Removal of a Mig1p Binding Site Converts a *MAL63* Constitutive Mutant Derived by Interchromosomal Gene Conversion to Glucose Insensitivity

Jianfan Wang and Richard Needleman

Department of Biochemistry, Wayne State University School of Medicine, Detroit, Michigan 48201

Manuscript received July 24, 1995
Accepted for publication September 28, 1995

ABSTRACT

Maltose fermenting strains of *Saccharomyces cerevisiae* have one or more complex loci called *MAL*. Each locus comprises at least three genes: *MALx1* encodes maltose permease, *MALx2* encodes maltase, and *MALx3* encodes an activator of *MALx1* and *MALx2* (*x* denotes one of five *MAL* loci, with *x* = 1, 2, 3, 4, or 6). The *MAL43*^{r} allele is constitutive and relatively insensitive to glucose repression. To understand better this unique phenotype of *MAL43*^{r}, we have isolated several *MAL63*^{r} constitutive mutants from a *MAL6* strain. All constitutive mutants remain glucose repressible, and all have multiple amino acid substitutions in the C-terminal region, now making this region of Mal63^{r}p similar to that of Mal43^{r}p. These changes have been generated by gene conversion, which transfers DNA from the telomeres of chromosome *II* and chromosome *III* or *XVI* to chromosome *VIII* (*MAL6*). The removal of a Mig1p binding site from the *MAL63*^{r} promoter leads to a loss of glucose repression, imitating the phenotype of *MAL43*^{r}. Conversely, addition of a Mig1p binding site to the promoter of *MAL43*^{r} converts it to glucose sensitivity. Mig1p modulation of Mal63p and Mal43p expression therefore plays a substantial role in glucose repression of the *MAL* genes.

IN *Saccharomyces cerevisiae*, maltose fermentation depends upon the presence of any one of five nonallelic *MAL* loci: *MAL1* (chromosome *VII*), *MAL2* (chromosome *III*), *MAL3* (chromosome *II*), *MAL4* (chromosome *XI*), and *MAL6* (*VIII*). Each locus contains at least three genes required for maltose fermentation (NEEDLEMAN *et al.* 1984). At *MAL6*, the most extensively studied complex, these are designated *MAL61*, *MAL62*, and *MAL63*, where *MAL61* encodes maltose permease, *MAL62* encodes maltase, and *MAL63* specifies a *trans*-acting regulator required for maltose induction of *MAL61* and *MAL62*. An additional gene, *MAL64* highly homologous to *MAL63* is also present but not required for maltose fermentation. *MAL61*, *MAL62*, *MAL63*, and *MAL64* are induced by maltose (DUBIN *et al.* 1988; NEEDLEMAN 1991). Glucose affects expression in two ways: it inactivates maltose permease and represses mRNA synthesis from *MAL61*, *MAL62*, and *MAL63*. (LUCERO *et al.* 1993).

The Mal63p activator is a zinc finger-containing protein similar to Gal4p, Put3p, and Ppr1p (CHANG *et al.* 1988; KIM and MICHELS 1988). It is a dimer and activates *MAL61* and *MAL62* by binding to three sequences in the divergent *MAL61-62* promoter region with the parallel recognition motif c/a GC N_9 c/a GC/g (SIRENKO *et al.* 1995). However, the precise way in which Mal63p activates transcription and its role in mediating glucose repression remain unknown. The standard model of activation proposes that specific yeast activators like Mal63p contain regions that activate transcription by interacting with a more general set of transcription factors controlling the transcription of diverse genes. The precise nature of these interactions have been difficult to determine, and Mal63p in particular lacks regions often associated with activation (*e.g.*, acidic and glutamine rich regions) (MA and PTASHNE 1987; PFEIFER *et al.* 1989; THUKRAL *et al.* 1989; COOK *et al.* 1994).

While the *MAL* genes are highly homologous and phenotypically equivalent (*e.g.*, *MAL63 MAL11 MAL12* strains ferment maltose), an exception is the "wild-type" allele of *MAL43* (called here *MAL43*^{r}) found in strain 1403-7A. Unlike other *MALx3* genes, it causes constitutive expression of maltase and maltose permease; this expression is also relatively insensitive to glucose repression (NEEDLEMAN 1975; CHARRON and MICHELS 1987). Unfortunately, however, the sequence of the Mal43p activator provides few clues as to the origin of either its constitutivity or its insensitivity to glucose repression. A wild-type, inducible *MAL43* strain is not available for comparison and Mal43^{c}p differs in >20 amino acids from the sequence of Mal63p (GIBSON and MICHELS 1993).

To better understand the origin of these unique properties of Mal43^{c}p, we isolated constitutive mutants in a *MAL6* strain. All previously isolated *MAL6* constitutives are *MAL64*^{c} constitutives; despite repeated attempts in several laboratories, no *MAL63*^{r} constitutive mutants had been obtained.

We describe here the first isolation of *MAL63*^{r} mutants. Two of these, Mal63^{c}p-D8 and Mal63^{c}p-C38, contain multiple amino acid substitutions in the C-terminal

Corresponding author: Richard Needleman, Department of Biochemistry, Wayne State University School of Medicine, 540 E. Canfield, Detroit, MI 48201. E-mail: rneedle@moose.med.wayne.edu

TABLE 1

Yeast strains used in the study

Strains	Genotypes	Source
706-6A	*MATa MAL64::LEU2 MAL6 MAL12 MTP1 Mal3g ura3-52 leu2-3,112 trp1 his1 suc αmg*	This laboratory
706-6B	Isogenic to 706-6A but *MATα*	This work
612-1D	*MATα MAL6 MAL12 ura3-52 leu2-3,112 trp1 his1*	NEEDLEMAN *et al.* (1984)
715-1C	Isogenic to 612-1D but *MAL6::LEU2*	This laboratory
A9	Isogenic to 612-1D but *MAL63::URA3*	This laboratory
1403-7A	*MATa MAL43^c MAL1g MAL3g trp1 ura3*	This laboratory

region that are generated by gene conversion from "cryptic" DNA homologous to *MAL63* on chromosomes *II, III,* or *XVI.* Surprisingly, these new amino acids now make the C-terminal region of Mal63^{c}p-D8 identical to that of *MAL43^c* from leu-418 to the terminal proline at 470; Mal63^c-C38 is also identical to *MAL43^c* from lys-367 to the C-terminal pro-470 except that it lacks the val-388 found in *MAL43^c* and has two additional valines at positions 387 and 465 as well as an additional final residue. However, unlike *MAL43^c*, both *MAL63^c* mutants remain sensitive to glucose repression. We show that this insensitivity in *MAL43^c* is due to the presence of a Mig1p binding site which overlaps the transcriptional start site in the *MAL63* promoter; its removal yields *MAL63^c* mutants partially resistant to glucose repression. *MAL63^c* strains lacking the Mig1p binding site are constitutive, dominant, and glucose insensitive. Conversely, addition of a Mig1p binding site to the *MAL43^c* promoter restores glucose sensitivity. Taken together these observations provide an explanation for the phenotype, and perhaps the origin of *MAL43^c*.

MATERIALS AND METHODS

Strains and growth conditions: The yeast strains used in the study are listed in Table 1. The cells were grown on YEP medium (1% Yeast extract, 2% Bacto-Peptone) or SC synthetic medium (0.67% Bacto Yeast Nitrogen Base without amino acids) to which was added the indicated carbon source (2%); SC was supplemented as appropriate with required amino acids. Standard genetic analysis was as described previously (SHERMAN *et al.* 1986). Antimycin A (1μg/ml) was added to the YEPMal and YEPSuc plates.

PNPGase and maltose permease assay: PNPGase (p-nitrophenyl-α-D-glucopyranoside) activity was assayed as described previously (NEEDLEMAN and EATON 1974). We also use a filter paper assay as a qualitative method for detecting constitutive PNPGase activity in which cells were assayed after being permeabilized by drying on Whatmann 3MM paper. Cells were inoculated into 5 ml YEP medium plus the required carbon source and grown to stationary phase. Culture (100 μl) was pipetted to 3MM Chromatography paper and allowed to dry for ≥1 hr. To the dried cells, 40 μl of PNPG (3 mg/ml) and 40 μl of PNPG buffer (0.1 M potassium phosphate buffer, pH 7.4, 1 mM EDTA) were added. Under these conditions, constitutive strains produce a deep yellow color after a few minutes. The reaction was stopped by the addition of 100 μl of 1 M sodium carbonate.

