Curr Genet (2003) 44: 173–183
DOI 10.1007/s00294-003-0429-9

RESEARCH ARTICLE

Sara E. Danzi · Mehtap Bali · Corinne A. Michels

Clustered-charge to alanine scanning mutagenesis of the Mal63 *MAL*-activator C-terminal regulatory domain

Received: 4 June 2003 / Revised: 3 July 2003 / Accepted: 5 July 2003 / Published online: 24 September 2003
© Springer-Verlag 2003

Abstract The *MAL*-activator genes of *Saccharomyces cerevisiae* encode regulatory proteins required for the expression of the structural genes encoding maltose permease and maltase. Residues within the C-terminal region of the Mal63 protein required for negative regulation were previously identified. Evidence suggested that the C-terminal domain is also involved in positive regulatory functions, such as inducer responsiveness and transactivation in the context of a full-length protein. Charged-cluster to alanine scanning mutagenesis of the regulatory domain of *MAL63* and the constitutive *MAL43-C* were undertaken to identify distinct regions within Mal63p involved in positive functions and to define their roles in induction. Mutations that affect the ability to activate transcription in the inducible *MAL63* but have no effect in the constitutive *MAL43-C* define regions that function in induction. Those that affect both the inducible and constitutive alleles define regions involved in activation more generally. Mutations in *MAL63* fell into three classes, those that have little or no impact on activity, those that decrease activity, and those that enhance function. Mutations from these classes mapped to distinct regions of the protein, identifying a region of approximately 90 residues (residues 331–423) involved in maltose sensing and an approximately 50-residue region at the extreme C-terminus (residues 420–470) required for activation, such as the formation and/or maintenance of an active state. These studies support a model for *MAL*-activator function which involves complex protein–protein interactions and overlapping negative and positive regulatory regions.

Communicated by S. Hohmann

S. E. Danzi · M. Bali · C. A. Michels (✉)
Biology Department, Queens College and the Graduate School of CUNY, 65-30 Kissena Boulevard, Flushing, NY 11367 USA
E-mail: corinne_michels@qc.edu

Present address: S. E. Danzi
Department of Medicine, Division of Endocrinology, North Shore—LIJ Research Institute, 350 Community Drive, Manhasset, NY 11030 USA

Introduction

Saccharomyces strains require one of five unlinked *MAL* loci (*MAL1*, *MAL2*, *MAL3*, *MAL4* or *MAL6*) to ferment maltose. Each is a three-gene complex containing the *MAL* structural genes encoding maltose permease and maltase and a regulatory gene encoding the *MAL*-activator, a DNA-binding transcription activator required for maltose induction of the structural genes (for reviews, see Needleman 1991; Hu et al. 1999). The *MAL*-activator gene of *MAL6*, referred to as *MAL63*, encodes a predicted 470-amino-acid protein (Kim and Michels 1988; Sollitti and Marmur 1988). Functional domain analysis of Mal63p (Hu et al. 1999) and sequence analysis of various *MAL*-activator constitutive mutations (Gibson et al. 1997; Higgins et al. 1999) suggested that the C-terminal approximately 200 residues of Mal63p act negatively to regulate the response to maltose. Detailed analysis of *MAL*-activator constitutive mutant alleles by Danzi et al. (2000) localized residues required for this negative regulatory function to three short domains and showed that multiple missense mutations within any one of these domains is sufficient to produce the constitutive phenotype, but no single point mutation capable of producing a constitutive mutation was identified. The constitutive phenotype of these mutant alleles could be suppressed (that is, restored to an inducible phenotype) by secondary mutations at adjacent or more distant sites. These results suggest that complex protein–protein interactions are involved in *MAL*-activator regulation. It was proposed that residues in the C-terminal region of the protein are required for the formation and/or maintenance of a specific inactive conformation of the *MAL*-activator protein and that, in this conformation, the protein is poised to respond to inducer.

The N-terminal half of Mal63p contains the DNA-binding domain (residues 1–100) and the transcription activation domain (residues 60–283; Hu et al. 1999). No evidence supporting the presence of additional transactivation domains in the C-terminal half of Mal63p was obtained. Nonetheless, Gibson et al. (1997), Hu et al. (1999), and Danzi et al. (2000) all report that noninducible mutations frequently resulted from a few missense alterations or short deletions within the C-terminal regulatory domain. This suggests that, in addition to the negative regulatory role of the C-terminal domain, sequences in this region also play a positive role in induction. In order to identify residues involved in positive functions and to define their role in induction, a charged-cluster to alanine scanning mutagenesis was undertaken for both *MAL63*, encoding an inducible *MAL*-activator, and *MAL43-C*, encoding a constitutive *MAL*-activator. Two classes of noninducible mutations mapping to separate regions of the C-terminal regulatory domain were identified: those that cause a noninducible phenotype in *MAL63* but have no affect when introduced in the constitutive *MAL43-C* and those that cause a noninducible phenotype in both *MAL63* and *MAL43-C*. Studies of these mutants provide valuable insights into *MAL*-activator regulation and the mechanism of maltose induction.

Materials and methods

Yeast strains

The following strains were used in this study: YPH500 (*MATα AGT1 MAL12 mal13 MAL31 MAL32 mal33 ura3–52 his3*-Δ200 *leu2*-Δ1 *ade2–101 lys2–801 trp1*-Δ63; Sikorsky and Heiter 1989), 61-106 (a YPH500 derivative carrying several copies of a $MAL61_{promoter}$-*lacZ* reporter integrated at *LEU2*), and CMY1073 [a YPH500 derivative in which *mal13* and *mal33* were deleted by one-step gene replacement with Kan^R and Hyg^R genes, as described by Wang et al. (2002)].

Strain 61-106 was constructed as follows. A *Bam*HI fragment containing the *MAL61* promoter and a few codons of the *MAL61* open reading frame was cloned into the *Bam*HI site of YIp365, creating a $MAL61_{promoter}$-*lacZ* reporter gene (Hu et al.1995). This construct was targeted to integrate at the *leu2*-Δ1 gene of YPH500 by digestion at the unique *Bst*EII site. Integration was confirmed by Southern analysis. Several Leu^+ transformants were tested and transformant strain 61-106 exhibited an intense plasmid-sized (*Hin*dIII) fragment, indicating multiple copies.

Construction of pMAL63

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 to produce pUN30-MAL63 (Elledge and Davis 1988). Vector pUN30 is a yeast/*Escherichia coli* shuttle vector which contains the *Saccharomyces TRP1* gene, ARS1, CEN4, the polylinker derived from pUC18, and the F1 phage origin of replication in the plus orientation (which allows transformants to produce single-stranded template DNA when infected with F1 helper phage).

