% glycerol and 2% ethanol. The PNPase assay for maltose was described previously (19).

**Genomic Footprinting—**The wild type strain 332-5B contains two identical copies of the MAL62 promoter corresponding to MAL62 and MAL12; both are under the control of Mal63p. The genomic footprinting experiments therefore measure the occupancy of both promoters simulta-
taneously. In experiments accessing the effect of a site 1 mutation and the competition between Mig1p and Mal63p for binding, it is necessary to either introduce the site 1 mutation into both the MAL62 and MAL12 promoters or to introduce it into one of the promoters and to delete the other. We chose to introduce it into the MAL62 promoter and to delete the promoter of MAL12. The primer was CTTCTCTCTGGTATACA, and it is located at positions −480 to −460 of the MAL61–62 promoter (Fig. 1). It was labeled by T4 kinase (Life Technologies, Inc.) using [γ-32P]ATP (ICN) according to the supplier’s instructions. One-twentieth volume of 3 M sodium acetate (pH 5.2) was added, and the DNA was precipitated with 5 volumes of cold ethanol. The mixture was kept at −20°C for 30 min, centrifuged for 15 min at 10,000 × g, washed with 75% ethanol, and air-dried. The labeled primer was then dissolved in 5–10 μl of primer buffer (10 mM NaOH, 1 mM EDTA), and 3% volume of stop buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol FF) was added before the primer was gel-purified on a 15% denaturing polyacrylamide gel.

The procedures for genomic footprinting and primer extension were as described previously (17, 18). Briefly, Taq DNA polymerase was used for primer extension, with each primer extension reaction comprising 10–20 μg of DNA, 2 units of Taq DNA polymerase (Cetus), 5 μl of 10 × Taq polymerase buffer (Life Technologies, Inc.), 10 μl of 50 mM MgCl2, 5 μl of a nucleotide stock solution (1 mM dATP, 1 mM dTTP, 1 mM dCTP, and 1 mM dGTP), and 1–2 μl of 32P-labeled primer (specific activity of 3000–6000 Ci/mmol), and H2O to 50 μl. For genomic footprinting, we used 10 μg of chromosomal DNA; for genomic sequencing we used 2–5 μg of naked DNA. For the sequence standards, we used either 5 μl of a dideoxy-GTP stock (1 mM dATP, dCTP, dTTP, 100 μM dGTP, 500 μM dideoxy-GTP) or 5 μl of a dideoxy-ATP stock (1 mM dCTP, dGTP, dTTP, 25 μM dATP, 1 mM dideoxy-ATP). It was sometimes necessary to adjust the relative amounts of dGTP and dideoxy-GTP or dATP and dideoxy-ATP. PCR was carried out for 15 rounds of primer extension; the cycle was as follows: 94°C for 1 min, 95–55°C for 1.5 min, 55°C for 3 min, 55–72°C for 1.5 min, 72°C for 4 min, and 72–94°C for 1.5 min.

The product of the primer extension was precipitated by 3 volumes of ethanol, frozen at −20°C for 15 min, centrifuged for 15 min at 12,000 × g, and the pellet was washed with 75% ethanol and dried. The pellet was dissolved in 5 μl of TE buffer and 3 μl of sequencing stop buffer. It was heated at 85–100°C for 2 min and loaded onto a sequencing gel.

**RESULTS**

The elimination of a Mig1p site in the promoter (site C) of the constitutive MAL63–D8 allele leads to a substantial decrease in glucose repression (8). We therefore investigated whether the deletion of site C led to increases in Mal63p expression and, if so, whether the postulated increases could relieve glucose repression.

**Elimination of a Mig1p Binding Site in the MAL63 Promoter Leads to Increased Synthesis of Mal63p mRNA**—Since we were unable to obtain antibody of sufficient quality for determining the levels of Mal63p in vivo, we instead measured MAL63 mRNA during glucose growth in strain A9 (MAL63:URA3) transformed with one of three single copy plasmids: a MAL63 plasmid, a MAL63–D8 plasmid with a wild type site C, and a MAL63–D8 plasmid with a site C mutated to a sequence unable to bind Mig1p (Fig. 2; Refs. 19 and 20). Removal of site C acted as a promoter-up mutation, with a 2-fold increase in MAL63 mRNA. This strongly suggests that an increase in the concentration of Mal63p is responsible for the reduction in glucose repression in strain MAL63–D8 seen after mutation of site C (8).

