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MOLECULAR GENETIC ANALYSIS OF CONSTITUTIVE AND NONINDUCIBLE MUTANTS OF THE *SACCHAROMYCES MAL*-ACTIVATOR

by

SARA ELLEN DANZI

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

MOLECULAR GENETIC ANALYSIS OF CONSTITUTIVE AND NONINDUCIBLE MUTANTS OF THE *SACCHAROMYCES MAL*-ACTIVATOR

by

Sara Ellen Danzi

Advisor: Professor Corinne A. Michels

The C-terminal 220 amino acid residues of the *MAL*-activator protein function as a regulatory domain. Previous studies which characterized constitutive and noninducible *MAL*-activator mutations found several of both phenotypes which contained multiple alterations and deletions within this region suggesting that both negative and positive regulatory subdomains were present but the number, location, and function of these subdomains within this regulatory region were unknown. Using site directed *in vitro* mutagenesis of the inducible *MAL63* and *MAL63/23* genes, negative subdomains were identified at 2 sites (called NSD-1 and NSD-2). Multiple mutations within each of these regions relieve the negative regulation of *MAL*-activator activity in the absence of inducer, producing a constitutive phenotype. Intragenic suppressors of the constitutive mutations in NSD-2 and NSD-3, a third negative subdomain previously identified by Wang and Needleman (1996), also were identified at closely linked sites. Constitutive mutations of NSD-2 and NSD-3 are partially dominant and dominant, respectively. Additional suppressors of NSD-3 mutations were revealed at more distant sites within NSD-1. These results suggest that complex intramolecular interactions within this regulatory domain are required to maintain the inactive state of the *MAL*-activator in the absence of inducer. Clustered-charge to alanine mutagenesis of the C-

terminal regulatory domain of the inducible Mal63 *MAL*-activator was undertaken to identify positive subdomains. Noninducible mutations were obtained when residues in the region from 331-469 were altered. In order to distinguish a role for these residues in induction from other required functions of the activator, the noninducible alterations were introduced into the constitutive hybrid gene *MAL63/43*. Those mutations found to affect only the inducible activator localized to residues 331-423 and are proposed to be involved in induction. Mutations affecting both the inducible and the constitutive activators localized to residues 438-470 and are proposed to be involved in transactivation and/or maintenance of a transcriptionally active state. Three essential residues at 364, 367, and 391 also were identified. A model for maltose induction of the *MAL*-activator is proposed.

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Introduction

All cellular functions must be regulated for proper metabolism and growth. Diverse cell functions, such as differentiation, cell cycle regulation, and reproduction are regulated by differential gene expression. Gene expression in eukaryotes can be controlled at many levels, from transcription to post-translational modification. Of these mechanisms of control, transcriptional initiation is the most common. Some genes are unregulated; these genes are expressed constitutively throughout the cell cycle. Many genes, however, are expressed in a regulated manner and respond to environmental or developmental cues. The spatial and temporal expression of genes is controlled by regulatory proteins. Transcription factors are proteins which bind to specific DNA sequences and activate or repress transcription of linked genes. A fundamental question in molecular biology asks, "How is the regulator regulated?" The goal of my thesis project is to explore this question as it relates to the *MAL* gene transcription activator of *Saccharomyces cerevisiae*.

To a first approximation, individual segments or domains of these transcription factors are devoted to functions such as nuclear localization, DNA-binding, and transcription activation. Often times these functional domains are separable as is evident from studies which utilize gene fusions encoding the DNA-binding or transactivation of Gal4, LexA DNA-binding, and VP16 transactivation domains, among others. This implies that each domain is structurally and functionally independent and distinct from the others.

Regulation of the regulator can affect any, some, or all of these functions. Regulation can be achieved on many levels. Regulator expression itself can be regulated. Nuclear entry can be restricted. Post-translational modifications or additional positive or negative factors may be required to activate or inactivate the regulator. In addition, regulation may be achieved by altering the stability of the regulator, its activator or its inhibitor. This introduction focuses on a few examples of transcriptional activators whose mechanism of response to small inducer molecules has been defined in some detail. Examples from *Saccharomyces* and mammalian systems are described below.

Saccharomyces transcriptional activators

GAL4. A well studied example of regulation in *Saccharomyces* involves the genes required for galactose fermentation. Gal4 protein, an 881-amino acid molecule, regulates the transcription of *GAL1* (kinase), *GAL7* (transferase), *GAL10* (epimerase), *GAL2* (galactose permease), the structural genes required for fermentation of galactose, and *MEL1* (α-galactosidase) (Lohr *et al.*, 1995; Johnston, 1987). Three carbon source regulated states exist for the *GAL* structural genes: inactive-repressed (glucose grown cells); inactive but poised for induction (glycerol grown cells); and active (galactose grown cells). In the absence of galactose, Gal4p is associated with Gal80p, a repressor protein, and this complex is bound to the UAS_G, a cis-acting controlling sequence or upstream activating sequence, but does not activate transcription. In the presence of galactose, a conformational change is induced and Gal4p, still bound to Gal80p, activates transcription. Although the *GAL* gene family is one of the best studied

regulated systems in *Saccharomyces*, until recently very little was known about the mechanism of sensing galactose and the process of induction.

Gal4p binds DNA via a C_6 zinc cluster localized to the N-terminus of the protein (Lohr *et al.*, 1995). The C-terminal residues of Gal4p contain both an acidic transcription activation domain and a Gal80p binding domain (Leuther *et al.*, 1993). Circular dichroism spectroscopy indicates that the structure of this C-terminal region is likely β-sheet as opposed to the previously proposed α-helical structure, with the Gal80p binding domain on the "up" face and the activation domain on the "down" face (Van Hoy *et al.*, 1993). In this case, an anti-parallel β-sheet would allow for distinct and separate intermolecular interactions within the same region. Gal4p-Gal80p complex is suggested to be the predominant form of Gal4p found in all carbon sources. The complex is bound to DNA in uninduced, but not repressed conditions.

Gal3p mediates the induction of the *GAL* genes in the presence of galactose. Induction occurs within minutes in wild-type cells, but takes several days in a *gal3* mutant strain. Constitutively overexpressed Gal1p, a galactokinase and the first enzyme in galactose metabolism, can restore normal inducibility in *gal3* mutants (Lohr *et al.*, 1995). In the yeast *Kluveromyces lactis*, *LAC9* encodes a Gal4p homologue which regulates lactose fermentation, and Lac9p and KlGal80p are interchangeable with their homologues found in *Saccharomyces*. Interestingly, induction depends on KlGal1p, and there is no *GAL3* homologue. The *Saccharomyces cerevisiae* Gal3p and Gal1p share some sequence similarity, but Gal3p has no galactokinase activity, therefore, this activity is not required for induction. Moreover, overexpression of

Gal3p leads to constitutive expression of the *GAL* genes, however, the concomitant overexpression of Gal80p suppresses this constitutivity (Suzuki-Fujimoto *et al.*, 1996).

It was further demonstrated that Gal3p coimmunoprecipitates with Gal80p. This recent evidence suggests that Gal3p binds to Gal80p and thereby may interfere with the Gal80p inhibition of Gal4p. It has also been shown that Gal1p is able to bind Gal80p, indicating that it may function by a similar mechanism with respect to induction (Zenke *et al.*, 1996). The isolation of constitutive $GAL3^{C}$ alleles which are not the result of overexpression supports the hypothesis that galactose causes an alteration in Gal3p structure. These Gal3p mutants demonstrate altered Gal80p binding activity which causes constitutive transcription of Gal4p-activated genes (Blank *et al.*, 1997). Gal3p forms a complex with Gal80p *in vitro*, and complex formation is stabilized in the presence of galactose and ATP. The Gal3p/Gal80p complex is easily dissociated when washed with buffer lacking galactose. In addition, *gal80* mutants which confer a galactose-uninducible phenotype do not interact with Gal3p. These results suggest that Gal3p functions as the galactose sensor and transducer of the inducer signal. Based on the structural similarity of galactokinase and Gal3p, it is more reasonable to assume that galactose binds to Gal3p rather than Gal80p. The proposed model for galactose induction of the *GAL* genes involves two steps, one which establishes induction and another which is required to maintain the induced state. In this model, galactose and ATP bind Gal3p which may or may not dissociate from Gal80p once induction is initiated.

Phosphorylation of Gal4p at a critical site is proposed to be required for maintenance of induction (Yano and Fukasawa, 1997). This hypothesis is based on the following studies. It has been demonstrated that Gal4p is multiply phosphorylated at residues in both the N- and C- terminal regions, but phosphorylation of only one serine residue (699) is required for galactose-inducible transcription. This effect is only seen in *GAL80* cells. The phosphorylation of multiple residues in Gal4p, including serine 699, is stimulated by both galactose and glucose in *gal80* cells indicating that Gal80p prevents phosphorylation of Gal4p in the presence of glucose. Prerequisite DNA binding and the product of the *GAL11* gene play a role in the phosphorylation of serine 699. Gal11p is a nonessential component of the RNA polymerase II holoenzyme (Sadowski *et al.*, 1996). Therefore, association with the holoenzyme may influence the association between Gal4p and Gal80p, although the role of phosphorylation in this regulation remains unclear.

HAP1. *HAP1* encodes a 1483-residue protein which binds as a homodimer to asymetric DNA sites and activates the transcription of the structural genes encoding iso-1-cytochrome c (*CYC1*) and iso-2-cytochrome c (*CYC2*) (Zhang and Guarente, 1996; Sousa and Arcangioli, 1989). These cytochromes are components of the mitochondrial electron transport chain and their expression is oxygen-regulated. Regulation is mediated by the inducer, heme, which is abundant during periods of aerobic growth (Fytlovich *et al.*, 1993). It has been shown that Hap1p dimerizes when functional, and that DNA-binding does not occur without dimerization. In addition, the dimerization of

Hap1p is regulated by sequences immediately C-terminal to the DNA-binding domain which have been shown to bind heme.

The Hap1p DNA-binding domain consists of a cysteine-rich zinc-cluster and an adjacent leucine-rich region capable of forming a coiled coil. Hap1p fragments which are missing the coiled coil dimerization domain are unable to bind DNA. The essential sequences responsible for dimerization have been mapped to a minimal required region between residues 123 and 148. Experiments using the dihybrid method of Fields and Song (1989) show that heme regulates dimerization *in vivo* as well. These results suggested that the regulatory domain acts by preventing dimerization in the absence of heme. The heme responsive domain of Hap1p contains six repeats of a conserved peptide sequence, the heme regulatory motif (HRM), also found in enzymes of heme metabolism. The heme regulatory domain of Hap1p was mapped to residues 244 through 444, the region adjacent to the DNA-binding domain. Deletion of this region results in constitutive activity indicating that this region is negatively controlling Hap1p activity and that heme relieves this inhibition (Zhang *et al.*, 1993). Using a synthesized peptide which corresponds to the HRM, it was shown that heme binds directly to this short sequence (Zhang and Guarente, 1995; Zhang and Guarente, 1994).

Hap1p associates with a high molecular weight multiprotein complex and is sequestered in this complex in the absence of heme dimer (Zhang and Guarente, 1994). Deletion of the heme domain prevents this association and allows the activator to bind DNA even in the absence of heme. Overexpression of non-DNA-binding mutants of Hap1p titrates the high molecular weight complex and allows partial expression of

Hap1p regulated genes. The heme binding domain is involved in binding Hap1p in the complex. When heme levels reach a certain threshold, association of Hap1 with the complex is disrupted, Hap1p is released, and is able to form an active DNA-binding dimer (Zhang and Guarente, 1994). Thus, heme mediates the ability of Hap1p to bind DNA. A seventh HRM lies in the C-terminal portion of the Hap1 protein. Studies have shown that the HRM is capable of conferring heme regulation on a heterologous activator.

Unlike other zinc cluster family proteins which bind inverted triplet repeats, Hap1p homodimers bind asymmetric DNA sites containing a direct 5'-CGG-3' repeat. It is likely that an asymmetric interaction between the two Hap1 protein monomers enables them to recognize and bind these direct repeats. The data also suggests that this binding orientation acts cooperatively with dimerization elements and locks Hap1p-DNA complexes in the stable, dimeric conformation (Zhang and Guarente, 1996). In addition, evidence suggests that alterations in the sequence of the binding site alter or prevent activation without changing DNA binding. Conformation may be influenced by appropriate orientation of DNA binding (Ha *et al.*, 1996).

PUT3. Put3p is the activator which regulates expression of the structural genes of the proline utilization pathway (*PUT1*, encoding proline oxidase, and *PUT2*, encoding Δ^1-pyrroline-5-carboxylate dehydrogenase). Put3p is a 979 amino acid protein which is similar to Gal4p in several respects. Like Gal4p, Put3p has an N-terminal zinc cluster DNA binding domain and is constitutively bound to DNA. DNA-bound Put3p is inactive in the absence of proline. Put3p binds to the upstream

activating sequences of the *PUT1* and *PUT2* genes and activates transcription in the presence of inducer, proline, and in the absence of preferred nitrogen sources such as ammonia (Siddiqui and Brandriss, 1988). Put3p functions as a dimer. The dimerization element is a coiled coil motif and is contained within a thirty-residue segment just C-terminal to the DNA-binding zinc cluster. A central domain of Put3p, which is part of a region termed the middle homology region (MHR) in fungal activators, is involved in transcription activation and mutation of a conserved glycine residue at 532 interferes with activation but not with DNA binding (Schjerling and Holmberg, 1996; des Etages *et al.*, 1996; Axelrod *et al.*, 1991).

The C-terminal 24 residues of Put3p are not required for activation of *PUT1* and *PUT2*, but are required for regulation by proline. However, this region does not contain the proline responsive domain. It is possible that these C-terminal residues stabilize the protein. The mechanism for the induction in the presence of proline is unknown.

LEU3. Leu3p is a transcriptional regulator of genes encoding enzymes of the branched-chain amino acid biosynthetic pathways, and is the major regulator of *LEU1* and *LEU2*, the genes involved in amino acid biosynthesis. *LEU3* works in conjunction with *GCN4* to regulate the transcription of *LEU4*. *LEU3* encodes a zinc cluster DNA binding protein like *GAL4, PUT3, HAP1,* and the *MAL*-activator among others (Sze and Kohlhaw, 1993; Hu *et al.*, 1995b). It binds DNA as a homodimer to everted repeats (CCG-N4-CGG) in the upstream promoter of *LEU3* responsive genes. This is in contrast to Gal4p, Put3p, and Ppr1p which bind to palindromic CGG repeats, and

Hap1p which binds CGG in direct repeats (Hellauer *et al.*, 1996). Recently, Leu3p has been shown to regulate the expression of *GDH1*, an important gene in nitrogen assimilation. This suggests that Leu3p participates in other regulated pathways (Hu *et al.*, 1995b). *LEU3* provides a good example of direct transcriptional regulation in eukaryotes by a small effector molecule, α-isopropylmalate (α-IPM) (Sze *et al.*, 1992). The leucine biosynthetic pathway is controlled by both the general amino acid control system and specifically by leucine. *LEU4* encodes α-isopropylmalate (α-IPM) synthase, the first enzyme in the pathway, and is effected by both types of regulation.

Leu3p is an 886 residue protein which functions as both a repressor and an activator. The Leu3p activation domain has been characterized as one of the shortest known in eukaryotes, 30 C-terminal residues. This short segment in a heterologous gene fusion construct with the Gal4p DNA-binding domain has been shown to be sufficient to turn on transcription of a reporter gene (Sze *et al.*, 1993).

Regulation of Leu3p is controlled by a small molecule, α-IPM, which is an early intermediate in the enzymatic pathway and the reaction product of the Leu4p enzyme. Leu3p remains bound to DNA under all conditions (Kirkpatrick and Schimmel, 1995). It functions as a repressor in the absence of α-IPM and becomes an activator in the presence of α-IPM. In the absence of Leu3p, a basal level of transcription of *LEU1* and *LEU2* is established, and, in the presence of Leu3p and the absence of α-IPM, a lower repressed level of transcription is established.

Those sequences of Leu3p which are responsive to α-IPM have been mapped to two regions. The first region involves the middle portion of the protein and extends

from residue 174 to 773, and the second is the 30 residue C-terminal region which also contains the transcription activation domain. Mutations within these regions create constitutive activators (Friden *et al.*, 1989; Sze *et al.*, 1992; Sze *et al.*, 1993).