To measure maltose permease activity, cells were grown in YEP medium plus the indicated carbon source to log phase (~2 × 10^7 cells/ml or ~100 Klett units using a red filter). Of this, 20 ml was harvested, washed three times with water, and resuspended in 0.8 ml of 0.1 M tartaric acid buffer, pH 4.3; 100 μl cold maltose (20 mM) was added and the assays started by adding 100 μl uniformly labeled ^{14}C-maltose (2 μCi/ml). At intervals, 150-μl aliquots were removed to a prewashed manifold containing No. 31 glass filters (Schleicher and Schuell). The samples were filtered immediately and washed again with water. After drying the filters were counted in 5 ml of Aquasol (New England Nuclear).

Yeast transformation: Transformation was done using electroporation essentially as described by BECKER and GUARENTE (1991).

Selection of constitutive mutants: A lawn of strain 706-6A was prepared on YPD plates and 20 μl of a saturated solution of N-methyl-N″-nitro-N-nitrosoguanidine (NTG) in acetone placed in the center. After growth at 30° for 2 days, the lawn was replicated to YEPSuc plates. Colonies that grew in a ring closest to the mutagen were purified and screened for constitutivity using the filter paper assay.

Southern transfer analysis: Genomic DNA was prepared according to the rapid protocol of HOFFMAN and WINSTON (1987). One-tenth of the DNA obtained from a 10-ml stationary yeast culture was digested with restriction enzyme and separated on a 1% agarose gel. The DNA was transferred onto a nylon membrane (Hybond-N) by positive pressure. The probes were labeled by random priming with dAT^{32}P. After hybridization, the membrane was washed in 2× SSC-SDS buffer (0.3 M NaCl/0.03 M Nacitrate/1% SDS) at 65° and then washed in 0.1× SSC at room temperature.

Chromosome-sized yeast DNA molecules in solid agarose were prepared according to the protocol of KAISER *et al.* (1994). A CHEF-DR II apparatus was used to separate the chromosomal DNAs. Electrophoresis was carried out at 6 V/cm for 12 hr with a switching time of 60 sec and then for 18 hr with a switching time of 90 sec.

Cloning of *MAL43^c*: A DNA library was constructed by gel isolating an ~5-kb *Bam*HI-*Sal*I fragment that contains *MAL43^c* from 1403-7A. The DNA was ligated into the BlueScript SK plasmid (Stratagene) and the library screened by colony hybridization using *MAL63* as a probe. Positive colonies were checked by restriction and sequenced using Sequenase 2.0 (US Biochemical Corp.) according to the supplier's protocol. The presence of linked *SIR1* sequences confirmed that *MAL43* had been isolated. Sequence comparisons used the BLAST program developed by the National Center for Biotechnology Information at the National Library of Medicine (ALTSCHUL *et al.* 1990).

Plasmid construction: Plasmids pJW101 to pJW107 are clones containing DNA from wild type and constitutive strains in a p-Bluescript SKII cloning vector (Figure 1). pJW101 and pJW102 are from mutant D8 and pJW103 and pJW104 are

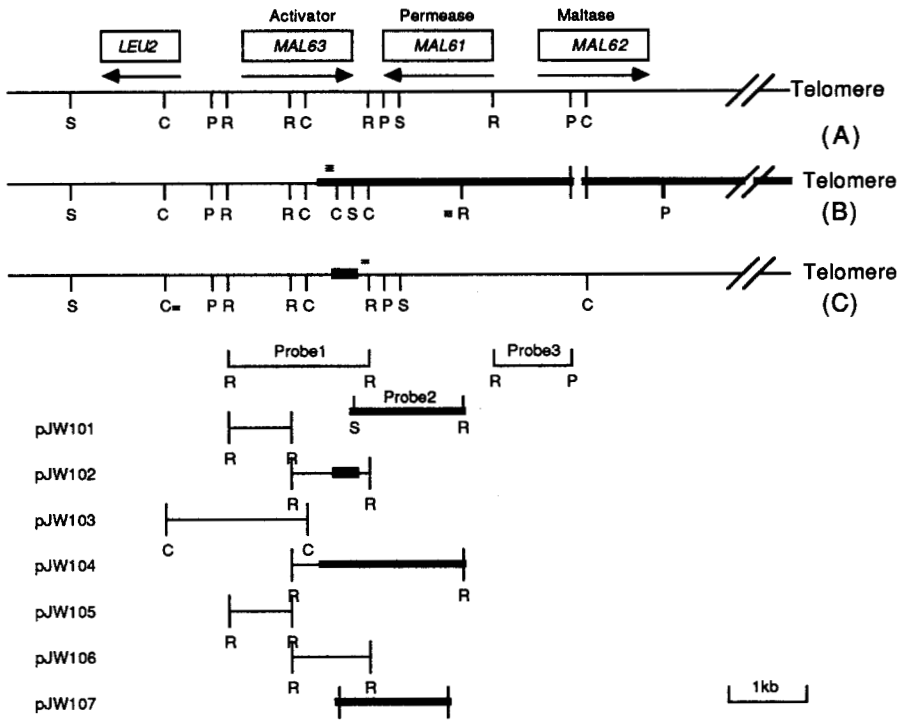

FIGURE 1.—Restriction map of *MAL6* in 706-6A (A), the "Southern abnormal" constitutives (B) and the "Southern normal" constitutive D8 (C). The arrow indicates the gene orientation. Probe 1 is the *MAL63* probe that includes two *Eco*RI fragments from *MAL63*; probe 2 is the *Sal*I-*Eco*RI fragment from C38 that contains a *SGE1*-homologous sequence; probe 3 is the *Pst*I-*Eco*RI fragment that contains *MAL61* and *MAL62* homologous sequences. ≡, primers used for amplification of *SGE1*-homologous sequences from wild-type 706-6A; =, primers used for amplification of the *MAL63* allele from D8. Solid bars indicate sequences foreign to the *MAL6* locus. Genomic DNA fragments cloned in p-Blue Script SK vector: pJW101 and pJW102 from D8; pJW103 and pJW104 from C38; pJW103 and pJW104 from 706-6A. pJW 107 contains a PCR product obtained from 706-6A genomic DNA. C, *Cla*I; E, *Eco*RI; P, *Pst*I; and S, *Sal*I.

from mutant C38. pJW105 and pJW106 are from the wild-type *MAL6* strain 706-6A; the insert in pJW107 was a PCR product obtained from the same strain. pJW1 was constructed by ligating a 2.5-kb *Kpn*I-*Sal*I fragment containing *MAL63* (wild type) from plasmid 9-26 (NEEDLEMAN *et al.* 1984) into phagemid pS1 (Promega). pJW2 was constructed by replacing the 1.0-kb *EcoRI* fragment in pJW1 with the same *Eco*RI fragment from *MAL63c*-D8 contained in pJW102. pJW3 was obtained from pRS316 (SIKORSKI and HIETER 1989) by eliminating the *Spe*I and *Xba*I sites. pJW4 was obtained by cloning a 2.9-kb *Bgl*II-*Sal*I fragment containing the wild-type *MAL63* into pJW3. pJW5 was constructed by replacing the *Spe*I fragment of pJW4 with the same *Spe*I fragment of pJW2. pJW7 was constructed by ligating the *Bgl*II-*Sal*I fragment of wild-type *MAL63* into *Bam*HI-*Sal*I sites of pRS315 (SIKORSKI and HIETER 1989). pJW9 was constructed by inserting the *Kpn*I-*Sal*I fragment containing *MAL63*^{r} from pJW5 into pS1. pJW20 was constructed by replacing the *Pst*I fragment containing the wild-type *MAL63* of pJW7 with the *Pst*I fragment from pJW9, which contains *MAL63*^{r}-D8.

Site-directed mutagenesis: Site-directed mutagenesis was carried out using the Promega mutagenesis kit according to the supplier's instruction. After mutations were made in pJW1, 2 or 9 the *MAL63* gene was subcloned into yeast *Escherichia coli* shuttle vectors pRS315 and pRS316 and sequenced.

PCR amplification of genomic DNA: Two synthetic oligonucleotides of 18 bp derived from pJW104 sequences were used as primers for PCR amplification to confirm the origin of *MAL63*^{r} sequence in the C-terminal region of 706-6A. The 5′ primer was GCCAAGGATATGTTACAG, and 3′ primer was GCCGAAGTCATGGAACTT. For confirmation of the D8 clones, we used a 5′ primer homologous to *LEU2*, GCAGACATAGGGGCAGACAT and a 3′ primer homologous to *MAL63*, GGAATTATGTCGTCTTCATC. Pfu polymerase (Stratagene) was used to amplify the genomic sequence. A reaction contained: 5 μl 5′ primer (10 μM), 5 μl 3′ primer (10 μM), 5 μl dNTP mix (1 mM each nucleotide), 5 μl buffer (10x = 200 mM Tris-HCl, 500 mM KCl, pH 8.4), 2–20 ng genomic DNA and 2.5 U Pfu polymerase. After the expected PCR product (~1.5 kb) was purified, it was cloned into the SK vector using the pCR-Script^{cm} SK(+) Cloning Kit (Stratagene).

