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Functional domain analysis of the *Saccharomyces MAL*-activator

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Abstract *MAL63* of the *MAL6* locus and its homologues at the other *MAL* loci encode transcription activators required for the maltose-inducible expression of the *MAL* structural genes. We carried out a deletion analysis of *LexA-MAL63* gene fusions to localize the functional domains of the Mal63 *MAL*-activator protein. Our results indicate that the sequence-specific DNA-binding domain of Mal63p is contained in residues 1–100; that residues 60–283 constitute a functional core region including the transactivation domain; that residues 251–299 are required to inhibit the activation function of Mal63p; and that the rest of the C-terminal region of the protein contains a maltose-responsive domain that acts to relieve the inhibitory effect on the activation function. Abundant overproduction of Mal63p does not overcome the negative regulation of *MAL* gene expression in the absence of maltose, suggesting that a titratable *MAL*-specific repressor similar to Gal80p is not involved in the negative regulation of the *MAL*-activator. A model for maltose-inducible autoregulation of the *MAL*-activator is presented.

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

Introduction

MAL63 encodes a positive regulatory protein, referred to as the *MAL*-activator, that is required for the maltose-inducible expression of the *MAL* structural genes encoding maltose permease and maltase in *Saccharomyces* (reviewed in Needleman 1991). *MAL63* is one of a family of five nearly identical genes each of which maps to one of the five complex *MAL* loci, *MAL1–4* and *MAL6*, and is referred to as *MAL13*, *23*, *33*, *43* or *63* depending upon the locus position (Charron et al. 1989). Mal63p is a predicted 470 amino-acid protein with a cysteine-rich amino-terminal region (residues 8–34) homologous to the cysteine-rich, zinc-cluster DNA-binding domains characterized in Gal4p and other yeast activators (Kim and Michels 1988; Solliti and Marmur 1988; Vallee et al. 1991; Marmorstein et al. 1992).

The *MAL6* structural genes, *MAL61* (maltose permease gene) and *MAL62* (maltase gene), are coordinately and divergently transcribed from a common 874-bp promoter region (Needleman et al. 1974; Levine et al. 1992). Deletion analysis of the bi-directional promoter defined a 68-bp region sufficient for maltose-dependent and Mal63p-dependent transcription of the *MAL* structural genes (Levine et al. 1992). This sequence is referred to as the UAS$_{MAL}$. DNA footprint analysis, using purified Mal63 protein isolated from *Saccharomyces* cells grown in uninduced conditions, identified three sites in the *MAL61-MAL62* intergenic region protected by Mal63p, two of which coincide with the UAS$_{MAL}$ defined by Levine et al. (1992) (Ni and Needleman 1990; Sirenko et al. 1995).

Based on a molecular genetic analysis of several inducible, noninducible, and constitutive *MAL*-activator alleles (Gibson et al. 1997), we reported previously that the C-terminal portion of the *MAL*-activator from residue 215 contains a maltose-responsive regulatory domain capable of inhibiting transcription activation by the *MAL*-activator in the absence of maltose. We also showed that extensive mutations within the C-terminal region, or deletion of the entire region C-terminal to residue 283, produce a constitutive *MAL*-activator. Our goal in the analysis reported here is to characterize further the functional domains of the *MAL63*-encoded

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MAL-activator, and to gain insight into the mechanism of maltose-inducible regulation.

Materials and methods

Yeast strains. Strain 340–2A (*mal13 AGT1 MAL12 ura3-52 ade*) was used as a *MAL*-activator-deficient tester strain, and is described in detail by Charron and Michels (1987). The *AGT1* and *MAL12* genes of 340-2A encode maltose permease and maltase, respectively, and map to the *MAL1* locus position (Charron and Michels 1988). No functional *MAL*-activator genes are present. Strain YPH500 (*MAT*α *AGT1 MAL12 mal13 MAL31 MAL32 mal33 ura3-52 his3-200 leu2-1 trp1-63 lys2-801 ade2-101*) was used as a *MAL*-activator-deficient tester strain for experiments measuring *LacZ* reporter gene expression (Sikorski and Heiter 1989). YPH500 does not ferment maltose, but does contain both *MAL1*-linked and *MAL3*-linked *MAL* structural genes encoding maltose permease (*AGT1* and *MAL31*) and maltase (*MAL12* and *MAL32*) (data not shown). Both 340-2A and YPH500 are complemented by plasmid-borne *MAL*-activator genes and transformants ferment maltose. Strain CMY1001 (*MAT***a** *MAL61/HA MAL12 MAL13 ura3-52 his3-200 leu2 trp1-63 lys2-801 ade2-101*) contains a complete *MAL1* locus (Medintz et al. 1996).

Recombinant DNA techniques. DNA preparation, digestion, ligation, transformation and sequencing were carried out according to standard protocols, or according to the manufacturer's recommendation when commercially available kits were used (Ausubel et al. 1998). In vitro oligonucleotide-directed mutagenesis was carried out using the BioRad Muta-Gene kit. All constructions which resulted from in vitro mutagenesis or PCR were sequenced to confirm that only the intended changes were present.

Construction of LexA-MAL63 hybrids. *LexA*-fusion constructions utilized plasmid pSH2-1, which contains codons 1–87 of the *Escherichia coli LexA* gene fused to the *Saccharomyces ADH1* constitutive promoter, or plasmid pBTM116 (obtained from Stan Fields), which contains codons 1–202 of the *LexA* gene fused to the *Saccharomyces ADH1* promoter. Both plasmids provide *Eco*RI, *Sal*I, and *Bam*HI sites positioned 3′ to the *LexA* sequence for fusion to inserted sequences (Brent and Ptashne 1985; Hanes and Brent 1989). Plasmid pLexA-MAL63$_{(2–470)}$ was constructed as follows. The *Bam*HI-*Sal*I fragment containing *MAL63/HA* (see below) was cloned into the *Bam*HI and *Sal*I sites of pSH2-1 (the *Bam*HI site is in the HA-tag sequence 5′ to codon 1 of *MAL63*). The reading-frame was corrected by linearizing the plasmid at its unique *Bam*HI site, digesting briefly with S1 nuclease, and self-ligating to create the fusion. The resulting plasmids were sequenced to determine the number of deleted base pairs and to insure that the correct reading frame had been established. Plasmid pLexA-MAL63$_{(2–470)}$ retains codon 2 of *MAL63* but has lost the *Eco*RI and *Bam*HI sites from the polylinker sequence between *LexA* and *MAL63*. pLexA-MAL63$_{(2–456)}$ was constructed from pLexA-MAL63$_{(2–470)}$ by replacing the *Eco*RI fragment containing the 3′-end of *MAL63* with one containing this same sequence but with a translation termination at codon 457. This nonsense codon was introducted by in vitro mutagenesis using oligonucleotide L24 (Table 1). pLexA-MAL63$_{(215–470)}$ was constructed by inserting the *Eco*RI fragment containing the 3′-end of *MAL63* from codon 215 into the *Eco*RI site of pSH2-1. pLexA-MAL63$_{(2–216)}$ was created by deleting the *Eco*RI fragment containing codons 216–470 and 3′ downstream sequences from pLexA-MAL63$_{(2–470)}$. This fuses co-

Table 1 The various oligonucleotides used in the construction of plasmids by PCR for in vitro site-directed mutagenesis, or for other purposes, are listed here and referred to in the text where appropriate