The C-terminal region of Leu3p includes a short cluster of tryptophan residues which play important regulatory roles. Trp861Ala and Trp864Ala mutations each make the Leu3p activator less sensitive to inducer and raise the basal level of *LEU1* and *LEU2* expression. The latter mutation results in almost constitutive activity. These experiments suggest that the C-terminal activation domain is bifunctional, and, in addition to activation, this region acts as a "modulation region-interactive function." It has been proposed that the activation region is masked by association with the central portion and that a conformational change induced by α-IPM unmasks this domain, thereby changing the function of Leu3p from a repressor to an activator. The mutations cited above might decrease the interaction between residues in the C-terminus and the central region of the protein relieving any repressor function (Sze and Kohlhaw, 1993; Zhou *et al.*, 1990). Leu3p retains regulatory properties when expressed in mammalian cells suggesting that the protein is autoregulated. A C-terminal mutation which appears to create a permanently masked activation domain was used to demonstrate an α-isopropylmalate-dependent interaction between the C-terminal activation domain and the middle region of the protein (Wang *et al.*, 1997). The mechanism by which α-IPM acts as an inducer of Leu3p is not known.

Recent studies suggest that Mot1p, a known TBP-associated protein, functions as a transcriptional co-repressor. Leu3p-dependent repression can be reconstituted *in*

vitro with Motlp and recombinant TBP and a mutation in *MOT1* leads to partial derepression of Leu3p-dependent genes (Wade and Jaehning, 1996).

Mammalian transcriptional activators

p53. Mutations in the *p53* gene have been implicated in more than half of human cancers. The p53 protein is a transcription factor which has been shown to either activate or suppress transcription of specific target genes. p53 protein is 393 amino acids in length. Genetic analysis has helped to dissect this protein into four distinct functional domains. The first 42 residues at the N-terminal end of the p53 protein contain a transcriptional activation function. Amino acids 13-23 contained within this region are identical in the p53 proteins of several diverse species. *In vitro*, these residues have been shown to bind to TATA-associated factors and thus are proposed to mediate interaction with the transcription machinery (Levine 1997; Lin *et al.*, 1995). The p53 acidic N-terminal region also contains binding domains for Mdm-2 and E1B 55-Kd protein, both of which negatively regulate p53. Genetic analysis of this region demonstrates that only 2-4 hydrophobic residues are required for transcription activity and for binding to the negatively regulating proteins identified above (Lin *et al.*, 1994).

Sequence specific DNA binding of p53 has been localized between residues 102 and 292. More than 90% of missense mutations in p53 localize to the DNA-binding domain. Two classes of mutations within this domain have been identified, one which causes defective DNA contacts and another which causes alterations in protein conformation (Cho *et al.*, 1994; Hollstein *et al.*, 1994). p53 binds DNA as a tetramer

which consists of a dimer of dimers. Each dimer contains two β-sheets and two α-helices and tetramerization forms a four-helix bundle held together along the surface of each helix and requires residues 324-355. A 37-residue flexible linker is located in between the DNA binding domain and the oligomerization domain (Jeffrey *et al.*, 1995; Prives, 1994).

The C-terminal region of p53 has been implicated in the regulation of DNA binding. This short C-terminal negative regulatory domain, 26 amino acids in length, locks the p53 tetramer in an inactive state. Relief of this negative regulation can be accomplished in several ways: phosphorylation by caseine kinase II or protein kinase C, deletion of the entire regulatory domain, or binding to a monoclonal antibody targeted to the C-terminal residues. Short single strands of DNA which interact with the C-terminal domain also activate DNA binding, but longer double strands of DNA have the opposite effect (Jayaraman and Prives, 1995; Lee *et al.*, 1995; Taneka *et al.*, 1995; Hupp *et al.*, 1995; Delphin and Baudier, 1994; Hupp and Lane, 1994; Hupp *et al.*, 1993; Hupp *et al.*, 1992). This indicates that the C-terminus regulates DNA binding either sterically or allosterically. The current model for p53 protein function proposes that the C-terminal regulatory domain interacts with another portion of the protein, possibly the DNA binding domain, or core domain, and negatively regulates the DNA-binding function. This model is supported by experiments which demonstrate that small peptides which interact with the C-terminus are capable of activating DNA binding. These peptides may interfere with intra- or intermolecular interactions in order to activate the DNA binding function (Hupp *et al.*, 1995). In addition, genetic

suppressor elements have been used to dissect the functional domains of p53 protein. Only those elements directed toward the C-terminus functioned to inhibit transcriptional activation by p53. Using this technique, various cellular effects attributed to p53 function have now been assigned to different regions within the p53 protein (Ossovskaya *et al.*, 1996).

Glucocorticoid receptor. The glucocorticoid receptor is a nuclear receptor which functions as a transcriptional regulatory protein in mammalian cells. It belongs to the family of steroid receptors which includes the progesterone, estrogen, androgen, and dioxin receptors. These receptors function as direct signal transduction systems by binding intracellular hormone which promotes DNA binding by the receptor-hormone complex (Bohen *et al.*, 1995; Rutherford and Zuker, 1994; Pratt, 1993). Separate domains function in DNA binding and inducer response, or hormone binding. Deletion of the C-terminal hormone binding domain yields a constitutively active transcription activator. The hormone binding domain is responsible for several functions. It negatively regulates transcriptional activation; it is required for inducer responsiveness and binds hormone directly; and it has a positive function in the derepression process following hormone binding (Pratt, 1993). Studies have shown that optimal steroid receptor activity requires other proteins, primarily molecular chaperones. In the absence of hormone, the glucocorticoid receptor is associated in a high molecular weight complex. Together, this aporeceptor complex consists of one receptor monomer, a dimer of the heat shock protein Hsp90, one 56-kDa protein, and may include other factors (Bohen *et al.*, 1995; Bohen and Yamamoto, 1993).

Glucocorticoid receptor is a phosphoprotein, as is the aporeceptor. Additional phosphorylation occurs upon hormone binding (Krstic *et al.*, 1997; Orti *et al.*, 1992; Hoeck and Groner, 1990; Orti *et al.*, 1989; Grandics *et al.*, 1984; Housley and Pratt, 1983). The direct binding of hormone to the receptor was demonstrated by *in vitro* binding assays using labeled hormone. In addition, studies using mutant receptors with single amino acid alterations exhibiting decreased hormone binding demonstrated a correlation between defective binding and decreased levels of transcription activation. Glycine567 plays an important role in both functions (Warriar *et al.*, 1994).

Binding to Hsp90 is mediated by the hormone-binding domain, and this association is crucial for proper functioning of the glucocorticoid receptor in transcriptional regulation. *Saccharomyces* yeast were made steroid responsive by the introduction of a receptor gene, and receptor function was measured using a reporter containing a steroid response element fused to LacZ. In this strain, reporter gene expression was found to be dependent upon the yeast Hsp90 protein, providing strong evidence that this complex is required for derepression of transcriptional activation by the glucocorticoid receptor as well as by the estrogen receptor, another of the intracellular steroid receptors (Rutherford and Zuker, 1994; Bohen and Yamamoto, 1994; Picard *et al.*, 1990). In addition, mutant steroid receptors which are constitutively active do not bind Hsp90, whereas regulated receptors are bound to Hsp90. When the hormone-binding domain of the glucocorticoid receptor was fused to an unrelated protein, the E1A adenovirus gene product, activity of the fusion protein was glucocorticoid dependent. Other steroid receptor hormone-binding domains have

been shown to impart hormonal control onto structurally different fusion proteins. The Myc oncoprotein was made estrogen-dependent by fusion to the estrogen receptor hormone binding domain. It is unlikely that the hormone-binding domain is capable of inactivating different protein structures by direct interaction, but rather by associating with a second protein which provides this regulatory function. The hormone-binding domain appears to confer hormonal control on fusion proteins by regulating binding to Hsp90 which inhibits the function of the fusion protein. In the case of the steroid receptors, proper interaction with Hsp90 is essential for efficient hormone binding and induction (Bohen *et al.*, 1995; Bohen and Yamamoto, 1993; Pratt, 1993; Scherrer *et al.*, 1993; Bresnick *et al.*, 1989; Eilers *et al.*, 1989; Picard *et al.*, 1988; Picard and Yamamoto, 1987).

Recent work has focused on the activation capacity of the glucocorticoid receptor and interactions between the glucocorticoid receptor and the Ada adaptor complex. This complex has been identified previously and shown to function as an adaptor for some acidic transactivation domains, bridging interaction between these proteins and those of the transcriptional machinery. Ada proteins have been shown to bind directly to the transactivation domains of VP16 and Gcn4p as well as to TBP (TATA-binding protein) (Henriksson *et al.*, 1997).

Regulation of maltose fermentation in *Saccharomyces.*

***MAL* locus structure and function.** *Saccharomyces* strains require one of five unlinked *MAL* loci in order to ferment maltose: *MAL1* (chromosome *VII*, right telomere), *MAL2* (chromosome *III*, right telomere), *MAL3* (chromosome *II*, right

telomere), *MAL4* (chromosome *XI*, right telomere), and *MAL6* (chromosome *VIII*) (reviewed in Needleman, 1991). The *MAL* loci have been shown to be structurally and functionally highly homologous (Charron *et al.*, 1989). Each locus consists of three genes (see Figure 1). Genes 1 and 2 encode the structural genes, maltose permease and maltase, respectively. Gene 3 encodes a transcription factor, the *MAL*-activator, required for the transcription of genes 1 and 2. The genes at each locus are named according to the locus position and gene number. At *MAL6*, the permease gene is *MAL61*, the maltase gene is *MAL62*, and the *MAL*-activator gene is *MAL63*. At *MAL3* and *MAL6* there are nonfunctional homologues of the *MAL*-activator on the centromere proximal side of the locus, *MAL34* and *MAL64*, respectively.

The *MAL* structural genes are induced by maltose and repressed by glucose, despite the presence of maltose (Hu *et al.*, 1995a; Chang *et al.*, 1989; Charron *et al.*, 1986; Needleman *et al.*, 1984). Both maltose induction and certain aspects of glucose repression are mediated by the *MAL*-activator (Hu, 1997; Hu *et al.*, 1995a; Charron *et al.*, 1986; Needleman *et al.*, 1984). It has been shown that the *MAL*-activator binds to the UAS_{MAL}, a short bidirectional sequence element in the shared promoter of the structural genes, and binding is required for the transcription of the structural genes (Levine, *et al.*, 1992; Kim, 1992). Figure 1 diagrams the *MAL6* locus structure and regulation.

***MAL*-activator structure and function.** The *MAL63* open reading frame encodes a predicted 470-residue protein (Kim and Michels, 1988; Sollitti and Marmur, 1988). The sequences of *MAL23* and *MAL43* were reported by Gibson *et al.* (1997).

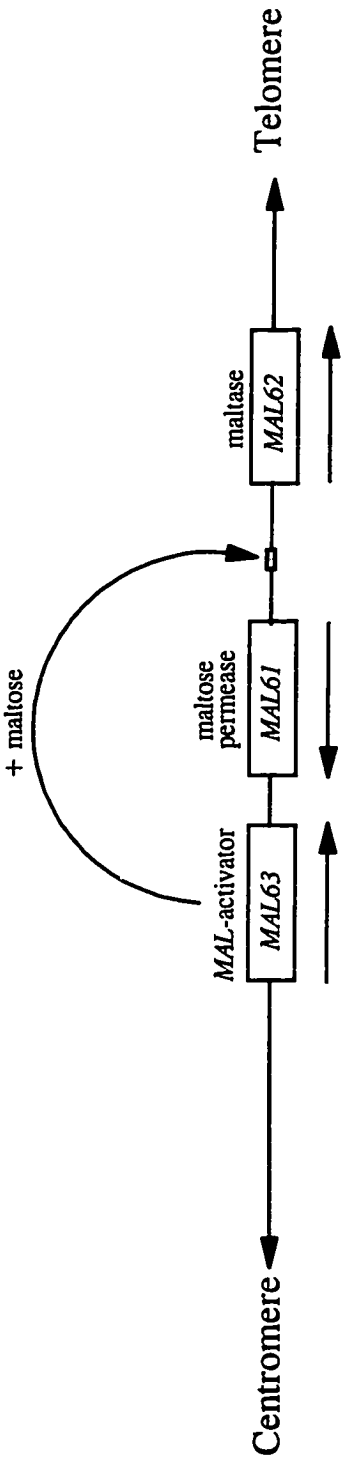


Figure 1. Schematic representation of the structure and function of the *MAL6* locus. Open rectangles represent the coding region of each gene and give the gene name. Written above each ORF is the protein product encoded by the corresponding gene. Arrows below indicate the direction of gene transcription. The *MAL*-activator binds to the bidirectional sequence element called the UAS$_{MAL}$ (small gray box) in the shared promoter of *MAL61* and *MAL62*, and, in the presence of maltose, activates transcription of the structural genes.

Mal23p is inducible as is Mal63p, and the deduced protein sequences of Mal63p and Mal23p are 95% identical. *MAL43-C* is a constitutive allele of *MAL43*; an inducible allele has not been identified at this locus. Mal43-Cp is 93% identical to Mal63p.

Mal63p and each of the other sequenced *MAL*-activators contains a C_6 zinc cluster DNA-binding domain between residues 8 and 34. The *MAL*-activator is a member of the family of fungal transcription factors containing the C_6 zinc cluster DNA-binding domain (Schjerling and Holmberg, 1996). Mutation analysis of the zinc cluster region of Mal63p clearly demonstrated that it is involved in DNA binding (Kim, 1992). *In vitro* mutagenesis was used to introduce the alterations Cys18Leu, Cys27Ser, and Cys34Gly, each of which produces a noninducible phenotype. Gel mobility shift DNA-binding assays were carried out using a Mal63p fragment containing residues 1-111 and a 40-base pair DNA fragment containing the UAS_{MAL}. None of the mutant proteins binds DNA *in vitro*. Another mutant, *MAL63Pro23Leu*, in a conserved proline residue of the zinc cluster region is noninducible in a single copy, but transformants carrying two copies of the mutant gene induce poorly. However, the Mal63p N-terminal fragment containing this Pro23Leu alteration does not bind DNA *in vitro*.

LexA-Mal63 fusion proteins were created by fusing sequences encoding the DNA-binding domain (residues 1-87) of the *E. coli lexA* gene to the full length *MAL63* gene and these were used to investigate the functional domains of the *MAL*-activator (Gibson, 1995). The wild-type LexA-Mal63 fusion protein was able to activate maltose inducible expression of the *MAL* structural genes as well as the $lexA_{operator}$-*lacZ* reporter. Several deletion mutants were made in this construct. Strains carrying *lexA*-

MAL63Δ1-37 as the sole *MAL*-activator activate maltose-inducible expression of the reporter but the *MAL* genes are not expressed. This mutant fusion protein lacks the C_6 zinc cluster region and probably is not able to bind the UAS_{MAL}. Similar results were obtained for mutants *lexA-MAL63Δ40-66* and *lexA-MAL63Δ60-99*. The region immediately C-terminal to the C_6 zinc cluster DNA-binding domain of other members of the fungal transcription activator family has been implicated in DNA-binding sequence specificity (Reece and Ptashne, 1993). In many members of this family, including Gal4p, Leu3p and Hap1p, this region is believed to form a coiled-coil dimerization domain (Schjerling and Holmberg, 1996). It is possible this region of Mal63p also forms a coiled-coil and is involved in the sequence specificity of DNA-binding, and the results described above are consistent with this possibility. DNA binding studies by Ni and Needleman (1990) and Sirenko *et al.* (1995) using purified protein suggest that Mal63p binds DNA as a dimer to direct triplet repeats.

Constitutive mutations have been isolated at all of the *MAL* loci (Winge and Roberts, 1950; Kahn and Eaton, 1971; Charron and Michels, 1987; Zimmerman and Eaton, 1974; Rodicio, 1986; Ten Berge *et al.*, 1973; Dubin *et al.*, 1986; Wang and Needleman, 1996; Gibson *et al.*, 1997). At the *MAL6* locus, dominant constitutive mutations isolated as revertants of nonfermenting *mal6* strains were mapped to the centromere proximal side of *MAL63*, to a nonfunctional *MAL63* homologue called *mal64* (Dubin *et al.*, 1986 and 1988). Wild type *mal64* does not complement *mal63* mutations, but *mal64* can be activated by mutation to encode a constitutive *MAL*-activator. Two *MAL64-C* constitutive alleles, *MAL64-C2* and *MAL64-R10*, encode

truncated proteins of 306 and 282 amino acids, respectively (Gibson *et al.*, 1997). Surprisingly, *mal63-283NS* has a noninducible phenotype, but several findings suggest that this is the result of the instability of the truncated protein and is not indicative of a fundamental functional difference between the Mal63-283NS and Mal64-R10 (also a nonsense mutation at codon 283) activators. When overexpressed, the LexA-Mal63(1-283) fusion protein is a constitutive activator of the *MAL* genes and the *lexA-lacZ* reporter. Alteration of residues Thr247Ala, Arg172Gln, or Arg117Cys in *mal63-283NS* is sufficient to produce a constitutive phenotype (Gibson *et al.*, 1997). In addition, when *mal63-283NS* is expressed in a *doa4Δ* strain which is deficient in ubiquitin-dependent proteolysis, the truncated activator has a constitutive phenotype (Papa and Hochstrasser, 1993; Zhen Hu, unpublished results).