RESULTS

Selection of *MAL63* constitutive mutants: To avoid the isolation of *MAL64*^{c} mutants, we used a strain in which *MAL64* and the region upstream of the *Bgl*II site at −583 bp in the *MAL63* promotor were replaced by *LEU2* (strain 706-6A, Figure 1). In addition, this strain contains *MTP1* (Maltose Triose Permease), which encodes a maltose permease of broad specificity linked to *MAL12* (PERKINS *et al.* 1988), and additional Malg information at the *MAL3* locus (MAL3g;) The Malg designation indicates that functional *MALx1* and *MALx2* gene(s) are present; 706-6A ferments maltose and galactose but not sucrose or α-methylglucoside.

The selection is based on the following considerations: (1) Sucrose is an excellent substrate of maltase, (2) sucrose does not induce maltose, maltose permease (Mal61p), or the maltotriose permease Mtp1p, (3) sucrose can be transported by Mal61p and/or Mtp1p, and (4) maltose induces sucrose transport in *MAL63 MAL61 MTP1* strains (PERKINS and NEEDLEMAN 1988; KHAN *et al.* 1973). We therefore isolated Mal63p constitutive mutants (*i.e.*, *MAL63*^{r}) by selecting for growth on sucrose. Because *MAL63*^{r} cells constitutively transport sucrose and constitutively synthesize maltase, constitutives cells should grow on sucrose, unlike the wild-type *MAL63* strains. This strategy was successful, and from ~300 mutants able to grow on sucrose, 16 strains constitutive for maltase were isolated. Using a *MAL63* ura3 strain containing 1463, a CEN ARS *URA3* plasmid car-

TABLE 2

Maltase activity of various cells after growth on different carbon sources

Strains	Maltase activity (nmPNPG/min/mg protein)			
	Glucose	Maltose	Sucrose	Galactose
706-6A	8	1292	75	34
D10	29	1450	1479	1706
D8	19	2056	1617	1513
C1	27	1673	1446	1669
E40	53	1615	1483	1275
C37	28	1256	1327	2124
C38	31	1226	1248	1024
D8x706-6B	20	1573	ND[a]	1491
C38x706-6B	33	1227	ND	931
C38xC38	24	968	ND	1236
706-6Apdm3u (1463-4[c])	22	1799	1396	999

The cells were grown and assayed as described in MATERIALS AND METHODS.

[a] ND, not determined.

TABLE 3

Maltose permease activity of 706-6A and the constitutive C38 after growth on different carbon sources

Strains	Maltose permease (nmol/mg dried cells/min)			
	Glucose	Maltose	Sucrose	Galactose
706-6A	<0.1	2.7	0.2	0.1
C38	<0.1	3.0	2.3	1.8

Cells were grown on different carbon sources as in Table 2, the assays were done according to MATERIALS AND METHODS.

rying *MAL63* (706-6Apdm3 ura$^-$[p1463]), the same method was used to isolate a plasmid linked constitutive mutant, 1463-4^c.

Maltase and maltose permease levels in the constitutive mutants: The PNPGase (maltase) activity of all 17 constitutive mutants isolated from 706-6A and 706-6Apdm3 ura$^-$ was measured after growth on galactose, glucose, maltose, and sucrose. All had high levels of PNPGase on galactose, maltose, and sucrose, but were still repressed by glucose (see Table 2 for representative data). The permease was measured in one mutant, C38; it also had high permease levels after growth on galactose (Table 3).

Constitutivity segregates as a single gene difference and is dominant: To determine if the constitutivity is linked to *MAL6*, we crossed the mutants with 612-1D (*MAL6 MAL12*). In the cross C38 × 612-1D, 18 tetrads were analyzed, and constitutivity: inducibility segregated 2:2 consistent with a single gene difference. Moreover, all *LEU2* cells were constitutive. Dominance was tested by backcrossing two mutants, C38 and D8, to a strain isogenic to 706-6A except for mating type. Constitutivity was dominant, in contrast to the semirecessive nature of *MAL64c* strains (Table 2; DUBIN *et al.* 1988).

Constitutivity is accompanied by the loss of function in one or more genes of MAL6: Surprisingly, and in contrast to the cross of 612-1D with the wild-type strain from which mutants were derived (706-6A), the segregation of maltose fermentation in the cross C38 × 612-1D was not strictly 4:0 as expected from a *MAL6* × *MAL6* cross, but yielded seven 4:0, eight 3:1, and three 2:2 tetrads for MAL:mal, consistent with maltose fermentation requiring a gene(s) at *MAL6* and either one of two independently segregating genes. This interpretation is suggested from the observation that 706-6A contains two partially active *MAL* loci each having genes for maltose permease and maltase but lacking the maltose Malx3p regulator (*i.e.*, Malg). Alternatively the *MAL6* gene could have been translocated to another chromosome, though in this case, we would expect fewer 4:0 tetrads.

Whatever the precise cause, it appeared that the genetic events in mutant C38 occurred with the loss of function of one or more of the genes at *MAL6*.

Constitutivity is linked to *MAL6*: Before investigating the precise nature of the changes at *MAL6* revealed by the C38 × *MAL6* cross, we determined whether constitutivity was linked to *MAL6*, taking into account the possibility of gene rearrangements. Because *LEU2* was linked to constitutivity (see above), linkage of constitutivity to *MAL6* would be demonstrated if *LEU2* remained at its original position (*i.e.*, *MAL64 LEU2*) on chromosome *VIII*.

C38 (*MAL64::LEU2*) was therefore crossed to strain 715-1C (*MAL6::LEU2 leu2-3, 112*). In all 15 tetrads, LEU:leu segregated 4:0, demonstrating that in mutant C38 the *LEU2* gene remained at *MAL6*. The gene conferring constitutivity in C38 was therefore linked to *MAL6*.

Genetic properties of the other mutants: Similar tetrad analysis of five other independently isolated constitutive mutants showed that four (C1, C2, C30, and D10) had the same genetic properties as C38. The exception was the fifth mutant chosen, D8, which when crossed to 612-1D, showed 4:0 MAL:mal segregation in 14 tetrads. In D8 constitutivity was also linked to *MAL6*.

Fifteen of 16 chromosomal mutants have abnormal Southern transfer patterns when probed with *MAL63*: In our laboratory, fusions between the *MAL64* promoter and *MAL63* were recovered that had increased expression of maltase and maltose permease (CHANG 1994). Southern transfer analysis was therefore used to see if there were large structural changes at *MAL63*. Fifteen of the constitutive mutants showed altered Southern transfer patterns when cut with four restriction enzymes (*Cla*I, *EcoR*I, *Pst*I, *Sal*I) and one, D8 showed the same pattern as the wild type (Figures 2 and 3); only the *EcoR*I pattern is shown in Figure 3. In the 15 mutants with an altered pattern, the changes

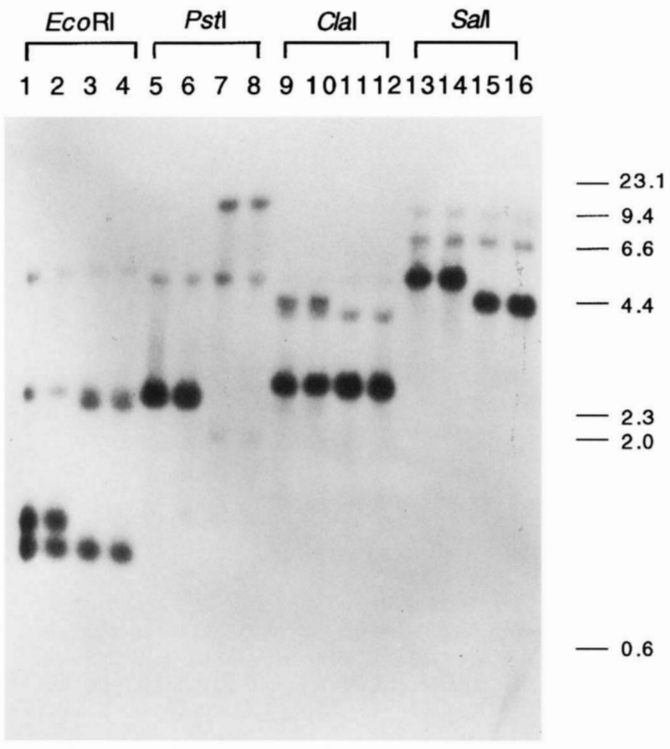

FIGURE 2.—Southern transfer analysis of the wild-type *MAL6* strain and the constitutive mutants D8, C1 and C38. Lanes 1, 5, 9, 13 are from wild-type 706-6A DNA; 2, 6, 10, 14 from D8; 3, 7, 11, 15 from C1 and 4, 8, 12, 16 from C38. Genomic DNA was restricted as indicated and probed with the *MAL63 Eco*RI fragments shown in the Figure 1.

