The Constitutive, Glucose-Repression-Insensitive Mutation of the Yeast *MAL4* Locus Is an Alteration of the *MAL43* Gene

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Manuscript received September 29, 1986
Revised copy accepted February 12, 1987

ABSTRACT

Mutations resulting in constitutive production of maltase have been identified at each of the five *MAL* loci of Saccharomyces yeasts. Here we examine a dominant constitutive, glucose-repression-insensitive allele of the *MAL4* locus (*MAL4-C*). Our results demonstrate that *MAL4-C* is an alteration in the *MAL43* gene, which encodes the positive regulator of the *MAL* structural genes, and that its product is *trans*-acting. The *MAL43* gene from the *MAL4-C* strain was cloned and integrated into a series of nonfermenting strains lacking a functional regulatory gene but carrying copies of the maltose permease and maltase structural genes. Expression of the maltase structural gene was both constitutive and insensitive to glucose repression in these transformants. The *MAL4-C* allele also results in constitutive expression of the unlinked *MAL12* gene (encoding maltase) in this strain. In addition, the cloned *MAL43* gene was shown to be dominant to the wild-type *MAL63* gene. We also show that most of the glucose repression insensitivity of strains carrying the *MAL4-C* allele results from alteration of *MAL43*.

FERMENTATION of maltose by Saccharomyces strains utilizes two enzymes: maltose permease and maltase, which cleaves the disaccharide into two molecules of glucose. Synthesis of these enzymes is subject to dual regulatory control: synthesis of both enzymes is induced approximately 30-fold over basal levels by growth in the presence of maltose (DE KROON and KONINGSBERGER 1970; OUWEHAND and VAN WIJK 1972); in the presence of glucose, maltase expression is repressed (catabolite or glucose repression) (VAN WIJK *et al.* 1969; GORTS 1969).

Saccharomyces strains able to ferment maltose contain at least one of five unlinked *MAL* loci (reviewed in BARNETT 1976). Genetic and physical analyses of the *MAL6* locus of *Saccharomyces carlsbergensis* have demonstrated that the locus includes three genes: *MAL61*, encoding maltose permease; *MAL62*, encoding maltase; and *MAL63*, encoding a positive *trans*-acting regulator required for induction of the two structural genes (NEEDLEMAN *et al.* 1984; COHEN *et al.* 1985; DUBIN *et al.* 1985; Y. S. CHANG, R. A. DUBIN, C. A. MICHELS and R. B. NEEDLEMAN, unpublished data). Mutations in *MAL63* result in a maltose nonfermenting phenotype, and such strains are unable to induce the expression of the structural genes above basal levels (TEN BERGE, ZOUTEWELLE and VAN DE POLL 1973; Y. S. CHANG, R. A. DUBIN, C. A. MICHELS and R. B. NEEDLEMAN, unpublished data). Several *MAL6*-linked constitutive mutations were isolated as maltose fermenting revertants of *mal63* mutations (TEN BERGE *et al.* 1973; TEN BERGE, ZOUTEWELLE and NEEDLEMAN 1974; DUBIN *et al.* 1986). All but one of these constitutive revertants are recessive to the wild-type *MAL6* allele and to *mal63* nonfermenting mutations and are sensitive to glucose repression. Surprisingly, detailed genetic analysis of two *MAL6* constitutive revertants revealed that they map to *MAL64*, a gene lying outside the *MAL61-MAL62-MAL63* complex (DUBIN *et al.* 1986).

Constitutive mutations of other *MAL* loci appear to be fundamentally different from those at *MAL6* (WINGE and ROBERTS 1950; KAHN and EATON 1971; NEEDLEMAN and EATON 1974; ZIMMERMAN and EATON 1974). All five *MAL2*-linked constitutive revertants of a *mal2* mutant are dominant to the wild-type allele, and three are glucose repression insensitive (ZIMMERMAN and EATON 1974). Similarly, a constitutive allele of *MAL4* is dominant to the wild type and glucose repression insensitive (KAHN and EATON 1971). The dominant nature of these *MAL2*- and *MAL4*-linked constitutive mutations as well as their resistance to glucose repression implies that their genetic basis is fundamentally different from that of the *MAL6*-linked constitutives (described by TEN BERGE *et al.* 1973; TEN BERGE, ZOUTEWELLE and NEEDLEMAN 1974; DUBIN *et al.* 1986).

In order to investigate the genetic basis of the dominant constitutive, glucose-repression-insensitive type of *MAL* regulatory mutation, we undertook an analysis of the *MAL4*-constitutive strain described by KAHN and EATON (1971). We demonstrate that the alteration(s) leading to both the constitutive and glucose-repression-insensitive phenotypes of this strain lies in the *MAL43* gene. Based on the results presented

here on the functional and structural homology of *MAL43* to the *MAL63* gene, we conclude that *MAL43* encodes a positive *trans*-acting regulatory protein.

MATERIALS AND METHODS

Strains and growth conditions: Yeast strains used in this study are listed in Table 1. Plasmids were propagated in *Escherichia coli* strain RR1. Strain MCY100-3A was derived from the constitutive *MAL4-C* strain 1403-7A originally described by KAHN and EATON (1971). Utilizing the type of genetic analysis described in MICHELS and NEEDLEMAN (1983), the *MAL1g* and *MAL3g* loci were found to be present in 1403-7A and were crossed out of the strain. The *MAL* genotype of MCY100-3A is *MAL4-C mal1*^{0}. In addition to containing the *MAL4*-linked constitutive mutation MCY100-3A also contains a partially functional allele of the *MAL1* locus which has been shown to contain only a functional *MAL12* gene encoding maltase. Strain MCY111-2 was isolated, following selection of Mal$^-$, Ura$^+$, Trp$^-$, Leu$^-$ isolates, from a random spore analysis (DAWES and HARDIE 1974) of diploid MCY111 (MCY100-3AΔ43-1 and 332-5A).