A 0.5-kb *Kpn*I fragment containing upstream sequences of *MAL63* plus some vector sequences was removed from pUN30-MAL63 by digestion with *Kpn*I and self-ligation. Approximately 200 bases of upstream sequences remain, sufficient for full expression of *MAL63*. This construct, pUN30-MAL63ΔKpnI, contains three *Eco*RI sites, one in the polylinker upstream of *MAL63*, one at codon 216, and one downstream of the open reading frame. In order to be able to exchange different 3′ fragments (codons 216–470) of *MAL*-activator genes, the upstream *Eco*RI site in the polylinker was mutagenized by in vitro mutagenesis, using primer R1 (5′-AGGAATTATGTCGTCTTCATCTTT-3′) in pUN30-*MAL63*ΔKpnI, to create pMAL63. This construct was used to create hybrid *MAL*-activator genes.

Construction of pMAL63/43-C

Plasmid pMAL63 was digested with *Eco*RI, releasing the 1.1-kb fragment containing the 3′ region of *MAL63* from codon 215/216. The digested plasmid was treated with alkaline phosphatase and the vector-containing fragment was gel-purified. This fragment, composed of the pUN30 vector sequences and the promoter and 5′ region of *MAL63* to codon 215/216, was used for the construction of the *MAL63/43-C* fusion gene by ligation with the 1.1-kb *Eco*RI fragment containing the 3′ region of *MAL43-C*. YCp50-MAL43, carrying *MAL43-C*, was used as the source of this 1.1-kb *Eco*RI insert (Gibson et al. 1997). Transformants were screened by PCR and sequencing to confirm the construction.

Construction of *LexA-MAL63* fusion genes

mal63 mutations generated in vitro were introduced into a *LexA-MAL63* fusion gene as follows. Plasmid pLexA(1–87)-MAL63 (2–216) derived from plasmid pSH2-1 contains codons 1–87 of *lexA* (encoding the DNA-binding domain) fused to codons 2–216 of *MAL63* driven by the constitutive *ADH1* promoter (Hu et al. 1999). It retains a unique *Eco*RI site at codon 215/216 of the *MAL63* sequence; and the *Eco*RI 1.1-kb fragment containing codons 216–470 of *MAL63* from each of the in vitro mutants described below were inserted into this site in the correct orientation.

Site-directed in vitro mutagenesis

Site-directed in vitro mutagenesis was carried out using the BioRad Muta-Gene kit according to the manufacturer′s protocol. Mutagenic primers are listed in Tables 1 and 2. Mutagenized genes were sequenced in their entirety to confirm that only the desired alteration was present.Random mutagenesis of *MAL63* codons 454–470 was carried out as follows.

Random sequence oligonucleotides were generated using the DNA synthesizer, as described by Leuther et al. (1993), in which each nucleotide supply bottle contained 1% of each of the other three nucleotides. Given this level of intentional contamination, each 63-mer oligonucleotide made is predicted to contain 1–2 incorrect bases. These randomly mutagenic oligonucleotides were then used for in vitro mutagenesis, using pMAL63-D466A,D467A DNA as a template. The newly generated double-stranded plasmids were transformed into *E. coli* strain DH5α. Plasmid DNA was prepared from a pool of approximately 15,000 transformant colonies and transformed into the $MAL61_{promoter}$-*lacZ* reporter strain 61–106; and transformants were selected on synthetic minimal (SM) medium lacking tryptophan and containing 2% maltose, 3% glycerol, 2% lactate. Approximately 20,000 yeast transformant colonies were screened for *lacZ* expression, using the X-gal plate assay described below, and plasmids carrying a potential *mal63* mutation were rescued from the white transformants for further analysis. The full open reading frame of each mutant was sequenced.

Enzyme assays

Cells were grown in SM medium containing the indicated carbon source and lacking appropriate nutrients for plasmid selection.

Table 1 List of site-directed mutations introduced into *MAL63*. The annealing site open reading frame begins with base pair number 1

mal63 allele	Amino acid replacement	Oligo sequence (5′–3′)	Annealing site
mal63-244	K244A	CGTGACACACGCAATATATAC	740–720
mal63-259	E259A, D263A	AAGACGAGGGGCTGTTACAACCGCTGGTAGTGG	797–765
mal63-265	R265A, E269A	AAGGAAGCTTGCTAGAGAAAGAGCAGGGTCTGT	815–783
mal63-273	E273A, R276A	AGTGAAAACTGCAATCACCGCAAGGAAGCT	836–807
mal63-283	K283A, D287A	AGCCAAAGCAGCATAAAAACACGCTCCTGGTAT	869–837
mal63-295	D295A, D296A	GGTGCAGGAAAGCAGCGACACAGTT	896–873
mal63-300	E300A, D301A, K304A	CCGTATCCTTGCTAGAGAGGCTGCGGTGCAGGA	920–888
mal63-305	R305A, R307A, E309A	GGTATGAAGTGCGTTCGCTATCGCTTTTAGAGA	935–903
mal63-316	D316A, E318A	AGACCATGGCGCTATAGCAAGTGATGT	962–936
mal63-331	R331A, R335A	CGCTAGTGTCGCGACCCAATGCGCCGAAAACAG	1,013–981
mal63-346	K346A, R349A	AAAATTCATCGCCATACCTGCCATATGAAG	1,055–1,026
mal63-364	E364A, R367A	CAACATGTCTGCAGCAATTGCGACTGGTAT	1,109–1,080
mal63-368	D368A, D372A	TAAAAACGTGGCTCCCAACATGGCTCTAGCAAT	1,124–1,092
mal63-378	K378A, D382A	ACCATGTACAGCATACAGGTTTGCCGGAGTTAA	1,154–1,122
mal63-391	K391A, E394A	ATTGGCTATTGCTAATGCCGCCATCGGTAT	1,190–1,161
mal63-401	D401A, K405A	GTGATCATACGCATTTACGACAGCTACCAATGC	1,223–1,191
mal63-405	K405A, D407A	CATATTGTGAGCATACGCATTTACGAC	1,229–1,203
mal63-411	K411A, E413A	ATTCCAAGCTGCCAACGCCATATTGTG	1,247–1,221
mal63-420	D420A, K423A	AAAAACAAACGCGGATACAGCATACAAAAC	1,277–1,248
mal63-434	K434A, D437A, R438A	TTTCGTTGAAAATGCGGCGAACATTGCATTATTGCA	1,325–1,290
mal63-438	R438A, K442A	ACCTTGACATGCCGTTGAAAATGCGTCGAACAT	1,334–1,302
mal63-463	K463A, D464A, E465A, D466, 467A	TTAAGGAATTATGGCGGCTGCAGCTGCGGAGTTATCATT	1,412–1,374
Mal63-464	D464A, E465A	TATGTCGTCGCAGCTTTGGAGTT	1,403–1,380
mal63-466	D466A, D467A	AGGAATTATGGCGGCTTCATCTTT	1,409–1,386
mal63-460	D460A, K463A	GTCTTCATCTGCGGAGTTAGCATTTAATTG	1,397–1,368
mal63-462	S462A	GTCGTCTTCATCTTTGGCGTTATCATTTAATTGCAA	1,400–1,365
mal63-467S9V	D467S, I469V	AAATTAAGGAACTATGGAGTCTTCATC	1,415–1,389