Mutagenic oligonucleotides (altered sequence underlined, restriction sites in bold)	
A18	CCGTATCCTTTTTAGAGAGTC**AGATCT**TAGGCTCGTGACACACTT
A19	AGTATTCGAAAGAAAATT**AGATCT**TTCGGTGCAGGAATCATC
A20	ATACTTATTTACGACATC**AGATCT**CATCCGCATACCTTTCAT
A21	CAAAGGTTTAGAAATGGG**AGATCT**TACCAATGCATTGGCTAT
K21	GATGTATCCGTAAGA**CCAGGG**CTCTATATCAAGTGATGT
K22	AGACTGTTTCGCAATAC**CCATGG**TATTATATGATAAATTTTCTG
K23	ACATGGTTTATTCCTGTCA*AG*CTTTACTCGACGAACGC
K24	GTTCAAATTGCGCAAGTTTAACGCCTGAAT*CG*ATCGATTACATGGTTTATT
K25	TTTCAACGGTTGAAGATAAGT*AC*TGTTCAAATTGCGCTGAAT
K26	CTGAATGCAGCGATTACA*AA*G*C*TTATTCCTGTCACACTTTAC
L22	TATCGATTAATA**CCATGG**TTTAAATGTACCCATACGACGTTCCAGATTAC
L23	TATCGATTAATA**CCATGGATCC**GGGACCACCCAAGCTAGCGTAATCTGGAAC
L24	GTCGTCTTCATCTTTGGAG*GGATCCTTTAATTA*CAAAGGTTTAGAAA
L25	GTCTGTAAAGTGGAAAA*CTCGAG*AACCCAGTTTTTTAGCTGCCTG
PCR primers (Restriction sites in bold, stop codon underlined)	
A10	CG**GGATCC**TTCTTCAACCGTTGAAAAAGAGA
A11	CG**GGATCC**CAATGCTTTCCTACGATGATCTT
A12	CG**GGATCC**AGCAGTTGTGCACTCTTTGCATG
A13	GGG**AATATT**CAACGGTTGAAGATAAGT
A14	CG**GGATCC**CCATCTGCACTTCCGCTAT
A15	CG**GGATCC**CTGGCCAAATTACATATAAGTT
A27	CG**GGATCC***TTA*CTTTCCTGGTATAGTGAA
A28	CG**GAATTC**ATGGGTATTGCGAAACAGTCT
A29	GC**GGATCC**CACACTCTATCAGTATATCTATC
B33	GG**GAATTC**AAAATGTTCGACAGATTTTCAACG
B34	GG**GGATCC**TCAGATGGTGAGTATGAA
L3	G**GGATCC**GGATGCTTTCCTACGATGAT
L4	G**GGATCC**ATCTAGGCTCGTGACACA
L5	G**GGATCC**GTAGGCGCAACG
Z11	GG**GGATCC**GTATGGGTATTGCGAAACAGTCT
UAS$_{MAL}$ oligonucleotides	
K27	GTTTACAGGATTTATCCGGAAATTTTCGCGG
K28	TAGGCCTTTAAAAGCGCCTGGGGTGTG

don 216 to vector sequences and results in the C-terminal addition of 24 amino-acid residues (FPGIRRPAAKLIPGEFLMIYDFYY) before an in-frame termination codon is reached. Plasmids pLexA-MAL63$_{(\Delta 251–299)}$, pLexA-MAL63$_{(\Delta 302–349)}$, pLexA-MAL63$_{(\Delta 352–399)}$ and pLexA-MAL63$_{(\Delta 402–449)}$ were created in a two-step process. Oligonucleotides A18, A19, A20, and A21 (Table 1) were used to delete codons 251–299, 302–349, 352–399, and 402–449, respectively, from the *Eco*RI fragment of *MAL63* containing codons 216–470 using site-directed mutagenesis. A *Bgl*II site was inserted at the site of each deletion. The alterations were confirmed by completely sequencing the *Eco*RI fragments, and the mutagenized *Eco*RI fragments were then cloned into LexA-MAL63$_{(2–216)}$ in the correct orientation. The following plasmids were constructed in plasmid pSH2-1 using a PCR product containing the indicated codons of *MAL63* amplified with the oligonucleotide primers listed in Table 1. Oligonucleotide primers A27 and A28 were used for the construction of pLexA-MAL63$_{(1–283)}$ and the PCR product was digested with *Eco*RI and *Bam*HI and inserted into *Eco*RI/*Bam*HI-digested pSH2-1. Similarly, oligonucleotide primers A28 and L5 were used for the construction of pLexA-MAL63$_{(1–120)}$ and the PCR product was digested with *Eco*RI and *Bam*HI and inserted into *Eco*RI/*Bam*HI-digested pSH2-1. Plasmid pLexA-MAL63$_{(215–283)}$ was created from pLexA-MAL63$_{(1–283)}$ by digestion with *Eco*RI. pLexA-MAL63$_{(37–470)}$, pLexA-MAL63$_{(99–470)}$, and pLexA-MAL63$_{(150–470)}$ were constructed using oligonucleotides A10, A11, and A12 as the upstream primers and A29 as the downstream primer. pLexA-MAL63$_{(\Delta 41–66)}$ was created by three-fragment ligation using *Eco*RI/*Bam*HI-digested plasmid pSH2-1, the PCR product containing codons 1–40 of *MAL63* amplified using oligonucleotide primers A28 and primer A13, and the 1.2-kb *Sst*I/*Bam*HI fragment from pLexA-MAL63$_{(37–470)}$ containing *MAL63* codons 66–470. pLexA-MAL63$_{(\Delta 62–98)}$ was created from pLexA-MAL63$_{(99–470)}$ by a similar three-fragment ligation using *Eco*RI/*Bam*HI-digested pSH2-1, the PCR product containing codons 1–61 of *MAL63* produced using oligonucleotide primers A28 and A14, and the 1.2-kb *Bam*HI fragment from pLexA-MAL63$_{(99–470)}$ containing codons 99–470. The *Eco*RI fragments from pLexA-MAL63$_{(\Delta 41–66)}$ and pLexA-MAL63$_{(\Delta 62–98)}$ containing codons 1–215$_{(\Delta 41–66)}$ and codons 1–215$_{(\Delta 62–98)}$, respectively, were subcloned into the unique *Eco*RI site of pLexA-MAL63$_{(215–283)}$ to create pLexA-MAL63$_{(1–283\Delta 41–66)}$ and pLexA-MAL63$_{(1–283\Delta 62–98)}$, and the proper orientation confirmed by PCR. pLexA-MAL63$_{(\Delta 99–150)}$ was created by three-fragment ligation using the PCR product containing codons 1–99 of *MAL63* amplified with oligonucleotides A28 and A15, the 1.0-kb *Bam*HI fragment from pLexA-MAL63$_{(150–470)}$ containing codons 150–470, and *Eco*RI/*Bam*HI digested pSH2-1. A fragment containing codons 100–250 was PCR-amplified using primers L3 and L4, both of which contain a *Bam*HI site, and then inserted into the *Bam*HI site of plasmid pLexA (a *TRP1* derivative of pSH2-1, generating pLexA-MAL63$_{(100–250)}$. An *Eco*RI fragment containing codons 100–215 from pLexA-MAL63$_{(100–250)}$ was inserted into pLexA-MAL63$_{(215–283)}$ digested with *Eco*RI to create pLexA-MAL63$_{(100–283)}$. pLexA-MAL63$_{(100–250)}$ was digested with *Eco*RI to release a fragment containing codons 100–215 and the remaining fragment self-ligated to generate pLexA-MAL63$_{(215–250)}$. An *Eco*RI fragment containing codons 1–215 from pLexA-MAL63$_{(1–283)}$ was inserted into pLexA-MAL63$_{(215–250)}$ digested with *Eco*RI to create pLexA-MAL63$_{(1–250)}$. To create pLexA$_{(1–202)}$-MAL63, an *Eco*RI-*Bam*HI fragment containing codons 215–470 was ligated into plasmid pBTM116 digested with *Eco*RI and *Bam*HI. The resulting pLexA$_{(1–202)}$-MAL63$_{(215–470)}$ was then digested with *Eco*RI to allow the insertion of an *Eco*RI fragment containing codons 1–215, creating pLexA$_{(1–202)}$-MAL63. A fragment containing codons 1–283 was amplified using primer Z11 and A27, both of which contain a *Bam*HI site, and was inserted into the *Bam*HI site of pBTM116 to create pLexA$_{(1–202)}$-MAL63$_{(1–283)}$. pLexA$_{(1–202)}$-MAL63$_{(100–250)}$ and pLexA$_{(1–202)}$-MAL63$_{(100–283)}$ were created by inserting the *Bam*HI fragments containing codons 100–250 and codons 100–283, respectively, into the *Bam*HI site of pBTM116. pLexA$_{(1–202)}$-MAL63$_{(434–470)}$ was constructed from the PCR amplification product of primers B33 and A29 containing codons 434–470 between the *Eco*RI and *Bam*HI sites of pBTM116. pLexA$_{(1–202)}$-MAL63$_{(60–283)}$ was created by inserting the *Bam*HI fragment containing codons 60–283 produced by PCR amplification with primers B34 and A27 into the *Bam*HI site of pBTM116.