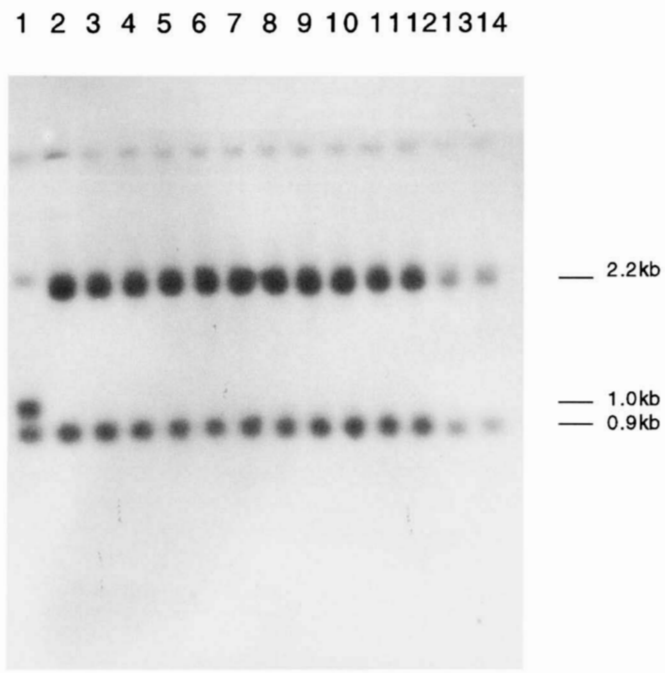

FIGURE 3.—Southern transfer analysis for 13 mutants. Genomic DNA prepared from wild-type and 13 mutants were cut with *Eco*RI. The probe used in the analysis is the same as in the Figure 2. Lane 1 is 706-6A; 2–14 are C2, C6, C18, C19, C21, C30, C37, C41, D10, E5, E23, E40, and F22, respectively.

were not consistent with a gene fusion, because the Southern transfer analysis indicated that only DNA encoding the C-terminal region of Mal63p was altered (Figures 1 and 2).

Constitutive mutants with an abnormal Southern pattern also have alterations in *MAL61* and *MAL62*: Two observations demonstrate that a functional *MAL61* gene is not present on chromosome *VIII* in the "Southern abnormal" mutants. First, in crosses between 612-1D (*MAL6 MAL12*) and all constitutive mutants except for D8, LEU^+ mal^- constitutive spores are recovered. Because this cross is homozygous for *MAL12*(encoding maltase), and because *LEU2* is linked to constitutivity as assayed by maltase levels, this suggests that *MAL61* is altered. Second, altered Southern patterns were seen when genomic DNA from all constitutives but D8 was digested by *Bgl*II, *Cla*I, *Eco*RI, *Pst*I and *Sal*I and probed with *MAL61*. Similarly, when chromosomes from nine constitutive strains were separated on CHEF gels and probed with *MAL61-62*, no homology was detected on chromosome *VIII* but the *MAL12* locus was still present on chromosome *VII* (Figure 4). Taken together this indicates that *MAL61* has been grossly altered and that a portion of *MAL62* has been lost from chromosome *VIII*. In contrast, the single mutant with a normal Southern pattern (D8) has functional *MAL61* and *MAL62* genes as determined by genetic analysis.

The constitutives therefore fall into two classes, represented by C38 and D8 for the chromosomal mutants. Further investigation was therefore confined to the representative strains C38 and D8.

Cloning of *MAL63*^{c}-C38 and *MAL63*^{c}-D8: Because several chromosomes have *MAL* homologous sequences (both by genetic analysis and the CHEF gels in Figure 4), it is necessary to ensure that the cloned sequences do in fact represent the sequences present at *MAL6* responsible for constitutivity.

The isolation of *MAL63*^{c}-C38 and *MAL63*^{c}-D8 therefore involved two steps: the cloning and sequencing of DNA restriction fragments with homology to *MAL63*, and the subsequent identification of these fragments as coming from *MAL63* and not from other *MAL* loci.

The isolation of *MAL63*^{c}-C38 began with the cloning of a 2.5-kb *Cla*I fragment from C38 homologous to *MAL63*. This was sequenced to identify the 5′ sequences of *MAL63*^{c}-C38 (Figure 1). The fragment contains a sequence from *LEU2* and the sequence encoding the N-terminal region of Mal63p-C38; the *MAL63*^{c} sequences are identical to wild type. To isolate the sequences encoding the C-terminal region of Mal63p-C38, we noted that *MAL63*^{c}-C38 (and all constitutives but D8 and 1463^c) has a new 2.2-kb *Eco*RI fragment (Figures 2 and 3). The linkage of this fragment to *LEU2* was tested by isolating six LEU^+ and six leu^- random spores from the diploid, C38 × 612-1D (*MAL6 MAL12 leu2-3, 112*) and testing them for the presence of this new *Eco*RI fragment using a *MAL63* probe. All LEU^+ spores contained the frag-

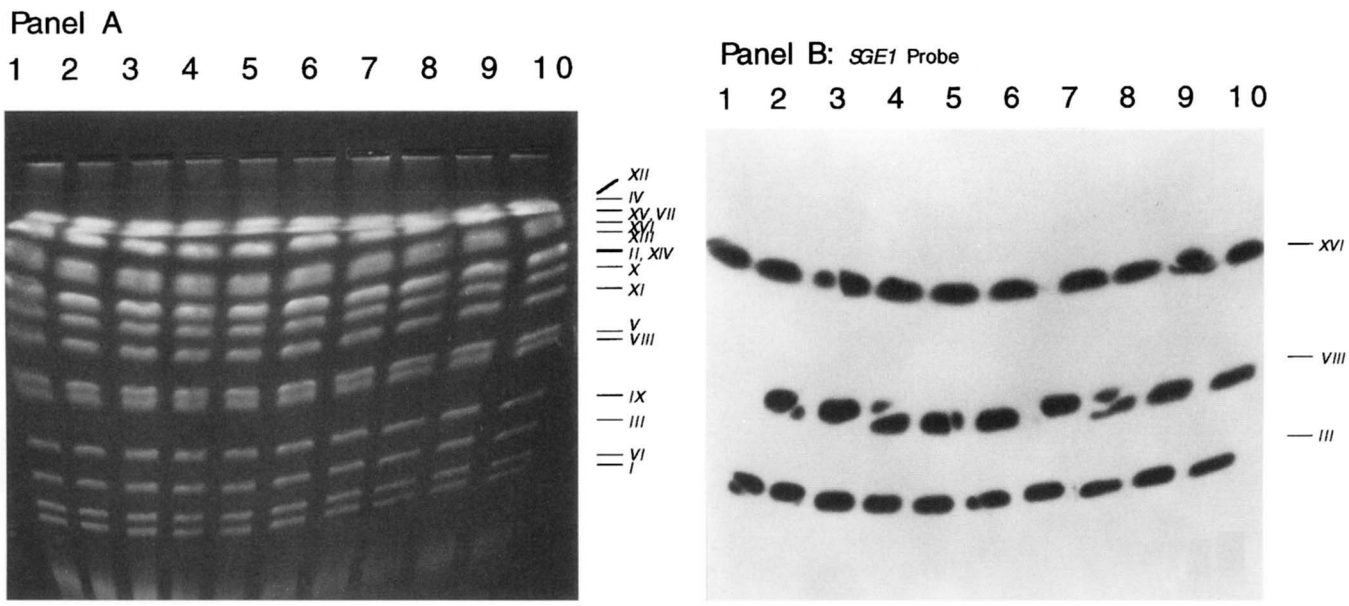

FIGURE 4.—Southern hybridization analysis of chromosomal DNA from the wild-type 706-6A and the "Southern abnormal" constitutive mutants. (A) The ethidium bromide stained CHEF gel is shown. (B and C) Results are shown of the Southern analysis of the chromosomal DNAs probed with the indicated probe: B, *SGE1* probe and C, *MAL61-62* probe (see Figure 1). The strains are: wild type 706-6A (lane 1), C1 (lane 2), C2 (lane 3), C18 (lane 4), C30 (lane 5), C37 (lane 6), C38 (lane 7), C41 (lane 8), D10 (lane 9) and E40 (lane 10).

ment; all leu$^-$ spores lacked it (data not shown). This fragment is therefore linked to *LEU2* (*MAL64::LEU2*) and derives from the *MAL6* locus.