**Increased Mal63p Expression Relieves Glucose Repression Conferred by Mig1p**—To directly test if increased Mal63p expression relieves glucose repression in strains containing Mig1, we manipulated the plasmid copy number of the MAL63 and MAL63. If the model is correct, then increasing the amount of activated Mal63p by introducing multiple-copy MAL63 plasmids should also relieve glucose repression.

We first tested the effect of Mig1p on glucose repression in our standard strain, 332-5B. Table II shows that deletion of Mig1p in the wild type MAL6 strain leads to an approximately 15-fold increase in maltase under glucose-repressed conditions.
with the final levels equal to those observed in wild type strains after derepressed growth in galactose (i.e. 20–60 units). Therefore, in the absence of Mig1p there is no glucose repression.

Maltase levels were then measured under repressed, nonrepressed, and induced conditions in strains having different copy number plasmids carrying MAL63, MAL63*, and MIG1 (Table II). Increases in the gene dosage of MAL63 and MAL63* relieve glucose repression as follows. 1) While strains ΔMAL63 (containing plasmid MAL63, CEN) and MAL63 have the same repressed level of maltase (repressed roughly 15-fold), increasing the gene dosage of MAL63 largely eliminates glucose repression in strain ΔMAL63 containing plasmid MAL63, 2μ, with the levels in glucose essentially the same as those in strains deleted for MIG1. 2) If Mal63p is in its activated, constitutive form, Mal63p, it not only relieves glucose repression mediated by the Mig1p, but also activates the expression of MAL62 in glucose media (e.g. compare strain ΔMAL63 containing plasmid MAL63, 2μ with strain ΔMAL63 containing plasmid MAL63*, 2μ). 3) The degree of MAL62 glucose repression depends upon the concentration of both wild type activator and constitutive. Increased MAL63 and MAL63* gene dosage (and, therefore, increases in the levels of Mal63p) relieve glucose repression.

An Increase in Mig1p Concentration Causes Glucose Repression by a Pathway Independent of Its Effect on Mal63p Synthesis—Table II shows that a MIG1 ΔMAL63 strain is glucose-repressed and that this repression is totally absent in a ΔMIG1 ΔMAL63 strain. One constitutive mAL63* strain, strain 1403-7A, is relatively insensitive to glucose repression and lacks the equivalent of site C in the MAL63* promoter. Introduction of a high copy MIG1 plasmid into this strain severely inhibits the synthesis of maltase during glucose growth; PNPGase is 378 nmol of PNPG/min/mg of protein for cells having no plasmid, and 34 nmol of PNPG/min/mg of protein for cells with the high copy pMIG1 plasmid. Mig1p must therefore confer glucose repression by another pathway independent of its effect on Mal63p synthesis. Since high levels of Mal63p can totally overcome glucose repression in MIG1 strains, this pathway must also be antagonized by increases in Mal63p. Maltase levels in glucose-grown cells are therefore likely to be set by the relative amounts of Mig1p and Mal63p.

**Competition Binding Model for Glucose Repression of MAL62**—Since the Mig1p binding site, site A, is adjacent to the Mal63p binding site, site 1 (Fig. 1; Refs. 8, 9, 19, and 20), one possible explanation for the dependence of MAL62 expression of glucose-grown cells on the relative levels of Mal63p and Mig1p is that Mal63p and Mig1p compete for binding in the MAL62 promoter. High levels of Mig1p would repress by inhibiting Mal63p access to the UASMAL; raising the levels of Mal63p would overcome the inhibition. If binding competition between Mig1p and Mal63p is indeed partially responsible for the relief of glucose repression in a MIG1 (containing plasmid MAL63, 2μ) strain, increased expression of Mal63p in a ΔMIG1 strain should not affect maltase levels in glucose medium; i.e. we expect that maltase levels in a ΔMIG1 and a ΔMIG1 (containing plasmid MAL63, 2μ) strain would be equal. Within experimental error, this is precisely what we observe (Table II).

MAL62 Expression Is Transcriptionally Controlled—The competition binding model requires that maltase levels measured in the gene dosage experiments (Table II) reflect changes in the transcription of MAL62. We therefore used RNA protection assays to measure MAL62 mRNA isolated from some of these strains. The results of some of these assays are presented in Fig. 3. They confirm that the enzyme activity shown in Table II accurately reflects altered transcription.