Yeast strains were grown on YEP medium [1% (wt/vol) yeast extract/1% (wt/vol) peptone] plus the indicated amount of a specified carbon source. Maltose fermentation is defined as the production of acid and gas in 1–3 days after inoculation and determined in 5 ml of YEP plus 2% (wt/vol) maltose medium in Durham tubes.

Selection of the *ura3* mutant MCY111-2R4 was performed by the method of BOEKE, LACROUTE and FINK (1984).

Yeast Transformation: Yeast transformation was performed by the method of ITO *et al. (1983)* using lithium acetate. All transformants were screened for the stability of the selective marker by a modification of the method of GRUNSTEIN and HOGNESS (1975) as described by HINNEN, HICKS and FINK (1978) and by passage through non-selective media [YEP medium plus 2% (wt/vol) glucose] to determine whether the plasmid was being maintained in an integrated state or episomally. Only those transformants containing single copy integrants of the plasmid as determined by Southern gel transfer analysis were selected for further analysis.

Measurement of *p*-nitrophenyl-α-D-glucopyranoside (PNPGase) activity: Determination of maltase activity, measured as the rate of release of *p*-nitrophenol from *p*-nitrophenol-α-D-glucopyranoside, was performed by a modification of the method of KAHN and EATON (1967) as described in DUBIN *et al.* (1986).

DNA isolation and analysis: DNA isolation as well as Southern analysis were carried out as previously described (NEEDLEMAN *et al.* 1984).

Gene disruptions and plasmid rescue: For gene disruptions (ROTHSTEIN 1983) and for site-directed integration (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1983), transformants which stably maintained the selective marker were further screened by Southern gel transfer analysis and by standard genetic analysis (MORTIMER and HAWTHORNE 1966).

Gene disruption plasmids were constructed using cloned *MAL6* DNA sequences. Plasmids pDM3 and pRD3 have been previously described and used in the construction of strain A9 (a *MAL63* deletion/disruption) and 332-5A/Δ61/62-9 (a *MAL61/MAL62* deletion/disruption strain) (DUBIN *et al.* 1986; CHARRON, DUBIN and MICHELS 1986; Y. S. CHANG, R. A. DUBIN, C. A. MICHELS and R. B. NEEDLEMAN, unpublished data). These plasmids are used here for the deletion/disruption of *MAL4*-linked sequences. The construction of a *MAL43::URA3* strain utilizing pDM3 is described in the Results. Deletion of *MAL41-MAL42* was carried out using pRD3. Plasmid pRD3 was constructed from *MAL6* sequences by replacing a portion of the 5′ end of the *MAL61* and *MAL62* genes along with the intergenic sequences with the *LEU2* gene as described in DUBIN *et al.* (1986). Because of the homology between the *MAL* loci we can use this plasmid to delete *MAL4* sequences. Plasmid pRD3 (Figure 1) was digested with *PstI* and used to transform strain MCY133-1C. Stable Leu$^+$, maltose non-fermenting transformants were isolated and shown by Southern analysis to have disrupted the 7.6-kb *Hin*dIII fragment of the *MAL4* locus (NEEDLEMAN and MICHELS 1983; data not shown).

Plasmid pY6-RΔC contains a 3.9-kb *Hin*dIII-*Cla*I fragment derived from the *MAL6* locus containing the *MAL61* gene and the 5′ end of the *MAL62* gene cloned into YIp5. This plasmid was integrated at the *MAL4* locus following linearization of the plasmid with *Bgl*II. Plasmid pMJC4B was later isolated following digestion of genomic DNA from the strain MCY100-3A:pY6-RΔC-2 with *Bam*HI according to the method of ORR-WEAVER, SZOSTAK and ROTHSTEIN (1983).

RESULTS

Nomenclature: Our comparative studies involving isolated sequences from the five *MAL* loci (*MAL1, MAL2, MAL3, MAL4* and *MAL6*) indicate that each is organized identically to the *MAL6* locus, as described in NEEDLEMAN *et al.* (1984), CHARRON, DUBIN and MICHELS (1986) and M. J. CHARRON and C. A. MICHELS (unpublished data). In view of the homology to *MAL6* (which extends for approximately 9.0 kb and includes the *MAL61, MAL62* and *MAL63* genes) we have devised a system for naming the homologous genes at each of the loci. Gene 1 encodes maltose permease, gene 2 encodes maltase and gene 3 encodes the positive *trans*-acting regulatory protein. To designate the locus position of the particular gene, the locus number is inserted before the gene number. For example, the gene encoding maltase at the *MAL6* locus is *MAL62*. At the *MAL4* locus, it is the *MAL42* gene. In this way, information regarding both the locus position and the function encoded by a particular gene is given.