Construction of mutations in the cysteine-rich region of MAL63. A *Sal*I fragment from plasmid p40Leu containing *MAL63* from the *Bgl*II site approximately 350 bp upstream of the ORF to the *Sal*I site approximately 300 bp downstream from the 3′ end of the ORF was cloned into M13 for in vitro mutagenesis (Kim and Michels 1998). Oligonucleotides K21 and K22 (Table 1) were used to remove the *Nco*I site at codons 319/320 of *MAL63* and to create a *Nco*I site at codon 1, respectively. This was done so as to simplify expression of *MAL63* using the T7 expression system (Studier and Moffatt 1986; Rosenberg et al. 1987). No alteration in protein sequence resulted from these changes. The *Sal*I fragment was then subcloned into vector YCp50, transformed into strain 340-2A, and full functional activity of the altered *MAL63/N* gene confirmed. The following mutations were created in *MAL63/N* using the indicated oligonucleotide from Table 1: Cys18Leu, K23; Cys27Ser, K24; Cys34Gly, K25; Pro23Leu, K26.

Gel-mobility shift assays. The T7 expression system was used to synthesize an N-terminal fragment (residues 1–111) of Mal63p and the mutant proteins (Studier and Moffatt 1986; Rosenberg et al. 1987). An *Nco*I-*Xba*I fragment from *MAL63/N* and from each of the mutant alleles *mal63/N-C18L*, *mal63/N-C27S*, *mal63/N-C34G*, and *mal63/N-P23L* was cloned into the expression vector pET-8c so as to fuse the T7 gene ten upstream sequences to codon 1 of *MAL63* at the *Nco*I site. The *Xba*I site in pET-8c is in vector sequences and an in-frame translation termination is reached 21 codons downstream from the site. Strain BL21(DE3) pLysS was used as the *Escherichia coli* host. Synthesis of the fragment proteins was induced and extracts prepared according to the published procedures. SDS-PAGE gels were used to estimate the concentration of the fragment in the cell extract and approximately equal amounts of the fragment proteins were used for the gel-mobility shift assays of DNA-binding. DNA-binding activity was assayed with the standard gel-mobility shift assay described in Ausubel et al. (1998) using a 40-bp oligonucleotide synthesized as follows. Two partially overlapping, complementary oligonucleotides, K27 and K28 (Table 1), were annealed and the ends filled in with radiolabeled nucleotides. The sequence of this oligonucleotide is derived from base pairs −548 to −587 of the *MAL61-MAL62* UAS$_{MAL}$ (Levine et al. 1992). Binding of the wild-type Mal63(1–111) fragment protein to this DNA sequence is specific since it was not competed by a 16-fold excess of a 30-bp oligonucleotide from the *MAL61-62* promoter (base pairs −516 to −547) or by a 40-fold excess of salmon sperm DNA, but was competed by an equal quantity of unlabeled oligonucleotide of the same sequence (data not shown).

Over-expression of MAL63 in yeast. Over-expression was accomplished as described in Mylin et al. (1990). Levels of Mal63p synthesis were monitored by Western analysis of hemagglutinin-tagged Mal63p with the 12CA5 anti-HA specific antibody (Fields et al. 1988). Using the *MAL63/N* allele described above, a DNA fragment with *Nco*I ends encoding the peptide MYPYDVPDYASLGGP (including the HA epitope sequence plus some linker amino acids) was inserted into the *Nco*I site at the 5′-end of the *MAL63/N* ORF placing the HA tag-sequence in-frame with codon 1 of *MAL63/N*. This DNA fragment was formed by annealing oligonucleotides L22 and L23, filling in the ends with the Klenow fragment, and digesting with *Nco*I. The position of a *Bam*HI site within this fragment is indicated in the oligonucleotide sequences in Table 1. A *Xho*I site was introduced 60 bp upstream of codon 1 of *MAL63/HA* using oligonucleotide L25. This *Xho*I-*Sal*I fragment was subcloned into Yep51 at the *Sal*I site, thereby placing *MAL63/HA* downstream from the *GAL10* promoter to form plasmid YEpMAL63/HA. YEpMAL63/HA was transformed into yeast strain SC340, containing an integrated *GAL10promoterGAL4* cassette (Mylin et al. 1990).

Western-blot analysis. Protein extracts were prepared as outlined in Mylin et al. (1989). The samples were size-separated in SDS-PAGE gels and transfered to nitrocellulose for probing with the appropriate antibody using standard procedures (Ausubel et al. 1998). The LexAp fusion protein blots were probed using rabbit anti-LexA antibodies (obtained from Roger Brent) followed by alkaline phosphatase-conjugated, goat anti-rabbit antibodies (Sigma). The Mal63/HA blots were probed with the anti-HA monoclonal antibody (Berkeley Antibody Company) followed by goat anti-mouse IgG (Sigma) and mouse PAP antibody (Sigma). Protein bands were visualized using the Enhanced Chemiluminescence technique (ECL-kit, Amersham).

Enzyme assays. β-Galactosidase activity was assayed according to the protocols of Ausubel et al. (1998) using crude cell extracts. Specific activity is expressed as nmol of o-nitrophenol liberated/min per mg of protein. Maltose fermentation and maltase activity were assayed as outlined by Dubin et al. (1985). Specific activity is expressed as nmol of PNPG hydrolysed/min per mg of protein.

Results

Overexpression of *MAL63*

Activation by Gal4p is specifically inhibited by interaction with Gal80p in the absence of galactose (reviewed in Johnston and Carlson 1993). The site of interaction lies near the C-terminal transcription activation domain of Gal4p, and in the presence of galactose this inhibitory effect is relieved by a conformational change of the complex which is mediated by Gal3 protein. Since Gal80p is present in limiting quantities, its negative effects can be overcome by increasing the concentration of the activator molecules. Johnston and Hopper (1982) showed that overexpression of *GAL4* using a high-copy plasmid vector resulted in constitutive, partially glucose-repression-insensitive expression of the *GAL* structural genes. In contrast, constitutive *PUT3* mutations with alterations near the C-terminal transactivation domain of the protein have also been isolated, but overexpression of the Put3 activator protein does not result in constitutive expression of the target genes (Marczak and Brandriss 1991).