Table 2 List of site-directed mutations introduced into the constitutive *MAL63/43-C* allele. The indicated alterations were introduced into the constitutive *MAL63/43*-C *MAL*-activator gene. This gene fuses the promoter and codons 1–215 of inducible *MAL63* to codons 216–470 of constitutive *MAL43-C*. The resulting fusion gene, *MAL63/43-C*, is constitutive. Alleles *mal63/43-411* and *mal63/43-467S9V* were made by in vitro mutagenesis with oligos *mal63-411* and *mal63-467S9V* (see Table 1)

mal63/43 allele	Amino acid replacement	Oligo sequence (5′–3′)	Annealing site
mal63/43-283	K283A, D287A	AGCCAAAGCAGCATAAAAACACGCTCCAGGTAC	870–838
mal63/43-331	R331A, R335A	TGCTAGAGTCGCGACCCAGTGCGCAGAAAAGAG	1,014–982
mal63/43-364	E364A, K367A	TAACATATCCGCGGCTATTGCGACTGGAAT	1,110–1,081
mal63/43-391	K391A, E394A	ATTGGCTATTGCTAGCGCCGCCATCGGTAC	1,191–1,162
mal63/43-401	D401A, K405A	GTGATCATACGCACTTACGACGGCTACCAATGC	1,224–1,192
Mal63/43-411	K411A, E413A	ATTCCAAGCTGCCAACGCCATATTGTG	1,247–1,221
mal63/43-420	D420A, K423A	GAAAACGAACGCAGATACAGCGCACAAAAT	1,278–1,249
mal63/43-438	R438A, K442A	ACTCTGACATGCAGTTGAAAACGCTTGAAACAT	1,335–1,303
mal63/43-457NS	Δ457–470	GGAATCATCATTTAGTTACAGTGGTCTAGAGAT	1,386–1,354
Mal63/43-467S9V	D467S, I469V	AAATTAAGGAACTATGGAGTCTTCATC	1,415–1,389
mal63/43-467A9N	D467A, I469N	AAATTAAGGATTTATGGCGTCTTCATC	1,416–1,390

Maltase activity was determined using total cell extracts prepared from mid-log phase cells (optical density at 600 nm = 0.3–0.5), as described by Dubin et al. (1985). Maltase activity is reported as the rate of *p*-nitrophenol released from *p*-nitrophenyl-α-D-glucopyranoside (PNPG) and is expressed as *x* nmol PNPG min^{-1} mg^{-1} protein. The protein concentration of the extracts was measured with the BioRad protein assay dye reagent. Assays were done at least in duplicate on three independent transformants, with an average error of about 15%.

Maltose transport activity was measured as the rate uptake of C^{14} maltose as described by Cheng and Michels (1991) and Medintz et al. (1996). The results are reported as *x* nmol maltose min^{-1} mg^{-1} protein.

Fermentation was determined by the number of days required for the initial appearance of gas bubbles in inverted Durham tubes, following inoculation of approximately 10^5 cells into 5 ml of SM medium plus 2% maltose.

The plate assay of *lacZ* expression was carried out as described by Bohen and Yamamoto (1993). Yeast strains were grown at 30 °C as colonies or patches on plates containing 2% maltose, 3% glycerol, 2% lactate. These were overlaid with 10 ml of a mixture containing 0.5% agarose, 0.5 M sodium phosphate (pH 7), 0.1% SDS, 2% dimethyl-formimide, 0.5% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. The plates were observed over time and the rate of blue dye formation was noted. Live cells could be recovered by piercing the overlay with a sterile toothpick.

Western blot analysis

Transformants carrying plasmid-borne *lexA-MAL63* fusion genes were grown in SM medium plus 2% maltose, 3% glycerol, 2% lactate and lacking histidine. Cells were harvested and protein

extracts prepared, as described by Ausubel et al. (2002). Total protein concentration of the extract was determined using the Bio Rad protein assay reagent dye. Western blot analysis was performed according to Medintz et al. (1996). Membranes were probed with rabbit anti-LexA antibody (obtained from Roger Brent) as the primary antibody and horseradish peroxidase-linked donkey anti-rabbit immunoglobulin secondary antibody (Amersham). Binding was visualized using the enhanced chemiluminescence (ECL) reaction (with reagents obtained from Amersham) on ECL-Hyperfilm.

Results

Clustered-charge to alanine scanning mutagenesis of the regulatory domain of inducible *MAL63*

Strain 61-106 is derived from YPH500 and contains an integrated $MAL61_{promoter}$-*lacZ* reporter. The strain was created to screen potential *mal63* mutations generated by random in vitro mutagenesis and enabled us to follow reporter expression as another measure of *MAL*-activator function. A total of 29 mutations were made in *MAL63* by in vitro mutagenesis. Twenty-five of these (*mal63-244* through *mal63-466*) were generated based on the clustered-charge to alanine scanning method. In charged-cluster to alanine scanning mutagenesis, clusters of two or more charged residues in a window of five are changed to alanine by site-directed in vitro mutagenesis (Wertman et al. 1992). Charged regions of a protein are likely to be on the surface of the protein and could be involved in interactions with other proteins and/or in intramolecular interactions. Moreover, because of their surface location, charged-cluster to alanine alterations should be less likely to produce drastic alterations in protein conformation, which would make the mutant protein a target for degradation. The specific basepair changes introduced to create each *mal63* mutation are listed in Table 1. The mutant alleles are named by indicating the number of the first altered residue of the cluster. Figure 1a, b indicate the location of each mutant alteration in the Mal63 protein sequence,