**Mig1p Binds in Vivo to a Site Adjacent to the Mal63p Binding Site 1 in the MAL62 Promoter**—We next sought direct evidence for the modulations of transcription in glucose by competition for promoter binding between Mal63p and Mig1p. In particular, it was necessary to identify the Mig1p and Mal63p binding sites in the MAL62 promoter in vivo. We used in vivo genomic footprinting to determine if Mig1p binds to the consensus site previously identified in vitro, site A (Fig. 1; Refs. 19 and 20). Fig. 4a shows that Mig1p does bind to site A in glucose-grown cells. Genomic footprinting for glucose grown MAL63 MIG1 (332-5B) and mal63 MIG1 (strain 332-F) cells shows a protected region in the MAL62 promoter identical to

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**Table II**

*Maltase activity is dependent on the gene dosage of the MIG1 and MAL63 alleles*

| Genomic genotypesa | Plasmid genotypes | Maltase activity |
|--------------------|------------------|-----------------|
|                    |                  | Glucose | Galactose | Maltose  |
|                    |                  | nmol PNPG/min/mg protein |
| WT                 |                  | <2     | 28       | 1200    |
| Δ MAL63            |                  | <1     | 5        | 12b     |
| Δ MAL63            | MAL63, CEN       | 4      | 24       | 1784    |
| Δ MAL63            | MAL63, CEN       | 35     | 1125     | 1228    |
| Δ MAL63            | MAL63, 2μ        | 22     | 59       | 2051    |
| Δ MAL63            | MAL63*, 2μ       | 417    | 1125     | 2260    |
| Δ MIG1             |                  | 30     | 26       | 1880    |
| Δ MIG1 Δ MAL63     |                  | 22     | 25       | 20c     |
| Δ MIG1 Δ MAL63     | MAL63, CEN       | 23     | 32       | 1268    |
| Δ MIG1 Δ MAL63     | MAL63*, CEN      | 811    | 1118     | 3079    |
| Δ MIG1 Δ MAL63     | MAL63, 2μ        | 41     | 32       | 2268    |
| Δ MIG1 Δ MAL63     | MAL63*, 2μ       | 1717   | 4000     | 4880    |
| site1              |                  | <1     | 7        | 118a    |
| site1              | MAL63, 2μ        | <1     | 6        | 220b    |

a The strains are WT (MAL63 MIG1), 332-5B; Δ MAL63, 332-F; Δ MIG1, 332 ΔMIG1; ΔMIG1 Δ MAL63, 332-F ΔMIG1; site1 332-J1.

b Cells were grown on the glycerol/ethanol + maltose media.
that seen in vitro for Mig1p footprinting at the SUC2, GAL4, and GAL1–10 promoters. In addition, both in vivo and in vitro footprints show a hypersensitive site at the 3′-end of the GC box (Fig. 4A, lanes 5 and 6; Refs. 2 and 3). To avoid the possible interference from the binding of Mal63p to site 1, we used the strain 332-F, which, unlike the A9 deletion strain, has an early mal63 frameshift mutation (Fig. 4A, lane 6); this mutation eliminates the region of Mal63p required for DNA binding (see “Experimental Procedures”; Table I). Inspection of the same region in the otherwise isogenic control strain, ΔMIG1 (MAL63ΔMIG1; Fig. 4A, lane 4), clearly shows that the footprint is due to Mig1p (Fig. 4A, lane 5). The Mig1p footprint is most pronounced in strains lacking Mal63p, but it is still apparent when MAL63 is present. In a separate experiment, we performed genomic footprinting in a glucose-grown Mal63p strain containing a multicopy MIG1 plasmid (pMIG1, Fig. 4B). Again the same protected region and hypersensitive band seen in vitro are present in vivo, as expected, increased MIG1 copy number gives increased protection. This increased binding is consistent with the reduction in maltase levels seen in glucose-grown cells in the presence of pMIG1 (data not shown).

At high concentrations of Mig1p or in glucose-grown cells lacking Mal63p, a new footprint was observed, with the protected sequence, CTGAACTCCCGTG (bottom strand), overlapping site 3 of the UAS_MAL (Fig. 1, site X, and Fig. 4). This site resembles the consensus Mig1p binding site but has not been shown to bind Mig1p in vitro (2, 3).

Mal63p Binds to Sites 1, 2, and 3 in Vivo—To obtain the clearest genomic footprint of Mal63p without any possible interference of Mig1p, we performed genomic footprinting in a MIG1:URA3 strain grown in maltose and containing a multicopy MAL63 plasmid. Figure 5 shows that all three binding sites identified previously in vitro are protected in vivo (Fig. 5, lane 7; Refs. 9 and 21). In the absence of the multicopy MAL63 plasmid, binding to site 1 is still apparent, but the occupancy of the other sites is lower (Fig. 5, lane 6). Once again the enhanced band characteristic of Mal63p binding in vitro is clearly seen (Fig. 1; Refs. 9 and 21).