To examine whether a Gal80p-like negative regulator may act on the *MAL*-activator, we overexpressed *MAL63* using the *GAL10* promoter. To monitor expression levels, we used a hemagglutinin epitope-tagged allele of *MAL63*. The *GAL10promoter-MAL63/HA* fusion construction contains 60 bp of sequence upstream of the *MAL63/HA* ORF, and was transformed into strain SC340 carrying a regulated *GAL10-GAL4* cassette (Mylin et al. 1990). Strain SC340 does not ferment maltose because it lacks a *MAL*-activator gene, but transformants carrying the *GAL10promoter-MAL63/HA* fusion on plasmid YEp51 ferment maltose even in the absence of galactose, indicating that the basal levels of *MAL63/HA* protein expressed from this YEp51-based construct containing only 60 bp of the *MAL63/HA* promoter sequence are adequate to mediate normal activation of the *MAL* structural genes (data not shown). Nevertheless, even though the expression of Mal63/HAp is adequate to support maltose fermentation, the protein is not detectable in the absence of galactose (see Fig. 1 A, time 0).

Transformants of strain SC340 carrying the *GAL10promoter-MAL63/HA* fusion gene were grown to mid-log phase in media containing 3% glycerol and 2% lactate and then induced with galactose in the absence and presence of 2% maltose. Samples were taken at the indicated times to assay the level of Mal63/HA protein (Fig. 1 A) and maltase expression (Fig. 1 B). Galactose induced the production of Mal63/HAp, as is demonstrated in Fig. 1 A, and expression is unaffected by maltose in the medium (data not shown). Despite the abundant level of Mal63/HAp, which is far greater than normally found in strains capable of fermenting maltose, Fig. 1 B shows that no increase in maltase expression is seen in the absence of maltose. Moreover, maximal maltose-induced levels of maltase expression never exceed 1.5–2-times those found in induced SC340 cells carrying single-copy *MAL63* expressed from the natural promoter. Consistent with these data, the induced level of maltase expressed by YPH500 transformants carrying the overexpressed LexA-Mal63$_{(2–470)}$ fusion gene are only approximately 3-times the induced level of maltase found in YPH500 transformants carrying the wild-type *MAL63* gene (see Table 3 below; data not shown).

LexA-MAL63 deletion analysis

Our previous results show that residues 1–283 of certain *MAL*-activator alleles are sufficient to activate maltase expression while residues 215–470 contain a negative regulatory domain (Gibson et al. 1997). To localize further the functional regions of *MAL63*, we carried out a deletion analysis of the *LexA-MAL63* fusion gene. We used site-directed mutagenesis and PCR to create N-terminal, C-terminal and internal deletions in *MAL63*, and tested their function by fusing the mutant alleles, in-frame, to the DNA-binding domain of the *E. coli LexA* gene (codons 1–87), using plasmid pSH2-1, or to full-length *LexA* gene (codons 1–202) containing the DNA-binding domain and the dimerization domain of LexA, using plasmid pBTM116 (Brent and Ptashne 1985; Hanes and Brent 1989). Plasmids carrying the *LexA-MAL63* fusion genes were transformed into strain YPH500. Strain YPH500 lacks *MAL*-activator gene function, and thus does not ferment maltose. *MAL1*- and *MAL3*-linked genomic copies of the *MAL* structural genes encoding maltose permease and maltase are present in this strain and the maltose nonfermenting phenotype can be complemented by plasmid-borne copies of a *MAL*-activator gene. All of the YPH500 transformants produced easily detectable and comparable amounts of all of the fusion proteins shown in Fig. 2, as determined by Western analysis using an anti-LexA specific antibody (data not shown).

The ability of the hybrid LexA-Mal63 proteins to activate expression of a *LacZ* reporter gene under the

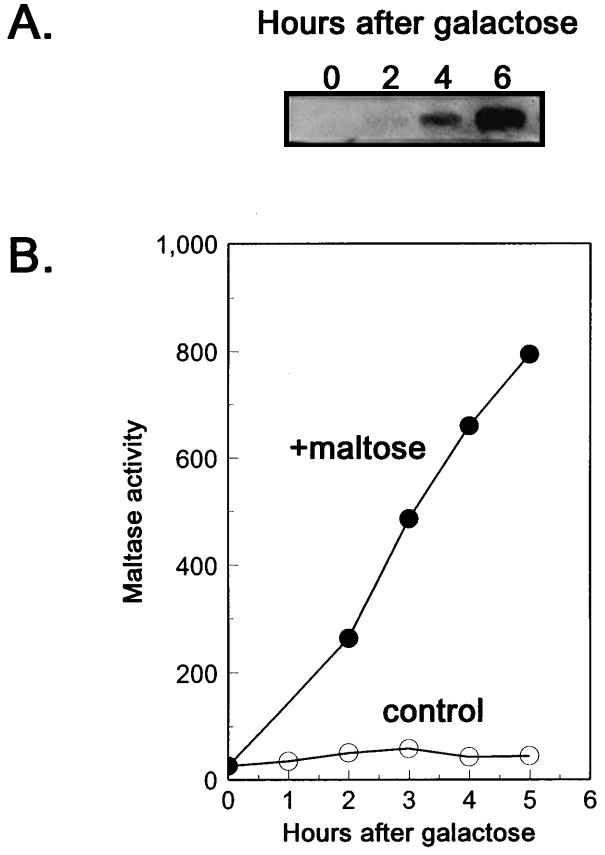


Fig. 1 A, B Over-expression of Mal63 protein. A hemagglutinin epitope-tagged allele of *MAL63* was expressed from the inducible *GAL10* promoter in strain SC340 which contains an integrated *GAL10-GAL4* cassette (Mylin et al. 1990). Strain SC340 lacks a *MAL*-activator gene. Transformants were grown to early log phase in selection medium on 3% glycerol and 2% lactate. At time 0, the cells were harvested and re-suspended in either 2% galactose or 2% galactose plus 2% maltose. Panel **A** shows the results of Western analysis of the galactose-induced expression of the Mal63/HA protein using anti-hemagglutinin antibody. Panel **B** presents the level of maltase expression during galactose-induction in the presence (*closed circles*) and absence (*open circles*) of maltose