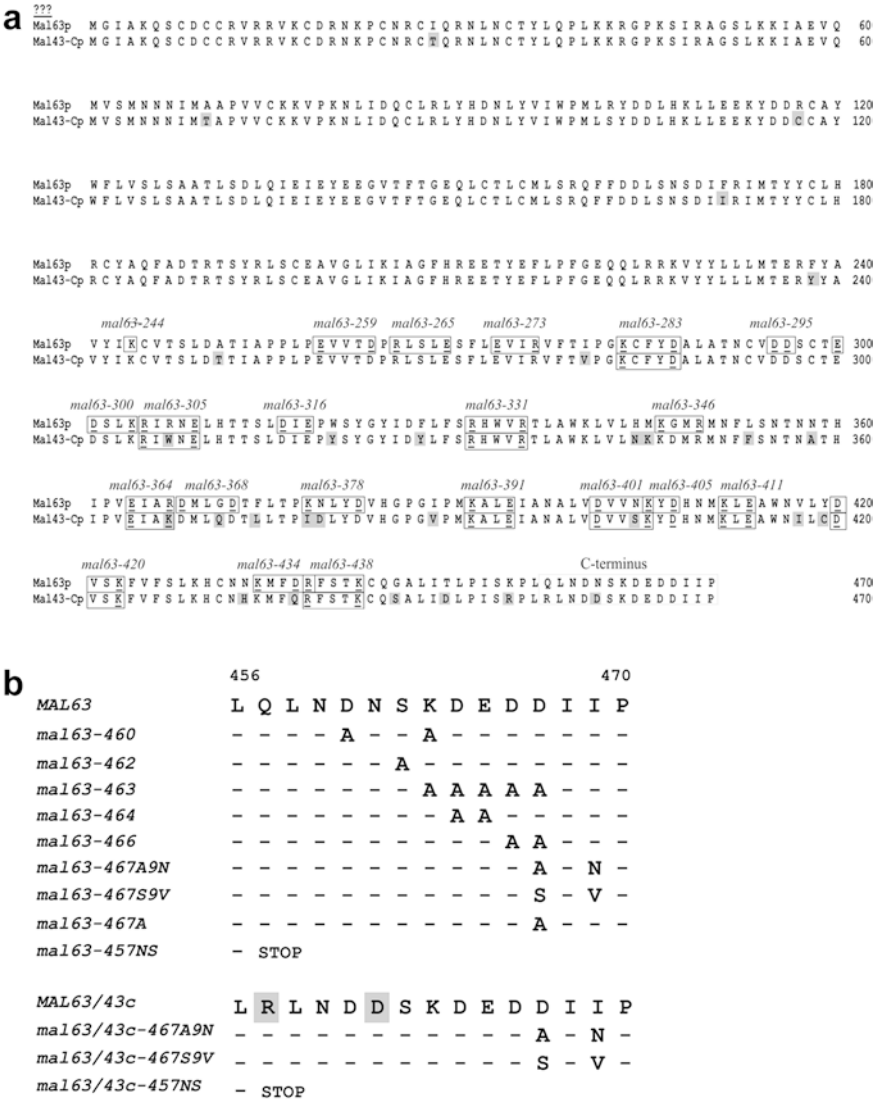

Fig. 1a, b In vitro mutations of *MAL63* and *MAL63/43*. **a** The complete amino acid sequence of the inducible Mal63 and constitutive Mal43-C activators is presented, with amino acid differences *highlighted in gray*. Clustered-charge to alanine mutations are *boxed* and the allele names are *indicated*. *The underlined residues* were changed to alanine. The mutations were introduced into *MAL63* and the *MAL63/43-C* hybrid gene. **b** Mutations within the C-terminal 14 residues of *MAL63* and the *MAL63/43-C* hybrid are *indicated below the amino acid sequence* for each activator. *Boxed residues* indicate amino acids in Mal63/43-Cp which differ from those of Mal63p

with the mutated charged residues underlined (see also Table 2). Several of the alanine-scanning mutations alter partially overlapping regions. This was done in regions where large clusters of charged residues are found. In one case, *mal63-244* (K244A), only one residue was changed.

In addition to the alanine-scanning mutations described, four other mutations were made. One serine (Ser-462), which is part of a potential casein kinase II site near the extreme C-terminus of the protein, was changed to alanine, to produce *mal63-462*. Two mutations, *mal63-467A9N* (D467A, I469N) and *mal63-467A* (D467A), were obtained by random mutagenesis of the last 17 residues of the protein, as described in Materials and methods. Finally, mutation *mal63-467S9V* (D467S, I469V) changed the indicated residues to those found at the C-terminus of the Mal64 protein, a noninducible Mal63p homologue encoded by a nonfunctional gene located immediately centromere-proximal to *MAL63* in the *MAL6* locus (Dubin et al. 1988; Gibson et al. 1997). Because the first altered residue is the same in each of these mutations, the allele name indicates all altered residues. For example, in allele *mal63-467A9N*, the D at residue 467 of wild-type Mal63p is A and the I at residue 469 is N. In allele *mal63-457NS*, codon 457 is a nonsense codon.

These mutations were introduced into *MAL63* carried on the CEN vector pUN30 and were transformed into strain 61-106, a YPH500 derivative lacking a functional *MAL*-activator gene and containing an integrated $MAL61_{promoter}$-*lacZ* reporter construct. Maltose fermentation ability, maltase expression, and *lacZ* reporter expression were determined for transformants grown in uninduced (3% glycerol, 2% lactate) and induced (2% maltose, 3% glycerol, 2% lactate) growth conditions. Figure 2 presents the results of maltase expression levels for each mutant allele in a linear array based on the position of the alteration in the Mal63 protein sequence, including a nonsense mutation at codon 457 (Hu et al. 1999). It should be noted that the nine mutations at the 3′ end of the gene are tightly clustered. The results of $MAL61_{promoter}$-*lacZ* reporter expression and maltose fermentation rates were carried out for each transformant strain shown in Fig. 2 and a clear correlation among all three measures of *MAL*-activator activity for all of the mutant alleles was evident (data not shown).

Generally, the mutations fall into three classes: those that have little or no impact on function, those that decrease activator activity (albeit to varying extents), and those that enhance function. More significantly, as can be seen from Fig. 2, mutations from these different classes largely map to a defined region of the protein. Seven of the 30 mutations are still inducible and transformants carrying these plasmid-borne mutations exhibit maltase levels in the normal range. These seven relatively silent mutations are located within residues 244 and 318, suggesting that this region of Mal63p does not play a significant role in induction or activator function. Mutation *mal63-283* is the only alteration in this region that exhibits significantly reduced levels of maltase expression; and this is addressed in the Discussion.

Almost every mutation in the last 140 residues of the protein (331–470) adversely affects *MAL* gene expression. Of the 21 mutations in this region, nine are noninducible (including *mal63-331*, *mal63-364*, *mal63-391*, *mal63-401*, *mal63-411*, *mal63-438*, *mal63-463*, *mal63-467A9N*), while ten others (*mal63-346*, *mal63-368*, *mal63-378*, *mal63-405*, *mal63-420*, *mal63-434*, *mal63-464*, *mal63-466*, *mal63-467A*, *mal63-467S9V*) are poorly inducible, with modestly to significantly decreased levels of maltase activity. All are maltose nonfermenters and

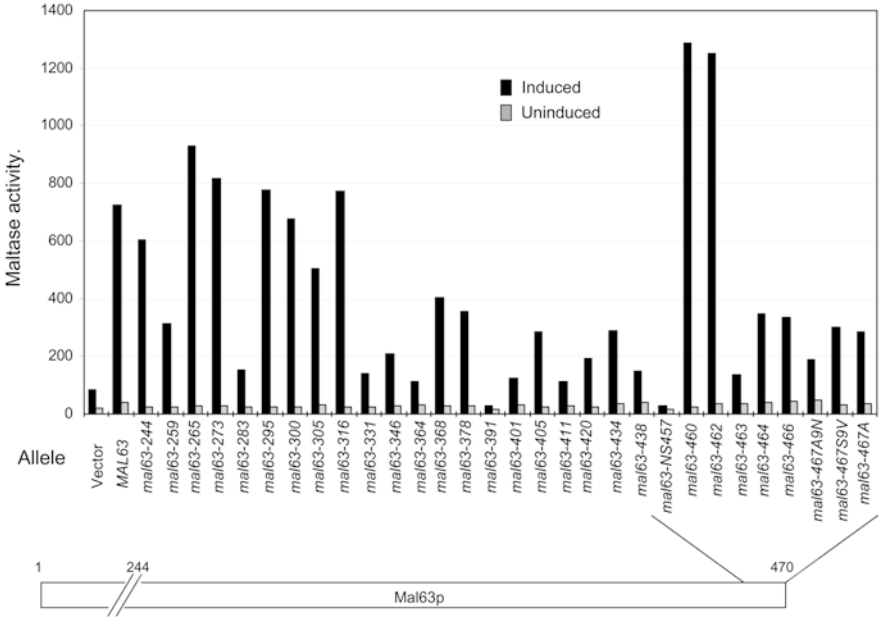

Fig. 2 Activity of clustered-charge to alanine scanning mutations in the regulatory domain of the Mal63 MAL-activator. The mutations are presented in a linear array according to the position of the alteration in the gene. *The open rectangle below* represents the C-terminal half of Mal63p from residue 244 to residue 470. The last nine mutations are tightly clustered between residues 456 and 469. The maltase activity of transformants carrying each of the *mal63* alleles was assayed in cells grown in conditions of induced (*black bars*) and uninduced (*gray bars*) growth

