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Alterations in the *Saccharomyces MAL*-activator cause constitutivity but can be suppressed by intragenic mutations

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Abstract The *Saccharomyces MAL*-activator regulates the maltose-inducible expression of the *MAL* structural genes encoding maltose permease and maltase. Constitutive *MAL*-activator mutant alleles of two types were identified. The first were truncation mutations deleting C-terminal residues 283–470 and the second contained a large number of alterations compared to inducible alleles scattered throughout the C-terminal 200 residues. We used site-directed in vitro mutagenesis of the inducible *MAL63* and *MAL63/23* genes to identify the residues responsible for the negative regulatory function of the C-terminal domain. Intragenic suppressors that restored the inducible phenotype to the constitutive mutants were identified at closely linked and more distant sites within the *MAL*-activator protein. *MAL63/mal64* fusions of the truncated mutants suggest that residues in the N-terminal 100 residues containing the DNA-binding domain also modulate basal expression. Moreover, a transcription activator protein consisting of LexA(1–87)-Gal4(768–881)-Mal63(200–470) allowed constitutive reporter gene expression, suggesting that the C-terminal regulatory domain is not sufficient for maltose-inducible control of this heterologous activation domain. These results suggest that complex and very specific intramolecular protein–protein interactions regulate the *MAL*-activator.

Key words *MAL*-activator · Constitutive mutations · Maltose fermentation · *Saccharomyces*

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Introduction

Maltose induction of the *Saccharomyces MAL* structural genes encoding maltose permease and maltase is mediated by the *MAL*-activator, a DNA-binding transcription activator (reviewed in Needleman 1991; Hu et al. 1999). *MAL63*, the *MAL*-activator gene of the *MAL6* locus, encodes a predicted 470-residue protein (Kim and Michels 1988; Sollitti and Marmur 1988). A cysteine-rich DNA-binding domain similar to the Gal4p DNA-binding domain and capable of sequence-specific binding to the UAS_{MAL} is found in the N-terminal 100 residues (Hu et al. 1999). Residues 60–282 are sufficient for high levels of transcription activation when fused to LexA residues 1–83 and no other activation domains are present (Hu et al. 1999). Genetic evidence suggests that the C-terminal approximately 200 residues contain negative regulatory sequences and this region of the protein responds to the presence of maltose.

To date, constitutive mutations have been isolated at all of the *MAL* loci (Winge and Roberts 1950; Kahn and Eaton 1971; ten Berge et al. 1973; Zimmerman and Eaton 1974; Dubin et al. 1986, 1988; Rodicio 1986; Wang and Needleman 1996). Of those that have been characterized, all contain alterations in the *MAL*-activator (Charron and Michels 1987; Wang and Needleman 1996; Gibson et al. 1997). C-terminal truncation mutations of the *MAL63* homologue *mal64* at codons 282 and 307 produce constitutive activators (Gibson et al. 1997). Activators encoded by constitutive alleles of *MAL23* and *MAL43*, *MAL*-activator genes at the *MAL2* and *MAL4* loci respectively, contain multiple alterations in the C-terminal region, compared to the inducible *MAL63* (Gibson et al. 1997). In *MAL43-C*, these 27 variant residues are distributed throughout the C-terminal region. Gibson et al. (1997) were unable to localize the alterations essential for the constitutive phenotype to smaller subdomains. Higgins et al. (1999) reported the sequence of three *MAL*-activator alleles isolated from an industrial baking strain. One of these

alleles is constitutive and differs from inducible Mal63p at multiple sites throughout the coding sequence. Hybrid constructions made with *MAL63* suggest that the effects of these alterations are additive and no single change or cluster of changes is sufficient to cause constitutivity, although certain residues were found to make a more significant contribution. Taken together, these results, along with those described in Hu et al. (1999), argue against the existence of a *MAL*-specific negative regulator, similar to Gal80p, that inhibits *MAL*-activator activity in the absence of maltose. Instead, it has been suggested that the *MAL*-activator is autoregulatory and undergoes a conformational change induced by the presence of maltose.

In contrast, Wang and Needleman (1996) isolated a constitutive *MAL63* allele that differs from Mal63p at eight C-terminal residues. These same eight changes can be found in the constitutive *MAL23-C* and *MAL43-C* alleles, but multiple additional alterations are also present. Surprisingly, the inducible *MAL23* allele also contains these same eight C-terminal changes plus 15 additional alterations mapping throughout the open reading frame (ORF; Gibson et al. 1997). This suggests that the effect of one or more of the additional 15 differences between Mal23p and Mal63p is responsible for suppressing the constitutive phenotype of the C-terminal mutations. In this report we explore the possibility of intragenic suppression of *MAL*-activator constitutive mutations. Our results identify residues important for maltose-regulated transcription activation. Some of these residues are clustered, particularly in the C-terminal region of the protein, but residues throughout the protein were found to modulate activity and the response to inducer.

Materials and methods

Yeast strains

Strain YPH500 (*MATα AGT1 MAL12 mal13Δ MAL32 mal33Δ ura3-52 his3-Δ200 leu2-Δ1 ade2-101 lys2-801 trp1-Δ63*; Sikorsky and Heiter 1989). This strain lacks a functional activator but contains copies of each of the structural genes. Strain 61-601 is a YPH500 derivative carrying several copies of a $MAL61_{promoter}$-*lacZ* reporter integrated at *LEU2*. A *Bam*HI fragment containing the *MAL61* promoter and a few codons of the *MAL61* ORF was cloned into the *Bam*HI site of Yip365, creating a $MAL61_{promoter}$-*lacZ* reporter gene (Hu et al. 1995). This construct was then targeted to integrate at the *leu2*-Δ1 gene of YPH500 by digestion at the unique *Bst*EII site. Integration was confirmed by Southern analysis; and transformant strain 61-106 exhibited an intense plasmid-sized fragment, indicating multiple integrated copies. Strain 340-2A *(mal13 AGT1 MAL12 ura3-52 ade)* is a maltose non-fermenting strain, because it lacks a functional *MAL*-activator gene; but it does contain two copies of each of the structural genes (Charron and Michels 1988; Gibson et al. 1997).