A 2.2-kb *Eco*RI fragment was cloned from C38 DNA after gel-isolation and hybridization screening with a *MAL63* probe. In addition to *MAL63* sequences, the clone contained DNA with homology to *SGE1* (see next section). We confirmed that the *SGE1* sequences found in the clone were present in the target 2.2-kb *Eco*RI fragment by Southern transfer analysis using a *SGE1* homologous probe (data not shown). We have therefore cloned *MAL63^c*-C38.

The isolation of *MAL63^c*-D8 was more difficult since D8 does not show any new restriction fragments. We therefore gel-isolated, cloned, and sequenced both *Eco*RI fragments that are homologous to *MAL63*. A restriction fragment containing the 3′ portion of *MAL63* and having mutational changes similar to C38 was isolated as a presumptive *MAL63^c*-D8 clone. To confirm the identity of the clone, we used the polymerase chain reaction to clone from nuclear DNA the region in D8 downstream of *LEU2*; the 5′ primer was homologous to *LEU2* and the 3′ primer homologous to the 3′ noncoding region of *MAL63*. The fragment obtained was of the predicted size and when completely sequenced, shown to be identical to that of the cloned fragment. We have therefore cloned *MAL63^c*-D8.

MAL-homologous sequences are also found on chromosome *IV*: In addition to DNA from the *MAL6* region, we also cloned a 2.5-kb *Cla*I fragment from mutant C1, which contained both *MAL63*-homologous sequences and the *COX9* gene (chromosome *IV*). Chromosome *IV* does not have a classical *MAL* gene. The DNA fragment encoded the same sequence as the C-terminal region of the wild-type Mal63p.

MAL63^c-C38 is linked to sequences homologous to *SGE1*: The larger *Eco*RI fragment from *MAL63^c*-C38 contained, in addition to the C-terminal region of Mal63^{c}p-C38, a 370-bp fragment identical to *SGE1*, a suppressor of *gal11* (AMAKASU *et al.* 1993; Figures 1 and 4). The C-terminal region of Mal63^{c}p-C38 is similar but not identical to that of constitutive D8 (Figure 5).

SGE1 has been mapped to chromosome *XVI* (AMAKASU *et al.* 1993). However, our CHEF gel analysis of strain 706-6A revealed two chromosomes containing *SGE1*-related sequences: one on chromosome *XVI* and the other on chromosome *III*. Southern transfer analysis of chromosomes separated on CHEF gels show that mutant C38 has an additional *SGE1* sequence on chromosome *VIII* further confirming the identification of the clone (Figure 4).

Mal63p-D8 has eight amino acid changes: The *MAL63* gene of the constitutive mutant D8, which has a normal Southern transfer pattern, was sequenced. D8 had eight amino acid substitutions in the C-terminal region of Mal63^c-D8p (Figure 5). The 166-bp sequence of D8 was found to be identical to an open reading frame on chromosome *II* (embl Z36166; frame YBR297W); it therefore might derive from *MAL3*-related sequences. The changes observed were identical to those found when the plasmid linked constitutive 1463-4^c mutant was sequenced. The insertion of a small foreign segment of DNA into the *MAL6* locus in *MAL63^c*-D8 suggests that it and 1463-4^c arose by gene conversion.

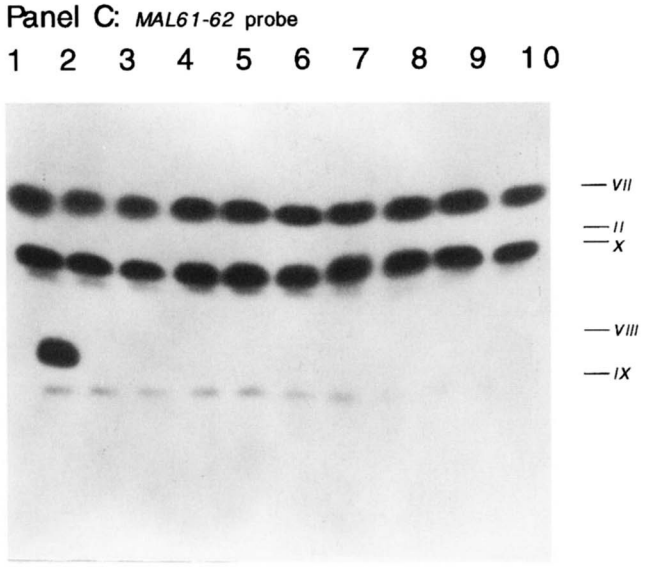

FIGURE 4.—*Continued*

Mal63^c-C38p has the same amino acid substitutions as Mal63^c-D8p but differs from wild type in 10 additional amino acid changes, including an extra C-terminal lysine (Figure 5).

C419 is necessary but not sufficient for constitutivity of D8: Our inability to isolate constitutive mutants with single amino acid changes suggested that more than one amino change was required; alternatively, the frequency of gene conversion stimulated by NTG was significantly higher than the single amino acid changes required (*i.e.*, no single amino acid changes were recovered in the screening of 17 mutants). pJW7-26 contains *MAL63*^{c}-D8 except with cys-419 of *MAL63*^{c}-D8 replaced by tyrosine. Conversely, plasmid pJW7–161 contains the wild-type *MAL63* but with tyr-419 replaced by cysteine. Both ARS CEN plasmids were transformed into a *MAL63::URA3* strain (A9) and both were inducible (Table 4). C419 is therefore necessary but not sufficient for the constitutivity of D8 suggesting that multiple amino acid changes may be required for the constitutivity of D8.

The constitutives showing altered Southern transfer patterns are generated by gene conversion: Two likely mechanisms for the generation of C38 and other members of its class can be suggested: reciprocal recombination in G2 followed by mitotic segregation or gene conversion. To distinguish these, we separated the chromosomes from nine mutants on CHEF gels, transferred the chromosomes to filters, and probed the filters with *MAL61-62* and *SGE1* homologous probes (Figure 4).

Reciprocal recombination in G2 would lead to half the mutants having *SGE1* on chromosome *XVI* (or *III*) and half having *MAL61 MAL62* on *XVI* (or *III*); all the mutants will, of course, have *SGE1* sequences on chromosome *VIII*. Figure 4 shows that all the mutants tested exhibit the same pattern: they have lost *MAL61 MAL62* sequences from chromosome *VIII*, *MAL61 MAL62* sequences are absent from chromosomes *XVI* and *III*, chromosomes *III* and *XVI* retain *SGEI*, and chromosome *VIII* gains *SGE1* homologous sequences.

The observation that none of the nine independent mutants tested have *MAL61 MAL62*-homologous sequences on chromosomes *III* or *XVI* is consistent with gene conversion as being the major mechanism generating the constitutives. However, some of the mutants may have been generated by reciprocal recombination; a larger sample size is required to determine the frequency of this mechanism.

To locate the donor sequences for the conversion event in C38, PCR was used to amplify the genomic DNA of 706-6A using a 5′ primer DNA homologous to *MAL63*^{c}-C38 in the beginning of the constitutive sequence in the C-terminal region and a 3′ primer homologous to *SGE1* (Figure 1). We obtained a DNA fragment with the expected size of 1.5 kb and this PCR product was cloned and sequenced; its sequence was the same as the 3′ sequence of *MAL63*^{c}-C38 as obtained from the clone, confirming the presence of *MALx3* sequences linked to *SGE1* in the wild-type strain.

For D8, the single mutant that retains *MAL61* and *MAL62*, we have no direct evidence that it is a result of gene conversion rather reciprocal recombination. However, because D8 has only a small segment of *MAL*-homologous sequence transferred, its "patchy" appearance suggests that it too originated by gene conversion.

Mal43^{c}p has the same eight amino acid changes common to Mal63p-C38 and Mal63p-D8: Strain 1403-7A is *MAL43*^{c}. However, because there is no "wild type" for the *MAL43*^{c} "mutant" the critical amino acids conferring constitutivity have not been identified. Unlike the *MAL63*^{c} strains, glucose repression is partially released in 1403-7A (CHARRON and MICHELS 1987; Table 2). *MAL43*^{c} was cloned from genomic DNA as described in MATERIALS AND METHODS. The amino acid sequence of the *MAL43*^{c} clone showed that the C-terminal region had the same eight amino acids found in *MAL63*^{c}-C38, D8 but absent from *MAL63* (Figure 5). This confirmed a sequence of this region generously provided to us before publication by CORINNE MICHELS (Queens College, CUNY).