When strain 332 ΔMIG1, a strain isogenic to wild type 332-5B except for a deletion-disruption of MIG1 (Table I), is grown on maltose, another new footprint was found at −245 to −230 (Fig. 5, lanes 6 and 7); the protected sequence is AAAAAAAGAAAAAG (Fig. 1, T-rich region, bottom strand).

Mal63p and Mig1p Do Not Bind Independently—Having defined the footprints of Mal63p and Mig1p each in the absence of the other, we then measured the occupancy of sites 1 and A when the concentration of both proteins was varied. By changing the gene dosage, and therefore the protein levels, it was possible to test the independence of Mal63p and Mig1p binding to each site in glucose.

High copy number MAL63 plasmids reduce the binding of Mig1p to site A in glucose (Fig. 6, lane 6). As a control, a multicopy plasmid containing only the Mig1p binding sites in the MAL63 promoter was introduced into wild type cells. It fails to reduce Mig1p binding to site A (Fig. 6, lane 7), ensuring that titration of Mig1p by plasmid binding sites present in the MAL63 promoter is not the basis for the reduction in the occupancy of site A.

Direct Competition for Binding and Not Squelching Is Involved in the Mig1p Inhibition of MAL62 Expression—The inhibition of Mig1p binding at site A by high copy number MAL63 plasmids could occur either through competition for binding sites or through direct Mig1p/Mal63p protein interaction that prevents Mig1p binding (i.e., “squelching”).

To determine which mechanism was operative, we mutated site 1 at two bases that were previously shown by in vitro methylation protection to be part of the consensus Mal63p binding site (9) while the Mig1p binding site A was left intact. Fig. 7 shows in vitro DNase I footprinting of the mutant and wild type site 1 using Mal63p activator from the heparin-
agarose purification step described elsewhere (9). The mutated site 1 does not bind Mal63p
in vitro even at high concentrations of Mal63p.

We then asked if overproduction of Mal63p could still inhibit Mig1p binding but, in vivo, if site 1 could not be occupied by Mal63p. Strain 332-J1 has a site 1 mutation in the MAL62 promoter. Strain 332-J1 was transformed with a multiple-copy plasmid carrying MAL63. If squelching occurs, overproduction of Mal63p should have the same effect on Mig1p binding in strains with the wild-type site 1 as in strains with the mutated site 1. Fig. 8 shows that this is not the case; despite increases in the concentration of Mal63p, site A remains fully occupied only in the strain having the mutated site 1. Therefore, the competition between Mal63p and Mig1p for DNA binding occurs only between bound Mal63p and Mig1p.

Site 1 Is Critical for MAL62 Expression—For the model above to be biologically relevant, site 1 must be important for the control of MAL62 expression. Maltase was assayed in strain 332-J1 (mutated site 1) after growth in glycerol/ethanol plus maltose, and the maltase (PNPGase) levels were 10% of the levels obtained in similarly induced wild-type cells (Table II). Since 332-J1 is ΔMAL12 (see “Experimental Procedures”), the normalized value is 20% of wild type (i.e. 332-5B). Binding of Mal63p to site 1 is therefore critical for normal MAL62 induction. In addition, glucose repression in 332-J1 (mutated site 1) was unaffected by expression of Mal63p from a multi-copy plasmid (Table II).

Fig. 6. Mal63p competes with Mig1p for binding in vivo. The genotypes are indicated as in Fig. 4, except that in the 2Δ lane the cells were transformed with a multiple-copy plasmid containing the indicated gene or site C alone. The growth conditions and the first three lanes are the same as in Fig. 4A.

Fig. 7. A mutated site 1 does not bind Mal63p in vitro. In vitro footprinting was done using heparin-purified Mal63p (30). WT indicates that the wild type MAL61–62 promoter was the template used for the footprinting assay, and mutant site 1 indicates the use of a MAL61–62 promoter, which contains a 2-base pair change in site 1 (see “Experimental Procedures”). The numbers at the top indicate the amount in ng of Mal63p used; 0 indicates that no protein was added.