Plasmid construction

For construction of pUN30MAL63, a 3-kb *Sal*I fragment containing a hemagglutinin-tagged allele of *MAL63* (Hu et al. 1999) was subcloned into the *Sal*I polylinker site in the CEN vector pUN30 (Elledge and Davis 1988). pUN30MAL63ΔKpnI-RI was constructed by deleting a 0.5-kb *Kpn*I fragment containing upstream sequences of *MAL63* plus some vector sequences, thereby deleting an upstream *Eco*RI site. An additional *Eco*RI site in the polylinker was mutagenized by in vitro mutagenesis using primer R1 (5′-AGGAATTATGTCGTCTTCATCTTT-3′). This plasmid, pUN30MAL63ΔKpnI-R1, contained two remaining *Eco*RI sites, one at codon 216 and one downstream of the ORF; and it was used to exchange *Eco*RI fragments containing the 3′ end of *MAL23* genes to construct 63/23 hybrids.

Construction of *MAL63*/*mal64* chimeras

Construction of the hybrid $mal64_{promoter}$-*mal63-NS283* gene is described in Gibson et al. (1997). The truncated *mal63-NS283* gene was fused to the *mal64* promoter, using PCR-based methods and cloned into the *Escherichia coli*/yeast shuttle vector YCp50. Plasmid pMAL64-NS282 carries the constitutive *MAL64-R10* gene (nonsense mutation of *mal64* at codon 282) in YCp50. These two plasmids were used to construct the other *MAL63*/*mal64* chimeras. Plasmid pMAL64(1–215)-MAL63(215–283) was constructed by replacing the *Eco*RI fragment containing the 3′ end of the *mal64* ORF with the comparable fragment from *mal63-NS283*. The *Xba*I-*Bam*HI fragment from this plasmid was used to replace the comparable fragment in plasmid pMAL64-NS282 to construct pMAL63(1–110)-MAL64(111–215)-MAL63(216–282). Plasmid pMAL64(1–100)-MAL63(101–282) was constructed by PCR-based methods, using pMAL64-NS282 and *mal63-NS283* as target sequences for amplification.

Construction of *LexA* fusion genes

The *LexA* fusion constructions utilized plasmid pSH2-1, which contains codons 1–87 of the *E. coli LexA* gene fused to the *Saccharomyces ADH1* promoter (Brent and Ptashne 1985; Hanes and Brent 1989). To construct plasmid pLexABD-Gal4AD, codons 768–881 of the *GAL4* ORF were amplified from the target plasmid pGAL424 (Invitrogen Two-hybrid kit) using PCR and inserted between the *Eco*RI-*Sal*I sites of pSH2–1 located 3′ of the *LexA* sequence, so as to create an in-frame fusion gene. This plasmid was used to construct plasmid pLexABD-Gal4AD-Mal63(200–470) by amplifying codons 200–470 of *MAL63*, using appropriate PCR primers and inserting this fragment between the *Sal*I-*Bam*HI sites at the 3′ end of the *LexA-GAL4AD* fusion gene. Plasmid pSH18-18, which expresses *LacZ* from a promoter containing six copies of the LexAp binding site, was used as a reporter to assay transcription activation.

Site-directed mutagenesis

Site-directed in vitro mutagenesis was carried out using the BioRad Muta-gene kit, according to manufacturers directions. Table 1 lists the oligonucleotides used to create the mutations.

Enzyme assays

Maltase activity was assayed as outlined by Dubin et al. (1985), with specific activity expressed as nanomoles of *p*-nitrophenol-α-D-glucopyranoside hydrolyzed per minute per milligram of protein. β-Galactosidase activity was assayed according to the protocols of Ausubel et al. (1999) using crude cell extracts and expressing specific activity as nanomoles of *o*-nitrophenol liberated per minute per milligram of protein. Enzyme assays were carried out at least on duplicate cultures and on two or three independent transformants. The error was about 20%.

Table 1 List of mutagenic oligonucleotides. The various oligonucleotides used to create the indicated mutation are listed and the annealing site in the open reading frame begins at base pair 1 of the sequence reported by Kim and Michels (1988)

MAL6/23 allele	Oligonucleotide sequence (5′–3′)	Annealing site
MAL63/23-W307R	AAGTTCGTTCCGTATCCTTTT	909–929
MAL63/23-S392A, V395I	TGCATTGGCTATTTCCAATGCTTTCATTGG	1,164–1,193
MAL63/23-T251A	CGCTATTGTTGCATCTAGGCT	741–761
MAL63/23-I402V	CTTATTTACGACATCTACCAA	1,194–1,214
MAL63(1)	GTCGATATTTCGTTTTCGAGGCATTGGATCAGGGCA	973–1,008
MAL63(2)	TTTCAAATGAATGGTACGAAGTTTTTTTCGAATGCTAATAATGCA	1,027–1,077
MAL63(3)	CTAGTCGAAATTGCTAAAGACATGTTGGATGA CATCTTTTTAACTCCGAAT	1,084–1,134

Results

Intragenic suppression of *MAL23*

Wang and Needleman (1996) reported the isolation of several constitutive *MAL63* alleles, each of which appeared to result from gene conversion-like events between the plasmid-borne *MAL63* and partially homologous, functionally inactive *MAL*-activator gene sequences contained in the genome of the host yeast strain. The shortest of these conversion events introduced an eight-amino acid substitution at the extreme C-terminus of the protein and is referred to as *MAL63-D8*. These same eight alterations were found in the constitutive activators encoded by *MAL43-C* and *MAL23-C* and surprisingly also in the inducible *MAL23* activator (Gibson et al. 1997). It was also found that, compared to Mal63p and the Mal63 constitutive activators, Mal23p contains 15 other amino acid differences scattered throughout the protein. This suggests that in Mal23p, one or more of the 15 altered residues is suppressing the otherwise constitutive phenotype of the eight C-terminal alterations.