MAL43-C encodes a 470 residue constitutive *MAL*-activator with multiple amino acid substitutions compared with Mal63p. Twenty-six out of 31 differences lie in the carboxy terminal 220 residues (250-470). A series of hybrid molecules between *MAL43-C* and *MAL63* was made which exchanged one or several of the residues which differ between them in an attempt to identify which residues are responsible for the constitutive or inducible phenotype, respectively (Gibson *et al.*, 1997). None of the *MAL63* hybrid molecules which contain blocks of *MAL43-C* alterations was constitutive and likewise, none of the *MAL43-C* hybrid molecules which contain *MAL63* alterations became inducible. Thus, the constitutive phenotype of *MAL43-C* could not be localized to a smaller region with this C-terminal region. Sequence analysis of a constitutive allele of *MAL23* revealed that Mal23-Cp differs from inducible Mal23p, at 18 residues

and contains a two-residue deletion between residues 322 and 378. Mal43-Cp contains 11 alterations within this same region when compared to Mal63p or Mal23p. Taken together, the results of the *mal64, MAL43* and *MAL23* constitutive mutations suggest that the C-terminal approximately 220 residues contain a maltose-responsive negative regulatory domain, and that deletion or extensive alteration throughout the entire region causes a loss of the negative regulatory function (Gibson *et al.*, 1997).

The C-terminal regulatory domain also contains positive regulatory elements whose function is required for induction (Gibson *et al.*, 1997). *mal64* and a *mal63/64* fusion at codon 215 are noninducible. The *mal63-13* allele containing as few as seven amino acid substitutions in this apparently nonessential region from codons 280 to 364 is noninducible. Moreover, a series of 50 codon deletion mutations in *lexA-MAL63* in the regulatory domain are also noninducible despite the fact that abundant protein is made. These results suggest a role in induction for this regulatory domain.

Residues 1-283 of Mal63p contain a transactivation domain, based on the *MAL64*, *MAL63* and *lexA-MAL63(1-283)* constitutive *MAL*-activators which each encode truncated proteins. The deletion series *lexA-MAL63Δ1-37, lexA-MAL63Δ40-66* and *lexA-MAL63Δ60-99* made in the full-length protein encode maltose-inducible activators of a *lexA-lacZ* reporter, and the latter two exhibit significantly increased induced expression levels compared to the wild-type fusion (Gibson, 1995; Gibson *et al.*, 1997). Additionally, LexA-MAL63(1-110) does not activate reporter expression (Zhen Hu, unpublished results). Taken together, these results suggest an essential function for residues 100-283 in these truncated proteins. This function could be

transcription activation, and, in fact, this region contains phenylalanines and other hydrophobic residues which have been identified as playing an important role in transcription activation in *GCN4* (Jackson *et al.*, 1996). Yet, surprisingly, the LexA-MAL63(100-283) fusion protein is unable to activate *lexA-lacZ* reporter expression despite the fact that it does bind *in vivo* to the *lexA* promoter, as determined in a repression assay (Zhen Hu, unpublished results). Additional experiments are required to more clearly define the activation domain(s) of the *MAL*-activator.

The C_6 zinc cluster family of fungal transcriptional regulatory proteins contains at least 79 proteins (Schjerling and Holmberg, 1996). Sequence information suggests that 60 of these proteins possess one or more coiled-coil dimerization domains just C-terminal to the zinc cluster region. Most of these proteins also share a second region of homology, the middle homology region (MHR), which is found in 50 of the 79 proteins, including the *MAL*-activator (Schjerling and Holmberg, 1996). The activation domains of only seven of these proteins have been identified. In all seven cases, the activation domain is acidic and located at the C-terminus of the protein. Interestingly, this study notes that residues 466 to 469 at the extreme C-terminus of Mal63p are highly acidic. Gal4p has six acidic residues within residues 862 and 873 at the extreme C-terminal tail of Gal4p. These residues are included in the region responsible for transcription activation and Gal80p binding. This homology suggests that the C-terminus of Mal63p may contain another transcription activation domain in addition to the one found in the N-terminal 283 residues. The MHR of Mal63p is contained within residues 152 and 252 encompassing an essential domain possibly involved in

transcription activation (see above). Since the MHR domain is only found in C_6 zinc cluster proteins, it is proposed to play a structural or functional role confined to this family of fungal proteins but this role remains undefined. Deletion of a large portion of the MHR of Leu3p produces a constitutive activator. Schjerling and Holmberg (1996) suggest that the MHR may regulate sequence-specifc DNA-binding.

A model for maltose induction of the *MAL*-activator. Several laboratories have isolated constitutive *MAL* mutants, usually as revertants of noninducible *MAL*-activator mutant strains. Only *MAL*-linked mutations have been obtained, and all map to *MAL*-activator genes. In addition, overexpression of the *MAL*-activator does not lead to constitutive expression of the *MAL* structural genes. These findings argue against the existence of a *MAL*-specific negative regulator protein similar to Gal80p, and suggest that the *MAL*-activator may be autoregulated. The genetic analyses discussed above indicate that this regulatory domain is a complex of both negative and positive functions. The goal of my thesis project is to define in greater detail the positive and negative subdomains within the regulatory domain, and to explore their role in the induction process.

Materials and Methods

Yeast strains. The following strains were used in this study: YPH500 (*MATα AGT1 MAL12 mal13Δ MAL31 MAL32 mal33Δ ura3-52 his3-Δ200 leu2-Δ1 ade2-101 lys2-801 trp1-Δ63*) (Sikorsky and Heiter 1989); PMY270 (*MATα AGT1 MAL12 mal13Δ MAL31 MAL32 mal33Δ doa4-Δ1::LEU2 his3-Δ200 ura3-52 lys2-801 trp1-1* (Papa and Hochstrasser 1993); and 61-106, a YPH500 derivative carrying several copies of a $MAL61_{promoter}$-*lacZ* reporter integrated at *LEU2*.

Construction of $MAL61_{promoter}$-*lacZ* reporter strain 61-106. 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.*,1995a). This construct was then targeted to integrate at the *leu2-Δ1* gene of YPH500 by digestion at the unique *Bst*EII site. Integration was confirmed by Southern analysis of *Hin*dIII digested total genomic DNA. Several leucine$^+$ transformants were tested and transformant strain 61-106 exhibited an intense plasmid-sized (*Hin*dIII) fragment indicating multiple integrated copies.

Bacterial growth and transformation. *E. coli* strain DH5α was used for plasmid amplification. Competent DH5α cells were purchased from Gibco/BRL. Cells were transformed according to manufacturer's protocol and transformants were selected as ampicillin resistant colonies on LB plates containing ampicillin. Transformants were screened by polymerase chain reaction (PCR) and/or digestion with restriction endonucleases.

Yeast growth and transformation. Yeast cells were grown at 30°C on rich media, YPD (1% yeast extract, 2% peptone, 2% dextrose), or synthetic minimal media, SM (Bio 101 Inc.) containing an appropriate carbon source but lacking relevant nutrients for plasmid selection. Yeast cells were made competent using lithium acetate as described by Ito *et al.* (1983). Transformants were selected on synthetic minimal media lacking appropriate nutrient. Plasmid transformation was confirmed based on the ability of the transformants to lose the plasmid following growth under non-selective conditions.

Construction of pUN30MAL63. A 3-kb *SalI* fragment containing a hemagglutinin tagged allele of *MAL63* (Wojciechowicz, 1993) was subcloned into the *SalI* polylinker site in the CEN vector pUN30 (Elledge and Davis, 1988). The vector pUN30 is a yeast/*E.coli* shuttle vector which contains the *Saccharomyces TRP1* gene, ARS1, and CEN4; the polylinker derived from pUC18; and the F1 phage origin of replication in the plus orientation which allows transformant to produce single-stranded template DNA when infected with R408 helper phage.

Construction of pUN30MAL63ΔKpnI-RI. A 0.5-kb *Kpn*I fragment containing upstream sequences of *MAL63* plus some vector sequences was removed from pUN30MAL63 by digestion with *Kpn*I and self ligation. Approximately 200 bases of upstream sequences remain, sufficient for full expression of *MAL63*. This construct, pUN30MAL63ΔKpnI, 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'AGGAATTATGTCGTCTTCATCTTT3') in pUN30MAL63ΔKpnI to create pUN30MAL63ΔKpnI-RI. This construct was used to create hybrid *MAL*-activator genes.

***MAL63* conversion to *MAL23-C* sequence.** Mutations were made in pUN30-MAL63 by *in vitro* mutagenesis. These mutations were made in 3 blocks within codons 325 and 378. A block 1 conversion altered the coding sequence of *MAL63* within base pair 973-1008 to 5'-GTCGATATTTCGTTTTCGAGGCATTGGATCAGGGCA-3'. A block 2 conversion altered the coding sequence of *MAL63* within base pair 1027-1077 to 5'-TTTCAAATGAATGGTACGAAG TTTTTTTCGAATGCTAATAATGCA-3' and a block 3 conversion altered the coding sequence of *MAL63* within base pair 1084-1134 to 5'-CTAGTCGAAATTGCTAAAGACATGTTGGATGACATCTTTTT AACTCCGAAT-3'. Mutagenic oligonucleotides convert the Mal63p amino acid sequence to that of Mal23-C protein within the designated blocks.

Construction of pUN30MAL63/43 and pUN30MAL63/23. pUN30MAL63-ΔKpnI-RI was digested with *Eco*RI releasing the 1.1kb fragment containing the 3' region of *MAL63* from codon 215/216. The digested plasmid was treated with alkaline phosphatase and the vector-containing fragment gel purified using the Geneclean kit (Bio 101 Inc.). 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 *MAL63/43* and *MAL63/23* fusion genes by ligation with the 1.1 kb *Eco*RI fragment containing the 3' region of *MAL43-C* and *MAL23*, respectively. YCp50MAL43,

carrying *MAL43-C,* and pMAL23, carrying *MAL23*, were used as the source of this 1.1 kb *Eco*RI insert (Gibson, 1995). Transformants were screened by PCR and sequencing to confirm the construction.

Construction of pUN30MAL43. YCp50MAL43-C was digested with *Kpn*I and *Sal*I releasing a 2.5kb fragment containing the *MAL43-C* open reading frame and approximately 200 base pairs of upstream sequences. This was inserted into the *Kpn*I and *Sal*I sites of the pUN30 polylinker.

Construction of LexA/MAL63 fusion genes. *In vitro*-generated *mal63* mutations were introduced into a *LexA-MAL63* fusion gene as follows. Plasmid pLexA(1-87)MAL63(2-216) derived from plasmid pSH2-1 (Gibson, 1995) contains codons 1-87 of *lexA* (encoding the DNA-binding domain) fused to codons 2-216 of *MAL63* driven by the constitutive *ADH1* promoter. 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. The sequence to be mutagenized was carried in either the M13-mp19 phage vector or in the plasmid pUN30 (Elledge and Davis, 1988). Single-stranded uracil containing template DNA was generated in *E. coli* strain CJ236 (*dut,ung*). This strain allows the incorporation of uracil into the template DNA instead of thymine and contains an F+ origin for the production of viral particles containing single stranded DNA when infected with helper phage.

CJ236 is grown in LB broth with chloramphenicol. Cells were made competent in 0.1M $CaCl_2$ and transformation was done with 100µl aliquots, incubated on ice 20 minutes and heat shocked at 42°C for 4 min. These transformed cells were then incubated at 30°C for 1 hour with 400µl LB and plated on LB plates with ampicillin and chloramphenicol. Cells containing plasmid were grown in LB plus ampicillin and chloramphenicol and infected using R408 helper phage.

Oligonucleotides containing the desired sequence alteration were annealed to the uracil-containing template DNA, T4 DNA polymerase and ligase were used to convert the template to double-stranded DNA which was transformed into *E. coli* strain DH5α and ampicillin-resistant colonies selected. Double-stranded plasmid DNA was prepared from 5-10 transformants and screened by sequencing through the mutagenized region using the dideoxy method (Sanger *et al.*, 1977) and a kit purchased from Amersham/United States Biochemical (USB Version 2.0 Sequenase kit) and S^{35}-dATP (Amersham). The open reading frames of mutagenized genes whose phenotypes differed from wild-type (noninducible or constitutive) were sequenced in their entirety to confirm that the desired alteration was the only mutation. Some oligonucleotide primers used in mutagenesis, PCR and sequencing were made and purified using the Applied Biosystems Inc. DNA synthesizer Model 381A, and reagents purchased from ABI. However, most of the oligonucleotide primers used were purchased from Gibco/BRL.

Random mutagenesis of *MAL63* codons 454 to 470. Random sequence oligonucleotides were generated using the DNA synthesizer as described by Leuther *et*

al. (1993). For this, each nucleotide supply bottle contained 1% of each of the other three nucleotides. Given this level of intentional contamination, each 63-mer oligonucleotide made should contain 1-2 incorrect bases. These randomly mutagenic oligonucleotides were then used in *in vitro* mutagenesis using pUN30MAL63-D466A,D467A as a template. The newly generated double-stranded plasmids were transformed into DH5α, approximately 15,000 transformant colonies were pooled, plasmid DNA prepared, transformed into the $MAL61_{promoter}$-*lacZ* reporter strain 61-106, and plated directly onto synthetic minimal media containing 2% maltose, 3% glycerol, 2% lactate and lacking tryptophan. Approximately 20,000 yeast transformant colonies were screened using a β-galactosidase plate assays (for reference see below) in which expression of the *lacZ* reporter gene can be detected by conversion of X-gal to a blue dye. Transformants were screened for the inability to express the blue color. Plasmids were rescued from the white transformants for further analysis. Each plasmid carrying a potential *mal63* mutation was transformed into strain 61-106 and the noninducible phenotype confirmed by maltase assays and fermentation tests. The full open reading frame of each mutant was sequenced.

β-Galactosidase plate assays. Yeast were grown as colonies or patches on plates containing 2% maltose, 3% glycerol, 2% lactate at 30°C. These were overlayed with 10 ml of 0.5% agarose/0.5 M sodium phosphate, pH7/0.1% SDS/2% (vol/vol) dimethyl-formimide/0.5% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, as described by Bohen and Yamamoto (1993). The plates were observed over time and

the rate of blue dye formation noted. Live cells could be recovered by piercing the overlay with a sterile toothpick.

Measurement of maltase and maltose permease activities and maltose fermentation. Cells were grown in SM media containing the indicated carbon source and lacking appropriate nutrients for plasmid selection. Total cell extracts were prepared from mid-log phase cells (OD_{600} 0.3-0.5) and maltase activity measured as the rate of *p*-nitrophenol released from *p*-nitrophenyl-α-D-glucopyranoside as described by Dubin *et al.* (1985). Activity was normalized to the protein concentration of the extracts measured with the BioRad Protein Assay Dye Reagent. Activity is expressed as nmoles PNPG / minute / mg protein. Maltose permease activity was measured by uptake of C^{14} maltose as described previously (Cheng and Michels, 1991). Fermentation was determined by the number of days required for initial gas bubbles to form in Durham tubes after innoculation into YP plus 2% maltose.

Western Analysis. Transformants carrying *lexA-MAL63* fusion molecules were grown in SM plus 2% maltose, 3% glycerol, 2% lactate and lacking histidine. Cells were harvested and protein extracts prepared as described in Ausubel *et al.*, (1997). Cells were lysed by vortexing 8-10 times using glass bead disruption buffer, (20mM Tris-Cl, 10mM $MgCl_2$, 1mM EDTA, 5% glycerol, 1mM dithiothreitol, 0.3 M ammonium sulfate, including protease inhibitors and 1mM PMSF), and glass beads. Cell suspensions were then centrifuged and supernatants were assayed for protein concentration using the Bio Rad protein assay reagent dye. Approximately equal concentrations of protein were loaded onto a 10% acrylamide gel. Gel electrophoresis

and membrane transfer were 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 ECL reaction with reagents obtained from Amersham on ECL-Hyperfilm.

RESULTS

Chapter 1: Localization of negative regulatory sequence elements in the C-terminal domain of the *MAL*-activator

Localization of the constitutive alteration(s) in *MAL43-C*. A hybrid *MAL* activator gene was constructed by fusing the 5' end of *MAL63* including approximately 200 basepairs of the promoter and encoding the N-terminal 215 residues to the 3' end of the constitutive *MAL43-C* gene encoding the C-terminal residues from 215-470. This construct was transformed into strain 61-106, lacking a functional *MAL*-activator gene but containing the *MAL* structural genes, and maltase activity was determined in cells grown in inducing (YEP plus 2% maltose, 3% glycerol, 2% lactate) and noninducing conditions (YEP plus 3% glycerol, 2% lactate). The results in Table 1 demonstrate that *MAL63/43,* like *MAL43-C,* is constitutive and transformants carrying either allele express comparable high levels of maltase in the absence of inducer. In the presence of maltose, both transformant strains exhibit a 2-3 fold decrease in maltase expression to a level similar to that of maltose-grown transformants carrying *MAL63*. Therefore, the alterations contained in the C-terminal region of *MAL43-C* are sufficient for the constitutive phenotype. The sequence of Mal43-C protein was reported in Gibson *et al.* (1997) and is presented in Figure 2 along with that of the Mal63 activator for comparison. The C-terminal region of Mal43-Cp from residues 215-470 contains 27 altered residues compared to Mal63p.