Mig1p Occupancy of Site A Is Highest in Glucose—Having established that the binding of the Mal63p and Mig1p is not independent and that this competition is a major determinant in setting the level of expression of MAL62, we asked if the binding of Mig1p and Mal63p was affected by carbon source. In ΔMIG1 strains, Mal63p occupancy of site 1 is greater in maltose medium than in glucose medium (compare Fig. 5, lane 6,
Genomic Footprinting of Mig1p

Fig. 8. Competition for binding between Mal63p and Mig1p in vivo requires the presence of site 1. The cells were grown on glucose medium, and the genotypes and the symbols used are the same as in Fig. 6.

Fig. 9. Mig1p binding to site A depends upon carbon source. The strain used was 332-F, which contains an early mal63 frameshift mutation (see “Experimental Procedures”). Lanes 1, 2, and 3 and the symbols used are the same as in Fig. 4A. The carbon source used for growth is also indicated. GEM, glycerol/ethanol plus maltose.

with Fig. 4B, lane 4). This is the expected result, since the expression of the MAL63 is repressed by glucose and induced by maltose (7, 20).

To avoid interference by Mal63p, Mig1p binding was assayed in strain 332-F, which contains an early frameshift mutation of mal63. Fig. 9 shows the extent of Mig1p occupancy of site A when the cells were grown on 1) glycerol/ethanol plus maltose, 2) galactose, and 3) glucose. Growth in glucose gives a strong footprint and a clear hypersensitive band, growth in galactose a clear footprint but a reduced hypersensitive band, and growth in glycerol/ethanol plus maltose no footprint and no hypersensitive band. On longer exposure the lesser intensity of the hypersensitive band seen after growth on galactose as compared with glucose is more apparent (data not shown). The occupancy of site A by Mig1p is therefore modulated by the carbon source and is highest during growth on glucose.

Glucose Repression Is Not Modulated by Changes in Mig1p Levels—An increase in the synthesis of Mig1p in glucose could account for its increased occupancy of site A. Alternatively, the “glucose signal” that leads to increased Mig1p binding could be transduced by covalent modification of Mig1p, by changes in chromatin accessibility, or by other mechanisms independent of the steady state levels of Mig1p.

The observation that the levels of Mig1p mRNA are not affected by carbon source (data not shown) indicates that a mechanism in which Mig1p increases during glucose growth is unlikely; however, we have not measured messenger stability or translational efficiency. Since we lacked an antibody of sufficient quality to measure Mig1p directly, we used instead an indirect method in which Mig1p binding to site A was measured after a shift from induced conditions where binding is weak, to repressed conditions where binding is strong. The shift was done under conditions where new protein synthesis was prevented by high concentrations of cycloheximide (22). Cells were grown in maltose, washed, and resuspended in glucose plus 100 μg/ml cycloheximide. Despite a rapid and total inhibition of protein synthesis under these conditions (22), Mig1p binding at site A, which is undetectable in maltose grown cells, was restored within 30 min after the shift. The footprinting of the shifted cells is as clear as that seen in glucose-grown cells (Fig. 10). This experiment, coupled with the observation of the rapid establishment of Mig1p repression (23), demonstrates that carbon source-dependent changes in Mig1p levels are not involved in the increased binding of Mig1p to the MAL62 promoter in glucose. This increase in its “effective binding affinity” must be due to other factors: intrinsic changes in Mig1p binding affinity through covalent modification, chromatin accessibility changes, or alterations in other proteins that stabilize binding.

TUP1 and CYC8-SSN6 Have a Mig1-dependent and a Mig1-independent Mechanism in the Regulation of the MAL62 Expression—For convenience, we have used the term “Mig1p binding” with the understanding that for in vivo experiments it refers to the binding of the Mig1p-Cyc8p (Sn6p)-Tup1p protein complex (24). While the roles of Tup1p and Cyc8p in the Mig1p-Cyc8p (Sn6p)-Tup1p complex are not completely understood, it is possible that the competition between Mig1p and Mal63p does not require Cyc8p (Sn6p) or Tup1p. To see if Mig1p repression of the MAL62 promoter was mediated by Tup1p and Cyc8p (Sn6p), we measured maltase levels in cells lacking different subunits of the Mig1p-Cyc8p (Sn6p)-Tup1p complex.

The results in Table III show that Tup1p and Cyc8p (Sn6p) have effects on glucose repression that are independent of Mig1p. In both MAL63 ΔMIG1 and MAL63ΔΔMIG1 strains, maltase levels are essentially the same on glucose and galactose (Table II, 23 versus 32 nmol of PNPG/min/mg; 811 versus 1118 nmol of PNPG/min/mg), showing that deletion of MIG1 completely removes glucose repression. However, maltase levels after glucose growth are considerably higher in a ΔM1ΔΔTUP1 and ΔMIG1Δ CYC8 (SSN6) strains than in a ΔMIG1ΔΔTUP1 or ΔMIG1Δ CYC8 (SSN6) strains, indicating that Tup1p has a repressive effect even in the absence of Mig1p.