Disruption of *MAL43*: Because of the extensive homology among the *MAL* loci, one can utilize *MAL6* sequences to alter *MAL4* chromosomal sequences. Sequences homologous to the *MAL63* gene were deleted from the genomic *MAL4-C* locus using plasmid pDM3 (Figure 1). Plasmid pDM3 was cleaved with *Eco*RI and used to transform the *MAL4* strain MCY100-3A to Ura$^+$. Southern analysis of three transformants indicated disruption of the *MAL43* gene (CHARRON, DUBIN and MICHELS 1986). Genetic analysis of one of these confirmed disruption at the *MAL4-C* locus as follows. Strain MCY111-2 [*MAL43::URA3 mal1*^{0} (see MATERIALS AND METHODS)] was mated to a strain

TABLE 1

List of strains[a]

Strain	(Relevant *MAL*) Genotype	Source
1403-7A	*MATa MAL4-C MAL1g MAL3g trp1 ura3*	Berkeley Stock Center
212-3B	*MATα MAL4-C mal1^0 lys*	R. NEEDLEMAN
MCY100-3A	*MATα MAL4-C mal1^0 lys ura3-52*	This work
MCY133-1D	*MATα MAL4-C mal1^0 trp1*	This work
MCY133-1C	*MATa MAL4-C mal1^0 ura3-52 leu2-3,112 lys*	This work
8-2B	*MATa MAL6-C2 mal1^0 ura3-52 leu2-3,112 trp1 ade*	DUBIN *et al.* (1986)
332-5A	*MATa MAL6 mal1^0 ura3-52 leu2-3,112 trp1 his*	DUBIN *et al.* (1985)
348-1B	*MATa mal63-10 mal1^0 ura3-52 leu2-3,112 trp1*	Y. S. CHANG *et al.* (unpublished data)
340-2B	*MATa MAL1g ura3-52 trp1 lys met*	NEEDLEMAN *et al.* (1984)
347-2A	*MATa mal1^0 ura3-52 trp1 leu2-3,112*	R. NEEDLEMAN
349-6A	*MATa mal1^0 ura3-52 leu2-3,112 trp1*	R. NEEDLEMAN
MCY111-2	*MATa MAL43::URA3 mal1^0 leu2-3,112 trp1*	This work
A9	*MATa MAL63::URA3 mal1^0 leu2-3,112 trp1 his*	Y. S. CHANG *et al.* (unpublished data)
332-5A/Δ61/62-9	*MATa MAL61/62::LEU2 mal1^0 ura3-52 trp1 his*	DUBIN *et al.* (1986)
MCY100-2C	*MATα MAL4-C mal1^0 ura3-52 ade leu2-3,112*	This work
328-4A	*MATα mal1^0 ura3-52 trp1 ade*	NEEDLEMAN *et al.* (1984)
1-31	*MATa MAL1p met*	R. NEEDLEMAN
345-4A	*MATa MAL1p ura3-52 trp1 ade leu2-3,112*	NEEDLEMAN *et al.* (1984)

[a] Yeast strains described in the text as well as those used in genetic analysis.

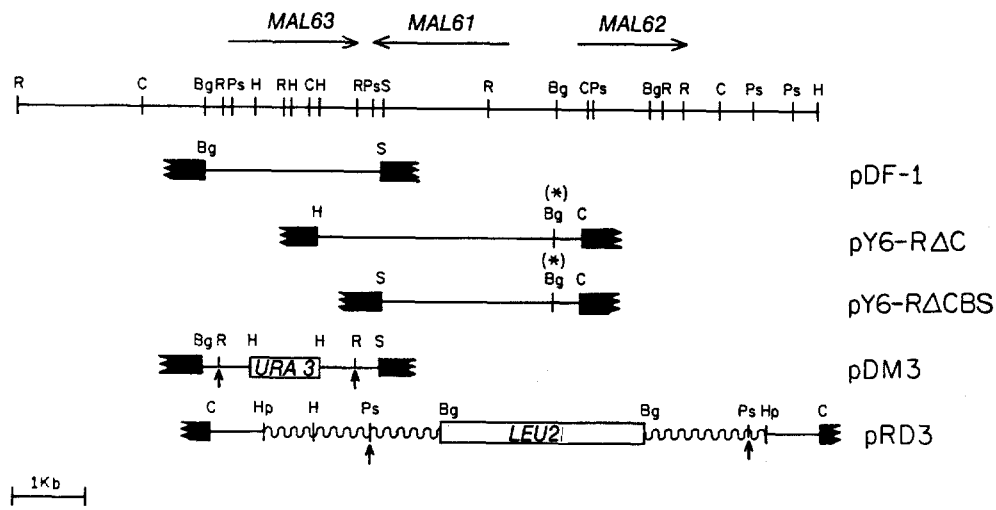

FIGURE 1.—Restriction endonuclease map of *MAL6*-derived plasmids used for deletion/disruption of the *MAL4* locus. A partial restriction map of *MAL6* and the location of *MAL61*, *MAL62* and *MAL63* is shown. Plasmid pDF-1 contains the *Bgl*II–*Sal*I fragment subcloned into pLC544 (NEEDLEMAN *et al.* 1984). Plasmids pY6-RΔC and pY6-RΔCBS contain the indicated regions subcloned into YIp5. Plasmid pY6-RΔCBS was formed by digesting plasmid pY6-RΔC with *Bam*HI and *Sal*I. The ends were filled-in with T4 DNA Polymerase prior to self-ligation and plasmid pY6-RΔCBS was isolated following transformation of *E. coli* strain RR1. Disruption plasmids pDM3 and pRD3 (described in MATERIALS AND METHODS) were digested with the indicated restriction endonucleases (↑) prior to transformation. Wavey lines indicate DNA sequences derived from the *MAL6* constitutive strain 8-2B (DUBIN *et al.* 1986). The symbol (*) is used to indicate the site of linearization of plasmids pY6-RΔC and pY6-RΔCBS. Recognition sites of restriction endonucleases are abbreviated as follows: Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; Ps, *Pst*I; R, *Eco*RI and S, *Sal*I.