Constitutive mutations of *MAL23*. Wang and Needleman (1996) reported the isolation of several constitutive *MAL63* alleles each of which appeared to result from

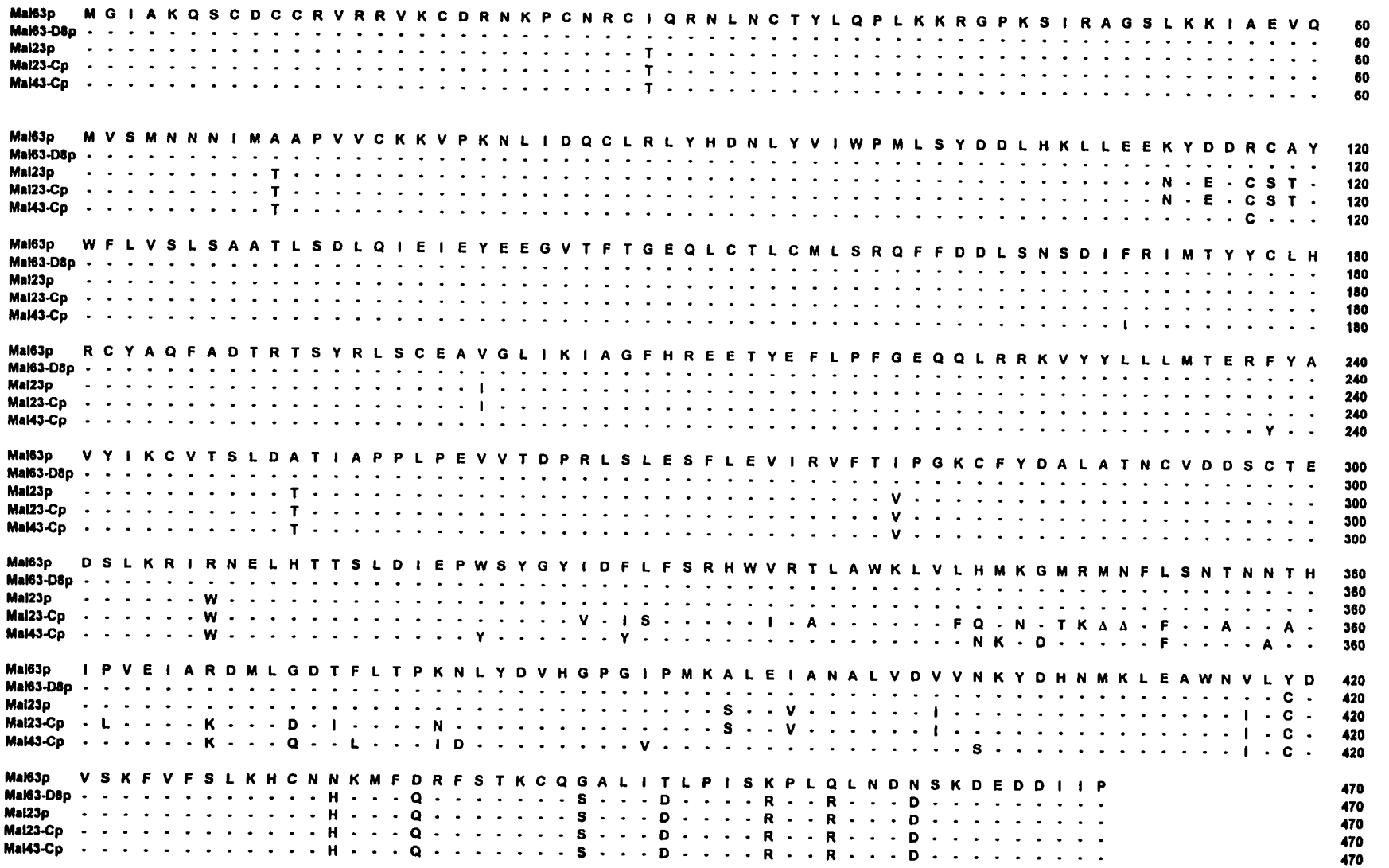
TABLE 1

Regulation of maltase expression by *MAL*-activator proteins

	Maltase Activity	
MAL-activator gene	Uninduced	Induced
Vector	21	86
MAL63	46	1525
MAL43-C	4390	1387
MAL63/43-C	3688	1087
MAL23	144	1695
MAL63/23	121	1054
MAL63/43(SVI)	3770	1745

The *Kpn*I-*Sal*I fragment from *MAL63, MAL43-C, MAL63/43-C, MAL63/23* and *MAL63(SVI)* containing the full ORF plus approximately 200 bases of upstream sequence (in CEN vector pUN30) and the *Bgl*II-*Sal*I fragment containing *MAL23* plus at least 500 bases of upstream sequence (also in pUN30) were transformed into strain 61-106 (pUN30-MAL23 was constructed by Zhen Hu). Maltase was assayed in at least two independent transformants in duplicate and the error was less than 20%. Cells were grown in minimal synthetic medium lacking the appropriate nutrient for plasmid selection in uninduced (3% glycerol, 2% lactate) and induced (2% maltose, 3% glycerol, 2% lactate) growth conditions.

Figure 2. Protein sequence alignment of several *MAL*-activators. The complete amino acid sequence of the Mal63 *MAL*-activator is given and compared to that of the constitutive mutant allele encoded by *MAL63-D8* (Wang and Needleman, 1996), the inducible Mal23 activator, and the constitutive Mal23-C and Mal43-C activators. Only amino acids which differ from Mal63p are indicated for the other activator sequences.



gene conversion-like events between the plasmid-borne *MAL63* and partially homologous functionally inactive *MAL*-activator gene sequences contained in the genome of the host yeast strain. The shortest of these conversion events introduced an eight-amino acid substitution at the extreme C-terminus of the protein and is referred to as *MAL63-D8*. Figure 2 shows the amino acid sequence of this allele and that of Mal63p from residues 251-470. These same eight alterations are found in the constitutive activators encoded by *MAL43-C* and *MAL23-C* and surprisingly also in the inducible *MAL23* activator (see Gibson *et al.*, 1997; Figure 2). Moreover, Mal43-Cp, Mal23-Cp, and Mal23p all retain the Y419C alteration which Wang and Needleman (1996) demonstrated is necessary but not sufficient for constitutivity in the *MAL63* constitutive mutations they describe. As shown in Figure 2, Mal23p contains 15 other amino acid differences compared to Mal63p and the Mal63 constitutive activators scattered throughout the protein. Eight of these 15 amino acid differences are located in the N-terminal 215 residues, five of which are clustered between residues 113 and 119. This suggests that in Mal23p, one or more of the 15 altered residues is suppressing the otherwise constitutive effects of the eight C-terminal alterations.

To test this, a *MAL63/23* fusion was constructed using the *Eco*RI site at codons 215/216 of both genes. This hybrid contains the promoter and first 215 codons of *MAL63* fused to codons 216-470 of *MAL23*, and includes seven of the 15 residue differences between the *MAL63-D8* constitutive mutation and inducible *MAL23*, all in the C-terminus. Plasmids carrying this *MAL63/23* fusion gene or *MAL23* were transformed into strain 61-106 and maltase expression was determined in cells grown in

uninduced and maltose-induced conditions (Table 1). The hybrid Mal63/23p is inducible, and maltase expression in uninduced conditions is comparable to that exhibited in strains expressing Mal23p, that is, three to five-fold higher levels of basal activity than strains expressing Mal63p. This result indicates that the eight-residue differences between Mal63p and Mal23p in the N-terminal region (residues 1-215) are not necessary to suppress the constitutivity of the eight C-terminal alterations. The *MAL63/23* construct retains seven residues in the C-terminal region of Mal23p as possible suppressors (see Figure 2).

Site-directed mutagenesis of *MAL63/23* was used to convert residues in the C-terminal Mal23p sequence to those found in Mal63p. The sequences of the oligonucleotides used to introduce these changes are given in Table 2. Figure 3 depicts these changes and the resulting phenotype of these conversions expressed in strain 61-106. In all, five of the seven residues were changed, most individually and some in combination. Up to a two-fold increase in maltose-induced levels of maltase expression can be seen for some of these conversions. However, grown in uninduced conditions, these alterations can be seen to increase or decrease maltase expression dramatically. Conversions S392A and V395I or I402V are sufficient to relieve the proposed suppression and increase basal (uninduced) maltase expression four to five-fold to approximately 30-40% of fully induced levels. The triple conversion containing all three alterations combined is fully constitutive.

Conversion of T251A or W307R in Mal63/23p causes a repression of the basal maltase expression by approximately three to four-fold while having no significant effect on maltose-induced expression levels (Figure 3). This basal expression level is

TABLE 2

MAL63/23 in vitro mutagenesis

MAL63/23 allele	Oligo sequence (5'-3')	Annealing site*
MAL63/23-W307R	AAGTTCGTTCCGTATCCTTTT	929-909
MAL63/23-S392A, V395I	TGCATTGGCTATTTCCAATGCTTTCATTGG	1193-1164
MAL63/23-T251A	CGCTATTGTTGCATCTAGGCT	761-741
MAL63/23-I402V	CTTATTTACGACATCTACCAA	1214-1194

* Open reading frame begins with base pair number 1.

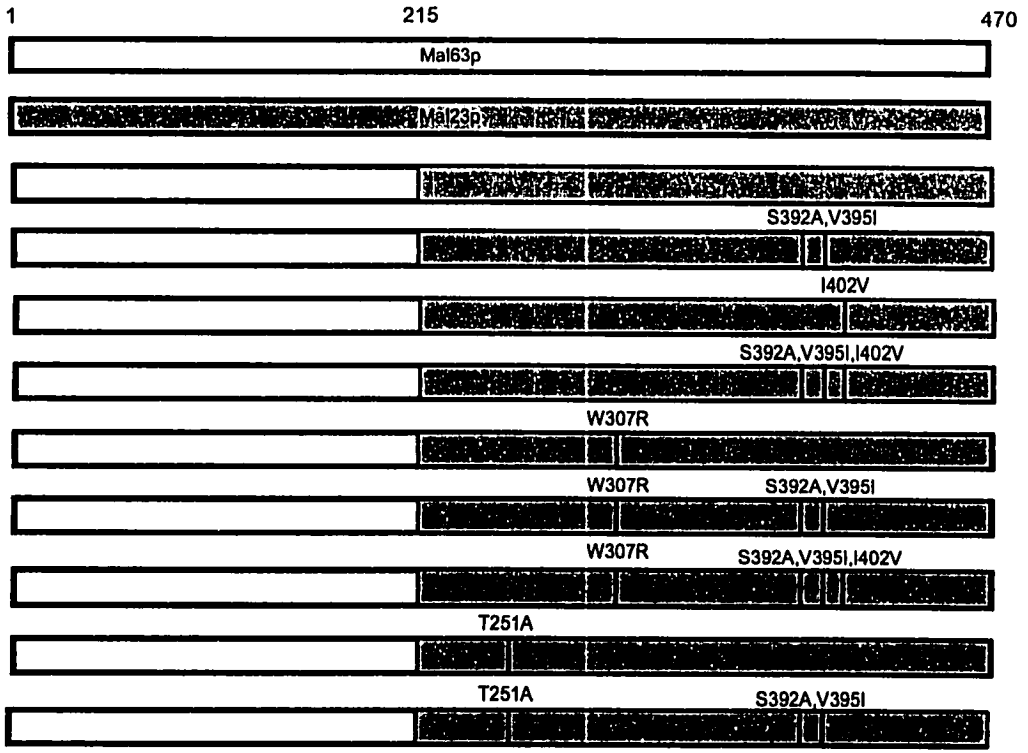
Figure 3. Constitutive mutations of Mal63/23p. The sequence alignment at the top of the figure indicates the amino acid differences between Mal63p, Mal63-D8p, and Mal23p from residue 251-470. Underlined residues indicate the amino acids converted by *in vitro* mutagenesis to the residues found in Mal63p. These conversions were made in a *MAL63/23* hybrid gene with the junction site at codons 215/216 (see Materials and Methods) contained in vector pUN30. The constructs were transformed into strain 61-106 and maltase expression assayed. Rectangles represent the sequence of residues 1-470 in Mal63/23p, Mal63p, or Mal23p, and vertical lines indicate the position of residue(s) converted from the Mal23p sequence to that found in Mal63p. Shaded rectangles indicate Mal23p residues and open rectangles indicate Mal63p residues. Maltase was assayed as described for Table 1.

```
Mal63p   251 ATIAPPLPEVVTDPRLSLESFLEVIRVFTIPGKCFYDALATNCVDDSCTEDSLKRIRNEL 310
Mal63-Cp     ------------------------------------------------------------
Mal63/23p    T-----------------------------V-----------------------W-----

Mal63p       HTTSLDIEPWSYGYIDFLFSRHWVRTLAWKLVLHMKGMRMNFLSNTNNTHIPVEIARDML 370
Mal63-Cp     ------------------------------------------------------------
Mal63/2p     ------------------------------------------------------------

Mal63p       GDTFLTPKNLYDVHGPGIPMKALEIANALVDVVNKYDHNMKLEAWNVLYDVSKFVFSLKH 430
Mal63-Cp     ----------------------------------------------------C-------
Mal63/23p    ----------------------S--V------I-----------------I-C-------

Mal63p       CNNKMFDRFSTKCQGALITLPISKPLQLNDNSKDEDDIIP                     470
Mal63-Cp     --H---Q-------S---D----R--R---D--------
Mal63/23p    --H---Q-------S---D----R--R---D--------
```



Maltase Activity

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

comparable to that expressed by transformants carrying *MAL63* (Table 1). When W307R is introduced along with S392A and V395I in *MAL63/23*, the constitutive phenotype of the S392A, V395I construct is suppressed and this triple mutant exhibits a repressed basal activity which is like that of W307R alone (Figure 3). A similar result is obtained when W307R is introduced into the fully constitutive S392A, V395I, I402V construct, but suppression is not complete. T251A also is able to suppress the constitutive phenotype of the S392A, V395I conversion, but not to the same extent as W307R.

Finally, residues alanine-392, isoleucine-395 and valine-402 are identical in Mal63p and Mal43-Cp. These 3 residues were converted to the Mal23p sequence (that is, A392S, I395V, V402I) in the constitutive activator *MAL63/43* to determine if these amino acid substitutions are sufficient to suppress the constitutive phenotype of this hybrid *MAL*-activator. As seen in Table 1, maltase expression of transformants carrying this allele is unaffected by these amino acid substitutions.

Isolation of *MAL63* constitutive mutants. The sequence of the constitutive activator Mal23-Cp as reported by Gibson *et al.* (1997) contains 18 amino acid substitutions and two deleted residues in the C-terminal region from residues 325 to 378 compared to the inducible Mal23p. Mal43-Cp contains 11 alterations in the same region, only three of which are common to both constitutive alleles. It is possible that this region represents a second negative subdomain.

In order to test this possibility, inducible Mal63p was converted to the sequence of Mal23-Cp within this region (residues 325 to 378) using the oligonucleotides

described in Materials and Methods. The region was divided into three sections, referred to as blocks, and amino acid substitutions were converted as individual blocks (that is, block 1, 2 or 3) or in combination, including one conversion which included all three blocks of sequence changes which produces an allele which is identical to the Mal23-Cp sequence in this region. Block 1 converts residues within codons 325 and 336 (I325V, F327I, L328S, V334I, T336A). Block 2 substitutions contained within codons 343 and 359 and include eight amino acid substitutions and two deletions (L343F, H344Q, K346N, M348T, R349K, M350Δ, N351Δ, L353F, T356A, T359A). Block 3 converts five residues within codons 362 and 378 (P362L, R367K, G371D, T373I, K378N). The various constructs were introduced into strain 61-106, and transformants were assayed for maltase expression following growth in inducing and in uninducing conditions. The results are summarized in Figure 4.