DISCUSSION

In this report we explore the role of Mig1p-Cyc8p (Sn6p)-Tup1p in the glucose repression of MAL62. Cyc8p (Sn6p)-Tup1p is a general repressor of yeast genes including those involved in fermentation (MAL, GAL, and SUC; Refs. 1, 4, 20, 23, and 25), anaerobic growth (26, 27), and mating type (24, 28). In each case, Cyc8p (Sn6p)-Tup1p complexes with a DNA binding protein that conveys the appropriate specificity (Mig1p
Genomic Footprinting of Mig1p

The results presented here show that at least two interrelated mechanisms are involved in the glucose repression of MAL62: modification of Mig1p binding ability by carbon source and competition between Mal63p and Mig1p for DNA binding. We propose that glucose repression of MAL62 occurs in the following way.

In the absence of Mal63p, Mig1p-Cyc8p (Ssn6p)-Tup1p inhibits by an unknown mechanism the basal expression of MAL62. In the presence of Mal63p and maltose, activated Mal63p is bound to sites 1, 2, and 3, which form the UAS, and less Mig1p-Cyc8p (Ssn6p)-Tup1p is bound to site A, allowing increased MAL62 transcription. During induction, activated Mal63p (Mal63p) synthesis is high due to both autoregulation of MAL63 (7) and to a decreased Mig1p occupancy of site C in the MAL63 promoter. The latter is ascribed to the decreased binding of Mig1p in nonrepressing carbon sources. The addition of glucose to maltose-grown cells decreases maltose uptake by decreasing the expression of the maltose permease and by inactivating preexisting permease. This reduction of maltose availability prevents the conversion of the Mal63p to its activated form and ensures that glucose is used in preference to maltose when both sugars are present. The addition of glucose also increases the Mig1p-Cyc8p (Ssn6p)-Tup1p occupancy of sites C and A, decreasing Mal63p levels and also decreasing the amount of Mal63p to site 1. However, some Mal63p remains bound to site 1 even in glucose. In nonrepressing sugars, less Mig1p-Cyc8p (Ssn6p)-Tup1p is bound to sites A and C, and consequently, MAL62 expression is higher than on glucose.

Mig1p Binding to Site A Is Dependent upon Carbon Source—To explore the carbon source dependence of Mig1p binding we measured the occupancy of site A (Mig1p) and site 1 (Mal63p) in vivo. Previous information on the binding sites for Mal63p and Mig1p had been obtained exclusively from in vitro experiments on naked DNA templates (7, 9). To determine these sites in chromatin we used genomic footprinting to identify the sequences protected by Mal63p and Mig1p; to avoid interference between the proteins through either solution binding or competition on the template, the binding of each protein was determined in cells lacking the other. The sensitivity of the genomic footprinting was aided by the appearance of enhanced bands indicating the binding of Mig1p and Mal63p; for both regulators, both the sequences protected, and the positions of the enhanced bands were the same as those observed in vitro.

Genomic footprinting clearly shows that the affinity of Mig1p for site A in the MAL62 promoter depends upon the carbon source (Fig. 9). Mig1p binding to site A was highest during glucose growth and reduced on nonrepressing carbon sources like galactose. The maltose to glucose shift experiments show that the increase in Mig1p binding to site A is due to changes in the concentration of Mig1p.

The relationship between Mig1p binding and glucose repression was recently investigated by Treitel and Carlson (29) using artificial promoters containing a LexA binding site and a protein fusion between the LexA DNA binding domain and Mig1p. In a construct containing a LexA binding site 5’ to a UAS, repression by LexA-Mig1p was dependent upon carbon source, with the cells showing a 10-fold (low glucose) to 50-fold (galactose) derepression. Since it is unlikely that LexA binding is dependent upon carbon source, the LexA-MIG1 fusion protein should also be bound in galactose media. Nevertheless, no repression is observed in galactose, suggesting, in contrast to the results presented here, that transcriptional inhibition occurs only in the presence of glucose and that Mig1p binding is not sufficient for repression.