Glucose repression in *MAL63*^{c}-D8 is strongly dependent upon the presence of a Mig1p binding site in the *MAL63* promoter: Although the 3′ sequences of C38 and D8 closely resemble those of *MAL43*^{c}, both *MAL63*^{c} mutants remain sensitive to glucose repression (Table 2). The difference in glucose repression of the two alleles could lie in other sequence differences in the *MALx3* gene, its promoter, or in differences in the genetic background of the strains.

GIBSON and MICHELS (1993) attempted to identify the sequences responsible for conferring glucose insensitivity and constitutivity in 1403-7A by making hybrid proteins between Mal63p and Mal43^{c}p. They were able to map the sequences required for constitutivity to

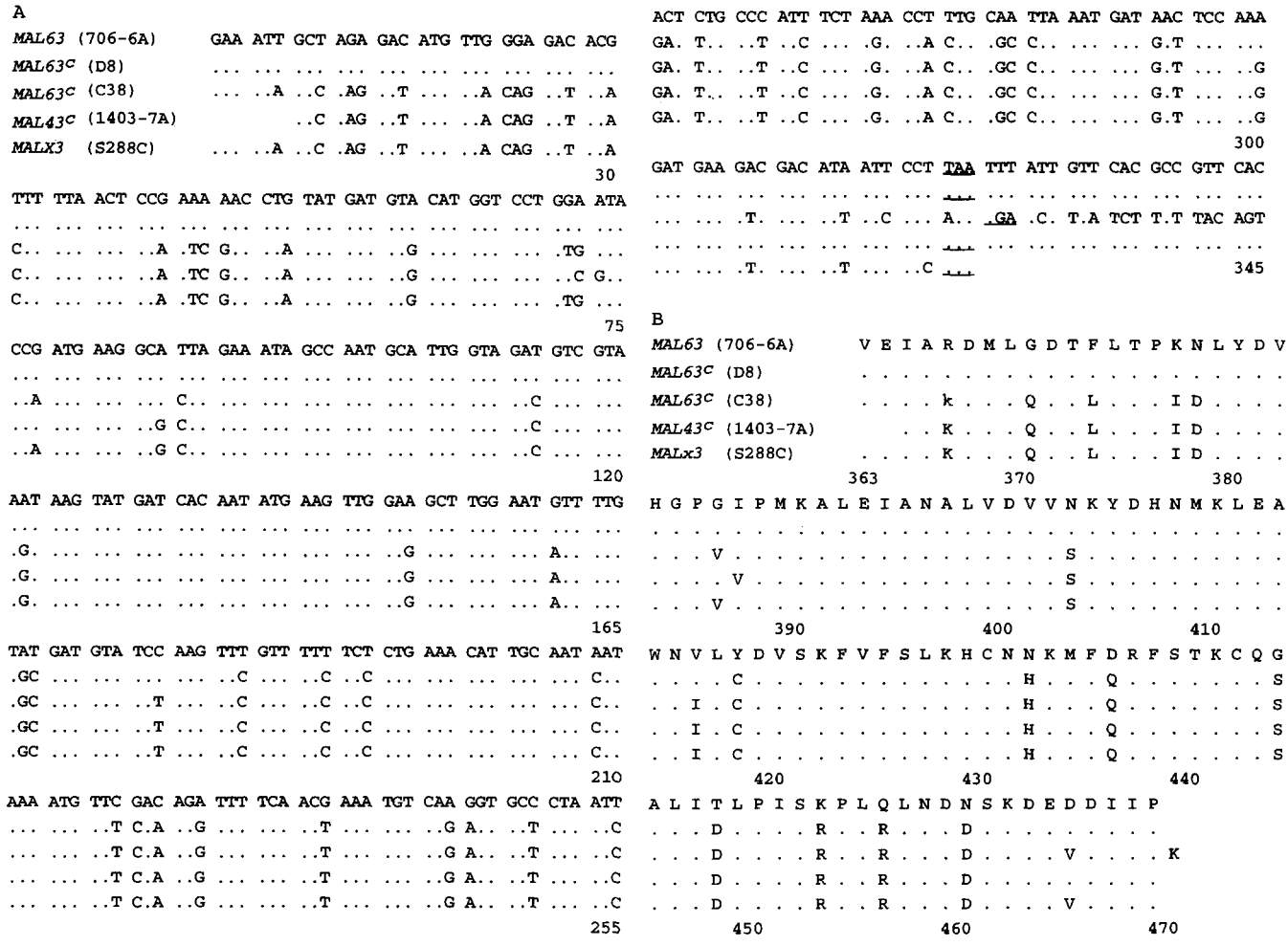

FIGURE 5.—DNA (A) and protein (B) sequences of *MAL63* (706-6A), *MAL63*^{c} (D8), *MAL63*^{c} (C38), *MAL43*^{c} (1403-7A), and *MALx3* (S288C). Only the sequence from the C-terminal region (amino acids 363–471) is shown. The stop codons are underlined.

amino acids 216–470 of Mal43^{c}p; however, they were not able to define the sequences more precisely nor could they separate the sequences required for constitutivity from those conferring glucose insensitivity.

An alternate hypothesis is that the glucose insensitivity of *MAL43*^{c} resides not in its amino acid sequence but rather in some aspects of its control—for example, in its controlling sequences. We have previously noted that the *MAL63* promoter contains a consensus binding site for Mig1p, a DNA binding protein that contributes to glucose repression, overlapping the transcriptional start site (NI 1992; LUNDIN *et al.* 1994). The *MAL43*^{c} promoter was partially sequenced and it was found that the *MAL63* and *MAL43* promoter sequences while highly homologous (~90%) differed at the Mig1p binding site—a critical cytosine residue is deleted in the Mig1p binding site of *MAL43*^{c} as compared with *MAL63* (Figure 6). To determine if the presence of a Mig1p binding site in the promoter of *MAL63*^{c}-D8 was responsible for the glucose sensitivity of this allele, we deleted a cytosine in the Mig1p binding site of *MAL63*^{c}-D8 (Figure 6), subcloned the gene on an ARS CEN vector, and transformed the plasmid into a9, a Δ*MAL63* strain. This strain now shows a release from glucose repression, strongly suggesting that the presence of a Mig1p binding site in the *MAL63*^{c} promoter is a major contributor to its glucose insensitivity (Table 5).

Loss of glucose repression in *1403-7A* is due to the absence of a Mig1p binding site in the *MAL43*^{c} promoter: Additional evidence for the importance of this Mig1p binding site was sought by attempting to convert the glucose insensitive *MAL43*^{c} allele to glucose sensitivity. A Mig1p binding site was added to the promoter of *MAL43*^{c} by making a single base change in the sequence at the same position that this binding site occurs in the *MAL63* promoter. The levels of maltase after growth in glucose show that this change largely restores glucose repression (Table 5).

The lack of a Mig1p binding site in *MAL43*^{c} therefore accounts for its loss of glucose repression, and the C-terminal region of Mal43^{c}p confers constitutivity.

DISCUSSION

Glucose repression and the *MAL* system: The repressive effect of glucose on gene expression plays a central

TABLE 4

Maltase activity of a Δ*MAL63* strain transformed with various *MAL63* alleles

Strains	Plasmid genotypes	Maltase activity (nmPNPG/min/mg protein)		
		Glucose	Maltose	Galactose
A9	*MAL63*	2	1785	24
	MAL63^c	35	1228	1125
	MAL63^c (C419Y)	4	2377	26
	MAL63 (Y419C)	6	1347	20

Transformants contained the indicated *MAL63* alleles on the ARS CEN plasmid pRS315 (SIKORSKI and HIETER 1989). They were inoculated into synthetic media lacking leucine and grown with the indicated carbon sources.

role in the regulation of carbon metabolism in yeast (RONNE 1995). The phenomenon has been most extensively studied for galactose and sucrose catabolism and only superficially for maltose utilization.

This lack of information about the *MAL6* system, while in part due to the greater popularity of the *GAL* and *SUC* systems, is more attributable to the unavailability of *MAL63^c* constitutive mutants. In the absence of *MAL63^c* constitutives it is impossible to assess the effects of glucose on *MAL6* gene expression independently of its effect on inducer uptake—the addition of glucose inactivates the maltose permease, thereby preventing maltose induction.