Table 3 Overexpression of *lexA-mal63* mutant activator fusions suppresses *MAL* structural gene expression. The *Eco*RI fragment containing codons 216–470 from several *MAL*-activator mutations was subcloned into plasmid pLexA(1–87)MAL63(2–216), creating a *lexA-mal63* fusion gene expressed from the *ADH1* promoter. These constructs were transformed into strain YPH500 carrying pSH18-18, a *lexAop-lacZ* reporter plasmid. Maltase activity and maltose transport rates were determined in duplicate on at least two different transformants grown in induced (2% maltose, 3% glycerol, 2% lactate) growth conditions. The maltose fermentation rate is reported as the number of days required for the appearance of gas production. *lexAop-lacZ* expression levels were monitored for up to 6 h by plate assay using X-Gal media, as described in the Materials and methods

MAL-activator fusion allele	Maltose Fermentation (days)	Maltose transport activity (units)	Maltase activity (units)	*lexAop-lacZ* reporter expression
lexA-MAL63	1–2	2.62	1,790	Blue (< 1 h)
lexA-mal63-283	3	1.87	1,574	White(< 6 h)
lexA-mal63-364	3	1.02	1,091	White(< 6 h)
lexA-mal63-391	> 7	0.05	67	White(< 6 h)
lexA-mal63-401	2–3	1.20	860	White(< 6 h)
lexA-mal63-411	2	1.32	621	White(< 6 h)
lexA-mal63-438	5–6	0.83	448	White (< 6 h)
lexA-mal63-463	4	0.81	940	White (< 6 h)
lexA-mal63-467A9N	4	0.78	497	White(< 6 h)
lexA-mal63-467S9V	2–3	1.02	955	White(< 6 h)
lexA vector	> 7	0.02	25	white(< 6 h)

lacZ expression is undetectable by plate assay (data not shown). Finally, transformants carrying two mutations, *mal63-460* (D460A, K463A) and *mal63–462* (S462A), exhibit significantly increased, inducible maltase expression and ferment maltose more rapidly than the wild type.

In order to determine whether these noninducible alleles are dominant or recessive, the pUN30 plasmids carrying the noninducible *mal63* mutations were co-transformed into strain 61-106 along with a second plasmid carrying wild-type *MAL63* and maltase assays were performed on transformants grown in growth-inducing conditions. As a control, strain 61-106 was transformed with two plasmids, each carrying wild-type *MAL63*. The noninducible mutations are all recessive; and the heterozygous double transformants express maltase at levels comparable with those of the homozygous *MAL63* double (data not shown).

Overexpression of noninducible mutant alleles as LexA fusion proteins

To exclude the trivial explanation that the noninducible mutations result in unstable mutant proteins, ten *mal63* mutations exhibiting significant defects in maltase induction and maltose fermentation were introduced into a *lexA-MAL63* fusion gene construct using plasmid pSH2-1, as described by Hu et al. (1999). This construct fuses the DNA-binding domain of *lexA* (residues 1–87) to the full-length *MAL63*, all expressed under the control of the *ADH1* promoter. Fusion of *lexA(1–87)* to *MAL63* produces a bifunctional activator capable of activating maltose-induced expression of the *MAL* structural genes and a *lexA-lacZ* reporter (Hu et al. 1999). Plasmids carrying the mutant fusion genes were introduced into strain YPH500, which lacks a functional *MAL*-activator gene, along with the multicopy plasmid pSH18-18 containing a *lexA-lacZ* reporter; and the *lexA-MAL*-activator function was then assayed by measuring maltose fermentation, maltase activity, maltose transport activity, and *lacZ* reporter gene expression in transformants grown in induced conditions. The results are reported in Table 3. Western analysis of total cell extracts of all of the transformants demonstrated comparable, very abundant expression of every fusion protein and no significant differences in expression level were observed (data not shown).

Transformants carrying wild-type *lexA-MAL63* activate expression of the *lacZ* reporter and ferment maltose rapidly (within 1 day, as compared with 2 days observed when *MAL63* was expressed from its native promoter). All of the transformants carrying mutant fusion genes were able to ferment maltose, but to varying degrees and more poorly than transformants carrying the wild-type fusion gene. Significant *MAL* structural gene expression was observed in all transformants, except those carrying *lexA-mal63-391*. But the levels of expression of maltose permease and maltase were reduced, compared with the wild type at a comparable extent. Only transformants carrying the *lexA-mal63-283* mutant fusion restored *MAL* structural gene expression to wild-type levels.

Thus, with the possible exception of *mal63-391*, it would appear that abundant expression of the mutant protein suppresses the mutant phenotype, but instability of the mutant proteins is unlikely to be responsible for their noninducible phenotype at normal expression levels. If this were the case, it would be expected that *lacZ* reporter expression would similarly be restored by overexpression of the mutants. Surprisingly, this is not the case. As is quite evident from the results in Table 3, none of the transformants expressing any of the mutant LexA-mal63 fusion proteins is able to activate transcription from the *lexA-lacZ* reporter. No blue color develops, even after overnight incubation at room temperature. An explanation of this unexpected result is presented in the Discussion.

Constitutive maltose permease expression suppresses the noninducible phenotype of *mal63* mutations

Wang et al. (2002) reports that overproduction of maltose permease in strain YPH500 suppresses the nonfunctional, naturally mutant *MAL*-activator gene present in this strain, *mal13*, and allows for maltose-inducible expression of maltase to wild-type levels. They suggest that the activator encoded by *mal13* in YPH500 is defective in its ability to respond to inducer and that the rapid accumulation of intracellular maltose to very high concentrations produced by the constitutive presence of high levels of maltose permease is able to overcome this defect. We decided to test the possibility that some of the *mal63* noninducible mutants identified here might be similarly suppressed.