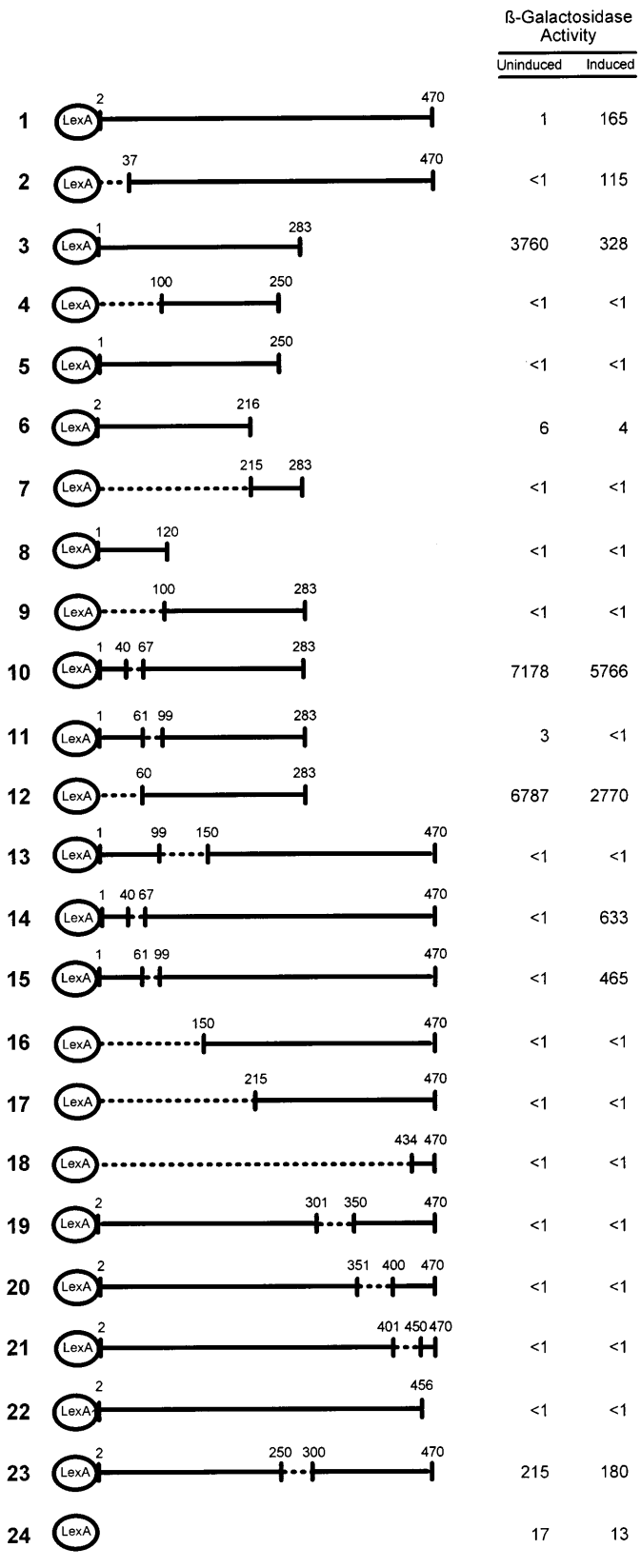
control of a promoter containing six LexA-binding sites (plasmid pSH18-18) was assayed in strain YPH500 grown under uninduced (3% glycerol, 2% lactate) and induced (2% maltose, 3% glycerol, 2% lactate) conditions. As can be seen in Fig. 2, the LexA-Mal63$_{(2–470)}$ fusion protein (construct 1) activates maltose-regulated *LacZ* reporter gene expression, and expression is induced over 100-fold by maltose. Deletion of the N-terminal 37 residues of Mal63p in the fusion activator (construct 2), which includes the entire cysteine-rich DNA-binding domain, does not affect the ability of this fusion activator to activate maltose-regulated reporter gene expression. Transformants expressing the truncated hybrid activator LexA-Mal63$_{(1–283)}$ (construct 3) express β-galactosidase constitutively. The uninduced levels of β-galactosidase are quite high, approximately 20-fold higher than the induced expression levels found for the full-length fusion activator LexA-Mal63$_{(2–470)}$, and this is repressed over 10-fold in maltose-grown cells. This repressed level of reporter expression is probably similar to the "maltose repression" that is seen in all strains carrying constitutive *MAL*-activators (see Discussion).

We intended to further define the activation domain within LexA-Mal63$_{(1–283)}$ which apparently activates transcription sufficiently. Constructs *LexA-MAL63*$_{(100–250)}$, *LexA-MAL63*$_{(1–250)}$, *LexA-MAL63*$_{(2–216)}$, and *LexA-MAL63*$_{(215–283)}$ all failed to activate reporter expression. Thus, residues 251–283 are necessary, but not sufficient, for activation function. The inability of constructs *LexA-MAL63*$_{(1–120)}$ and *LexA-MAL63*$_{(100–283)}$ to activate transcription suggests that residues N-terminal to 120 may also be required but are not sufficient for activation. Construct *LexA-MAL63*$_{(1–283Δ41–66)}$ activates reporter expression constitutively while construct *LexA-MAL63*$_{(1–283Δ62–98)}$ has no activation activity, suggesting that residues 67–98 are required for the activation function of *LexA-MAL63*$_{(1–283)}$. Thus, construct *LexA-MAL63*$_{(60–283)}$ was made and found to be a constitutive activator.

LexA-MAL63$_{(Δ100–149)}$ fusion is unable to activate transcription, supporting the idea that residues 60–283 contain an essential core region of the Mal63 protein. As expected, deletion construct *LexA-MAL63*$_{(Δ41–66)}$ functions as a maltose-inducible activator. However, surprisingly, the *LexA-MAL63*$_{(Δ62–98)}$ fusion also activates reporter expression in a maltose-dependent manner. Thus, the role of residues 62–98 in activation becomes essential only in the absence of residues C-terminal to 283.

We considered the possibility that the C-terminal region of Mal63p may contain a minor activation domain which could compensate for the loss of residues 60–99 in the full-length LexA-Mal63 protein, especially in light of the fact that several yeast zinc-cluster transcription activators contain an acidic activation domain at the C-termini and that Mal63p has an acidic region at its C-terminal end. Neither of the two constructs, *LexA-MAL63*$_{(150–470)}$ and *LexA-MAL63*$_{(215–470)}$, were able to activate expression of the reporter. In addition, *LexA*$_{(1–202)}$-*MAL63*$_{(434–470)}$, in which the C-terminal acidic region of Mal63p was fused to LexA, also exhibited no activation activity. We conclude from these results that the C-terminal half of Mal63p contains no activation domain.

To ensure that several fusion proteins which are important to our interpretation of the results are indeed entering the nucleus and binding to the *LexA* operator sites of the reporter, we carried out a transcription repression assay. Full-length *MAL63* and *MAL63*$_{(1–283)}$ when fused to *LexA*$_{(1–202)}$ behave the same as when they are fused to *LexA*$_{(1–87)}$ whereas protein products of *LexA*$_{(1–202)}$-*MAL63*$_{(100–250)}$ and *LexA*$_{(1–202)}$-*MAL63*$_{(100–283)}$ still fail to activate transcription (data not shown). The ability of these two fusion proteins to bind to the LexA sites was confirmed by a repression assay in the host strain which carries a



LacZ reporter gene expressed from the *GAL1* promoter containing two LexA binding sites positioned so as to interfere with Gal4p-activated expression of *LacZ*

◀

Fig. 2 Deletion analysis of *LexA-MAL63* fusions. Fusions of the LexA DNA-binding domain (residues 1–87) or the full-length LexA (residues 1–202) to regions of the Mal63 transcription activator were constructed as described in Materials and methods. All fragments of Mal63p were fused to $LexA_{(1–87)}$ except for constructs 12 and 18 in which $LexA_{(1–202)}$ was used. The *numbers* indicate the residues of Mal63 protein retained in the construction. Plasmids carrying these constructions were transformed into strain YPH500, a strain which lacks all *MAL*-activator genes, along with the *LacZ* reporter plasmid pSH18-18, which expresses *LacZ* from a promoter containing six copies of the LexAp binding site. β-Galactosidase expression levels were determined as described in Materials and methods. Uninduced medium contains 3% glycerol and 2% lactate, and 2% maltose was added to this for the induced medium

(Table 2). Both fusion proteins reduced reporter gene expression approximately 20–40%, implying that they enter the nucleus and bind to the *LexA* operator. The $LexA_{(1–202)}$-$Mal63_{(434–470)}$ fusion protein also binds to LexA sites effectively.

We found previously that extensive alterations in the C-terminal region of Mal63p produce a constitutive activator (Gibson et al. 1997). A series of fusion constructs containing deletions throughout the C-terminal region were generated in an effort to further define the negative regulatory domain. The LexA-$MAL63_{(Δ251–299)}$ fusion protein (construct 23) acts as a constitutive activator, indicating the disruption of a negatively regulatory function of the *MAL*-activator. Since *LexA-MAL63*$_{(1–250)}$ is inactive (construct 5) as compared to *LexA-MAL63*$_{(1–283)}$, the essential role of residues 251–283 in activation by LexA-$Mal63_{(1–283)}$p appears unnecessary in the presence of the region C-terminal to residue 301. In contrast to the constitutive LexA-$Mal63_{(1–283)}$, which lacks the entire C-terminal region, deletions of residues 302–349, 352–399, 402–449 and 457–470 (constructs 19–22) are all incapable of transcriptional activation. Since all of these fusions still retain residues 251–299, these data suggest that the region C-terminal to residue 300 may be required to relieve the inhibitory effect of residues 251–299 in response to maltose.