To test this, a *MAL63/23* fusion was constructed using the *Eco*RI site at codons 215/216 of both genes. This hybrid contains the promoter and first 215 codons of *MAL63* fused to codons 216–470 of *MAL23*, and includes seven of the 15 residue differences between the *MAL63-D8* constitutive mutation and inducible *MAL23*, all in the C-terminus. Plasmids carrying this *MAL63/23* fusion gene or *MAL23* were transformed into strain 61-106 (a YPH500 derivative lacking a functional *MAL*-activator gene) and maltase expression was determined in cells grown under uninduced and maltose-induced conditions (Fig. 1). The hybrid Mal63/23p is inducible and maltase expression under uninduced conditions is comparable to that exhibited in strains expressing Mal23p, that is, three- to five-fold higher levels of basal activity than strains expressing Mal63p. This result indicates that the seven-residue differences between Mal63p and Mal23p in the C-terminal region (residues 215–470) are sufficient to suppress the constitutivity of the eight C-terminal alterations.

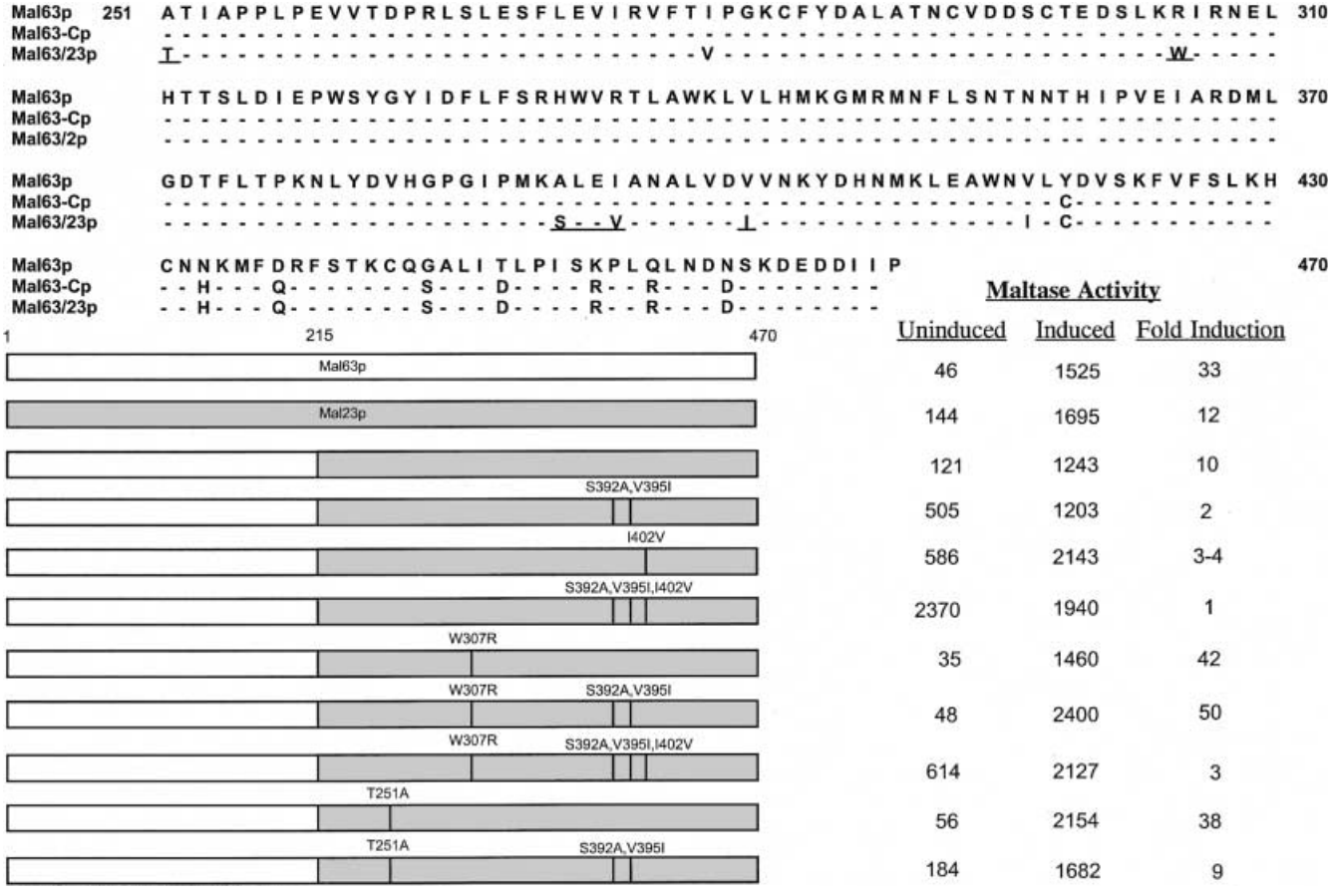
Site-directed mutagenesis of *MAL63/23* was used to convert residues in the C-terminal Mal23p sequence to those found in Mal63p. The changes are illustrated in Fig. 1. Plasmid-borne mutant alleles were introduced into strain 61-106 and maltase expression was assayed. The results are presented in Fig. 1. In all, five of the seven residues were changed, most individually and some in combination. These alterations can be seen to increase or decrease the uninduced level of maltase expression dramatically. Conversions S392A and V395I or I402V create a constitutive *MAL63/23* allele and increase basal (uninduced) maltase expression four- to five-fold, to approximately 30–40% of the fully induced levels. The triple conversion containing all three alterations combined is fully constitutive.