Seven noninducible *mal63* mutant alleles carried on a CEN vector were introduced into strain CMY1073 (YPH500 *mal13*Δ mal33Δ) either with or without a CEN plasmid-borne copy of *ADH1-MAL61* that fuses the constitutive high-level *ADH1* promoter to the open reading frame of *MAL61* encoding maltose permease (Wang et al. 2002). Maltase activity was determined in transformants grown in uninduced (1% raffinose), induced (1% raffinose, 2% maltose), repressed (1% raffinose, 2% glucose), and induced-repressed (1% raffinose, 2% maltose, 2% glucose) growth conditions. As seen in Table 4, all but two of the mutants exhibit some suppression of the inducible phenotype to approximately 25–45% of wild-type levels. Only mutations *mal63-411* and *mal63-391* do not respond to maltose permease overexpression. Based on the results reported in Tables 3 and 4, we suggest that the alteration in *mal63-391* (K391A, E394A) is essential for induction and that the alteration in *mal63-411* (K411A, E413A) is essential for the response to maltose.

Mutagenesis of selected residues in the constitutive *MAL63/43-C*

We reasoned that mutations decreasing the ability of the *MAL*-activator to undergo induction should have a reduced impact in a constitutive *MAL*-activator. In a constitutive activator, one might expect that only alterations affecting activator function and not activator induction per se would have an effect on *MAL* gene transcription. We use the term activator function to mean the ability to form and maintain and active conformation, not the ability to respond to inducer, because a constitutive activator does not require induction to be active. To test this, noninducible mutations were introduced into *MAL63/43-C*, encoding a constitutive *MAL*-activator. *MAL63/43-C* fuses residues 215–470 encoded by the constitutive *MAL43-C MAL*-activator gene to the promoter and 5′ end of *MAL63* encoding residues 1–215 (see Fig. 1a, b). The oligonucleotides used for these conversions are listed in Table 2. The mutation truncating the last 14 residues of Mal63p (*mal63/43-C-457NS*) is also included. Each of these plasmid-borne *mal63/43-C* mutations was transformed into strain 61-106 and maltase expression, fermentation, and reporter expression were determined. The results of maltase assays are shown in Fig. 3. A clear correlation among all

Table 4 Constitutive overexpression of maltose permease suppresses the noninducible phenotype of *mal63* mutant activators. Vector pUN70 carrying constitutively expressed maltose permease ($ADH1_{promoter}$-*MAL61*) and vector pUN30 carrying *MAL*-activator mutant alleles (expressed from *MAL63* native promoter) were transformed into strain CMY1073 (YPH500 carrying deletions of both *mal13* and *mal33* genes). Maltase activity was determined in uninduced (*1% raffinose*), repressed (*2% glucose, 1% raffinose*), induced (*2% maltose, 1% raffinose*), and induced/repressed (*2% glucose, 2% maltose, 1% raffinose*) growth conditions. Values are averages of two transformants assayed in duplicate

MAL-activator allele (plasmid-borne)	Permease overexpression plasmid	Maltase activity			
		1% raffinose	2% glucose, 1% raffinose	2% maltose, 1% raffinose	2% glucose, 2% maltose, 1% raffinose
MAL63	Vector	8	5	451	2
MAL63	pADH1-MAL61	28	2	805	25
mal63-331	Vector	7	3	83	2
mal63-331	pADH1-MAL61	16	2	385	5
mal63-364	Vector	9	4	72	2
mal63-364	pADH1-MAL61	18	2	313	3
mal63-391	Vector	16	2	28	2
mal63-391	pADH1-MAL61	16	3	43	5
mal63-401	Vector	13	3	47	1
mal63-401	pADH1-MAL61	15	3	242	6
mal63-411	Vector	18	2	31	1
mal63-411	pADH1-MAL61	21	3	17	5
mal63-438	Vector	30	3	48	2
mal63-438	pADH1-MAL61	23	2	197	4
mal63-463	Vector	18	2	42	3
mal63-463	pADH1-MAL61	9	2	226	3
mal63-467A9N	Vector	17	3	55	5
mal63-467A9N	pADH1-MAL61	10	2	220	6

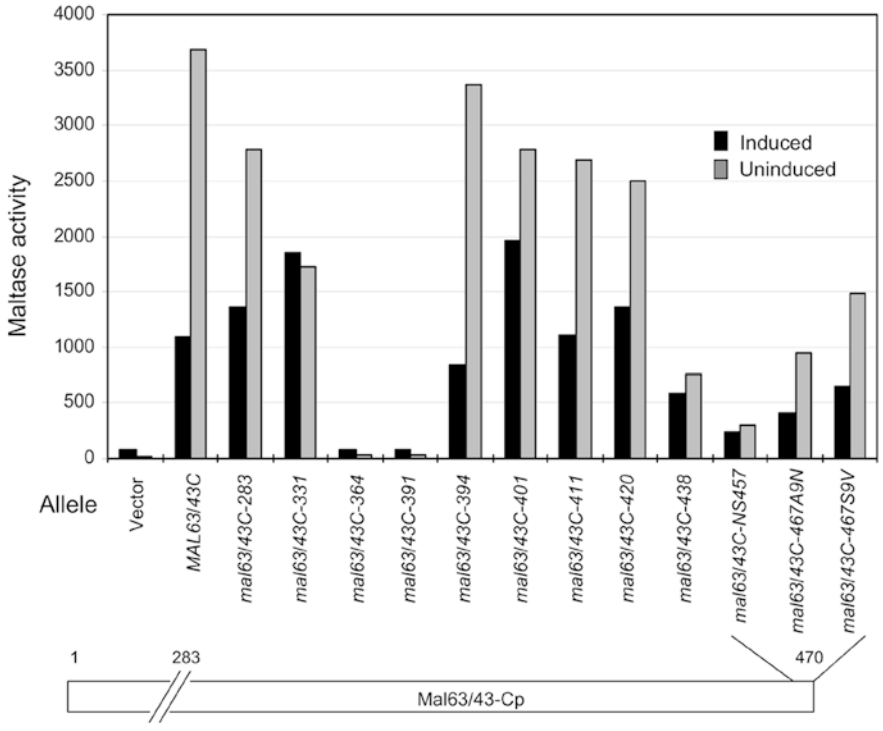

Fig. 3 Activity of clustered-charge to alanine scanning mutations in the regulatory domain of the constitutive *MAL*-activator, Mal63/43-Cp. The 12 mutations between codons 283 and 469 are represented in a linear array corresponding to their position in the gene. *The open rectangle below* represents the C-terminal half of Mal63/43p from residue 283 to residue 470. The last three mutations are clustered between residues 456 and 469. The maltase activity of transformants carrying each *mal63/43-C* allele was assayed in cells grown in conditions of induced (*black bars*) and uninduced (*gray bars*) growth

three measures of *MAL*-activator activity was evident (data not shown).

Five of the eight mutations located in the region encoding residues 283–423 have little or no effect on the constitutive phenotype of *MAL63/43-C* or on the level of maltase expressed in transformants grown under either induced or uninduced conditions (Fig. 3). Mutation *mal63/43-C-331* (R331A, R335A) decreases the uninduced levels of expression to approximately half of the wild-type *MAL63/43-C*, but expression is still constitutive. These results are consistent with the suggestion that the residues in the sequence at 331–423 are required for the response to maltose but not for activator function.