MAL63^c mutants are therefore critical for understanding glucose repression in particular and maltose regulation in general, but had not previously been isolated. Maltose constitutives have been isolated or found (*MAL43^c*) at other *MAL* loci (NEEDLEMAN and EATON 1974; ZIMMERMAN and EATON 1974; NEEDLEMAN 1975; RODICIO 1986; CHARRON and MICHELS 1987; DUBIN *et al.* 1988). However, no sequence comparisons have been made between inducible and constitutive *MALx3* alleles and, in the case of *MAL43^c*, no comparison is possible given the absence of an appropriate wild type.

Studies of the *MAL* gene system are hindered by the presence of multiple partially active *MAL* gene complexes, and analysis of regulation requires the use of genetically characterized strains. We therefore decided to use the best characterized system, that of *MAL6*, and to isolate constitutive mutants in genetically defined strains. In this we were successful and isolated *MAL63^c* constitutives by selecting for growth on sucrose, which, while not an inducer of the *MAL* system, is transported by one or more maltose inducible permeases (*MAL61, MAL31, MTP1*) and hydrolyzed by maltase.

We first discuss the isolation of the *MAL63^c* constitutive mutants, their origin, and their properties. We then return to discuss their relationship to the glucose insensitive *MAL43^c* allele and the insight they provide on the role of Mig1p in glucose repression.

Isolation and properties of the constitutive mutants: All the constitutive mutants isolated had high levels of maltase when grown on galactose; the one constitutive tested, C38, also had high levels of maltose permease under the same conditions. All constitutive mutants were dominant and sensitive to glucose repression. For reasons given in the text, we believe that 15 of the 17 isolated constitutive mutants were generated by the same recombinational event. The largest category of constitutive mutants is represented by C38, and the two remaining constitutives by D8. C38 and D8 were therefore chosen for further analysis.

The method used to isolate *MAL63^c* mutants was highly strain specific: we were unable recover any *MAL63* constitutives from a *MAL64::LEU2 MAL63 MAL61 MAL62 MAL12* strain (332-5B) but recovered them easily from a *MAL64::LEU2 MAL63 MAL61 MAL62 MTP1 MAL3g* strain (706-6A). A likely reason for this strain specificity was apparent after sequencing of the constitutives. Surprisingly these gain of function mutations occur by the replacement of sequences encoding the C-terminal region of Mal63p with sequences from defective *MALx3* alleles. The difficulty experienced by many laboratories in obtaining *MAL63^c* mutants might therefore be due to the necessity of obtaining multiple mutational changes by conversion of cryptic sequences, and be therefore highly strain dependent (*i.e.*, dependent upon the presence of appropriate ectopic sequences). Constitutives at *MAL1* obtained by reversion of a *mal1* strain also have altered Southern transfer patterns and are therefore likely to have arisen in a similar way (RODICIO 1986).

To identify the possible origin of donor sequences, DNA fragments with homology to the 3′ sequences of two *Mal63^c* mutants were cloned; these were linked to *SGE1* (mutant C38) and, from sequence comparisons with a known *mal33* allele, possibly to sequences at the telomere of chromosome *II* (mutant D8).

D8 is likely derived from *mal33* (chromosome *II*) by simple conversion, but we have not definitively identified its origin or the mechanism of its generation. The nature of *MAL63^c*-C38 is clearer, both in its likely origin and in the mechanism of its generation. In C38, the donor site was unaltered, but changes in *MAL61* and *MAL62* were apparent; ≥5 kb of *MAL61* and *MAL62* was replaced with DNA that includes *SGE1* (chromosome *XVI*, and in our strain chromosome *III*).

After the experiments reported here were completed, we learned of a newly sequenced *MALx3* allele from strain S288C that resides on chromosome *XVI* adjacent to *SGE1*. While the sequence differs greatly from that of *MAL63*, the sequence encoding the C-terminal region differs in only one base pair from that seen in the *MAL63^c* mutants (Figure 5). Many laboratory yeast strains have a pedigree in common with S288C, but 332-5B, the strain from which we were unable to obtain constitutive mutants, is descended from *S. carlsbergensis* (NCYC74) and "wild" strains collected in the former

```
MAL63 Promoter (706-6A)    GGTTC*GCAAT TGATGCATTT GAGAATTCTT TTAACTCAAT AGTAATATGC
MAL43c Promoter (1403-7A)  .....C.... .......... .......... .......... ..........
                          -280
ATTGTTCTTA TCTAAAAAAT TGCAGGTACC TGCAGACGAA TCCGGGTCAT GATCTGCGCT GCGCC*GTCAT
.......... .......... .......... .......T.. .......... .......CG. .....C.....

CCCACCCCGT GCTGCCTGCC TCTTAAAGCT ACCCCGGGTT TAATAATTCG TTCTTTAAGT TCTACAACTT AAATACAGGC
.......... .......... A...G..... ....Δ..... .......... .......... .......... ..........

AGCTAAAAAA CTGGGTTCGA GAGTTTTCCA CTTTACAGAC AAAAATAAAA ATACTGCCAG AAAATTTATC AT
.......... .......... .......... .....T.... .......... .......... .......... ..
                                                                          -8
```

FIGURE 6.—Comparison of the promoters of *MAl63* and *MAL43*^{c}. *, nucleotides missing in the *MAL63* promoter; Δ, nucleotide missing in the *MAL43*^{c} promoter. The Mig1p binding site is underlined.

Soviet Union by Genadi Naumov. In contrast, the pedigree of 706-6A from which constitutives were readily obtained, includes a sucrose negative derivative of S288C. This suggests that the recombination event responsible for generating C38 may have occurred between the *malx3* sequence on chromosome *XVI* (rather than the *mal23*) and *MAL63* on chromosome *VIII*; however, *mal23* can not be excluded as the donor. CHEF gels show that *malx3* alleles are present in strain 706-6A on chromosomes *III* and *XVI*; both must be cryptic because a deletion of *MAL63* makes the strain mal^{-} (data not shown).

Mechanism for the generation of the constitutive mutants—the "mosaic" organization of the telomeres: The five *MAL* genes (*MAL1, MAL2, MAL3, MAL4,* and *MAL6*) are present, respectively, on chromosomes, *VII, III, II, XI,* and *VIII*. The association of other polymeric gene families (*MEL, SUC,* and *PHO*) with the telomere and variation in the location of other telomere-associated sequences like *Y′* and *X* have suggested that the telomeres have evolved by active genetic exchange (CHARRON *et al.* 1989; NAUMOV *et al.* 1990, 1991, 1994; LOUIS *et al.* 1994). Our observation that the telomeric gene *SGE1* is located on two chromosomes lends additional support to the high recombinational activity of the telomeres. LOUIS and HABER (1990a,b) have demonstrated both gene conversion and reciprocal recombination between *Y′* elements present in different chromosomes. An active system for genetic exchange is also suggested by the frequent loss or mutation of *MAL* sequences found at the classical *MAL* loci; in a given strain, these loci often lack *MALx3* function and/or gene sequences, and the sequences present often fail to hybridize to any *MAL* probe (NEEDLEMAN and MICHELS 1983). This absence of *MAL* related sequences at the classical *MAL* alleles suggests either that large scale *MAL* deletions are common and/or that *MAL* sequences were only recently mobilized.

If the telomeres are particularly active sites of recombination, one would expect that *MAL* related sequences would be present on chromosomes not known to contain dominant *MAL* genes. However, an extensive investigation of 30 natural isolates of *S. cerevisiae* and *S. paradoxus* using Southern transfer analysis of CHEF separated chromosomes failed to identify *MAL* homologous sequences on chromosomes other than those known to contain dominant *MAL* genes (NAUMOV *et al.* 1994). In contrast to these findings, we show that our standard strain of *S. cerevisiae* (*carlsbergensis*) has *MALx3* homologous sequences on at least one chromosome lacking a dominant *MAL* gene-the sequence is linked to *COX9* on chromosome *IV*. Of the other cryptic sequences, one is on chromosome *II* (*mal33*) and the other linked to *SGE1* on chromosome *XVI*, or *III*. CHEF gels also show a sequence homologous to *MAL63* on chromosome *XIII* (data not shown). No classical *MAL* genes are found on chromosomes *IV, XIII* or *XVI*, although *MAL* homologous sequences have been recently found on chromosome *XVI*.