Alterations in block 1 alone, *MAL63(1),* have no effect on regulation and are not sufficient to cause constitutivity. Block 2 alone, *MAL63(2),* is sufficient to produce a constitutive phenotype. Interestingly, when block 3 alone, *MAL63(3),* is converted, the activator has a noninducible phenotype. *MAL63(1,2),* containing blocks 1 and 2 together, also is constitutive, but when block 3 is added, *MAL63(1,2,3),* the activator is again inducible. The basal level of *MAL63(1,2,3)* in uninduced conditions is higher than in *MAL63* and is more typical of levels observed in transformants containing *MAL63/23*. One construct of *MAL63(1,2)* included an unintentional alteration of P362L from the region included in block 3. This construct is inducible (*MAL63(1,2-L)*) suggesting that proline 362 is important in suppressing the constitutive phenotype of

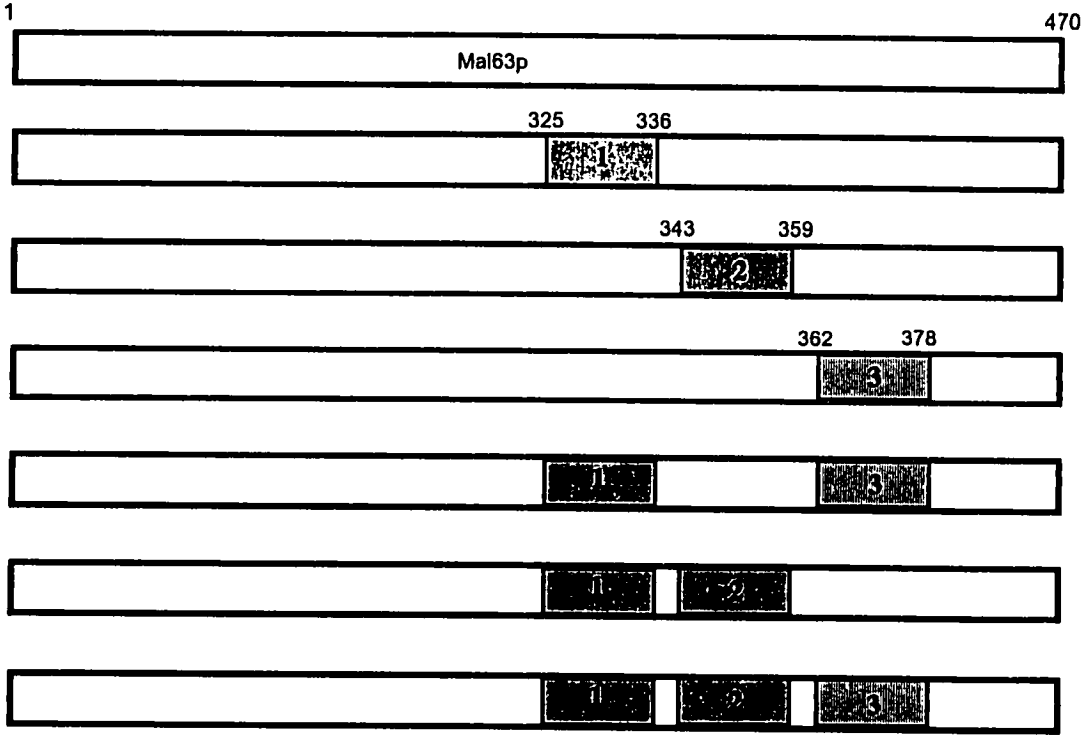
Figure 4. Constitutive mutations of Mal63p. The sequence alignment at the top of the figure indicates amino acid differences between Mal23p, Mal23-Cp, and Mal63p. Underlined sequences indicate those residues of Mal63p altered as a group (referred to as blocks 1, 2 or 3) to the residues found in Mal23-Cp. Open rectangles represent Mal63 protein from residues 1-470. Regions of *in vitro*-generated clustered alterations are indicated as shaded, numbered areas (blocks), and residue numbers indicate the endpoints of the clusters. Maltase assay conditions are the same as for Table 1.

```
Mal23p   251  T T I A P P L P E V V T D P R L S L E S F L E V I R V F T V P G K C F Y D A L A T N C V D D S C T E D S L K W I R N E L  310
Mal23-Cp      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Mal63p        A - - - - - - - - - - - - - - - - - - - - - - - - - - - - I - - - - - - - - - - - - - - - - - - - - - - - R - - - - -

Mal23p        H T T S L D I E P W S Y G Y I D F L F S R H W V R T L A W K L V L H M K G M R M N F L S N T N N T H I P V E I A R D M L  370
Mal23-Cp      - - - - - - - - - - - - - - - V - I S - - - - - I - A - - - - - - F Q - N - T K Δ Δ - F - - A - - A - - L - - - - K - - -
Mal63p        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
                                                  1                                   2                                   3

Mal23p        G D T F L T P K N L Y D V H G P G I P M K S L E V A N A L V D I V N K Y D H N M K L E A W N I L C D V S K F V F S L K H  430
Mal23-Cp      D - I - - - - N - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Mal63p        - - - - - - - - - - - - - - - - - - - - - - - - - - A - - I - - - - - - V - - - - - - - - - - - - - - V - Y - - - - - - - - - - -

Mal23p        C N H K M F Q R F S T K C Q S A L I D L P I S R P L R L N D D S K D E D D I I P                                  470
Mal23-Cp      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Mal63p        - - N - - - D - - - - - - - - G - - - T - - - - K - - Q - - - N - - - - - - - - - - - - - - - - - - - - - - - - -
```



Maltase Activity

	Uninduced	Induced	Fold-Induction
Mal63p	46	1525	33
1	32	1384	43
2	950	1277	1
3	26	83	3
1 3	23	351	15
1 2	1231	1206	1
1 2 3	126	1015	8

MAL63(1,2). Finally, the construct containing blocks 1 and 3 together, *MAL63(1,3)*, is poorly inducible, and transformants carrying this allele express levels about 25% of *MAL63/23* transformants grown in induced growth conditions.

In order to determine whether the constitutive mutations, *MAL63(2)* and *MAL23(S392A,V395I,I402V)* are dominant or recessive, strain 61-106 was co-transformed with plasmids carrying the mutant allele and *MAL63* and maltase assays were performed on cells grown in uninduced growth conditions. *MAL23(S392A,V395I,I402V)* is completely dominant and transformants express the same high uninduced levels of maltase in the presence or absence of the *MAL63* gene (Table 3). However, transformants carrying both *MAL63(2)* and *MAL63* exhibited reduced uninduced levels of maltase activity, approximately one third the level of that expressed in transformants carrying *MAL63(2)* alone (Table 3). Thus, *MAL63(2)* is partially dominant.

TABLE 3

Determination of dominance of constitutive *MAL*-activator mutations

MAL-activator	Maltase Activity	
	Control	*MAL63*
MAL63	39	44
MAL63/23(S392A, V395I, I402V)	2370	2418
MAL63(2)	950	280

Transformants of strain 61-106 containing *MAL63*, *MAL63/23(S392A, V395I, I402V)*, or *MAL63(2)* alone (in vector pUN30) or cotransformed with *MAL63* (in vector YCp50) were assayed for maltase expression in uninduced growth conditions (3% glycerol, 2% lactate).

Chapter 2: Defining residues involved in induction and activation

Based on sequence analysis of various *MAL*-activator constitutive mutations, Gibson *et al.* (1997) proposed that the C-terminal residues of Mal63p from residues 283 to 470 function to regulate the response to maltose. Chapter 1 of the Results localized short sequence regions within this regulatory domain that play negative regulatory roles in the absence of maltose. Mutant alleles containing missense alterations within these regions are constitutive. Moreover, intragenic missense suppressor mutations, identified at closely linked and more distant sites, restore the inducible phenotype to varying extents by significantly decreasing the uninduced levels of *MAL* structural gene expression. Deletion analysis of *MAL63* creating a series of in-frame 50 residue deletions from residue 250 to 470 did not reveal these negative regulatory sequence elements, but instead each of these deletion mutations resulted in a noninducible phenotype (Gibson, 1995). Additionally, this thesis (Chapter 1, block 3 mutation of *MAL63*), and Gibson *et al.* (1997) report on noninducible mutations resulting from a few (5-7) missense alterations within defined regions of this regulatory domain. Taken together, these results suggest that positive subdomains playing an active role in functions such as the induction process or transcription activation, are present within the regulatory domain and that regions involved in these positive functions overlap with the negative subdomains found within the regulatory domain. In order to define residues within this C-terminal regulatory domain involved in positive functions, a less invasive mutagenesis method was undertaken.

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). The theory is based on the assumption that 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 a drastic alteration in protein conformation which would make the mutant protein a target for degradation. This technique has been shown to produce stable, properly folded mutant proteins 90% of the time when used to study human tissue plasminogen activator (Bennett *et al.*, 1991). Clustered-charge to alanine mutagenesis was successfully used to systematically study the surface domains of proteins such as actin (encoded by *ACT1*), ubiquitin-conjugating enzyme (encoded by *CDC34/UBC3*), and protein phosphatase type-1 (encoded by *GLC7*) of *Saccharomyces cerevisiae* and identify sites of interaction with other components of multiprotein complex and target proteins (Wertman *et al.*, 1992; Pitluk *et al.*, 1995; Baker, *et al.*, 1997).

Clustered-charge to alanine scanning mutagenesis of the regulatory domain of the inducible *MAL63*. A total of 29 mutations were made in *MAL63* by *in vitro* mutagenesis (shown in Table 4 and Figures 5A and 5B). Twenty-five of these were generated based on the clustered-charge to alanine scanning method. Several of these mutations alter partially overlapping regions. This was done in regions where large clusters of charged residues are found. In one case, *mal63-25* (K244A), only one residue was changed. In addition, one serine (Ser-462), which is part of a potential

TABLE 4

MAL63 in vitro mutagenesis

mal63 allele	Amino acid replacement	Oligo sequence (5'-3')	Annealing site*
mal63-1	D464A,E465A	TATGTCGTCTGCAGCTTTGGAGTT	1403-1380
mal63-2	D466A,D467A	AGGAATTATGGCGGCTTCATCTTT	1409-1386
mal63-4	K463A,D464A,E465A, D466,467A	TTAAGGAATTATGGCGGCTGCAGCTGCGGAGTTATCATT	1412-1374
mal63-5	R438A,K442A	ACCTTGACATGCCGTTGAAAATGCGTCGAACAT	1334-1302
mal63-6	K434A,D437A,R438A	TTTCGTTGAAAATGCGGCGAACATTGCATTATTGCA	1325-1290
mal63-7	D420A,K423A	AAAAACAAACGCGGATACAGCATACAAAAC	1277-1248
mal63-8	K411A,E413A	ATTCCAAGCTGCCAACGCCATATTGTG	1247-1221
mal63-9	K405A,D407A	CATATTGTGAGCATACGCATTTACGAC	1229-1203
mal63-10	D401A,K405A	GTGATCATACGCATTTACGACAGCTACCAATGC	1223-1191
mal63-11	K391A,E394A	ATTGGCTATTGCTAATGCCGCCATCGGTAT	1190-1161
mal63-12	K378A,D382A	ACCATGTACAGCATACAGGTTTGCCGGAGTTAA	1154-1122
mal63-13	D368A,D372A	TAAAAACGTGGCTCCCAACATGGCTCTAGCAAT	1124-1092
mal63-14	E364A,R367A	CAACATGTCTGCAGCAATTGCGACTGGTAT	1109-1080
mal63-15	K346A,R349A	AAAATTCATCGCCATACCTGCCATATGAAG	1055-1026
mal63-16	R331A,R335A	CGCTAGTGTCGCGACCCAATGCGCCGAAAACAG	1013-981
mal63-17	D316A,E318A	AGACCATGGCGCTATAGCAAGTGATGT	962-936
mal63-18	R305A,R307A,E309A	GGTATGAAGTGCGTTCGCTATCGCTTTTAGAGA	935-903
mal63-19	E300A,D301A,K304A	CCGTATCCTTGCTAGAGAGGCTGCGGTGCAGGA	920-888
mal63-20	D295A,D296A	GGTGCAGGAAAGCAGCGACACAGTT	896-873
mal63-21	K283A,D287A	AGCCAAAGCAGCATAAAAACACGCTCCTGGTAT	869-837
mal63-22	E273A,R276A	AGTGAAAACTGCAATCACCGCAAGGAAGCT	836-807
mal63-23	R265A,E269A	AAGGAAGCTTGCTAGAGAAAGAGCAGGGTCTGT	815-783
mal63-24	E259A,D263A	AAGACGAGGGGCTGTTACAACCGCTGGTAGTGG	797-765
mal63-25	K244A	CGTGACACACGCAATATATAC	740-720
mal63-26	D460A,K463A	GTCTTCATCTGCGGAGTTAGCATTTAATTG	1397-1368
mal63-27	S462A	GTCGTCTTCATCTTTGGCGTTATCATTTAATTGCAA	1400-1365
mal63-64	D467S,I469V	AAATTAAGGAACTATGGAGTCTTCATC	1415-1389

* Open reading frame begins with base pair number 1.

Figure 5. *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, and amino acid differences are 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.

Figure 5A

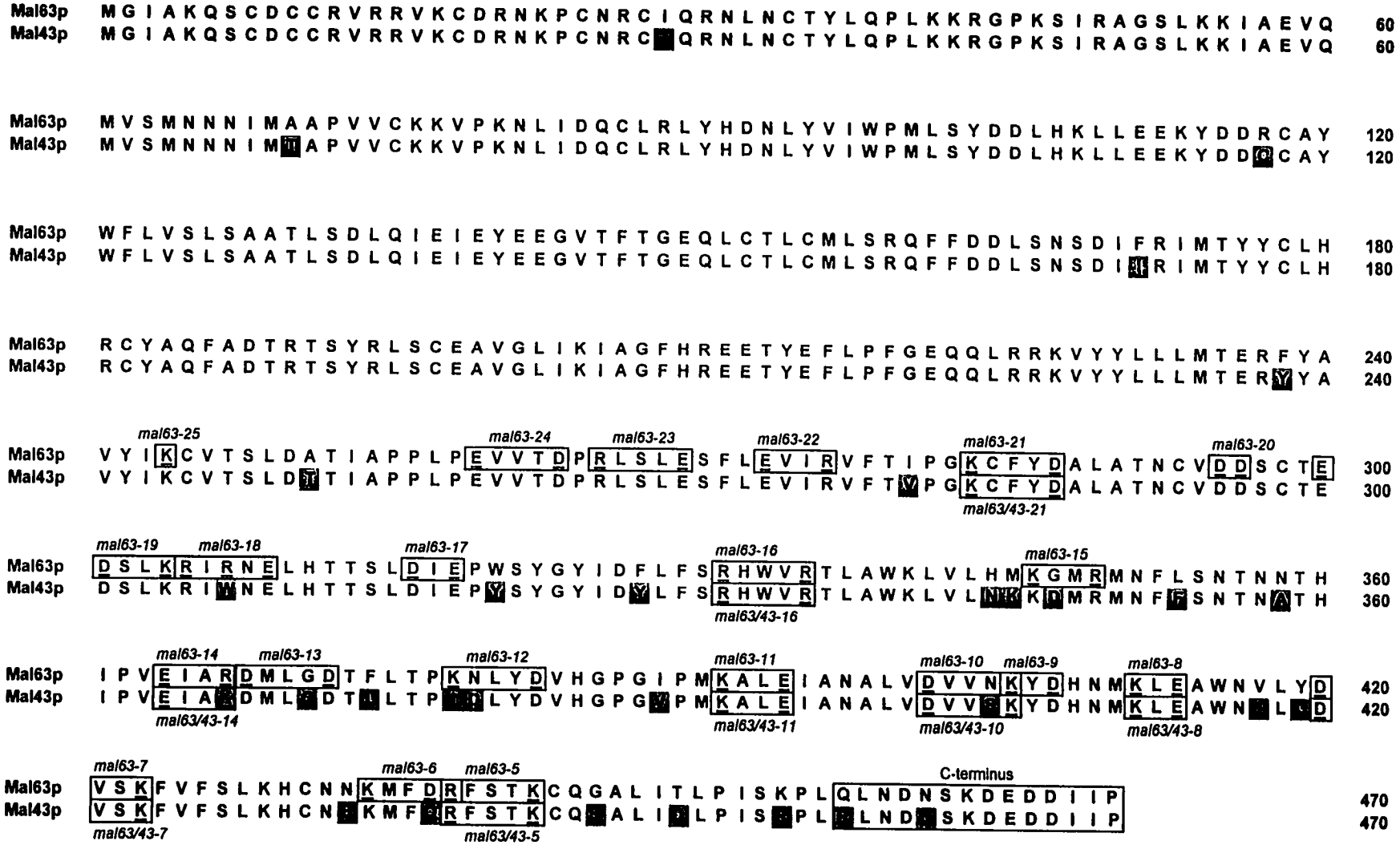


Figure 5B

	456														470
MAL63	L	Q	L	N	D	N	S	K	D	E	D	D	I	I	P
mal63-1	-	-	-	-	-	-	-	-	A	A	-	-	-	-	-
mal63-2	-	-	-	-	-	-	-	-	-	-	A	A	-	-	-
mal63-4	-	-	-	-	-	-	-	A	A	A	A	A	-	-	-
mal63-26	-	-	-	-	A	-	-	A	-	-	-	-	-	-	-
mal63-27	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-
mal63-39	-	-	-	-	-	-	-	-	-	-	-	A	-	N	-
mal63-64	-	-	-	-	-	-	-	-	-	-	-	S	-	V	-
mal63-317	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-
mal63-457NS	-	STOP													