Conversion of T251A or W307R in Mal63/23p causes a repression of the basal maltase expression by approximately three- to four-fold, while having no significant effect on maltose-induced expression levels. This basal expression level is comparable to that expressed by transformants carrying *MAL63*. When W307R is introduced along with S392A and V395I in *MAL63/23*, the constitutive phenotype of the S392A, V395I construct is suppressed and this triple mutant exhibits a repressed basal activity which is like that of W307R alone. A similar result is obtained when W307R is introduced into the fully constitutive S392A, V395I, I402V construct, but suppression is not complete. T251A also is able to suppress the constitutive phenotype of the S392A, V395I conversion, but not to the same extent as W307R.

Finally, residues alanine-392, isoleucine-395 and valine-402 are identical in Mal63p and Mal43-Cp. These three residues were converted to the Mal23p sequence (that is, A392 S, I395V, and V402I) in the constitutive activator *MAL63/43* to determine whether these amino acid substitutions are sufficient to suppress the constitutive phenotype of this hybrid *MAL*-activator. Maltase expression of transformants carrying this allele is unaffected by these amino acid substitutions (data not shown).

Analysis of constitutive mutations of *MAL23-C*

The sequence of the constitutive activator Mal23-Cp as reported by Gibson et al. (1997) contains 18 amino acid substitutions and two deleted residues in the C-terminal



Maltase Activity

	Uninduced	Induced	Fold Induction
Mal63p	46	1525	33
Mal23p	144	1695	12
	121	1243	10
S392A,V395I	505	1203	2
I402V	586	2143	3-4
S392A,V395I,I402V	2370	1940	1
W307R	35	1460	42
W307R S392A,V395I	48	2400	50
W307R S392A,V395I,I402V	614	2127	3
T251A	56	2154	38
T251A S392A,V395I	184	1682	9

Fig. 1 Mutagenesis of inducible *MAL63/23*. The sequences of Mal63p, Mal63-Cp (encoded by *MAL63-D8* described in Wang and Needleman 1996), and Mal23p are aligned over residues 215–470. Residues converted from the Mal23p sequence to that found in Mal63p by in vitro mutagenesis are *underlined*. The conversions were made in *MAL63/23*, a hybrid gene containing the 5′ half of *MAL63* and the 3′ half of *MAL23*, with their junction at the *Eco*RI site at codons 215/216. *Rectangles* represent the sequences of residues 1–470 of Mal63/23p, Mal63p, or Mal23p; and *vertical lines* indicate the position of the converted residue(s). The *MAL63/23* alleles were carried by vector pUN30 (Elledge and Davis 1988) and were introduced into strain 61-106, a derivative of YPH500 that lacks functional *MAL*-activator genes but contains the *MAL* structural genes. Maltase was assayed as described in Dubin et al. (1985) in cells grown in selective medium containing 3% glycerol and 2% lactate, either with 2% maltose (induced conditions), or without added maltose (uninduced conditions). All assays were done at least on duplicate cultures for at least two independent transformants. The error is about 20%

region at residues 325–378, compared to the inducible Mal23p. Mal43-Cp contains 11 alterations in the same region, only three of which are common to both constitutive alleles. This is in addition to alterations at the C-terminal end of the protein, identical to those discussed in the above section. We tested the possibility that alterations in this region alone (residues 325–278) are sufficient to cause constitutivity.

Inducible Mal63p was converted to the sequence of Mal23-Cp within this region (residues 325–378). The region was divided into three sections, referred to as blocks 1, 2, and 3. Amino acid substitutions were made as individual blocks (that is, the cluster of changes in block 1, 2, or 3) or in combination (such as blocks 1 and 2). One conversion includes all three blocks of sequence changes and produces an allele that is identical to the Mal23-Cp sequence in this region. Block 1 converts residues between positions 325 and 336 (I325V, F327I, L328 S, V334I, and T336A). Block 2 substitutions are located between positions 343 and 359 and include eight amino acid substitutions and two deletions (L343F, H344Q, K346N, M348T, R349K, M350Δ, N351Δ, L353F, T356A, and T359A). Block 3 converts five residues between positions 362 and 378 (P362L, R367K, G371D, T373I, and K378N). The various constructs were introduced into strain 61-106 and transformants were assayed for maltase expression following growth under inducing and non-inducing conditions. The results are summarized in Fig. 2.

Alterations in block 1 alone, *MAL63(1)*, have no effect on regulation and are not sufficient to cause constitutivity. Block 2 mutations alone, *MAL63(2)*, are sufficient to produce a constitutive phenotype. Interestingly, the block 3 mutations alone, *MAL63(3)*, result in a defective *MAL*-activator with a strongly diminished inducibility. *MAL63(1, 2)*, containing blocks 1 and 2 together, also is constitutive; but when block 3 is added, *MAL63(1, 2, 3)*, the activator is again inducible. The basal level of *MAL63(1, 2, 3)* is higher than in strains carrying *MAL63* and is more typical of levels observed