Interchromosomal recombination can be conversional or reciprocal; the precise result depends upon the substrate used for recombination. Interchromosomal recombination between two *his3-x his3-y* alleles is 90% conversional and 10% reciprocal (the latter leading to translocation; SUGAWARA and SZOSTAK 1983). However, when 5′ and 3′ truncated *his3* derivatives are tested, 14/15 events were reciprocal translocations (FASULLO and DAVIS 1987). An important question is the natural frequency of chromosomal translocations and

TABLE 5

Effect of the Mig1p binding sites of the *MAL63* and *MAL43*^{c} promoters on glucose repression

Strains	Plasmid genotypes	Maltase activity		
		Glucose	Maltose	Galactose
706-6A		5	1478	8
D8		19	2756	1004
1403-7A		378	1841	1241
A9	*MAL63*	7	1963	62
A9	*MAL63*^{c}-D8	35	1228	1125
A9	*MAL63*, *−Mig1p site*	7	1570	60
A9	*MAL63*^{c}, *−Mig1p site*	312	1963	955
A9	*MAL43*^{c}	777	2550	2478
A9	*MAL43*^{c}, *+Mig1p site*	100	2364	1538

Growth conditions and the plasmid used are described in Table 4. *−Mig1p site* indicates the removal of a Mig1p binding site in the *MAL63* promoter or in the *MAL63*^{c} promoter at −125; *+Mig1p* site indicates the addition of a Mig1p binding site to the *MAL43*^{c} promoter.

gene movement, but all studies using specific recombinational substrates for selection (including ours) are unable to answer this question.

While telomeric translocations are frequently postulated as the origin of high sequence variability of yeast telomeres, our failure to uncover such events suggests that gene conversion may more frequently contribute to the evolution of the *MAL* gene family. It is somewhat surprising to find not one but two unlinked cryptic sequences that can convert *MAL63* to *MAL63*^{c}. While it is impossible to determine whether they derive originally from a *MALx3*^{c} gene or a *malx3* gene, no natural *MALx3*^{c} genes have been described in standard laboratory strains. This is contrary to expectation because the ability to quickly utilize external maltose should be a particular advantage to the "domesticated" yeast strains used in brewing or baking; external maltose is not found in "the wild". This is also inconsistent with suggestions that the *MAL* genes evolved as a response to human activity.

Sequences and function of the MAL63^{c}p alleles: The common core of eight amino acid changes found in the C-terminal region of Mal63p-C38 and Mal63p-D8 might lead to constitutive expression of Mal61p and Mal62p by converting the C-terminal region to a constitutive activating sequence or by "uncovering" a preexisting Mal63p activating sequence. We prefer the later explanation Because "one-hybrid" experiments in which the "constitutive" C-terminal region was fused to a Gal4p binding site failed to activate a gene fused to the *GAL1-10* promoter (J. WANG and R. NEEDLEMAN, unpublished observations).

While it is impossible to assess in these experiments the relative frequencies of gene conversion and induction of single base changes at *MAL63,* it should be noted that the intensive mutagenesis and the strong positive selection for constitutive mutants employed here failed to recover any single mutational changes. Our previous attempts to isolate *MAL63*^{c} mutants by reversion of several point mutants of *mal63* were also unsuccessful. The simplest explanation for this is that inducer binding causes major conformational changes in Mal63p that lead to transcriptional activation, and these changes cannot be mimicked by single amino acid changes. A more interesting speculation is that multiple changes are required due to necessity of selecting for a very precise configuration that does more than simply "unmask" an activation domain. We have recently shown that *TUP1::URA3* Δ*MAL63* strains are constitutive for maltase (*MAL62*) but remain mal^{-} due to low permease (Mal61p) expression (unpublished). Mal63p must increase transcription of *MAL62* at least in part by reversing Tup1p repression, but may activate *MAL61* by a different mechanism. Two "types" of activation may therefore be necessary—one for turning on *MAL61* and another for turning on *MAL62.*

Origin of *MAL43*^{c}: *MAL43*^{c} is the dominant constitutive and glucose repression insensitive allele of *MAL43* found in strain 1403-7A. The genetic basis for these phenotypes and their connection has remained an open question (CHARRON and MICHELS 1987). *MAL43*^{c} was isolated after X-ray exposure so it too may have arisen by X-ray stimulated gene conversion (WINGE and ROBERTS 1950). It should be noted that reversion of certain *cyc1* alleles by the transfer of *CYC7* sequences has been reported and this transfer was also stimulated by mutagenic treatment (ERNST *et al.* 1981, 1982).

Role of Mig1p in glucose repression of *MAL6*: The *MIG1* gene isolated as a multicopy inhibitor of galactose fermentation also inhibits the utilization of sucrose and maltose (NEHLIN and RONNE 1990). Mig1p binds to DNA and regulates glucose repression, possibly by targeting the Cyc8p (Ssn6p)/Tup1p complex to glucose sensitive promoters (TREITEL and CARLSON 1995).

There are four Mig1p binding sites in the *MAL* gene complex, but only three are likely to be relevant for control of *MAL62* (NI 1992; LUNDIN *et al.* 1994); the fourth site lies between the UAS_{MAL} and the transcriptional start site of *MAL61.* Two of these sites are in the *MAL63* promoter; one overlaps the transcriptional start site at −125 (NI 1992), and the other is at −518 and is separated by 2 bp from a Mal63p binding site which we call site 4 (NI 1992). This later site, with the sequence GGAAAAACTGTGGGGAA (NI 1992) fits the definition of the consensus site (LUDLIN *et al.* 1994) but has not been definitively shown to bind Mig1p. The third site overlaps a Mal63p binding site in the *MAL61-62* promoter, binding site 1 (−305 relative to *Mal62*; NI and NEEDLEMAN 1990; SIRENKO *et al.* 1995). Why should the removal of only one of the Mig1p binding sites, the one at −125 in the *MAL63* promoter, lead to such a large relief of glucose repression (Table 5)?

Given the role of the concentration of Gal4p and other yeast activators in glucose repression (CZYZ *et al.* 1993; JOHNSTON *et al.* 1994), it is possible that differences between the promoters of *MAL43*^{c} and *MAL63*^{c} were critical in repression. *MAL* constitutive mutants resistant to glucose repression have been isolated for *MAL2,* and their existence suggests that the MALx3p regulator plays a significant role in glucose repression (ZIMMERMAN and EATON 1974). *MAL43* lacks the Mig1p site seen at the transcriptional start site of *MAL63* (Figure 6). Removal of this site from the promoter of *MAL63*^{c}-D8 leads to a significant release of glucose repression similar to that observed for *MAL43*^{c} (Table 5). Similarly, addition of a Mig1p binding site to the *MAL43*^{c} promoter restores glucose repression to a significant degree (Table 5).

We have recently shown that Mal63p is autoregulated (NI 1992). The location of the Mig1p binding sites in the *MAL63* promoter is suggestive of a direct modulation of Mal63p levels by Mig1p. This would occur by Mig1p preventing binding of Mal63p to site 4 and by Mig1p inhibiting the transcriptional apparatus by bind-

ing at −125 in the *MAL63* promoter. An additional mechanism affecting *MAL62* transcription would be the inhibition of Mal63p binding to site 1 by the adjacent Mig1p binding site. If this is the case, then an increase in the concentration of Mal63p is expected to overcome Mig1p inhibition at sites 1 and 4 but no such competitive mechanism is available for the other Mig1p site (−125) in the *MAL63* promoter. This latter site would therefore be the rate limiting site in the determining the concentration of Mal63p and Mal63^{c}p. High concentrations of activated Mal63p (*i.e.*, either Mal63p + maltose or Mal63^{c}p) would relieve the glucose repression of *MAL62*. However, the relief of glucose repression in *MAL63*^{c}-D8 seen after removal of the Mig1p binding site at −125 is not total, suggesting that control of activator concentration mediated through this site, while a major contributor to glucose repression, is not the only one.

The isolation of *MAL63*^{c} mutants has provided an explanation for the two phenotypes of the *MAL43*^{c} allele. The C-terminal region of MAL43^{c}p confers constitutivity and the lack of a Mig1p binding site in its promoter is responsible for its insensitivity to glucose repression. Glucose repression of *MAL* is conferred to a large degree by the Mig1p modulation of Mal63p levels, similar to the mechanism proposed for *GAL* and *LAC* regulation (CZYZ *et al.* 1993; JOHNSTON *et al.* 1994).

The origin of the *MAL63*^{c} mutants demonstrates that cryptic yeast sequences play a frequently postulated role in evolution—they can be a "storehouse" of genetic variability and can lead to the acquisition of novel phenotypes.

We thank CORINNE MICHELS for the complete unpublished sequence of *MAL43*^{c}, JOE VASQUEZ for performing a preliminary CHEF gel separation, LEON CARLOCK for the CHEF gel apparatus and advice, CRAIG GIROUX, and MAN CHANG for helpful discussions, and MARK JOHNSTON for pointing out the relevance of new sequence information from chromosome *XVI*.

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Communicating editor: M. JOHNSTON