(MCY100-3A:Y6-RΔC-2) that carries an integrated *URA3* gene at the *MAL4* locus (see below). The 4:0 segregation of Ura$^+$ to Ura$^-$ confirmed disruption at the *MAL4* locus. All three isolates are unable to ferment maltose, and one of these, MCY100-3AΔ43-1, neither constitutively expresses nor induces the synthesis of maltase (Table 2). The disruption mutation is therefore epistatic to the parental *MAL4-C* allele. This contrasts to the situation at the *MAL6* locus, where deletion of *MAL63* in *MAL6*-constitutive strains has no effect on the ability to constitutively express the maltose fermentative enzymes (DUBIN *et al.* 1986).

Disruption strain MCY100-3AΔ43-1 was mated to strains carrying the naturally occurring partially functional alleles of the *MAL1* locus: *MAL1g*, *MAL1p* and *mal1^0*. A complete structural and functional analysis of these *MAL1* alleles has been carried out (M. J. CHARRON and C. A. MICHELS, unpublished data). For the purposes of this study, it is sufficient to state that: the *MAL1p* allele encodes a functional activator but

TABLE 2

Maltase synthesis in *MAL43* and *MAL63* deletion strains transformed with the *MAL63* gene[a]

Host strain	*MAL genotype*	*Integrated plasmid (site)*	*Plasmid MAL* gene	Maltase activity (nM PNPG/min/mg protein) 2% Galactose grown	2% Maltose grown	2% Maltose/ 5% glucose grown
MCY100-3A	*MAL4-C mal1^0*			338	282	102
MCY100-3A-Δ43-1	*MAL43::URA3*			9	2	0
MCY111-2	*MAL43::URA3 mal1^0*			1	4	0
MCY111-2	*MAL43::URA3 mal1^0*	pDF-1 (*TRP1*)	*MAL63*	2	304	17
MCY111-2	*MAL43::URA3 mal1^0*	pLC544 (*TRP1*)		1	4	0
332-5A	*MAL6 mal1^0*			5	249	0
A9	*MAL63::URA3 mal1^0*			2	2	0
A9	*MAL63::URA3 mal1^0*	pDF-1 (*TRP1*)	*MAL63*	7	357	6
A9	*MAL63::URA3 mal1^0*	pLC544 (*TRP1*)		3	2	0
MCY133-1D	*MAL4-C mal1^0*			178	348	57
MCY133-1D	*MAL4-C mal1^0*	pDF-1 (*TRP1*)	*MAL63*	226	387	42
MCY133-1D	*MAL4-C mal1^0*	pLC544 (*TRP1*)		442	355	58

[a] Cells were pregrown in uninducing medium (YEP plus 2% galactose), inducing medium (YEP plus 2% maltose) or repressing medium (YEP plus 2% maltose/5% glucose), diluted into fresh YEP medium containing the indicated sugar additions and allowed to grow 8–9 hr (mid to late log phase). Maltase activity is determined by measuring the rate of hydrolysis of PNPG and is expressed as nmoles substrate split/min/mg protein at 30°. For these experiments and all others requiring a wild-type allele of the regulatory gene, we used plasmid pDF-1 (Figure 1). All transformants were shown by Southern analysis to contain a single integrated copy of the plasmid.

lacks functional structural genes for maltose fermentation; the *MAL1g* allele encodes functional structural genes but lacks the activator; and the *mal1^0* allele encodes only functional maltase (DUBIN *et al.* 1985; CHARRON, DUBIN and MICHELS 1986). The *MAL43* disruption mutation was complemented in strains carrying the *MAL1p* but not *mal1^0* or *MAL1g* alleles indicating that only the regulatory function present at *MAL4* had been altered by the disruption.

Transformation of MCY111-2 with the *MAL63* plasmid pDF-1 (Figure 1) (NEEDLEMAN *et al.* 1984), targeted to integrate at *TRP1*, restores the ability to ferment maltose, while the vector alone, pLC544, does not (CLARKE and CARBON 1980). While pDF-1 restores the ability to ferment, the transformant is inducible for maltase (Tables 2 and 3). For comparison purposes, the *MAL63* plasmid pDF-1 was inserted into a *MAL6* strain carrying a deletion of the *MAL63* gene (strain A9). Maltase synthesis in the resulting transformants is inducible (see Table 2, line 8). Taken together the results described above strongly suggest that the constitutive mutation lies in the *MAL43* gene. (A low but reproducible glucose insensitive synthesis of maltase is seen in maltose plus glucose grown *MAL63*-transformed cells. The significance of this is not clear.)

The undisrupted *MAL4-C mal1^0* strain MCY133-1D was also transformed with a single integrated copy of plasmid pDF-1 containing the *MAL63* gene. The resulting strain is constitutive for the production of maltase and insensitive to glucose repression, thus confirming the dominant nature of this *MAL4*-linked mutation (Table 2).

Cloning *MAL41* and *MAL43*: The possibility that the *MAL43* gene interacts with additional components in this strain to produce the constitutive phenotype could not be ruled out. To clarify this issue and to localize the mutation within *MAL4*, the *MAL43* gene was isolated from the *MAL4-C* mutant strain MCY100-3A. This was done by integrating a selectable plasmid at the *MAL4* locus and recovering this plasmid from the genome along with its flanking *MAL4* DNA. Based upon the demonstrated homology between the 7.3-kb *Hin*dIII fragment of the *MAL6* locus and that of the 7.6-kb *Hin*dIII fragment of the *MAL4-C* constitutive mutant, a subclone of the *MAL6* locus (see pY6-RΔC in Figure 1) was used to direct the integration of a yeast selectable plasmid to the *MAL4* locus of constitutive strain MCY100-3A (NEEDLEMAN and MICHELS 1983; M. J. CHARRON and C. A. MICHELS, unpublished results). Plasmid pY6-RΔC was directed to integrate at *MAL4* by digesting the plasmid with *Bgl*II (see (*) in Figure 1) prior to transformation. Integration at *MAL4* was confirmed for one Ura$^+$ transformant (strain MCY100-3A:Y6-RΔC-2) by Southern analysis. In strain MCY100-3A:Y6-RΔC-2 a 7.6-kb *Hin*dIII fragment having homology to *MAL6* structural gene sequences and shown to be linked to *MAL4* is altered in size in a way consistent with the integration of the plasmid at the *MAL4* locus (data not shown). Plasmid pMJC4B and flanking *MAL4* DNA (Figure 2) was then recovered from the genome of MCY100-3A:pY6-RΔC-2 as described in MATERIALS AND METHODS.