However, this system did not quite mimic the in vivo situation for the genes involved in sucrose, galactose, or maltose utilization; a tup1Δ strain showed no release of glucose repression, and when the LexA binding sites were 3’ to the UAS, no repression was seen (24, 29). In addition, as recognized by the authors, there was no quantitation of the binding of LexA-Mig1p, so that a carbon source-dependent binding of the fusion protein remains a possibility. They also showed that LexA-Mig1p is phosphorylated to different extents in repressing and nonrepressing media, suggesting that phosphorylation or other modifications could affect DNA binding of wild type Mig1p-Cyc8p (Ssn6p)-Tup1p.

The behavior of Mig1p reported here is conceptually similar.
to that observed for oxygen regulation, where repression is controlled by changing the concentration of Rob1p in response to oxygen levels (26); Mig1p occupancy of site A is increased in glucose-grown cells and decreased in cells grown on nonrepresing carbon sources. The reason for this increase in the “effective affinity” of Mig1p remains elusive, but in addition to phosphorylation it may involve regulation of Mig1p transport to the nucleus or glucose-induced modifications of chromatin structure.

**Competition between Mig1p and Mal63p**—We have previously shown that glucose repression is alleviated by a mutation in a Mig1p binding site present in the MAL63 promoter (site C; Ref. 8). Fig. 5 shows that this mutation increases the concentration of MAL63 mRNA and presumably Mal63p. This result supports the competition model.

The competition model requires that Mal63p and Mig1p compete for DNA binding, and in this model the signal affecting glucose repression can be transduced by decreasing the binding affinity of Mal63p, increasing the binding ability of Mig1p, or both. Changes in binding ability can be affected by changing the intracellular or nuclear concentration of Mal63p and Mig1p, or as indicated above, by modifications of these or other proteins. We believe that the glucose signal is transmitted through changing the “effective affinity” of Mig1p for its binding sites, and since the occupancy of site A is increases during a shift from inducing conditions to glucose even in the absence of protein synthesis (Fig. 10), changes in the level of Mig1p are not involved. This model is also attractive because it provides a simple explanation for the effect of the site C mutation on glucose repression (8).

For the proposed competition model to be of biological significance, the binding of Mal63p to site 1 must play a major role in the expression of MAL62. To test the importance of site 1 in the expression of MAL62, we measured maltase in a strain containing both a mutant site 1 and a deletion of the MAL12 promoter. This ensured that Mal63p could not bind to site 1 and that the maltase measured was due exclusively to MAL62 expression. Maltase levels were significantly lower than wild type for maltose-grown cells, demonstrating that site 1 is required for maltase induction. Maltase levels are also significantly decreased in the site 1 mutant when it is grown on glucose (Table II). The inhibition of Mal63p binding to site 1 by Mig1p is therefore consistent with the proposed mechanism for glucose repression of MAL62.

More direct evidence was then sought for competition for binding between Mig1p and Mal63p for site A and site 1, respectively. Figs. 4B, 5, and 6 show that increased concentrations of Mig1p inhibit binding of Mal63p and vise versa. The interference by Mig1p with the binding of Mal63p provides an explanation for the inhibition of fermentation by multicopy plasmids containing MIG1 during maltose and galactose growth in the absence of a specific “glucose” signal (2, 3). This suggests that increased occupancy of Mig1p binding sites is sufficient for glucose repression.

**Competition between Mig1p and Mal63p could occur by direct competition for binding or by direct interaction between Mig1p and Mal63p, which does not require DNA binding (i.e. squelching). Since no relief of glucose repression is seen in a site 1 mutant containing a high copy MAL63 plasmid, the squelching model can be excluded.**

**Competition between Mig1p and Mal63p is not the only mechanism for glucose repression, since Mig1p-Cyc8p (Ssn6p)-Tup1p inhibits basal transcription of MAL62 in the absence of Mal63p (i.e. in an early mal63 frameshift mutant) (Table II). Since competition for binding is not a factor here, the Mig1p-Cyc8p (Ssn6p)-Tup1p complex has intrinsic repressor activity as well.**

Reversal of glucose repression then involves two mechanisms. The first is that Mig1p binds more poorly in nonrepressing sugars; the second is that Mig1p binding to site A is further reduced by increases in the concentration of Mal63p.