Table 2 Transcription repression assay with *LexA*$_{(1–202)}$-*MAL63* fusions. Strain YPH500 was transformed with plasmids carrying the indicated *LexA*$_{(1–202)}$-*MAL63* fusion gene along with a reporter plasmid pJK101 (Forsberg and Guarente 1989). Cells were grown in selection medium lacking uracil and tryptophan with 2% galactose. Standard deviations were less than 10%

LexA-MAL63 fusion gene	β-Galactosidase activity
Vector	1925
LexA$_{(1–202)}$	1110
LexA$_{(1–202)}$-*MAL63*	1133
LexA$_{(1–202)}$-*MAL63*$_{(100–250)}$	1201
LexA$_{(1–202)}$-*MAL63*$_{(100–283)}$	1599
LexA$_{(1–202)}$-*MAL63*$_{(434–470)}$	163

In summary, our data leads to several conclusions. The region of Mal63p between residues 60 and 283 is able to function as an independent transcription activator. Both ends of this region, residues 67–98 and 251–283, are essential for its function but their roles can be compensated for by the presence of the C-terminal portion of Mal63p. Unlike several other yeast transcription activators with the same DNA-binding motifs, Mal63p does not appear to contain an activation domain at the C-terminus. The region covering residues 251–299 plays an essential role in inhibition of the activation activity of Mal63p, and the C-terminal portion between residues 300–470 appears to be required to relieve such negative regulation in response to maltose.

We next examined the ability of several active fusion proteins retaining the N-terminal DNA-binding zinc cluster to activate maltase expression. As shown in Table 3, *LexA-MAL63*$_{(2–470)}$ (construct 1 from Fig. 2) and *LexA-MAL63*$_{(1–283)}$ (construct 2) are inducible and constitutive activators of maltase expression, respectively. Strains expressing the hybrid activator proteins LexA-Mal63$_{(\Delta 41–66)}$ (construct 14) and LexA-Mal63$_{(\Delta 62–98)}$ (construct 15), in which residues adjacent and C-terminal to the Mal63p zinc-cluster DNA-binding domain have been deleted, exhibited no maltase gene expression suggesting that the region immediately adjacent to the zinc cluster is required for activation at *MAL* loci. Surprisingly, the LexA-MAL63$_{(\Delta 251–299)}$ fusion protein (construct 23) also fails to activate maltase expression, which suggests that residues 251–299, in addition to their negative regulatory role, also play an essential role in activating the *MAL* structural genes specifically.

Table 3 Regulation of maltase gene expression by *LexA-MAL63* fusions. Fusions of the LexA DNA-binding domain (residues 1–87) or the full-length LexA (residues 1–202) to regions of the Mal63 transcription activator were constructed as described in Materials and methods and shown in Fig. 2. Plasmids carrying these constructions were transformed into strain YPH500, a strain which lacks all *MAL*-activator genes but contains copies of the *MAL* structural genes encoding maltose permease (*AGT1 MAL31*) and maltase (*MAL12 MAL32*). The ability to ferment maltose and maltase expression levels was determined as described in Materials and methods. Uninduced medium contains 3% glycerol and 2% lactate, and 2% maltose was added to this for the induced medium

LexA-MAL63 allele	Maltose fermentation	Maltase activity	
		Uninduced	Induced
LexA	–	25	92
LexA-MAL63(2–470)	+ +	111	2338
LexA-MAL63(1–283)	+ +	1331	863
LexA-MAL63(37–470)	–	19	136
LexA-MAL63(1–470Δ 41–65)	–	21	88
LexA-MAL63(1–470Δ 62–98)	–	20	92
LexA-MAL63(2–470Δ 251–299)	–	21	105

Mutation analysis of the cysteine-rich domain of *MAL63*

Residues 8–34 of Mal63p are cysteine-rich and homologous to the Cys-6 class of zinc-cluster DNA-binding domains seen in several yeast activators (Kim and Michels 1988). We used site-directed mutagenesis of *MAL63* to individually alter three of these cysteines, the invariant proline which is found just N-terminal to the second pair of cysteines in Mal63p, and all of the Cys-6 DNA-binding domains sequenced to-date (see Fig. 3 A).

MAL63, *MAL63/N*, and each of the mutant alleles was cloned into the CEN vector pRS316, and introduced into strain 340-2A which lacks a *MAL*-activator gene. The ability of the transformants to ferment maltose and to induce the expression of maltase is shown in Table 4. The alterations introduced into *MAL63* to create *MAL63/N* (described in Materials and methods) do not affect the phenotype of this gene. None of the

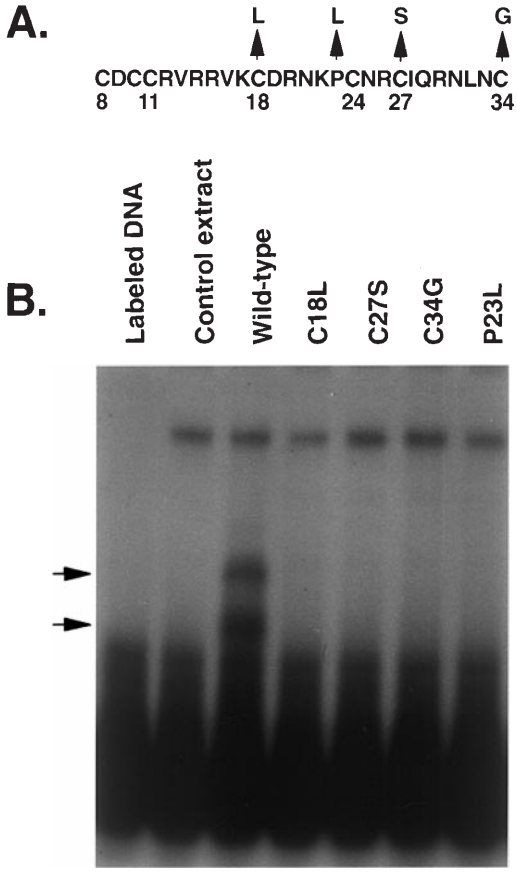


Fig. 3 A, B Gel-shift assays on the mutant Mal63 proteins containing alterations in the proposed zinc-cluster DNA-binding domain. Panel **A** the sequence of residues 8–34 containing the proposed zinc-cluster DNA-binding domain of Mal63p is given and the mutant alterations indicated. Panel **B** gel-shift assays were carried out using an extract from *E. coli* transformants expressing a fragment of Mal63p (residues 1–111) and a 40-bp fragment from the UAS$_{MAL}$ (Levine et al. 1992). The two DNA-protein complexes formed are indicated by *arrows*

Table 4 Regulation of maltase gene expression by *MAL63* mutations in the cysteine-rich proposed zinc-cluster region. Mutations in *MAL63* were constructed by in vitro site-directed mutagenesis as described in Materials and methods, subcloned into the CEN vector YCp50, and introduced into strain 340-2A which lacks a *MAL*-activator gene. Transformants were grown in selective medium with 3% glycerol and 2% lactate (uninduced) or 3% glycerol, 2% lactate and 2% maltose (induced) as the carbon source

MAL63 allele	Maltose fermentation	Maltase activity	
		Uninduced	Induced
Vector	−	17	25
MAL63	+ +	31	563
MAL63/N	+ +	35	819
mal63/N-Cys18Leu	−	13	20
mal63/N-Cys27Ser	−	15	25
mal63/N-Cys34Gly	−	14	25
mal63/N-Pro23Leu	+/−	22	291
mal63/N-Pro23Leu (2 copies)	+	26	386

transformants carrying the cysteine alterations ferment maltose, or exhibit maltose-inducible maltase expression. Interestingly, transformants carrying a single copy of the Pro23Leu mutant allele show dramatically reduced fermentation rates and maltase expression, but increasing the copy number of the mutant gene to 2 increases the level of maltase expressed significantly. A Pro to Leu mutation in the homologous residue of the Gal4p zinc-cluster DNA-binding domain fully disrupts in vivo function of Gal4p, but the mutant phenotype could be suppressed by high levels of $ZnCl_2$ in the medium (Johnston and Dover 1987). The *mal63-Pro63Leu* mutation is not zinc-suppressible (data not shown).