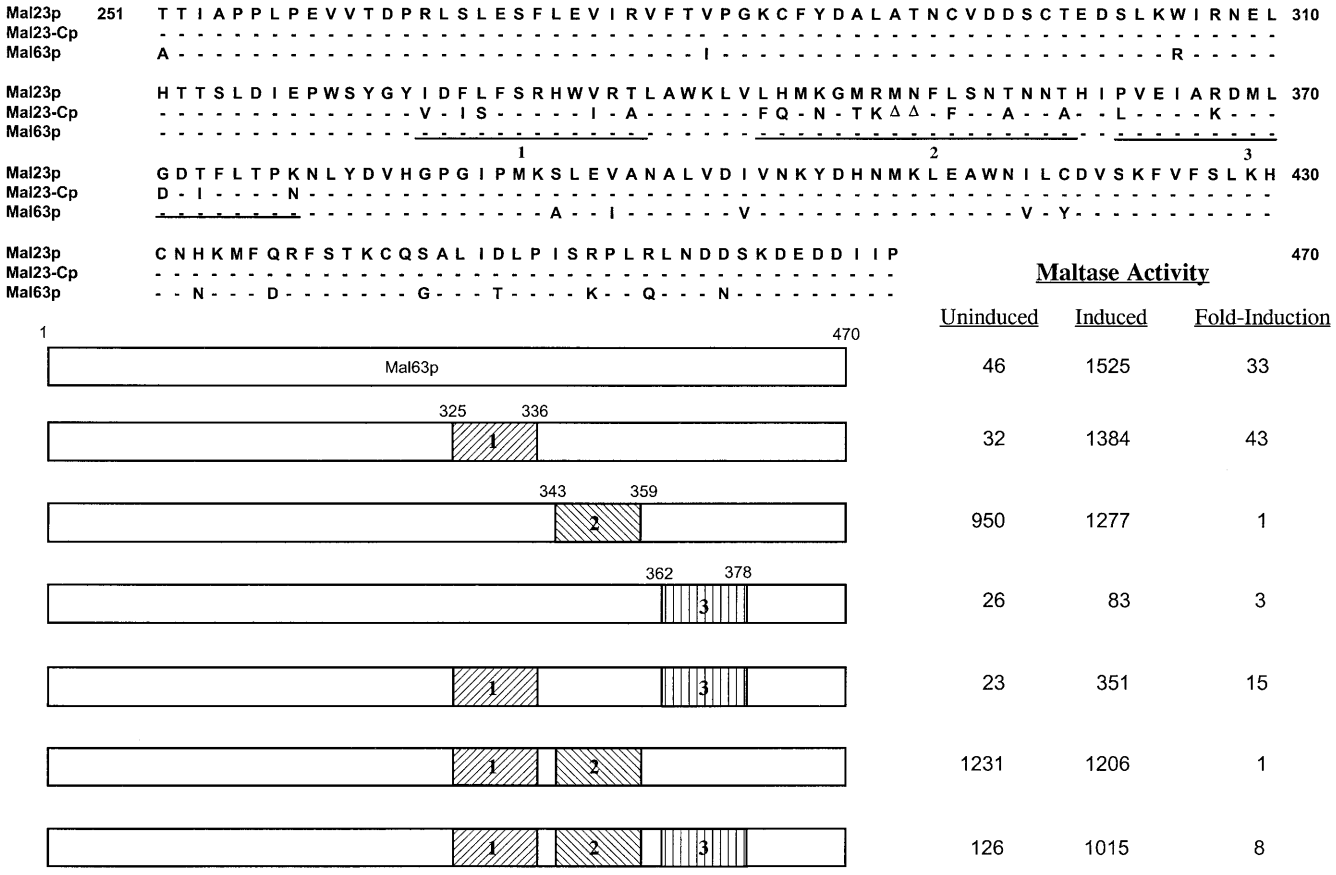


Fig. 2 Mutagenesis of inducible *MAL63* to *MAL23-C*. The sequences of Mal23p, MAL23-Cp, and Mal63p are aligned with differences indicated. *Underlined sequences* indicate the residues of Mal63p that were altered as a group (referred to as blocks 1, 2, and 3) to the residues found in Mal23-Cp. *Open rectangles* represent the Mal63 protein sequence at residues 1–470. Regions of in vitro-generated clustered alterations are indicated as *shaded numbered areas* (blocks), and *residue numbers* indicate the endpoints of the clusters. The size and position of these blocks is schematic and not to scale. Maltase assay conditions are the same as for Fig. 1

in transformants containing *MAL63/23*. One construct of *MAL63(1, 2)* included an unintentional alteration of P362L from the region included in block 3. This construct is inducible [*MAL63(1, 2-L)*] suggesting that proline-362 is essential for the constitutive phenotype of *MAL63(1, 2)*. Finally, the construct containing blocks 1 and 3 together, *MAL63(1, 3)*, is poorly inducible and transformants carrying this allele express levels about 25% of the levels of *MAL63/23* transformants grown under induced growth conditions.

In order to determine whether the constitutive mutations *MAL63(2)* and *MAL63/23* (S392A, V395I, and I402V) are dominant or recessive, strain 61-106 was co-transformed with plasmids carrying the mutant allele and *MAL63* and maltase assays were performed on cells grown under uninduced growth conditions. *MAL63/23* (S392A, V395I, and I402V) is completely dominant and transformants express the same high uninduced levels of maltase in the presence or absence of the *MAL63* gene (Table 2). However, transformants carrying both *MAL63(2)* and *MAL63* exhibited reduced uninduced levels of maltase activity, approximately one third the level of that expressed in transformants carrying *MAL63(2)* alone (Table 2). Thus, *MAL63(2)* is partially dominant.