Restriction enzyme mapping of this cloned *MAL4* region showed that it is similar to the *MAL61* and

TABLE 3

Maltase synthesis in strains transformed with the cloned *MAL43* gene of the *MAL4-C* strain[a]

Host strain	*MAL* genotype	Integrated plasmid (site)	Plasmid *MAL* gene	Maltase activity (nM PNPG/min/mg protein) 2% Galactose grown	2% Maltose grown	2% Maltose/ 5% glucose grown
MCY100-3A	*MAL4-C mal1^0*			338	282	102
MCY111-2	*MAL43::URA3 mal1^0*			1	4	0
MCY111-2	*MAL43::URA3 mal1^0*	pM43BS (ND)	*MAL43-C*	176	267	57
MCY111-2	*MAL43::URA3 mal1^0*	pLC544 (*TRP1*)		1	4	0
MCY111-2	*MAL43::URA3 mal1^0*	pM43S (*Ty*)	*MAL43-C*	211	317	63
MCY111-2	*MAL43::URA3 mal1^0*	YEp13ΔS (*Ty*)		1	2	0
MCY133-1D	*MAL4-C mal1^0*			178	348	57
MCY133-1D	*MAL4-C mal1^0*	pM43BS (*TRP1*)	*MAL43-C*	360	283	85
MCY133-1D	*MAL4-C mal1^0*	pLC544 (*TRP1*)		442	355	58
332-5A	*MAL6 mal1^0*			5	249	0
A9	*MAL63::URA3 mal1^0*			2	2	0
A9	*MAL63::URA3 mal1^0*	pM43S (*Ty*)	*MAL43-C*	382	422	100
A9	*MAL63::URA3 mal1^0*	YEp13ΔS (*Ty*)		4	6	0
348-1B	*mal63-10 mal1^0*			10	3	1
348-1B	*mal63-10 mal1^0*	pM43BS (ND)	*MAL43-C*	188	339	80
348-1B	*mal63-10 mal1^0*	pDF-1 (*TRP1*)	*MAL63*	8	210	2
348-1B	*mal63-10 mal1^0*	pLC544 (*TRP1*)		10	1	1
340-2B	*MAL1g*			1	2	2
340-2B	*MAL1g*	pM43BS (ND)	*MAL43-C*	170	204	60
340-2B	*MAL1g*	pDF-1 (*TRP1*)	*MAL63*	16	324	2
340-2B	*MAL1g*	pLC544 (*TRP1*)		15	3	0

[a] Growth and assay conditions were performed as described in Table 2. All transformants were shown by Southern analysis to contain a single integrated copy of the plasmid. In most cases, two independent transformants were screened and both gave similar results. The variability seen in the degree of glucose repression insensitivity and in the basal levels of activity of maltase appear to result from differences in genetic background. Strains MCY111-2 and A9 are *MAL4-C* and *MAL6* strains in which the linked regulatory gene, *MAL43* and *MAL63*, has been deletion/disrupted (Y. S. CHANG, R. A. DUBIN, C. A. MICHELS and R. B. NEEDLEMAN, unpublished data). Strain 348-1B is a *MAL6* strain carrying a point mutation in the *MAL63* gene (TEN BERGE, ZOUTEWELLE and VAN DE POLL 1973; Y. S. CHANG, R. A. DUBIN, C. A. MICHELS and R. B. NEEDLEMAN, unpublished data). Strain 340-2B contains the partially functional *MAL1g* allele encoding the *MAL* structural genes only (M. J. CHARRON and C. A. MICHELS, unpublished data). Plasmid pM43S was digested with *Bgl*II which opens the plasmid in the Ty element flanking the *LEU2* gene and directs integration to a Ty (ROEDER 1983). Digestion of plasmids pM43BS and pDF-1 with *Bgl*II was used to target integration to the *TRP1* gene (CLARKE and CARBON 1980). Digestion of plasmid pM43BS with *Xho*I, which cuts at a site flanking *MAL43* to the left, directs integration to this chromosomal site in *MAL4* containing strains. In other strains, such as *MAL6* or *MAL1g* strains, this *MAL4* chromosomal region is presumably lacking. Nonetheless, integration does occur at a reasonable rate implying some homologous sequences are present in these strains. In these cases, the exact site of integration is not known.

MAL63 genes of strain CB11 except for a few restriction site polymorphisms. The restriction map shown in Figure 2 is similar to the map of a DNA fragment from a *MAL4* constitutive strain isolated by RODICIO and ZIMMERMAN (1985). Hybridization between cloned DNA derived from the *MAL6* locus of CB11 and the *MAL4* locus of strain MCY100-3A confirmed the sequence homology between the two loci and enabled us to localize the *MAL43* and *MAL41* genes (see Figure 2).