The ability of these altered Mal63 proteins to bind DNA was determined using a gel-mobility shift assay. Protein extracts were prepared from *E. coli* transformants expressing a Mal63 fragment protein from residues 1 to 111, and used for binding assays with a 40-bp DNA fragment from the UASMAL of the *MAL61-MAL62* promoter containing the sequence from the *MAL62*-proximal end of this 68-bp region (Levine et al. 1992). Approximately equal amounts of wild-type and mutant Mal63 protein fragments were shown to be contained in the extracts used for the binding assays (data not shown). The wild-type Mal63p fragment binds to this 40-bp sequence forming two slower-migrating complexes (Fig. 3 B). Complex formation is specific for this oligonucleotide, and is not competed by the 40-bp adjacent sequence from the *MAL61*-proximal end of the *MAL61-MAL62* UASMAL or by salmon-sperm DNA (data not shown). Under the conditions of the assay, we could not detect binding with any of the mutant proteins, including the Pro23-Leu mutant protein. The reasons for this difference between the in vivo and in vitro results obtained with the Pro23Leu mutation are not clear, but suggest that the alteration has a greater impact on the DNA-binding affinity of the 1–111-residue fragment than it does in the full-length Mal63 protein.

Discussion

Maltose regulation of the *MAL*-activator does not involve a repressor protein

Our studies lead us to conclude that regulation of the *MAL*-activator does not involve a Gal80-like titratable negative regulatory protein. Abundant overproduction of the Mal63 *MAL*-activator does not result in a significant increase in the uninduced levels of maltase expression (Fig. 1). Moreover, the overexpressed LexA-$Mal63_{(2–470)}$ fusion protein is normally maltose regulated (Fig. 2, Table 3). The amount of Mal63 protein obtained with these expression systems is much greater than the undetectably low wild-type levels of Mal63p expression from the native promoter. Titration of Gal80p by Gal4p overproduction was achieved by using a multicopy YEp-type plasmid carrying *GAL4* (Johnston and Hopper 1982). It would be very surprising if a *MAL*-activator repressor protein would be synthesized in such abundance that that it would not be titrated by this level of Mal63p production, regardless of whether this repressor was, or was not, specific for the *MAL*-activators.

Our findings are consistent with genetic evidence accumulated over the past 50 years regarding the regulation of maltose fermentation in *Saccharomyces*. Extensive searches failed to uncover constitutive mutations in genes unlinked to the *MAL* loci (Winge and Roberts 1950; Khan and Eaton 1971; ten Berge et al. 1973; Zimmerman and Eaton 1974; Rodicio 1986; Dubin et al. 1986; Charron and Michels 1987; Dubin et al. 1988). All of the constitutive mutations identified and sequenced to-date are *MAL*-activator mutations, whether these were obtained directly in *MAL* inducible strains or indirectly by reverting *mal* nonfermenting mutant strains. Constitutive mutations would be expected to be rather common if a Gal80-like negative regulatory factor were involved in *MAL* gene regulation, but the genetic evidence alone is inconclusive because it does not exclude the possibilities that multiple repressor proteins are involved, that the repressor protein is encoded by multiple genes, or that the repressor protein serves an essential cellular function. Given our results and the genetic evidence, we feel rather confident that the regulation of the *MAL*-activator does not involve a *MAL*-activator specific repressor protein.

The DNA-binding domain of Mal63p

The mutation analysis reported here of the N-terminal cycteine-rich region of Mal63p, residues 8–34, clearly demonstrates that this region is required for DNA-binding to the UASMAL. Based on its sequence homology to the DNA-binding zinc-cluster region of Gal4p, this region of Mal63p is likely to form a similar binucleate metal-ion-binding cluster (Vallee et al. 1991). Alterations of three of the key cysteines of this structure in

Mal63p completely impair DNA-binding and *MAL* gene activation. A proline residue is commonly found N-terminal to cysteine-4 of the Cys-6 DNA-binding domain (Kim and Michels 1988; Pfeifer et al. 1989; Reece and Ptashne 1993; Schjerling and Holmberg 1996) and is essential for Gal4p DNA-binding (Johnston and Hopper 1982). Alteration of this proline of Mal63p results in a noninducible phenotype, but this phenotype is significantly suppressed by increasing the plasmid-borne gene copy number suggesting that the Pro23Leu alteration only reduces the DNA-binding affinity of the protein but does not elimitate binding completely.

The fact that the LexA-Mal63$_{(\Delta 41–66)}$ and LexA-Mal63$_{(\Delta 60–99)}$ fusion proteins activate reporter gene expression, but not *MAL* gene expression, suggests that the region adjacent to the cysteine-rich DNA-binding domain of Mal63p from residues 41 to 98 may be required for sequence-specific DNA-binding to the UAS$_{MAL}$. In a study of the Gal4p, Put3p and Ppr1p DNA-binding domains, all of which contain a Cys-6 sequence highly homologous to that in Mal63p, Reece and Ptashne (1993) reported that the residues adjacent and C-terminal to the zinc-cluster sequence confer sequence specificity to DNA-binding by the zinc-cluster region, and allow the different UAS sequences to be distinguished. This so-called specificity region includes a short 8–10-residue linker region (immediately C-terminal to cysteine-6), and the adjacent 40–60 residues. In Gal4p, Put3p and Ppr1p, the adjacent region is proposed to contain the dimerization domain of these proteins which is required for DNA-binding, and the region is postulated to form a coiled-coil type structure. Mal63 protein overproduced in *Saccharomyces* grown in uninduced conditions is purified as a homodimer, but there is no evidence regarding the structure of this dimer or the sequences involved in dimerization (Sirenko et al. 1995). Examination of residues 41–98 of Mal63p reveals no sequence, and little structural, homology to the putative linker-dimerization region of Gal4p, Put3p or Ppr1p. While this region of Mal63p is rich in leucines and other hydrophobic residues, there is no classic heptad repeat, and it is less likely that the region forms a coiled-coil structure and is involved in dimerization, although this possibility is not excluded (Schjerling and Holmberg 1996). We have made extensive efforts to demonstrate the dimerization of fragments from this region, residues 1–120, of Mal63p using two-hybrid analysis and all have been unsuccessful (Z. Hu, unpublished results).

The transactivation domain of Mal63p

The result showing that the LexA-MAL63$_{(1–283)}$ fusion protein functions as a constitutive activator is consistent with the work of Gibson et al. (1997) who reported the sequence of several *MAL*-activator constitutive alleles, and found that all were either truncations that deleted residues beyond residue 282 or were multi-site alterations in residues 300–470. Our data, presented in Fig. 2, localizes the Mal63p transcription activation domain approximately to residues 60–283 and, unlike several other well-known yeast transcription activators with similar DNA-binding domains, no activation domain is present at the C-terminal end of Mal63p. Both ends of the Mal63p transactivation domain, including residues 67–98 and 251–283, are necessary for activation but only in the absence of the C-terminal residues beyond residue 283 suggesting that these regions could serve some structural role that can be compensated for by other C-terminal sequences. However, LexA-Mal63 fusions lacking residues 62–98 and 251–299 (constructs 15 and 23) are unable to activate maltase expression. These regions apparently play essential roles in transcription activation specific to the *MAL* promoter and are indispensable even in the presence of the C-terminal sequences. Thus, Mal63p contains an essential core region between residues 60 and 283.