Two mutations, *mal63/43-C-364* (E364A, R367A) and *mal63/43-C-391* (K391A, I394A) are noninducible. Transformants carrying these alleles are nonfermenters and express very low levels of maltase when grown under both induced and uninduced conditions. During the construction of the *mal63/43-C-391* mutation, a partial conversion containing only the E394A alteration was obtained, called *mal63/43-C-394*. Transformants carrying this single mutation exhibited little or no alteration of the constitutive activity of *MAL63/43-C*. This suggests that K391 is responsible for the noninducible phenotype of *mal63/43-C-391*. Interestingly, overexpression of *lexA-mal63-364* (Table 3) and constitutive maltose permease expression (Table 4) both suppressed the noninducible phenotype of *mal63-364* but not that of *mal63-391*. This suggests that these alterations have distinctly different effects on *MAL*-activator function.

Mutations mapping to the C-terminal region of Mal63p (*mal63-438*, *mal63-467A9N*, *mal63-467S9V*, *mal63-457NS*) all exhibit dramatically reduced activator activity when introduced into *MAL63/43-C*, but the residual maltase expression is still constitutive.

Discussion

Clustered-charge to alanine scanning mutagenesis of *MAL63* was undertaken to identify regions of positive function within the C-terminal regulatory domain of the *MAL*-activator. Our results allow us to define an approximately 90-residue region (residues 331–423) that is involved in maltose sensing and an approximately 50-residue region at the C-terminus of Mal63p (residues 420–470) that is required for the formation and/or maintenance of the active state. Mutations affecting these positive functions are distributed throughout the C-terminal domain and overlap previously identified negative regulatory regions (Danzi et al. 2000).

With two exceptions, *mal63-460* and *mal63-462*, mutations altering residues 331–470 were found to significantly decrease the ability of Mal63p to activate transcription. We reasoned that mutations that affect the *MAL*-activator's ability to sense the presence of inducer should have a reduced impact in a constitutive activator. Of the six mutations altering residues 331–423 and producing a noninducible phenotype in *MAL63*, four have little or no effect on the constitutive phenotype of *MAL63/43-C* or its ability to activate maltase expression. Thus, we suggest this region of Mal63p is required for sensing inducer levels. It is not yet clear how the presence of maltose is sensed by *Saccharomyces*. Wang et al. (2002) found that intracellular maltose is sufficient to induce *MAL* gene expression in a

MAL-activator-dependent manner. Maltose could bind directly to the *MAL*-activator or indirectly via an as yet unidentified maltose-binding protein, comparable with Gal3p (Peng and Hopper 2000). The results presented here suggest that residues 331–423 are either the binding site of maltose itself or the binding site of this putative maltose-binding sensor. It should be noted that this maltose-responsive region overlaps one of the negative regulatory sites identified by Danzi et al. (2000), referred to as block 2 (residues 343–359). The sequence of block 2 in inducible Mal63p differs from that of constitutive activators Mal23-Cp and Mal43-Cp at ten and five residues, respectively. Moreover, the noninducible mutation *mal63-346* lies in the middle of block 2.

Two mutations in this maltose-responsive domain, *mal63-391* (K391A, E394A) and *mal63-364* (E364A, R367A), cause a noninducible phenotype when introduced into both *MAL63* and *MAL63/43-C*. With regard to *mal63-364*, our finding that constitutive overexpression of maltose permease is able to partially suppress the noninducible phenotype of *mal63-364* is consistent with the possibility that the defect might result from a reduced maltose-binding affinity. The *mal63-391* mutation is not suppressed by abundant overexpression (Table 3) or by constitutive overexpression of maltose permease (Table 4), indicating that the altered residues, or possibly K391 alone, are essential for the formation of an activated conformation.

All but two of the mutations in residues 438–470 significantly decrease the activity of both *MAL63* and *MAL63/43-C* activators. Nonetheless, the reduced level of maltase expression observed in transformants carrying the constitutive *mal63/43-C* mutants is still constitutive, suggesting this C-terminal region is involved in the formation and/or maintenance of the transcriptionally active state of the *MAL*-activator but not in maltose sensing. Interestingly, mutations *mal63-460* (D460A, K463A) and *mal63-462* (S462A) enhance the induced level of maltase expression. Perhaps these alterations increase the efficiency of active-state formation or stabilize the active state.

Residues in the region of 244–318 do not appear to be involved in induction or the response to maltose. Of the nine mutant alleles in this region, seven cause little or no defect in maltose induction or maltase expression. Only *mal63-283* exhibits significantly decreased maltose-inducible maltase activity. Overexpression of the LexA-mal63-283 fusion protein almost fully suppresses the maltose nonfermenting phenotype, suggesting that the noninducible phenotype of *mal63-283* may, at least in part, be the result of decreased protein stability. However, this does not fully explain the mutant phenotype since, despite the suppressing effect of overexpression of the LexA-mal63-283 fusion protein on *MAL* gene expression, the *lexA-lacZ* reporter expression is not similarly suppressed. Because this alteration lies in a region which we propose contains a negative region, we suggest the possibility that residues 283 and 287, which are altered in *mal63-283*, could strengthen this negative regulatory activity.

Figure 4 summarizes the results of the functional domain analysis by Hu et al. (1999), the analysis of *MAL*-activator constitutive mutations reported by Gibson et al. (1997) and Danzi et al. (2000), and the mutation analysis presented here. One should note that the negative regulatory domains defined by Danzi et al. (2000) overlap the maltose-responsive domain and the C-terminal domain described here. The 470-residue *MAL*-activator is about half the size of many other yeast transcription activators, which can range to over 1,000 residues in length. The overlap of regulatory domains observed in the *MAL*-activator could be necessitated by this restricted size, but we do not exclude the possibility that the overlap may have functional significance with regard to the mechanism of induction.

CaSUC1 encodes a transcription activator of the *Candida albicans SUC* genes (Kelly and Kwon-Chung 1992). *CaSUC1* expressed in *Saccharomyces* from the constitutive *ADH1* promoter complements the maltose nonfermentable phenotype of a *mal63 MAL*-activator mutation and restores maltose-inducible maltase expression. Pairwise alignment of the maltose-responsive region of Mal63p with CaSUC1p shows approximately 50% similarity and 25% identity between these proteins, particularly clustered in the region surrounding *mal63-331* and *mal63-391*. This homology does not extend beyond residue 420 of Mal63p. We were unable to identify any sequence homology of this maltose-responsive domain to known maltose-binding proteins, such as *E. coli* MalB.