Alteration(s) causing both the constitutivity and glucose repression insensitivity lie in *MAL43*: To determine if the *MAL43* gene cloned from the constitutive mutant is sufficient by itself to confer both the constitutive and the glucose-repression-insensitive phenotypes we constructed plasmids (pM43BS and pM43S) which contain only the *MAL43* gene (Figure 2).

The plasmid pM43BS or pM43S was introduced into a series of strains carrying the structural genes encoding maltose permease and maltase but lacking a functional positive regulator. Prior to transformation, the plasmid DNA was linearized in order to direct the integration of the plasmid near *LEU2*, *TRP1*, or *MAL4*. Several integrative transformants of each type were screened by Southern analysis and only strains carrying a single copy of the plasmid were analyzed further. Table 3 shows the level of maltase activity of the various strains and transformants following growth under uninduced, induced and glucose repressed conditions. Maltase synthesis is fully constitutive and at least partially glucose-repression-insensitive in each of the *MAL43-C* transformants (Table 3, lines 8, 12, 15 and 19). On the other hand, transformation of each of the strains with the isolated wild-type allele of *MAL63*, leads to inducible maltase synthesis (Tables 2 and 3). Thus the isolated *MAL43* gene from the *MAL4*-linked constitutive strain is sufficient to confer

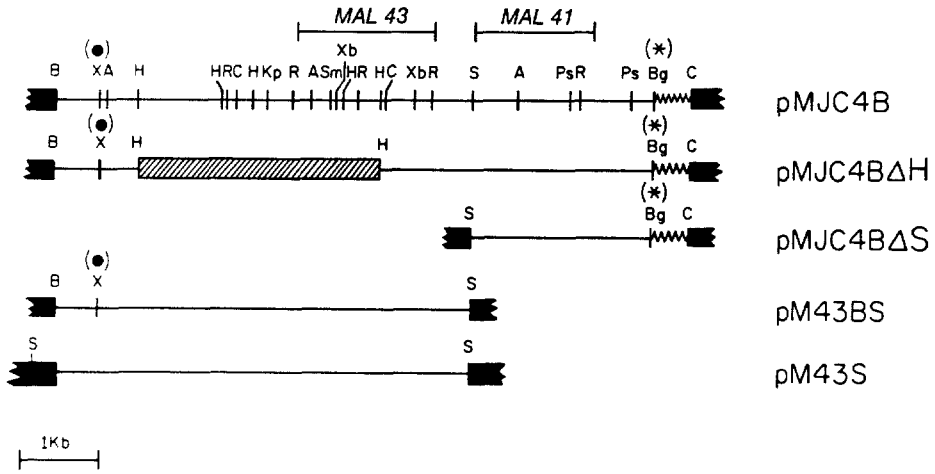

FIGURE 2.—Restriction endonuclease map of part of the *MAL4* locus. The approximate location of the *MAL41* and *MAL43* genes are indicated above the restriction map. Plasmid pMJC4B was isolated as described in MATERIALS AND METHODS. Plasmids pMJC4BΔH and pMJC4BΔS were isolated after digestion of plasmid pMJC4B with *Hin*dIII and *Sal*I, respectively, followed by self-ligation. Plasmid pM43BS contains the 6.1-kb *Bam*HI–*Sal*I fragment subcloned into pLC544 and plasmid pM43S contains the 6.4-kb *Sal*I fragment of plasmid pMJC4B subcloned into YEp13Δ*Sal*I. Jagged lines indicate sequences derived from the *MAL6* locus. The symbols (*) and (●) are used to indicate the site of linearization of various plasmids used in yeast transformations (described in text). Recognition sites for restriction endonucleases are abbreviated as in Figure 1 with the following additions: A, *Ava*I; B, *Bam*HI; Kp, *Kpn*I; SM, *Sma*I; X, *Xho*I and Xb, *Xba*I.

both constitutivity and glucose repression insensitivity. In addition, the *MAL43* gene product is clearly *trans*-acting.

As further proof of the *trans*-acting nature of this constitutive and glucose-repression-insensitive *MAL43* mutant allele, we attempted to determine whether or not the *MAL12* gene in strain MCY133-1C is also constitutively expressed, as was observed in the *MAL64* constitutive mutants (DUBIN *et al.* 1986). Additionally, we wished to confirm that the glucose repression insensitivity is also *trans*-acting. For this, the *MAL41* and *MAL42* genes were deleted from strain MCY133-1C utilizing plasmid pRD3 (see MATERIALS AND METHODS). One transformant selected for further analysis (strain MCY133-1C/Δ41/42-8) was shown to carry the *MAL41/42::LEU2* disruption. As can be seen by the results reported in Table 4, deletion of the *MAL41* and *MAL42* genes produces a nonfermenter with reduced, but significant levels of constitutively expressed maltase. Strain MCY133-1C/Δ41/42-8 was then mated to the *MAL63::URA3* disruption strain A9 and to the *MAL43::URA3* disruption strain MCY111-2. Both diploids ferment maltose and constitutively produce high levels of maltase. In addition, they are glucose repression insensitive (Table 4). In summary, these results clearly demonstrate that both constitutivity and glucose repression insensitivity at the *MAL4* locus are *trans*-acting functions.

Analysis of the *MAL43* flanking DNA sequences: To rule out the possibility that sequences outside of the *MAL43* gene contribute to the *MAL4-C* phenotype, the entire *MAL41* gene including flanking sequences was inserted into a *MAL6* strain deleted for sequences upstream of both *MAL61* and *MAL62* [using plasmid pRD3; see Figure 1 and DUBIN *et al.* (1986)]. This strain (332-5A/Δ61/62-9) therefore contains an intact copy of the *MAL63* gene and a functional *MAL12* gene (encoding maltase) and is a nonfermenter because it lacks functional maltose permease.