Analysis of MAL63/MAL64 truncated hybrids

Constitutive *MAL64-R10* is a nonsense mutation at codon 283 of the non-inducible *MAL*-activator

Table 2 Constitutive alleles *MAL63/23* (S392A, V395I, and I402V) and *MAL63(2)* are dominant to *MAL63*. Strain 61-106 was transformed with *MAL63*, *MAL63/23* (S392A, V395I, and I402V), or *MAL63(2)* either alone (in vector pUN30) or cotransformed with *MAL63* (in vector YCp50). Maltase expression was assayed in cells grown in selective medium with 3% glycerol and 2% lactate (uninduced conditions), as described in Fig. 1

MAL-activator allele	Maltase activity (uninduced growth conditions)	
	Vector	*MAL63*
MAL63	39	44
MAL63/23 (S392A, V395I, I402V)	2,370	2,418
MAL63(2)	950	280

homologue *mal64* (Gibson et al. 1997). A similar truncation mutation of *MAL63*, *mal63-NS283*, is only partially inducible but point mutations altering T247A, R172G, and R117C are constitutive (Fig. 3; Gibson et al. 1997). None of these changes in the full-length Mal63p is sufficient to produce a constitutive phenotype. Mal64-R10p differs from Mal63-NS283p at 43 residues, including an alanine at residue 247 and a cysteine at residue 117. We suggest that, even in the truncated Mal63-NS283 protein, some residual negative regulation is retained that is missing in Mal64-R10p as a result of the many sequence changes. Hybrid Mal63p/Mal64p would allow us to localize which changes in Mal64p may be involved in relieving the inhibition.

We constructed hybrids between truncated *mal63-NS283* and *MAL64-R10*. All hybrids were expressed from the *mal64* promoter to eliminate differences due to different expression patterns (Dubin et al. 1988). The plasmid-borne hybrids were introduced into strain 340-2A, which lacks a functional *MAL*-activator gene and maltase expression was assayed. The results are shown in Fig. 3. As can be seen, sequences in the N-terminal 100 residues of Mal64-R10p contribute significantly to the constitutive phenotype of the truncated allele. Mal63p and Mal64p differ at 20 residues in this region and nine are clustered at residues 66–84, in a region essential for transactivation but believed to play a structural role (Hu et al. 1999).

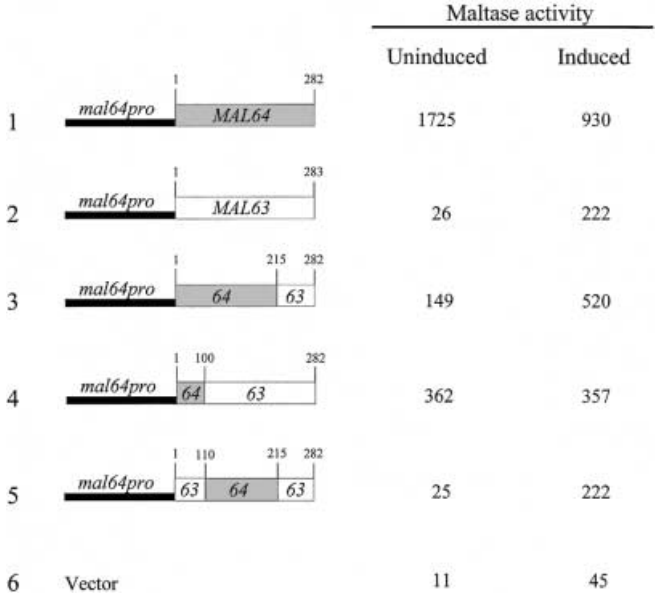


Fig. 3 Hybrid *MAL63/mal64* truncated alleles. *Construct 1* is *MAL64-R10*. *Construct 2* was made as described in Gibson et al. (1997). Construction of the others (*3–6*) is described in Materials and methods. All are carried by vector YCp50 and transformed into strain 340-2A, which lacks a functional *MAL*-activator gene but carries two copies of each of the *MAL* structural genes (Charron and Michels 1988; Gibson et al. 1997). Maltase assay conditions are the same as for Fig. 1

A LexABD-Gal4AD-Mal63RD heterologous activator is constitutive

We tested the ability of the *MAL*-activator C-terminal regulatory domain to regulate a heterologous activation domain. The DNA-binding domain of Mal63p does not appear to be involved in maltose regulation, since deletion of residues 1–37, 40–66, or 60–99 of the LexA-Mal63 fusion activator does not affect maltose-regulated reporter gene expression (Hu et al. 1999). An artificial activator was constructed in plasmid pSH2-1, using the DNA-binding domain of LexA (codons 1–87), the transactivation domain of Gal4p (residues 768–881), and the regulatory domain of Mal63p (residues 200–470). An activator consisting only of LexA(1–87)-Gal4(768–881) was used as control. These were introduced into strain YPH500 carrying a *lexA-LacZ* reporter and β-galactosidase activity was determined under maltose-induced and uninduced conditions (Fig. 4). While the strain carrying the triple fusion activator LexA(1–87)-Gal4(768–881)-Mal63(200–470) expressed about ten-fold higher levels of β-galactosidase than the strain carrying the control activator, LexA(1–87)-Gal4(768–881), both are constitutive.

Discussion

MAL-activator contains several regions important in negative regulation

We have identified three regions, referred to as regions 1, 2, and 3, of the Mal63 *MAL*-activator that play an important role in the negative regulation of activation. Region 1 (residues 251–307) was identified

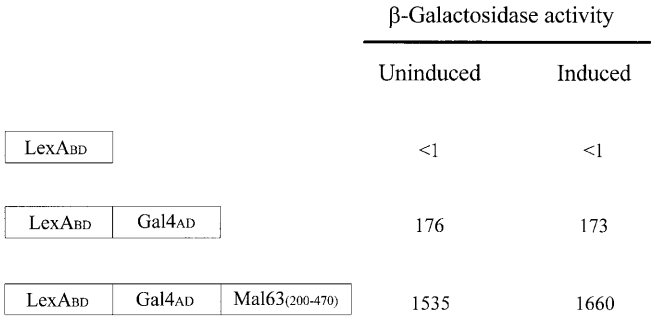


Fig. 4 Heterologous LexA-Gal4-Mal63 activator. Plasmid pSH2–1 carrying codons 1–87 of the *Escherichia coli LexA* gene encoding the DNA-binding domain was used to construct a heterologous activator containing the transactivation domain of Gal4 activator (residues 768–881) and the *MAL*-activator regulatory domain (residues 200–470) as described in Materials and Methods. These were transformed into strain YPH500 along with the *lexA(op)-LacZ* reporter gene in plasmid pSH18–18; and β-galactosidase activity was assayed in cells grown in selective medium, either under uninduced conditions (3% glycerol and 2% lactate), or under induced conditions (3% glycerol, 2% lactate, and 2% maltose) as described in Materials and methods. All assays were done at least on duplicate cultures for at least two independent transformants. The error is about 20%