**New Protected Region in the MAL61–62 Promoter**—An additional clue to the way in which Mig1p may affect gene expression on maltose media is the appearance in AMIG1 strains of a protected region downstream from site 1 (Figs. 1 and 5) at a T-rich region whose sequence is reminiscent of binding sites for the HMG proteins. During induction, bound Mig1p may interfere with the binding of an unknown protein at this site or change chromatin structure to make this site more accessible to DNase I. Since maltase is superinduced in maltose-grown MIG1::URA3 strains (see Table II), Mig1p influences MAL62 expression even during induction. Although not discussed by the authors, data in Levine et al. (30) show that a deletion immediately adjacent to this new footprinted region leads to superinduction, similar to what we report here; while this suggests that the T-rich sequence plays a role in maltase regulation, others have deleted this region with no effect, so its role remains uncertain (31).

**Role of Tup1p-Cyc8p**—The model proposed here is clearly incomplete. One problem is that we studied direct competition between Mig1p-Cyc8p (Ssn6p)-Tup1p and activator binding in a particular promoter context (one with adjacent Mig1p and activator binding sites), and this is not a universal arrangement. For example, the UASMAL62 and Mig1p binding sites are adjacent, the UASGAL and Mig1p binding sites are separated, and SUC lacks UAS sites. Of course, site interaction does not require proximity, and competition for binding between widely separated Mig1p and activator binding sites may still take place. Another, and more critical problem is that the model is silent on the role of Cyc8p (Ssn6p)-Tup1p in glucose repression.

Represors like Mig1p-Cyc8p (Ssn6p)-Tup1p can act at any stage of transcription initiation or RNA polymerase clearance and can involve interference with activator binding, interference with the interaction between bound activator and the transcriptional apparatus (quenching), direct inhibition with RNA polymerase, or interference by titration of unbound activator (squelching) (10, 32). These mechanisms are obviously not mutually exclusive, and our results show that Mig1p-Cyc8p (Ssn6p)-Tup1p represses both by interfering with activator binding and by an activator-independent mechanism. Tup1p-Cyc8p may interact with the transcriptional apparatus and/or be used to convey repression (in part by modulating binding affinity of the complex) by an unknown glucose-generated signal through the Snf1p kinase or other upstream proteins.

In contrast to another report (29), our observations suggest that Mig1p-Cyc8p (Ssn6p) does not activate MAL62 transcription. A ΔMIG1 ΔTUP1 strain has the same PNPGase activity as a ΔTUP1 strain (which has Mig1p-Cyc8p (Ssn6p)) when these cells are grown on glucose media, suggesting that Mig1p-Ssn6p is not an activator. Similarly, a ΔMIG1 ΔCYC8 (Ssn6p) strain (which has only Tup1p) has the same PNPGase activity as a ΔCYC8 (Ssn6p) strain (which has Mig1p-Tup1p).

Glucose-grown ΔMIG1 ΔTUP1 strains show an approximately 10-fold increase of maltase over the levels in ΔMIG1 strains (Table III). The maltase level in ΔMIG1 ΔTUP1 strains grown in glucose is also 10 times higher than seen in galactose-grown MAL6Δ MIG1Δ TUP1 wild type strains. Deletion of TUP1 therefore effects an increase that is independent of glucose repression mediated by Mig1p. Tup1p may therefore play two roles in the repression of MAL62, one by participating in a complex with Mig1p and another independent role possibly involving chromatin structure.

**Conclusion**—To complement previous in vivo studies of arti-
ficial promoters and Mig1p binding, we looked at glucose repression at a natural promoter and one that was regulated by induction as well as by repression. In several ways our results support previous results in the GAL system, suggesting that despite the particular arrangement of Mig1p and activator binding sites studied in MAL62, the mechanism we propose may be applicable to other glucose-repressed yeast genes.

For example, deletion of MIG1 is sufficient for a complete release of glucose repression, maltase levels for MAL6 ΔMIG1 on glucose being equal to those in MAL6 MIG1 strains grown on galactose (Table II). Maltase levels are also the same for MAL63 ΔMIG1 grown in glucose and MAL63 MIG1 strains grown in galactose. This result is identical to that observed in the GAL system, where deletion of MIG1 relieves all the glucose repression in a constitutive strain (23). Other similarities exist between glucose repression in GAL and MAL. Similar to the release of repression seen in strains deleted for site C in the MAL63 promoter, an increased concentration of Gal4p overcomes glucose repression in a gal80 strain (23); both mechanisms presumably work by increasing the occupancy of the UASMGL or the UASGAL. Mathieu and Felenbok (33) have demonstrated that the CREA protein, the Aspergillus nidulans analogue of Mig1p, competes for binding with the ALCR activator in vitro, again suggesting that the mechanism proposed for the glucose repression of MAL62 may be a general one.

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