Strain 332-5A/Δ61/62-9 was transformed separately with three different plasmids: pMJC4BΔH, containing the *MAL41* gene, the *MAL41–MAL42* intergenic region (and a portion of the 5′ end of *MAL62*) (Figure 2); plasmid pMJC4BΔS, containing the *MAL41* gene, the *MAL41–MAL42* intergenic region (and a portion of the 5′ end of *MAL62*) (Figure 2); and plasmid pY6-RΔCBS, containing the *MAL61* gene, the *MAL61–MAL62* intergenic region (and a portion of the 5′ end of *MAL62*) (see Figure 1). In each case, the plasmid was directed to integrate at the *MAL12* gene, which was confirmed by Southern analysis. Each of the above plasmids encodes maltose permease (*MAL41* or *MAL61*) and, as expected, all transformants are maltose fermenters. One can now ask, is the expression of *MAL12* inducible or constitutive? Maltase synthesis in three independent transformants containing the *MAL41* gene and flanking sequences is fully inducible, as is also found for two independent transformants containing the *MAL61* gene and its flanking sequences. This indicates that the constitutive phenotype is mediated by the *MAL43* gene alone with little or no contribution from structural gene upstream sequences. This is again confirmed by the results shown in the last two lines of Table 5. Plasmid pMJC4B, containing the *MAL43-C* gene, the *MAL41* gene and sequences extending into the *MAL42* gene was transformed into strain 332-5A/Δ61/62-9 and integrated at either the *MAL12* gene

TABLE 4

Maltase synthesis in strains containing the *MAL41/42* deletion disruption[a]

Strain	*MAL* genotype	Maltase activity (nM PNPG/min/mg protein)		
		2% Galactose grown	2% Maltose grown	2% Maltose/5% glucose grown
MCY133-1C	*MAL4-C mal1^0*	365	426	76
MCY133-1C/Δ41/42-8	*MAL41/42::LEU2 mal1^0*	209	226	31
A9	*MAL63::URA3 mal1^0*	2	2	0
MCY111-2	*MAL43-C::URA3 mal1^0*	1	4	0
MCY111-2 × MCY133-1C/Δ41/42-8	*MAL41 MAL42 MAL43-C::URA3 mal1^0* / *MAL41/42::LEU2 MAL43-C m*	393	583	95
MCY133-1C/Δ41/42-8 × A9	*MAL41/42::LEU2 MAL43-C MAL6ϕ mal1^0* / *MAL4ϕ MAL61 MAL62 MAL63::URA3 mal1^0*	343	582	82

[a] Growth and assay conditions were carried out as described in Table 2. Construction of strain 332-5A/Δ61/62-9 is described in DUBIN *et al.* (1986).

TABLE 5

Maltase synthesis in the *MAL61/62* deletion disruption strain 332-5A/Δ61/62-9 transformed with *MAL41*, *MAL61* and *MAL41 MAL43-C* containing plasmids[a]

Host strain	*MAL genotype*	*Integrated plasmid (site)*	*Plasmid MAL* gene	Maltase activity (nM PNPG/min/mg protein)		
				2% Galactose grown	2% Maltose grown	2% Maltose/5% glucose grown
332-5A	*MAL6 mal1^0*			5	249	0
332-5A/Δ61/62-9	*MAL61/62::LEU2 mal1^0*			2	7	0
332-5A/Δ61/62-9	*MAL61/62::LEU2 mal1^0*	pMJC4BΔH (*mal1^0*)	*MAL41*	4	357	2
332-5A/Δ61/62-9	*MAL61/62::LEU2 mal1^0*	pMJC4BΔS (*mal1^0*)	*MAL41*	4	150	8
332-5A/Δ61/62-9	*MAL61/62::LEU2 mal1^0*	pY6-RΔCBS (*mal1^0*)	*MAL61*	14	247	0
332-5A/Δ61/62-9	*MAL61/62::LEU2 mal1^0*	pMJC4B (*mal1^0*)	*MAL41 MAL43-C*	332	315	74
332-5A/Δ61/62-9	*MAL61/62::LEU2 mal1^0*	pMJC4B (ND[b])	*MAL41 MAL43-C*	170	300	28

[a] Growth and assay conditions were performed as described in Table 2.
[b] ND = site of integration not determined; unlinked to *MAL1* or *MAL4*.

of the *mal1^0* locus or at an undetermined site unlinked to either *MAL1* or *MAL4* (by linearization at the *Xho*I site in the DNA sequence flanking *MAL43* to the left). In both sets of transformants, the strains are constitutive and glucose-repression-insensitive. These results clearly indicate that the glucose-repression-insensitive phenotype of the *MAL4-C* mutant is mediated by the *MAL43-C* allele. Since these transformants contain both the *MAL43-C* gene and the genomic *MAL63* gene, these results also confirm that the mutation(s) in the *MAL43-C* gene is (are) dominant and *trans*-acting.

DISCUSSION

KAHN and EATON (1971) described a yeast strain carrying an allele of the *MAL4* locus causing constitutive *MAL* gene expression. They showed the constitutive phenotype to be dominant to the wild-type inducible glucose repression sensitive phenotype, with both traits being tightly linked. The origin of this *MAL4-C* mutation is not clear but other reports indicate that it was originally isolated by WINGE and ROBERTS (1950) either spontaneously or by x-ray mutagenesis. Nevertheless, the dominant constitutivity and glucose repression insensitivity of this mutation make it important for us to understand the genetic basis of these phenotypes in order to understand more fully the mechanisms controlling maltose fermentation in Saccharomyces.