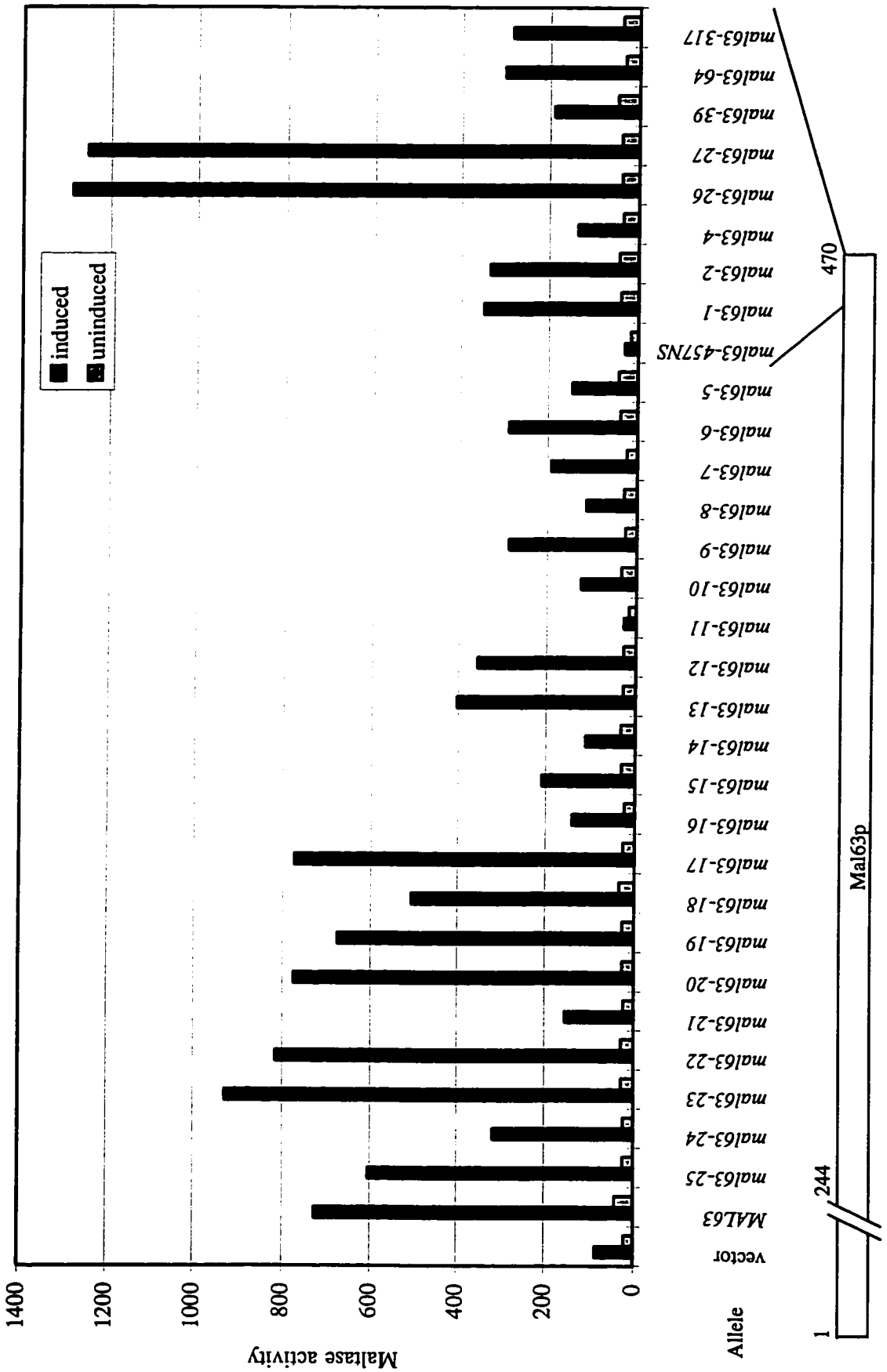
MAL63/43	L	R	L	N	D	D	S	K	D	E	D	D	I	I	P
mal63/43-39	-	-	-	-	-	-	-	-	-	-	-	A	-	N	-
mal63/43-64	-	-	-	-	-	-	-	-	-	-	-	S	-	V	-
mal63/43-457NS	-	STOP													

casein kinase II site near the extreme C-terminus of the protein, was changed to alanine. Two mutations, *mal63-39* (D467A,I469N) and *mal63-317* (D467A) were obtained by random mutagenesis of the last 17 residues of the protein as described in Materials and Methods. Finally, mutation *mal63-64* (D467S,I469V) changes the indicated residues to those found at the C-terminus of Mal64 protein, a noninducible Mal63p homologue encoded by a nonfunctional gene found upstream of *MAL63* in the *MAL6* locus (Dubin *et al.*, 1988).

These mutations were introduced into *MAL63* carried on the CEN vector pUN30, and were transformed into strain 61-106. Maltose fermentation ability, maltase expression, and $MAL61_{promoter}$ *-lacZ* reporter expression were determined for transformant strains grown in uninducing (3% glycerol, 2% lactate) and inducing (2% maltose, 3% glycerol, 2% lactate) growth conditions. Figure 6 presents the results for each mutant allele in a linear array based on the position of the alteration. Also included is a nonsense mutation at codon 457 constructed by L. A. Wojciechowicz (Wojciechowicz, 1993). It should be noted that the nine mutations at the 3' end of the gene are tightly clustered. The results of all three measures of *MAL*-activator activity are presented in Figure 6, and there was a clear correlation among these for all of the mutant alleles.

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 Figure 6, mutations from these different classes are alterations in a defined region of

Figure 6. Clustered charge to alanine scanning mutagenesis of the regulatory domain of Mal63p. 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 470. The last nine mutations are tightly clustered between residues 456 and 469. Maltase activity of transformants carrying each of the *mal63* alleles was assayed in cells grown in induced (black bars) and uninduced (gray bars) growth conditions.



the protein. Seven of the 30 mutations are inducible and transformants carrying these plasmid-borne mutations exhibit maltase levels in the normal range. The seven relatively silent mutations are located within the region encoding residues 244 and 318 suggesting that this region of Mal63p does not play a significant role in the induction process or transcription activation. Mutation *mal63-21* is the only alteration in this region which exhibits significantly reduced levels of maltase expression, and this will be addressed below.

Almost every mutation in the last 140 residues of the protein (331-469) adversely affects *MAL* gene expression. Of the 21 mutations in this region, 9 are noninducible (including *mal63-39, mal63-4, mal63-5, mal63-8, mal63-10, mal63-11, mal63-14, mal63-16)* while 10 others (*mal63-1, mal63-2, mal63-317, mal63-6, mal63-7, mal63-9, mal63-12, mal63-13, mal63-15, mal63-64*) are poorly inducible, slow fermenters with modestly to significantly decreased maltase activity. Finally, transformants carrying two mutations, *mal63-26* (D460A,K463A) and *mal63-27* (S462A), exhibit significantly increased, inducible maltase expression and ferment more rapidly than 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 inducing growth 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 levels comparable to those of the homozygous *MAL63* double (data not shown).

Overexpression of noninducible mutant alleles as LexA fusion proteins. All of the mutations described above were constructed using a hemagglutinin epitope tagged allele of *MAL63*. Despite this tag, we are unable to detect expression of even wild-type Mal63 protein from its native promoter by Western analysis. Therefore, to exclude the trivial explanation that the noninducible mutations result in unstable mutant proteins, the ten most severe mutations (which gave a nonfermenter phenotype, including *mal63-21*) were introduced into a *lexA-MAL63* fusion gene which fuses the DNA-binding domain of *lexA*, residues 1-87, to the full-length *MAL63*, all under the control of the *ADH1* promoter. The 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 (Gibson, 1995). Plasmids carrying the mutant fusion genes were introduced into strain YPH500 which lacks a functional *MAL*-activator gene, along with plasmid pSH18-18 containing a *lexA-lacZ* reporter, and fusion activator function was assayed by measuring maltose fermentation, maltase levels, transport activity and reporter gene expression. The results are shown in Table 5. Despite repeated attempts, for reasons that are unclear, I was unable to construct pLexA-mal63-16. Western analysis of total cell extracts of all of the transformants demonstrated comparable abundant expression of the fusion proteins (results not shown).

TABLE 5

Relative activity of *lexA-MAL63* fusion alleles

MAL-activator fusion allele	Maltose transport rate		Maltase activity	
lexA/MAL63	2.62	(100%)	1790	(100%)
lexA/mal63-4	0.81	(31%)	940	(52%)
lexA/mal63-5	0.83	(32%)	448	(25%)
lexA/mal63-8	1.32	(50%)	621	(35%)
lexA/mal63-10	1.20	(46%)	860	(48%)
lexA/mal63-11	0.05	(2%)	67	(4%)
lexA/mal63-14	1.02	(39%)	1091	(61%)
lexA/mal63-21	1.87	(71%)	1574	(88%)
lexA/mal63-39	0.78	(30%)	497	(28%)
lexA/mal63-64	1.02	(39%)	955	(53%)

The *Eco*RI fragment containing codons 216 to 470 from several *MAL*-activator mutations was cloned into plasmid pLexA(1-87)MAL63(2-216) creating a *lexA-mal63* fusion gene as described in Materials and Methods. These constructs were transformed into strain YPH500. Maltase activity and maltose transport rates were determined on at least two different transformants grown in induced (2% maltose, 3% glycerol, 2% lactate) growth conditions. Transport assays were done by Igor Medintz.

Transformants carrying wild type *lexA-MAL63* activate expression of the *lacZ* reporter as well as ferment maltose in one day. All of the transformant strains carrying the mutant fusion genes are able to ferment maltose to varying degrees, but despite the abundance of protein, *MAL* structural gene expression is still moderately to significantly reduced compared to that of the wild-type fusion gene. Thus, while abundant overproduction of the mutant protein does relieve the noninducible phenotype of these *mal63* mutations, it seems unlikely that the noninducible phenotype of these alterations can be due entirely to an increase in the instability of the mutant protein. Mutation *mal63-21* may be an exception. Abundant expression of the LexA-mal63-21 mutant protein appears to almost fully suppress the noninducible phenotype suggesting that this mutant protein is unstable. It is important to note that none of the transformants expressing the mutant LexA-mal63 fusion proteins is able to activate transcription from the *lexA-lacZ* reporter (data not shown).

Plasmids carrying the noninducible *MAL63* mutations were transformed into PMY270, a *doa4Δ* strain. *DOA4* encodes an enzyme which functions in ubiquitin recycling. Deletion of *DOA4* results in depletion of available ubiquitin, decreased ubiquitin-dependent proteolysis, and an accumulation of proteins which are normally degraded by the ubiquitin pathway. Preliminary results in this lab suggested that the ubiquitin pathway is involved in degradation of the *MAL*-activator (Z. Hu, unpublished results). The PMY270 transformants were tested for their ability to ferment maltose. Those mutations, which when overexpressed by fusion to *lexA* were able to allow maltose fermentation, also were able to support fermentation in PMY270, but again at

rates significantly slower than wild-type in PMY270 and even slower than the same mutations when expressed as *LexA* fusion genes in YPH500. These results are consistent with those reported above and, support the suggestion that protein instability is not the cause of the noninducible phenotypes, with the possible exception of *mal63-21*.

Clustered-charge to alanine scanning mutagenesis of selected residues in the constitutive *MAL63/43*. 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 transcription activation or the maintenance of the active state would have an effect on activity. To test this, the nine noninducible mutations discussed above and two poorly inducible mutations (*mal63-7* and *mal63-64*) were introduced into *MAL63/43*, encoding a constitutive activator which fuses residues 215-470 of the constitutive Mal43-C *MAL*-activator to residues 1-215 of Mal63p (see Figure 5A and 5B). The oligonucleotides used for these conversions are listed in Table 6. The mutation truncating the last 14 residues (*mal63/43-457NS*) was included.

Five of the eight mutations located in the region encoding residues 283 to 423 have little or no effect on the constitutivity of *MAL63/43-C* or on the level of maltase expressed in transformants grown in either induced or uninduced conditions (Figure 7). Mutation *mal63/43-16* (R331A,R335A) decreases the uninduced levels of expression to approximately half of wild-type *MAL63/43* but the mutation is still constitutive. These

TABLE 6

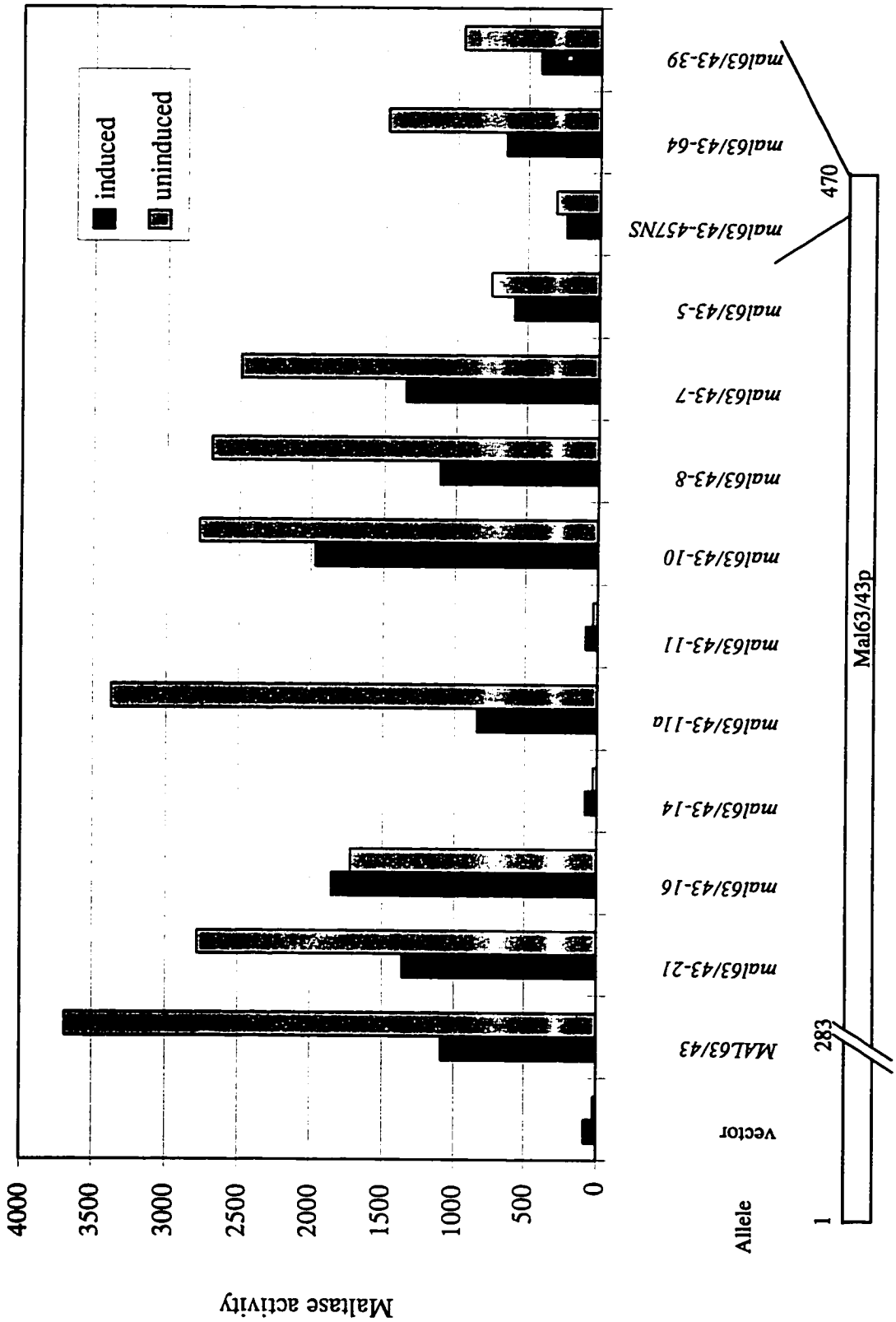
MAL63/43 in vitro mutagenesis

mal63/43 allele	Amino acid replacement	Oligo sequence (5'-3')	Annealing site*
mal63/43-5	R438A,K442A	ACTCTGACATGCAGTTGAAAACGCTTGAAACAT	1335-1303
mal63/43-7	D420A,K423A	GAAAACGAACGCAGATACAGCGCACAAAAT	1278-1249
mal63/43-10	D401A,K405A	GTGATCATACGCACTTACGACGGCTACCAATGC	1224-1192
mal63/43-11	K391A,E394A	ATTGGCTATTGCTAGCGCCGCCATCGGTAC	1191-1162
mal63/43-14	E364A,K367A	TAACATATCCGCGGCTATTGCGACTGGAAT	1110-1081
mal63/43-16	R331A,R335A	TGCTAGAGTCGCGACCCAGTGCGCAGAAAAGAG	1014-982
mal63/43-21	K283A,D287A	AGCCAAAGCAGCATAAAAACACGCTCCAGGTAC	870-838
mal63/43-457NS	Δ457-470	GGAATCATCATTTAGTTACAGTGGTCTAGAGAT	1386-1354
mal63.43-39	D467A,I467N	AAATTAAGGATTTATGGCGTCTTCATC	1416-1390
mal63/43-SVI	A392S,I395V,V402I	CTTACTTACGATGTCTACCAATGCATTGGCTACTTCTAGC GACTTCATCGG	1215-1165

* Open reading frame begins with base pair number 1.