previously by Hu et al. (1999), who found that a LexA-Mal63 fusion activator lacking residues 251–299 constitutively activated expression of a *lexA-LacZ* reporter. We found that strains expressing Mal23p exhibited higher basal maltase expression than those expressing Mal63p. Alteration of W307R or T251A in Mal23p reduced this basal expression to levels found in *MAL63* strains, consistent with this region of the *MAL*-activator being involved in negative regulation.

Sequences in region 2 (residues 343–359) are altered in *MAL23-C* and *MAL43-C* but the specific changes are different with the exception of phenylalanine-353, which is common to both constitutive mutations. Interestingly, an inducible *MAL*-activator isolated by Higgins et al. (1999), encoded by *MALx3-VH9*, is identical to Mal23-Cp in this region. In fact, it differs from Mal23-Cp at only six residues throughout the entire length of these proteins and, at all of these residues, Malx3-VH9p is identical to Mal63p. Four of the residues that differ between Mal23-Cp and Malx3-VH9p are located in our block 3 adjacent to region 2. We find that Mal63p block 3 in the context of the Mal23p sequence produces a non-inducible phenotype and, in the context of the Mal23-Cp sequence, produces an inducible phenotype. Thus, we propose that residues in the Mal63p block 3 region suppress the constitutive phenotype of mutations in region 2. In addition, a sequence analysis undertaken by Gibson et al. (1997) failed to identify residues involved in the constitutive phenotype of *MAL63/43-C*. Conversion of residues within three regions of the C-terminus (344–358, 367–379, and 445–461) separately and in combination was insufficient to convert either the inducible *MAL63* to a constitutive allele or the constitutive *MAL63/43-C* to an inducible allele. This illustrates the complex nature of the intramolecular suppression we have demonstrated here. Moreover, it is now clear why all constitutive *MAL*-activator alleles differ from inducible alleles at multiple residues.

Region 3 was identified by Wang and Needleman (1996). Several constitutive alleles of *MAL63* were isolated and all appeared to be produced by a gene conversion-like event involving a cryptic *MAL*-activator sequence. *MAL63-D8* contained the fewest alterations and changed eight residues at the C-terminus of the protein, residues 419–461. Results in Fig. 1 clearly indicate that alterations at residues linked to region 3 (including S392A, V395I, and I402V) suppress the constitutive phenotype of mutations in region 3.

N-terminal sequences of the *MAL*-activator also contribute to regulation

Results reported in Fig. 3 suggest that sequences in the N-terminal region of the *MAL*-activator contribute to negative regulation. The truncated *mal63-NS283* allele is poorly inducible but a similar nonsense mutation in *mal64* (*MAL64-R10*) creates a constitutive activator (Gibson et al. 1997). Mutations at residues 117, 172, and 247 in *mal63-NS283* convert this allele to a constitutive activator but only in the truncated allele (Gibson et al. 1997). In the full-length protein, these same alterations have no effect on inducibility. We show here that a Mal63/Mal64 truncated hybrid activator containing residues 1–100 of Mal64p is constitutive, while those with the Mal63p sequence are poorly inducible. Moreover, our finding in Fig. 4, which shows that the hybrid activator LexA(1–87)-Gal4(768–881)-Mal63(200–470) is constitutive, indicates that the *MAL*-activator C-terminal regulatory domain is not sufficient to impose negative regulatory control over a heterologous activation domain and may require specific interactions with residues in the N-terminal region. These results are consistent with those of Higgins et al. (1999), who constructed hybrid *MAL*-activators from constitutive and regulated alleles and found that alterations throughout the ORF affected regulation.

Mechanism of *MAL*-activator induction

The results reported here, taken together with previous genetic analysis and the results reported by Gibson et al. (1997), Higgins et al. (1999), and Hu et al. (1999) offer compelling evidence that negative regulation of the *MAL*-activator depends on the formation/maintenance of a specific inactive conformation that is exquisitely sensitive to sequence alterations, particularly in the C-terminal region of the protein. Although specific residues in each of the three negative regulatory regions identified here are essential for the formation of this inactive conformation, the C-terminal region (residues 200–470) alone is insufficient (Fig. 4) and it appears that sequences elsewhere in the protein also make a contribution (Fig. 3).

Intramolecular negative regulatory interactions were reported for *Saccharomyces* Leu3p, the transcription activator of the branched-chain amino acid biosynthetic pathway. Molecular genetic analysis of Leu3p suggests that the C-terminal activation domain is masked in the absence of inducer, α-isopropylmalate; and that masking is accomplished by an intramolecular interaction between the middle region of the protein and the short C-terminal activation domain (Zhou et al. 1990; Wang et al. 1997). Intragenic suppressors of a non-inducible Leu3p allele with mutations in the activation domain map to the middle region of the protein. One of the second site suppressor mutations is constitutive when separated from the original non-inducible mutations. In addition, an interaction between the C-terminal activation domain and the middle region could be demonstrated using a modified two-hybrid system (Wang et al. 1997). Mutations at many sites in Leu3p have a significant effect on modulating activation, suggesting that the effect is due to impaired masking rather than better induction.