For this, we isolated DNA sequences from the *MAL4* locus extending from the coding region of the *MAL42* gene to the DNA sequences flanking *MAL43* [including both *MAL41* and *MAL43* (Figure 2)]. Using these segments in a variety of strain constructions, we demonstrate the following: 1) The alteration(s) in this *MAL4-C* locus which lead to the constitutive phenotype clearly map to the *MAL43* gene (encoding the *MAL4*-linked positive *trans*-acting regulatory protein

homologous to the *MAL63* gene of the *MAL6* locus). Preliminary results of similar experiments performed on a *MAL2*-linked dominant, constitutive mutant are in agreement with those described above (M. J. CHARRON and C. A. MICHELS, unpublished results). In addition, an alteration(s) responsible for the glucose repression insensitivity of this strain maps to *MAL43*. 2) Both the constitutive and the glucose-repression-insensitive phenotypes caused by this *MAL43-C* mutation are dominant to the wild-type *MAL63* allele. 3) The mutation is *trans*-acting. Our results do not exclude the possibility that additional mutations at this *MAL4-C* locus also could be contributing to the glucose repression insensitivity but the results do not support this possibility.

These results are in contrast to those of DUBIN *et al.* (1986) regarding the *MAL6*-linked constitutive mutations. In their study, two constitutive revertants of *mal63* mutations were analyzed in detail and shown to map to a gene, called *MAL64*, which lies 2.3 centamorgans to the left of *MAL63* and not to *MAL63* itself which encodes the positive regulator of maltose fermentation. These and other studies have shown that constitutive mutations in *MAL64* are recessive to the wild-type *MAL64* allele and to various *mal63* mutant alleles and that they are glucose repression sensitive (TEN BERGE *et al.* 1973; TEN BERGE, ZOUTEWELLE and NEEDLEMAN (1974). Clearly, the genetic basis of the constitutive mutations at *MAL6* is different from that found here for the *MAL4-C* constitutive allele.

MAL4-C mutants are fully constitutive but only partly insensitive to glucose repression. It is not clear whether the partial insensitivity to glucose repression is a characteristic of this particular mutant allele of *MAL43* or if the glucose repression of maltase synthesis is mediated by a number of independent systems. Genetic analysis indicates that three control circuits are involved in the glucose repression of the galactose fermentative enzymes (MATSUMOTO, YOSHIMATSU and OSHIMA 1983). We feel that glucose may have similar multiple pathways in controlling maltose fermentation.

Identification of dominant constitutive mutations in the *MAL43* gene is not surprising. The product of the *MAL63* gene, and therefore, by homology, the product of the *MAL43* gene, is a positive regulator controlling the expression of the maltose fermentative enzymes (Y. S. CHANG, R. A. DUBIN, C. A. MICHELS and R. B. NEEDLEMAN, unpublished data). Similar dominant constitutive mutations have been reported in other yeast positive regulators. DOUGLAS and HAWTHORNE (1966) and MATSUMOTO *et al.* (1980) describe extensive genetic analyses of mutations in the *GAL4* gene, encoding the positive activator controlling the synthesis of the galactose fermentative enzymes. Dominant constitutive mutations were obtained, many by reverting noninducible *gal4* mutations. None of the *GAL4*-constitutive mutations described show resistance to glucose repression. The glucose-repression-insensitive phenotype of the *MAL43-C* mutation described here is therefore unique and makes the detailed genetic analysis of this mutation, as well as the *MAL2*-linked constitutive glucose-repression-insensitive mutations described by ZIMMERMAN and EATON (1974) important for any analysis of the mechanisms of glucose repression of the maltose fermentative enzymes in yeast. It is interesting to note that sequence analysis of a dominant mutation of the *ADR1* gene, which encodes a positive regulator controlling the glucose sensitive expression of *ADH2*, has found the alteration to lie within a putative recognition site for a cyclic AMP-dependent kinase (CIRIACY 1979; DENIS and GALLO 1986). Analysis of the sequence of the *MAL* regulatory genes is underway in this laboratory and should prove quite fruitful.

One of the most interesting issues raised by the results reported here is why *MAL64*-like constitutives are not obtained at *MAL4*. Comparisons by Southern analysis between the cloned *MAL4* and *MAL6* loci including several kb of flanking DNA sequences, show that the homology is limited to the approximately 9.0-kb region containing the structural genes and the activator (*MAL61-62-63* and *MAL41-42-43*) (M. J. CHARRON and C. A. MICHELS, unpublished data). Thus, a gene equivalent to *MAL64* does not appear to be present at the *MAL4* locus. The *MAL64* gene has been localized to a site about 3.0 kb from *MAL63* in a region showing significant sequence homology to *MAL63* and, like *MAL63*, *MAL64*-constitutive mutants encode an activator of *MAL* structural gene transcription (R. A. DUBIN, M. J. CHARRON, R. B. NEEDLEMAN and C. A. MICHELS, unpublished data). Obtaining dominant constitutive mutations similar to *MAL43-C* in genes encoding activators is not surprising. What is unusual is that constitutive mutations in *MAL63* have not yet been isolated. A comparison of the isolated *MAL63*, *MAL64* and *MAL64-C* sequences should give some insight into the reason for the absence of this class of mutations.

We wish to thank RICHARD B. NEEDLEMAN for generously providing yeast strains and for critical reading of this manuscript. We also wish to thank GEORGE BAURIES for assisting in tetrad dissections and SYLVIA SCHAFFEL for secretarial assistance.

This work was supported by the National Institutes of Health grant GM28216 to C.A.M. Additional support was obtained from the Board of Higher Education of the City University of New York-Professional Staff Congress Research Award Program.

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Communicating editor: I. HERSKOWITZ