Alleles *mal63/43-8* and *mal63/43-64* were made by *in vitro* mutagenesis with oligos *mal63-8* and *mal63-64* (see Table 4).

Figure 7. Clustered-charge to alanine scanning mutagenesis of the regulatory domain of Mal63/43-Cp. The 12 mutations within 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 470. The last three mutations are clustered between residues 456 and 469. Maltase activity of transformants carrying each *mal63/43-C* allele was assayed in cells grown in induced (black bars) and uninduced (gray bars) growth conditions.



results are consistent with the results reported above suggesting that the region which includes residues 331-423 is required for the process of induction. Two mutations, *mal63/43-14* (E364A,R367A) and *mal63/43-11* (K391A,I394A) are still noninducible and transformants carrying these alleles are nonfermenters and express very low levels of maltase. Interestingly, during the construction of the *mal63/43-11* mutation, a partial conversion containing only the E394A alteration, called *mal63/43-11a*, was obtained. Transformants carrying this single mutation exhibited little to no alteration of the constitutive activity of *MAL63/43*. This suggests that K391 is responsible for the noninducible phenotype of *mal63/43-11*. The three missense mutations in the C-terminal residues 438-470 and the nonsense mutation at codon 457 in *MAL63/43-C* all exhibit dramatically reduced levels of maltase expression, but it is important to note that the residual maltase expression is still constitutive.

Discussion

The C-terminal 220 residues of the *MAL*-activator protein function in regulation. This regulatory domain contains positive as well as negative functions as evidenced by previous work in this laboratory outlined in the Introduction of this thesis. Negative subdomains function to inhibit activation in the absence of inducer. Mutational disruption of negative subdomains should relieve the inhibition and bring about an increase in the basal (or uninduced) level of activation, albeit to varying degrees. Positive subdomains function in induction (response to inducer) or in transcription activation, including those residues which function in maintaining a transcriptionally competent state. Mutational disruption of positive subdomains should interfere with the activators ability to function under any growth conditions. In the work presented here, I have defined regions involved in negative regulation, called negative subdomains, and in positive regulation, called positive subdomains. The positive subdomains have been further characterized to identify those involved in the induction process or in transcription activation/maintenance.

***MAL*-activator constitutive mutations.** Wang and Needleman (1996) isolated constitutive mutations of *MAL63* in a host strain lacking *mal64* and all other functional copies of *MAL*-activator genes. Fifteen of 16 alleles exhibited an abnormal restriction endonuclease digestion pattern in the chromosomal region around *MAL63* and sequence analysis of two alleles demonstrated multiple alterations at the C-terminus of Mal63p. The constitutive activator encoded by *MAL63-D8* had the fewest alterations, eight residues (Figure 2). The other allele, *MAL63-C38*, shared these same eight C-terminal

alterations but contained an additional eight changes N-terminal to this region. All of the changes in Mal63-D8p are also found in Mal43-Cp and 15 of the 16 changes in Mal63-C38p are present in Mal43-Cp. It is unlikely that these constitutive mutations resulted from multiple independent events. Wang and Needleman (1996) proposed that gene conversion events between *MAL63* and specific genomic sequences gave rise to these mutations, and demonstrated that the strain used for the selection contained three potential donor sequences which exhibited homology to the 3' end of *MAL63* at low stringency.

Similarly, Gibson *et al.* (1997) found that two independent *MAL23* constitutive mutations shared the same 20 alterations. These two *MAL23* constitutive alleles were isolated by Zimmermann and Eaton (1974) as revertants of *mal2* noninducible mutant strains. Only five revertants were obtained, and the two analyzed by Gibson *et al.* (1997) both appear to have resulted from gene conversion-like events with the same donor sequence. A candidate donor sequence for these exchange events is open reading frame YBR296w which is linked to *MAL31* and *MAL32*, is highly sequence homologous to the *MAL*-activator (probably a *mal33* variant allele), and is identical to the sequence of the *MAL23-C* alleles in this region.

Finally, the *MAL43-C* allele isolated by Winge and Roberts (1950) was obtained by X-ray mutagenesis of a maltose nonfermenting strain. While an inducible *MAL43* allele is not available for comparison, it seems reasonable to assume that this allele also was the result of a gene conversion-like event, particularly given the mutagenic agent used.

Thus, based on analysis of these constitutive alleles, the rarity of the events, and their dependence on recombination with cryptic sequences, it would appear that single-residue alterations of the inducible *MAL*-activator alleles are not sufficient to produce a constitutive phenotype. Based on the results reported here and by Wang and Needleman (1996) and Gibson *et al.*, (1997), we conclude that protein-protein interactions are required for negative regulation of the *MAL*-activator in the absence of maltose and that single residue changes are not sufficient to disrupt these interactions.

Negative subdomains of Mal63p. Here we make use of these existing constitutive *MAL*-activator mutations and the natural variation of the *MAL*-activators encoded by *MAL63* and *MAL23* to define shorter negative regulatory subdomains in Mal63 protein. Our results support those of Wang and Needleman (1996) which propose that the C-terminal residues 419 to 461 contain a negative subdomain (NSD-3). Additionally, of the 54-residue region of clustered alterations in *MAL23-C*, we were able to define a 17-residue core (block 2) and found that alterations in this core sequence alone were sufficient to relieve negative regulation of Mal63p in the absence of maltose. It is important to note that similar experiments were undertaken by Gibson *et al.* (1997) in which multiple clustered changes found in the constitutive Mal43-C activator in this same block 1, 2, 3 region were introduced into Mal63p. None of these clustered alterations, which included up to ten changes, was sufficient to produce a constitutive allele.

The region between residues 251 and 307 modulates the level of activity of the *MAL*-activator and the two-residue differences between Mal63p and Mal23p in this

region (A251T and R307W) appear to be responsible for the high uninduced maltase expression in strains carrying the *MAL63/23* activator. Additional genetic evidence points to this region (residues 250-299) as having a negative regulatory function. A LexA-Mal63 fusion protein containing a deletion of residues 250-299 exhibited partially constitutive $lexA_{promoter}$-*lacZ* reporter expression (Gibson, 1995). However, this fusion was unable to activate *MAL* gene expression. These results suggest that residues 250-299 contain a negative regulatory function and also may play a role in sequence specific DNA binding.

Two negative subdomains, NSD-2 and NSD-3, are themselves modulated by alterations at closely linked sites. Alterations in NSD-3 are suppressed by changes in neighboring residues, specifically A392S, I395V, V402I. In addition, the constitutive phenotype of *MAL23(S392A,V395I,I402V)* is suppressed by alterations in residues within NSD-1 (T251A and W307R). NSD-2 is affected by neighboring residues as well. Alterations in this negative subdomain, sufficient to produce a constitutive phenotype in Mal63p, are suppressed when combined with blocks 1 and 3, restoring an inducible phenotype.

Taken together, these results offer compelling evidence that negative regulation of the *MAL*-activator in the absence of maltose involves the formation of complex intramolecular interactions as opposed to intermolecular interactions with other negative regulatory proteins. These negative intramolecular interactions are significantly weakened by sequence alterations in three negative subdomains, referred to as NSD-1 (251-307), NSD-2 (343-359), and NSD-3 (419-461), and NSD-2 and NSD-3 are both

essential for maintaining the inactive structure in the absence of maltose. Changes in NSD-1 alone were not tested in this study. Moreover, despite the important role of these negative subdomains, their effects are modulated by closely linked and even more distant sites in the regulatory region suggesting that other portions of the protein contribute significantly to the formation of the inactive conformation. Reports in the literature suggest that Mal63p forms a homodimer when overproduced in *Saccharomyces* (Sirenko *et al.*, 1995). The model of negative autoregulatory interactions proposed here for the *MAL*-activator does not exclude the possibility that these interactions occur between the subunits of the homodimer, and the finding that the *MAL63(2)* constitutive exhibits partial dominance is consistent with such intra-dimer interactions.

Similar intramolecular negative regulatory interactions have been reported for Leu3p, the transcription activator of the branched-chain amino acid biosynthetic pathway. Molecular genetic analysis of Leu3p suggests that the C-terminal activation domain is masked in the absence of inducer, α-isopropylmalate, and that masking is accomplished by an intramolecular interaction between the middle region of the protein and the short C-terminal activation domain. Intragenic suppressors of a noninducible Leu3p with mutations in the activation domain map to the middle region of the protein. One second site suppressor mutation was constitutive when separated from the original noninducible mutations. In addition, an interaction between the C-terminal activation domain and the middle region was demonstrated using a modified two-hybrid system (Wang *et al.*, 1997). Mutations at many sites in Leu3p had a significant effect on

modulating activation suggesting that the effect was due to impaired masking rather than better induction. Additional experiments are underway that are designed to look at these intramolecular interactions in the *MAL*-activator.

Fine structure functional analysis of the Mal63 *MAL*-activator regulatory domain. Genetic analysis of *MAL63* clearly indicates that the C-terminal negative regulatory domain plays a required role in inducible activation. The noninducible *mal63-13* allele isolated by ten Berge *et al.* (1973) contains seven altered residues including a short cluster of four changes from 355 to 358 (Gibson *et al.*, 1997). A hybrid *MAL63/mal64* gene fusing codons 1-215 of *MAL63* to codons 215-470 of *mal64*, a nonfunctional homologue exhibiting 85% identity to *MAL63*, is noninducible while the reverse construction, *mal64/MAL63*, is maltose inducible (Gibson *et al.*, 1997). This result indicates that the 33 variant residues in the C-terminal regulatory domain interfere with induction. A nonsense mutation at codon 457 of *MAL63* is noninducible despite abundant expression (Wojciechowicz, 1994). Finally, a series of 50 codon deletions of *MAL63* through the regulatory domain was noninducible even when abundantly expressed as *lexA-MAL63* fusions (Gibson, 1995). These results are to be contrasted with those reported by Gibson *et al.* (1997) which describe constitutive alleles of *mal64*, *MAL63*, and a *LexA-MAL63* fusion gene which are nonsense mutations at codons 283 and 307, 284, and 284 respectively (Gibson, 1995). In summary, it appears that not only does the C-terminal regulatory domain contain negative regulatory elements which inhibit activator function in the absence of inducer, but it also contains other sequences that function to relieve this inhibition in the presence of inducer.

These elements could play a role in sensing and/or responding to the presence of inducer. Additionally, residues in the regulatory domain could be required for maintaining the induced and transcriptionally active state. Finally, we considered the possibility that the *MAL*-activator, like several other yeast transcription activators might have additional activation domains located in the C-terminal region (Jackson *et al.*, 1996; Johnston, 1987; Thukral *et al.*, 1989). The clustered-charge to alanine scanning mutagenesis of *MAL63* was undertaken to distinguish between the positive functions of the C-terminal regulatory domain without altering negative regulation.

Residues in the region of 244 to 318 do not appear to be required for a positive function. *mal63-21* is the only mutation in this region which exhibits significantly decreased maltose-inducible maltase activity. Overexpression of the LexA-mal63-21 fusion protein almost fully suppresses the maltose nonfermenting phenotype suggesting that the noninducible phenotype of this *mal63* mutation 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 on *MAL* gene expression, *lexA-lacZ* reporter expression remains uninducible. Because this alteration lies in a region which we suggest contains a negative subdomain, NSD-1, we suggest the possibility that residues 283 and 287 which are altered in *mal63-21* could strengthen this inhibitory function.

With two exceptions, *mal63-26* and *mal63-27*, the mutations altering residues 331 to 470 significantly decrease the transcriptional activity of Mal63p. In order to distinguish between the positive functions of induction, maintenance, and activation,

these mutations were introduced into the constitutive *MAL63/43*. We reasoned that mutations which affect the activators ability to undergo the induction process would have a reduced impact in a constitutive activator. Conversely, those mutations which cause reduced transcription activation in both an inducible and a constitutive activator might specify residues involved in maintenance or activation. The constitutive fusion activator *MAL63/43* was used for this analysis.

Of the six mutations altering residues 331 to 423, four have little or no affect on the constitutive phenotype of *MAL63/43* or on its transcription activation activity. Thus, we suggest that this region of Mal63p is required for sensing inducer levels and/or actively participates in carrying out the process of induction. It is not yet clear how the presence of maltose is sensed by *Saccharomyces*, but two alternate possibilities can be proposed. Maltose could bind directly to the *MAL*-activator or maltose could activate a signal transduction pathway with the *MAL*-activator as the downstream target. The results presented here suggest that residues 331-423 are either the binding site of maltose or the target site of the signal transduction pathway. It should be noted that this region overlaps NSD-2 and NSD-3 (Figure 8).

Two mutations in this induction domain, *mal63-11* (K391A,E394A) and *mal63-14* (E364A,R367A), cause a noninducible phenotype in *MAL63* and *MAL63/43* indicating these alterations could inhibit induction or are essential for activation. Mutation *mal63/43-11a* which includes the alteration of E394A, but not the mutation converting K391A, exhibits essentially normal activation activity, suggesting that the K391A alteration of *mal63/43-11* alone is responsible for the nonfunctional phenotype.

Figure 8. Location of positive and negative subdomains within the regulatory domain. A. Negative subdomains are located at 3 sites, NSD-1 within residues 251-307, NSD-2 within residues 343-359, and NSD-3 within residues 419-461. Residues within 359 and 419 also function to modulate the basal level of activity. B. The positive function of induction involves residues 331 to 423. C. Transcription activation and/or maintenance of the active state involves residues 438-470, and specific residues within 364-394.

Our studies of the negative subdomains described above suggest that induction involves a change in conformation of the regulatory domain, and residues in this region could be required for this restructuring. Transformants carrying *mal63-11* exhibit dramatically reduced maltase expression in both induced and uninduced conditions, even lower levels than in the vector control transformants. It would appear that this mutant Mal63p could repress rather than activate transcription. It is of note that this mutation, which introduces two alanine residues, lies in a region that is already rich in alanine. With this mutation, the region now includes five alanine residues in a span of eight amino acids and may represent an alanine-rich repression motif (Hanna-Rose and Hansen, 1996).

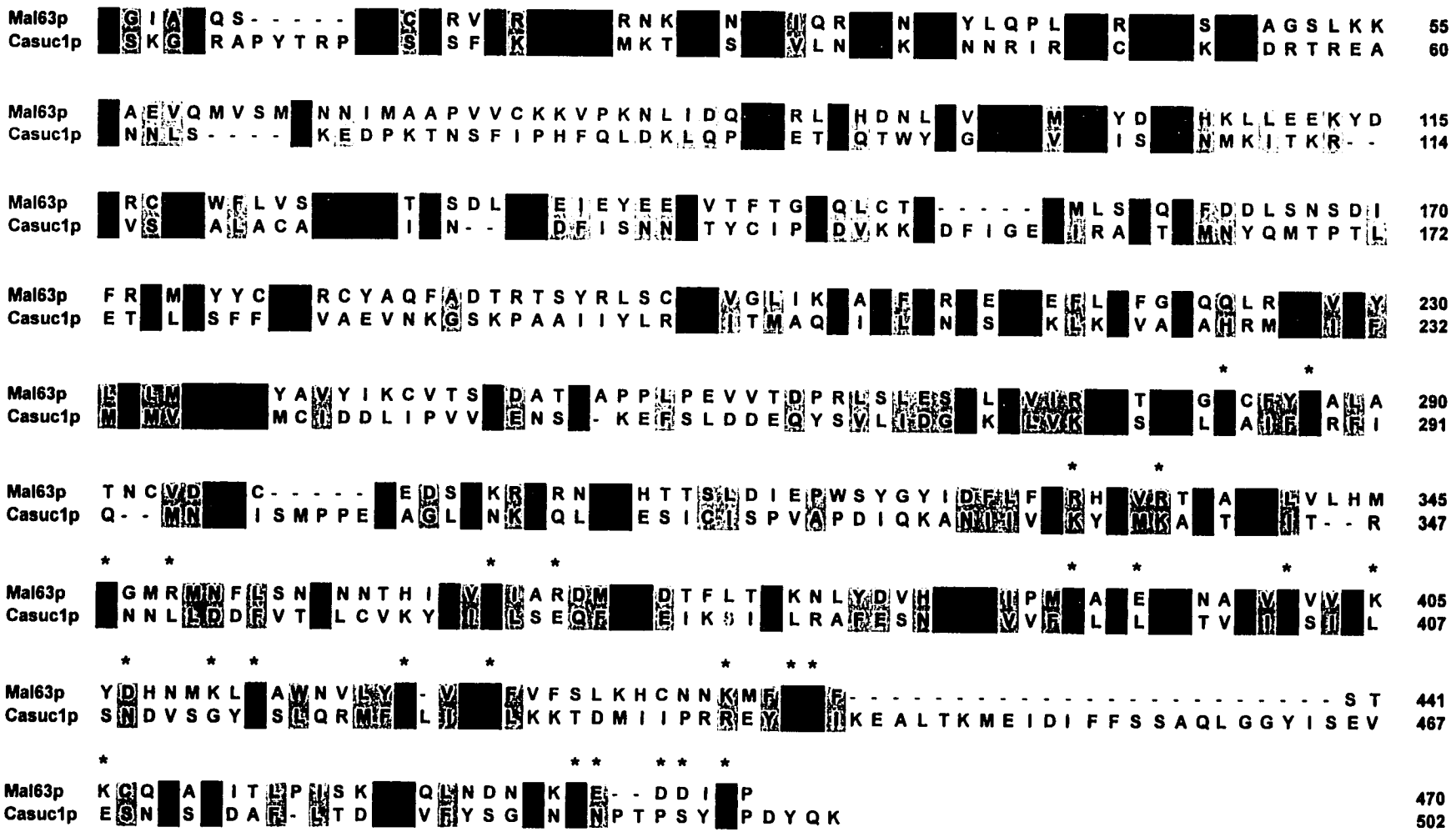
Most of the mutations in residues 438-470 significantly decrease the activity of both *MAL63* and *MAL63/43* activators indicating that this C-terminal region is involved in maintenance of the transcriptionally active state or activation. Consistent with this is the finding that transformants carrying these mutations in the constitutive *MAL63/43* exhibit reduced but constitutive maltase expression. Moreover, two mutations, *mal63-26* (D460A,K463A) and *mal63-27* (S462A) enhance the maltose-inducible transcription activation by the mutant Mal63p activator. These changes could stabilize the active state of Mal63p or allow it to interact more productively with the transcription machinery.