Eukaryotic transcription-activation domains appear to consist of several subdomains which function in concert to achieve high levels of activation (reviewed in Fraenkel and Kim 1991). Detailed sequence analysis of subdomains from various "acidic" activator proteins indicates that the acidic residues are found in association with an essential core phenylalanine residue and other bulky hydrophobic residues (Regier et al. 1993; Blair et al. 1994; Drysdale et al. 1995). Residues 99–250 of Mal63p contain several such clusters of acidic residues each associated with one or more phenylalanines and other hydrophobic residues with bulky sidechains: residues 114–126, Phe122; residues 133–149, Phe146; residues 161–177, Phe161, Phe162, Phe170; residues 183–189, Phe186; residues 208–221, Phe208, Phe216, Phe219; and possibly residues 238–243, Phe238. These regions are likely to represent subdomains of the Mal63p transcription-activation domain, but only detailed mutation analysis can determine which ones contribute to transactivation activity and to what extent.

Transformants expressing the *LexA-MAL63*$_{(1–283)}$ fusion lacking the C-terminal residues from 284–470 synthesized β-galactosidase and maltase constitutively. The levels of both enzymes are reduced by growth in maltose. The mechanism of this maltose-dependent partial repression has not been explored, but maltase expression in other strains carrying constitutive *MAL*-activator alleles is also repressed by maltose, and we believe that this effect results from a repression of the *MAL* genes by the high intracellular levels of glucose produced during maltose hydrolysis in fermenting constitutive strains (Hu et al. 1995; Gibson et al. 1997). The promoter sequence of the *LacZ* reporter gene carried by plasmid pSH18-18 also contains elements responsive to glucose repression (Brent and Ptashne 1985).

The regulatory domain of Mal63p

We found previously (Gibson et al. 1997) that constitutive *MAL*-activator mutations fell into two classes:

nonsense mutations that truncate the encoded *MAL*-activator at codon 283 [comparable to the LexA-Mal63$_{(1–283)}$ constitutive activator described in this study] and multi-site sequence alterations that alter several residues in the region from approximately 300 to 470. In the present study, we identify a region covering residues 251–299 which is required for negative regulation of Mal63p activity since deletion of these residues produces a constitutive activator. This region includes residues 251–283, which appear to be essential for the constitutive activation activity of LexA-Mal63$_{(1–283)}$ and are apparently required for activation at the *MAL* locus. This could explain why none of the constitutive mutations of the full-lenth *MAL*-activators isolated so far are located within this inhibitory region. Truncation at codon 283 may destroy, or significantly impair the inhibitory function of this region while not affecting its activation function significantly.

Constitutive mutations of various *MAL*-activator alleles have been found throughout the C-terminal portion from residue 300 to the end (Gibson et al. 1997). In our original analysis, these mutations were proposed to have destroyed the negative regulatory function of the C-terminal region. However, we find that in-frame 48-residue deletions and a nonsense mutation at codon 457 which cover almost the entire C-terminal region between residues 300 and 470, are all noninducible (constructs 19–22 in Fig. 2). Moreover, short clusters of point mutations through this region also are noninducible (Gibson et al. 1997; S. Danzi, unpublished results). The fact that none of these deletion mutations produces a constitutive activator suggests that the C-terminal portion of the *MAL*-activator between residues 300 and 470 plays a positive role instead; that is, it is needed to relieve the inhibitory effects on the functional core by residues 251–299 in response to maltose. Thus, the constitutive mutations identified within the C-terminal portion may actually alter this domain such that it acts to relieve the inhibition of the 251–299 domain without the need for maltose. On the other hand, one might argue that the C-terminal portion of the *MAL*-activator could contain multiple negative elements each of which is able to function independently, and, as a result, a single deletion of 50 residues is not sufficient to eliminate the negative regulation completely. While this is a possibility, it seems less likely in view of the recent finding that a cluster of alterations within a short region of the C-terminal regulatory domain can also result in a constitutive Mal63p (Wang and Needleman 1996; S. Danzi, unpublished results).

A model for the inducible-regulation of the *MAL*-activator

The mechanism of regulation of a number of eukaryotic transcription activators is known in detail. Nuclear entry, DNA-binding, accessibility of the transactivation domain to the transcription machinery, activator stability, as well as other activator functions, can be regulated in response to the controlling signal. Some examples follow. The yeast Pho4p and the mammalian NF-KB activators are regulated by controlling nuclear entry (Alkalay et al. 1995; O'Neill et al. 1996) but via quite different mechanisms. In the case of Pho4p, nuclear entry/exit depends on its phosphorylation state, while NF-KB is sequestered in the cytoplasm by interaction with I-KB in the absence of inducing conditions. Sequence-specific DNA-binding by p53 is regulated in response to the inducer, which is suggested to stimulate a structural change in the protein that reveals the DNA-binding domain (Jayaraman and Prives 1995). In contrast, Gal4p is bound to DNA even in the absence of the inducer but is repressed by Gal80p, which appears to obscure its transactivation domain (reviewed in Johnston and Carlson 1993). Induction, which requires Gal3p, involves a reorganization of the Gal4p-Gal80p-Gal3p complex to reveal the transactivation to the transcription machinery (Bhat and Hopper 1992; Blank et al. 1997; Platt and Reece 1998). In Leu3p, intramolecular interaction between the transactivation domain and other parts of the protein prevents transactivation; inducer-binding to Leu3p unmasks the transactivation domain (Wang et al. 1997). The yeast Hap1p and mammalian steroid receptors complex with heat-shock proteins in the absence of the inducer and, in the presence of inducer, are released in a transcriptionally active DNA-binding competent conformation (reviewed in Pratt 1993; Zhang and Guarente 1994, 1995; Zhang et al. 1998). Gcn4 protein, an activator of the amino-acid and purine biosynthetic enzymes, is rapidly degraded by the proteasome pathway in rich media but is stabilized under starvation conditions (Kornitzer et al. 1994). The studies reported here define the functional domains of the *MAL*-activator and allow us to begin to explore the mechanisms regulating *MAL*-activator induction.

Our finding that the inducible LexA$_{(1–202)}$-Mal63$_{(2–470)}$ fusion binds to the reporter promoter in uninduced conditions (Table 3) suggests that nuclear entry and promoter binding are not sufficient for the *MAL*-activator to activate transcription. Instead, we suggest that the transactivation activity of the *MAL*-activator is inhibited in the absence of maltose. This, of course, does not necessarily mean that *MAL*-activator DNA-binding is constitutive, and it is still possible that induction leads to both DNA-binding and transactivation, perhaps as the result of a major structural reorganization.

The simplest model consistent with the data reported here is that the *MAL*-activator is autoregulatory; that is, in the absence of maltose, negative regulation is achieved by protein-protein interactions between the regulatory domain and sites elsewhere in the *MAL*-activator protein, possibly within the functional core. Fusion activators encoded by *LexA-MAL63* deletion mutations which remove residues from the N-terminal 98 residues (residues 1–36, 41–66, or 62–98) are still normally maltose-regulated, suggesting that none of these regions contains

sequences necessary for maltose-inducible regulation. Two-hybrid analysis and genetic analysis of constitutive and noninducible *MAL*-activator mutations is being used to define potential regions of intramolecular interaction but with little success to-date. Thus, this simple model remains an open question and other possibilities are being considered.

We propose the following model describing induction of the *MAL*-activator. In the absence of maltose, the region including approximately residues 251–299 negatively regulates transactivation. This could occur by blocking the transactivation domain from interacting with coactivators or the transcription machinery, but other mechanisms are possible. Induction involves a maltose-stimulated conformational change, or re-folding, requiring the C-terminal domain between residues 300 and 470 to counteract the effect of this inhibitory region. Residues in the C-terminal domain participate in this structural reorganization, and we find that, when mutated, either induction is inhibited or constitutivity is generated (Gibson et al. 1997). Detailed molecular genetic analysis of the *MAL*-activator is underway to explore this process.

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