We have been unsuccessful in our efforts to demonstrate direct interaction between specific regions of the *MAL*-activator using two-hybrid analysis. Over 20

different sections of *MAL63* have been used, including both large and small sequences, sequences derived from different regions of the gene, and sequences from inducible and non-inducible alleles. None provide any evidence of a physical interaction. Perhaps the native conformation cannot be formed by the protein fragments, or perhaps the interaction is too weak to be detected by the methods employed. Clearly other approaches will be needed to demonstrate these potential interactions and the postulated conformational differences between the inducible and constitutive forms.

The simplest interpretation of these results is that intricate intramolecular interactions between different regions of the *MAL*-activator contribute to the formation of an inactive conformation and inhibit activator function in the absence of inducer. In the presence of inducer, the *MAL*-activator folds (or refolds) into an active conformation capable of entering the nucleus and/or activating transcription. Moreover, alterations at various sites throughout the protein, but especially in the three regions identified here, destabilize the inactive conformation and allow the *MAL*-activator to fold into the active conformation, even in the absence of the inducing signal.

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References

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1999) Current protocols in molecular biology. Wiley, New York

Berge AMA ten, Zoutewelle G, Poll KW van de, Bloemers HPJ (1973) Regulation of maltose fermentation in *Saccharomyces carlsbergenesis* II. Properties of a constitutive *MAL6*-mutant. Mol Gen Genet 125: 130–146

Brent R, Ptashne P (1985) A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. Cell 43: 29–736

Charron MJ, Michels CA (1987) The constitutive glucose-repression-insensitive mutation of the yeast *MAL4* locus is an alteration of the *MA43* gene. Genetics 11: 23–31

Charron MJ, Michels CA (1988) The naturally occurring alleles of *MAL1* in *Saccharomyces* species evolved by various mutagenic processes including chromosomal rearrangement. Genetics 115: 23–31

Dubin RA, Perkins EL, Needleman RB, Michels CA (1985) Identification of the structural gene encoding maltase within the *MAL6* locus of *Saccharomyces carlsbergensis*. J Bacteriol 164: 605–610

Dubin RA, Perkins EL, Needleman RB, Michels CA (1986) Identification of a second *trans*-acting gene controlling maltose fermentation in *Saccharomcyes carlsbergensis*. Mol Cell Biol 6: 2757–2765

Dubin RA, Charron MJ, Haut SR, Needleman RB, Michels CA (1988) Constitutive expression of the maltose fermentative enzymes in *Saccharomyces carlsbergensis* is dependent upon the mutational activation of a nonessential homolog of *MAL63*. Mol Cell Biol 8: 1027–1035

Elledge SJ, Davis R (1988) A family of versatile centromeric vectors designed for use in the sectoring-shuffle mutagenesis assay in *Saccharomyces cerevisiae*. Gene 70: 303–312

Gibson AW, Wojciechowicz LA, Danzi SE, Zhang B, Kim JH, Hu Z, Michels CA (1997) Constitutive mutations of the *Saccharomyces cerevisiae MAL*-activator genes *MAL23*, *MAL43*, *MAL63*, and *mal64*. Genetics 146: 1287–1298

Hanes SD, Brent R (1989) DNA specificity of the bicoid activator protein is determined by homeodomain recognition helix residue 9. Cell 57: 1275–1293

Higgins VJ, Braidwood M, Bissinger P, Dawes IW, Attfield PV (1999) Leu343Phe substitution in the Malx3 protein of *Saccharomyces cerevisiae* increases the constitutivity and glucose insensitivity of *MAL* gene expression. Curr Genet 35: 491–498

Hu Z, Nehlin JO, Ronne H, Michels CA (1995) *MIG1*-dependent and *MIG1*-independent glucose regulation of *MAL* gene expression in *Saccharomyces cerevisiae*. Curr Genet 28: 258–266

Hu Z, Gibson AW, Kim JH, Wojciechowicz LA, Zhang B, Michels CA (1999) Functional domain analysis of the *Saccharomyces MAL*-activator. Curr Genet 36: 1–12

Kahn NA, Eaton NR (1971) Genetic control of maltase formation in yeast I. Strains producing high and low basal levels of enzyme. Mol Gen Genet 112: 317–322

Kim JH, Michels CA (1988) The *MAL63* gene of *Saccharomyces* encodes a cysteine-zinc finger protein. Curr Genet 14: 319–323

Needleman RB (1991) Control of maltase synthesis in yeast. Mol Microbiol 5: 2079–2984

Rodicio R (1986) Insertion of non-homologous DNA sequences into a regulatory gene causes a constitutive maltase synthesis in yeast. Curr Genet 11: 235–241

Sikorsky RS, Heiter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122: 19–27

Sollitti P, Marmur J (1988) Primary structure of the regulatory gene from the *MAL6* locus of *Saccharomyces carlsbergensis*. Mol Gen Genet 213: 56–62

Wang J, Needleman RB (1996) Removal of a Mig1p binding site converts a *MAL63* constitutive mutant derived by interchromosomal gene conversion to glucose insensitivity. Genetics 142: 51–63

Wang D, Hu Y, Zheng F, Zhou K, Kohlhaw GB (1997) Evidence that intramolecular interactions are involved in masking the activation domain of transcriptional activator Leu3p. J Biol Chem 272: 19383–19392

Winge O, Roberts C (1950) The polymeric genes for maltose fermentation in yeast and their mutability. C R Trav Lab Carlsberg Ser Physiol 25: 35–81

Zhou K, Bai Y, Kohlhaw GB (1990) Yeast regulatory protein *LEU3*: a structure–function analysis. Nucleic Acids Res 18: 291–298

Zimmerman FK, Eaton NR (1974) Genetics of induction and catabolite repression of maltase synthesis in *Saccharomyces cerevisiae*. Mol Gen Genet 134: 261–272