Residues 1-283 of the *MAL*-activator are capable of constitutive activation of *MAL* genes and thus contain at least one activation domain. Deletion analysis of the *lexA-MAL63* fusion gene suggests that the region from 100-283 is essential, but residues

100-283 fused to the LexA DNA-binding domain are not sufficient to activate transcription despite the fact that the LexA-Mal63(100-283) activator is able to repress expression from another, so-called repression, reporter *in vivo*. Thus far we have been unable to demonstrate that this region contains an activation domain and deletion analysis of this region is ongoing. Several fungal transcription factors have been shown to have acidic activation domains within their most C-terminal residues, including Gal4p, Leu3p, Pdr3p, and Hap1p (Schjerling and Holmberg, 1996). The C-terminal residues of the *MAL*-activators encoded by *MAL63*, *MAL23* and *MAL43-C* are highly acidic and similar to Gal4p. The results reported here suggest the possibility that, like several other fungal activators, the primary activation domain in the full length *MAL*-activator is located in the C-terminus of the protein. As in Leu3p, this C-terminal acidic domain is approximately 30 residues long, and overlaps with a negative regulatory subdomain, NSD-3, of Mal63p. The possibility that residues 438-470 of Mal63p contain an activation domain are being tested using a LexA-MAL63(438-470) construct. Figure 8 summarizes the analysis of negative and positive subdomains in Mal63p.

Homology to Casuc1p. The *Candida albicans SUC1* gene (*CASUC1*), encoding the activator of the sucrose fermentation genes, complements *Saccharomyces cerevisiae MAL*-activator mutations and is maltose inducible (Kelly and Kwon-Chung, 1992). *CASUC1* encodes a zinc cluster DNA-binding protein with 28% sequence identity and 50% similarity to *MAL63* at the amino acid level (see Figure 9). These proteins share sequence homology primarily in three regions: 1) the zinc cluster DNA-

Figure 9. Protein sequence alignment of Mal63p and Casuc1p. The complete amino acid sequence of the Mal63 *MAL*-activator is given and compared to that of Casuc1p of *Candida albicans*. Dark grey areas indicate identical residues, light grey areas indicate similar residues. Asterisks are placed above residues which, when mutated in Mal63p, significantly decrease transcription activation activity (that is, transformants which carried these *mal63* mutations fermented in 4 or more days).



binding domain (residues 8-49; 54% identical, 62% similar), 2) the region adjacent to the DNA-binding domain, which may play a role in sequence-specific DNA binding or activation (residues 95-154; 35% identical, 55% similar), and 3) the middle homology region of Mal63p which appears to contain a transcription activation domain (residues 198-238; 44% identical, 71% similar) (Kelly and Kwon-Chung, 1992). Additional regions within the C-terminal half of the protein share homology as well, but overall the regions of homology are on average shorter and more dispersed, a surprising finding considering that this activator responds to maltose induction in *Saccharomyces*. The regions of greatest homology to Mal63p include residues 268-287 (40% identical, 80% similar), a region defined as a negative subdomain; and residues 381-403 (42% identical, 71% similar) which is included in the induction domain. Mutations in Mal63p which significantly decrease transcription activation are noted in Figure 9 with an asterisk. Most strikingly, the C-terminal acidic region of Mal63p is not conserved in Casuc1p despite the fact that this is an essential domain of Mal63p. It is difficult to make a correlation between conserved sequences in the C-terminal region of these proteins and the location of the noninducible *mal63* mutations. The extent of the divergence between these two proteins perhaps argues against the direct binding of maltose to the activator.

Model of maltose induction of the *MAL*-activator. Generally, transcription activators fall into two classes, those that bind to DNA constitutively, that is, in both the transcriptionally active and inactive states, and those that bind DNA only in the

active state. For the first type, which includes Gal4p, Put3p and Leu3p, induction involves the unmasking of the transcription activation domain probably by changes in protein conformation. For the second type, induction occurs prior to binding DNA and only the induced activator is capable of DNA binding. Our results suggest that the *MAL*-activator is of the second type.

Our first piece of evidence comes from the *lexA-MAL63* fusion constructs originally tested to explore the role of protein instability. The *lexA-mal63* mutant hybrid genes produce abundant levels of mutant fusion proteins which are able to turn on expression of the structural genes, although not to the same extent as wild-type *lexA-MAL63*, but all are seemingly unable to turn on the *lexA-lacZ* reporter gene. The ability to activate *MAL* gene expression clearly demonstrates that these mutant fusion activators are present in the nucleus, therefore nuclear exclusion is not a factor. Instead we suggest that binding to the *lexA* operator is constitutive. LexA-Mal63p binds DNA in a repression assay under uninduced growth conditions, (Zhen Hu, unpublished results), while binding to the UAS_{MAL} requires the conformational change associated with induction. Thus, out of the abundant pool of fusion protein, only those few which have undergone induction and achieved the active conformation are able to bind to the UAS_{MAL} and activate expression. The bulk of the fusion proteins has not undergone induction, because of the mutation, and are not capable of activating transcription but none the less are bound to the *lexA* operator. Binding of inactive protein blocks the transcription of the reporter gene. Thus, the selection process that occurs at the UAS_{MAL} site which allows only those activator proteins which are in the active

conformation to bind allows for the expression of these genes. On the other hand, the *lexA* promoter which indiscriminately binds inactive and active fusion proteins is not expressed because, in the absence of a selection for the active state, the small proportion of the proteins in the active state in combination with the limited number of available promoter binding sites is responsible for the lack of reporter expression.

Additional evidence that the *MAL*-activator binds only after induction comes from our finding that the *mal63* mutations are fully recessive to wild-type. The Mal63p activator appears to form a dimer even under uninduced conditions, based on the purification work of Needleman and coworkers (Sirenko *et al.*, 1995) and on the partial dominance of the constitutive *MAL63(2)* mutation. Given this, if DNA binding of the *MAL*-activator were constitutive, we would expect that the noninducible *mal63* mutations would exhibit some dominance. This was not seen in this study nor in previous studies in which noninducible *mal63* mutant proteins were overproduced (Gibson, 1995; Wojciechowicz, 1993).

Thus, we suggest the following model of induction diagrammed in Figure 10. The *MAL*-activator is negatively autoregulated by intramolecular or intra-homodimer interactions involving the negative subdomains in the C-terminal regulatory domain. In the absence of maltose, these interactions block DNA-binding to the UAS_{MAL}. The uninduced conformation involves an interaction between NSD-2 and NSD-3. If both of these negative subdomains are disrupted, as in Mal23-Cp, the suppressing residues at 362-378 and at 392-402 do not suppress the constitutive phenotype. However, if only NSD-

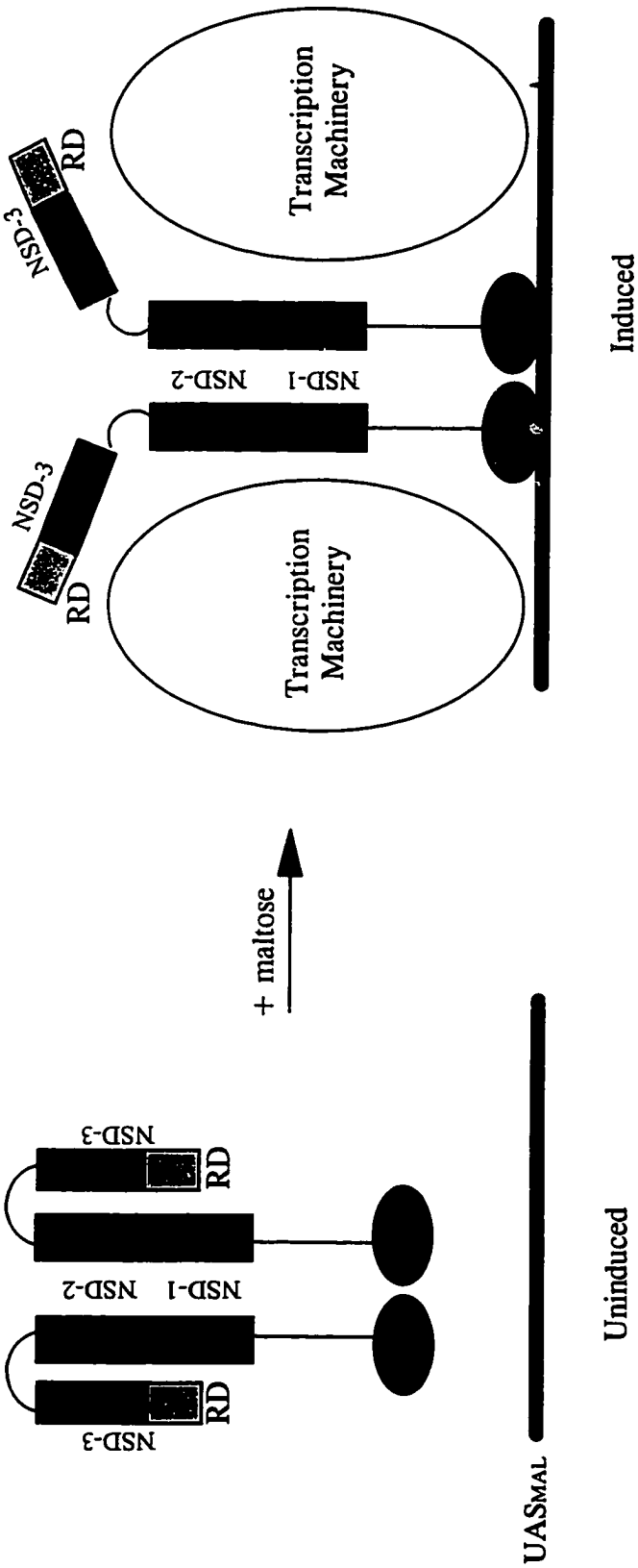


Figure 10. Model of Induction. The *MAL*-activator dimerizes in the absence of maltose, (uninduced growth conditions), but is not competent for binding to the UAS$_{MAL}$ or activating transcription. In the presence of maltose, (induced growth conditions), a conformational change causes NSD-2 and NSD-3 to dissociate, allows the *MAL*-activator to bind to the UAS$_{MAL}$, and makes the activation domain accessible to the transcription machinery. RD = Required Domain for activation/maintenance

2 is disrupted, and NSD-3 remains intact, as in Mal63(1,2,3)p, suppressors at residues 362-378 restore inducibility, possibly by tightening binding. Additionally, if only NSD-3 is disrupted, and NSD-2 remains intact, as in the inducible Mal23p, suppressors at residues 392-402 also restore inducibility, probably in the same way. Maltose stimulates a change in the tertiary conformation of the activator, relieving the interaction between NSD-2 and NSD-3, making it competent for binding the UAS_{MAL} and capable of interacting with the transcription machinery. Thus, the maltose-induced change in conformation regulates both functions. Interaction with the transcription machinery does not require binding to the UAS_{MAL} since the full-length LexA-Mal63 fusion activator is normally maltose regulated although bound to the *lexA* operator sites in the the promoter of the reporter gene.

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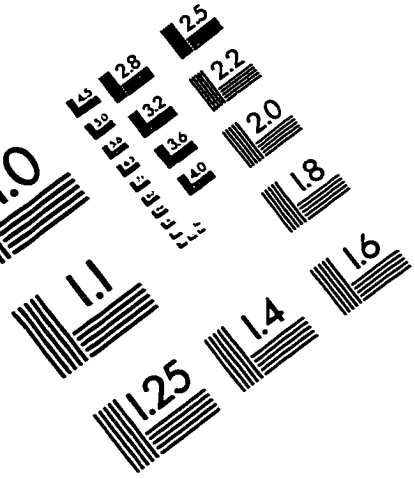
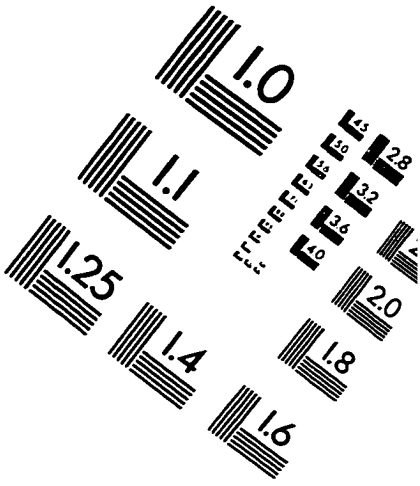
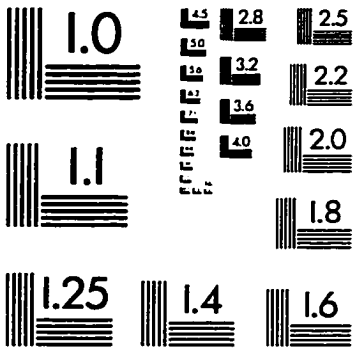
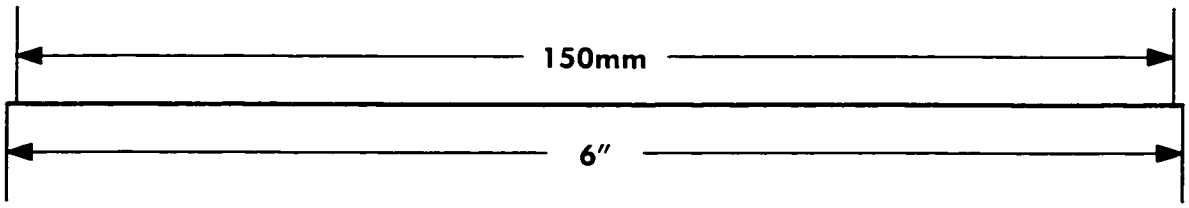
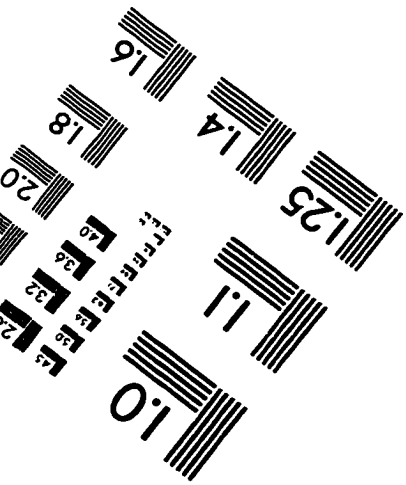
IMAGE EVALUATION TEST TARGET (QA-3)

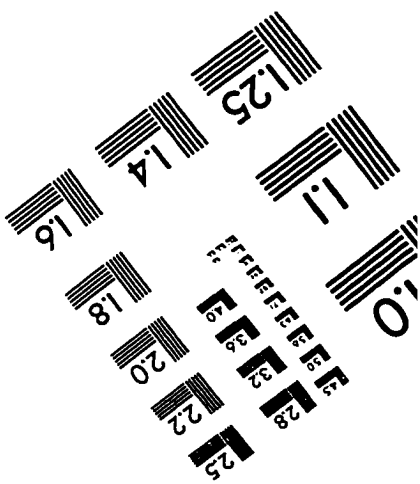












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