The apparently contradictory finding reported here that overproduction of many of our *mal63* mutant *MAL*-activator proteins restores *MAL* gene expression but not $lexA_{promoter}$-*lacZ* reported expression is consistent with our previous conclusion that the *MAL*-activator undergoes a conformational change upon induction (Danzi et al. 2000). The results reported here can best be explained if one concludes that only the properly folded form of the mutant *MAL*-activator proteins is capable of binding both the UAS_{MAL} and activating *MAL* gene transcription. The basis for this

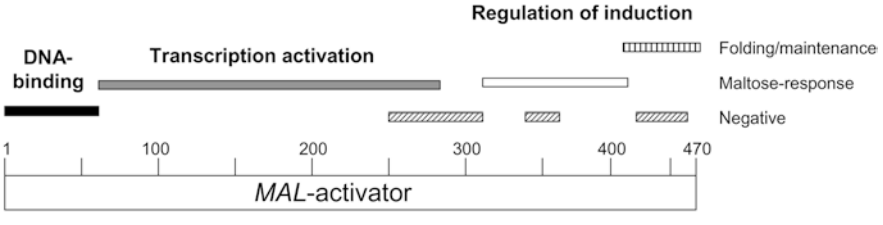

Fig. 4 *MAL*-activator functional domains. The diagram summarizes the results of this report and those of Gibson et al. (1997), Hu et al. (1999), and Danzi et al. (2000)

assumption is as follows. Clearly, the LexA-Mal63 mutant activator fusion proteins are localized to the nucleus because *MAL* gene expression can be induced, so the lack of reporter expression cannot be explained by nuclear exclusion. Instead, we propose that, of the abundant pool of LexA-mal63 mutant fusion protein, the bulk of the protein is not correctly folded into a transcriptionally active conformation and is unable to bind to the UAS_{MAL}. Nonetheless, this inactive protein is still capable of binding to the *lexA* operator, thereby blocking expression of the reporter. Despite this, within this abundant pool of mutant *MAL*-activator proteins, a few activator molecules have been able to fold into a transcriptionally active conformation in response to maltose; and these are capable of binding to the UAS_{MAL} and activating maltose-induced *MAL* gene expression. While inducible *MAL* gene expression is restored, it should be noted from the results in Table 3 that it is still defective.

Taken together, the results reported here and in previous publications support the following model of *MAL*-activator induction (Gibson et al. 1997; Hu et al. 1999; Danzi et al. 2000). In the absence of inducer, the *MAL*-activator is involved in complex protein–protein interactions that keep it in an inactive conformation but poised for induction. Maltose induction results in a conformational change in the *MAL*-activator so as to produce the activated state capable of binding to the UAS_{MAL} and activating *MAL* gene transcription. Based on previous studies by Hu et al. (1999) and Danzi et al. (2000), we believe these interactions are most probably intermolecular interactions and not intramolecular interactions between separate regions of the *MAL*-activator itself. Similar conformational changes have been suggested for other yeast and mammalian transcription activators, including *Saccharomyces* Put3p (the DNA-binding activator of genes of the proline utilization pathway), Lys14p (the DNA-binding activator of the *LYS* genes), Hap1 (the heme-responsive activator of genes involved in respiratory functions and control of oxidative damage), the mammalian steroid receptors, and the tumor suppressor p53 (Des Etages et al. 2001; El Alami et al. 2002; Lee et al. 2002; Pratt and Toft 2003). A potential candidate for a *MAL*-activator interacting protein comes from unpublished studies from our laboratory (M. Bali, B. Zhang) carried out in collaboration with Kevin Morano (University of Texas Houston Medical School) that demonstrate that Mal63 *MAL*-activator co-precipitates with Hsp90 and appears to be a Hsp90 chaperone client protein. The results of *MAL*-activator mutation analysis and structure–function studies reported here and by Gibson et al. (1997), Hu et al. (1999), and Danzi et al. (2000) are more easily interpretable in light of this finding; and we are actively engaged in additional studies along these lines, using our array of noninducible and constitutive *MAL63* mutants.

Acknowledgements This project was supported by a grant to C.A.M. from the National Institute of General Medical Sciences (GM28216). The work was carried out in partial fulfillment of the requirements for the PhD degree from the Graduate School of the City University of New York (S.E.D., M.B.).

References

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (2002) Current protocols in molecular biology. Wiley, New York

Bohen SP, Yamamoto KR (1993) Isolation of Hsp90 mutants by screening for decreased steroid receptor function. Proc Natl Acad Sci USA 90:11424–11428

Cheng Q, Michels CA (1991) *MAL11* and *MAL61* encode the inducible high-affinity maltose transporter of *Saccharomyces cerevisiae*. J Bacteriol 173:1817–1820

Danzi SE, Zhang B, Michels CD (2000) Alterations in the *Saccharomyces MAL*-activator cause constitutivity but can be suppressed by intragenic mutations. Curr Genet 294:233–240

Des Etages SA, Saxena D, Huang HL, Falvey DA, Barber D, Brandriss MC (2001) Conformational changes play a role in regulating the activity of the proline utilization pathway-specific regulator in *Saccharomyces cerevisiae*. Mol Microbiol 40:890–899

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

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 non-essential homolog of *MAL63*. Mol Cell Biol 8:1027–1035

El Alami M, Feller A, Pierard A, Dubois E (2002) The proper folding of a long C-terminal segment of the yeast Lys14p regulator is required for activation of *LYS* genes in response to the metabolic effector. Mol Microbiol 43:1629–1639

Elledge SJ, Davis RW (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

Hach A, Hon T, Zhang L (1999) A new class of repression modules is critical for heme regulation of the yeast transcriptional activator Hap1. Mol Cell Biol 19:4324–4333

Higgins VJ, Braidwood M, Bissinger P, Dawes IW, Attfield PV (1999) Leu343Phe substitution in the Malz3 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*. Gurr 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

Kelly R, Kwon-Chung KJ (1992) A zinc finger protein from *Candida albicans* is involved in sucrose utilization. J Bacteriol 174:222–232

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

Lee HC, Hon T, Zhang L (2002) The molecular chaperone Hsp90 mediates heme activation of the yeast transcriptional activator Hap1. J Biol Chem 277:7430–7437

Leuther K, Salmeron J, Johnston S (1993) Genetic evidence that an activation domain of *GAL4* does not require acidity and may form a β sheet. Cell 72:575–585

Medintz I, Jiang H, Han EK, Cui W, Michels CA (1996) Characterization of the glucose-induced inactivation of maltose permease in *Saccharomyces cerevisiae*. J Bacteriol 178:2245–2254

Needleman RB (1991) Control of maltase synthesis in yeast. Mol Microbiol 5:2070–2084

Peng G, Hopper JE (2000) Evidence for Gal3p′s cytoplasmic location and Gal80p′s dual cytoplasmic–nuclear location implicates new mechanisms for controlling Gal4p activity in *Saccharomyces cerevisiae*. Mol Cell Biol 20:5140–5148

Pratt WB (1998) The Hsp90-based chaperone system: involvement in signal transduction from a variety of hormone and growth factor receptors. Proc Soc Exp Biol Med 217:420–434

Pratt WB, Toft DO (2003) Regulation of signaling protein function and trafficking by the *hsp90*/*hsp70*-based chaperone machinery. Proc Soc Exp Biol Med 228:111–133

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 27: 509–516

Wang X, Bali M, Medintz I, Michels CA (2002) Intracellular maltose is sufficient to induce *MAL* gene expression in *Saccharomyces cerevisiae*. Eukaryot Cell 1:696–703

Wertman KF, Drubin DG, Botstein D (1992) Systematic mutational analysis of the yeast *ACT1* gene. Genetics 132: 337–350