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Structural and functional comparison of the *Saccharomyces cerevisiae* maltose-inducible transcription activator encoded by *MAL63* and its nonfunctional homologue encoded by *MAL64*

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STRUCTURAL AND FUNCTIONAL COMPARISON OF THE *SACCHAROMYCES CEREVISIAE* MALTOSE-INDUCIBLE TRANSCRIPTION ACTIVATOR ENCODED BY *MAL63* AND ITS NONFUNCTIONAL HOMOLOGUE ENCODED BY *MAL64*

by

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ABSTRACT

STRUCTURAL AND FUNCTIONAL COMPARISON OF THE *SACCHAROMYCES CEREVISIAE* MALTOSE-INDUCIBLE TRANSCRIPTION ACTIVATOR ENCODED BY *MAL63* AND ITS NONFUNCTIONAL HOMOLOGUE ENCODED BY *MAL64*

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Lori Wojciechowicz

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Maltose fermentation in *Saccharomyces* yeasts requires one of five, unlinked complex loci (*MAL1-4* and *MAL6*) each containing the three genes required for fermentation. At the *MAL6* locus, *MAL63* encodes a DNA-binding protein which promotes the maltose-inducible transcription of the two structural genes, *MAL61* (encoding maltose permease) and *MAL62* (encoding maltase). Yeast strains carrying noninducible *mal63* mutations can revert to maltose fermenters but structural gene expression in these revertant strains is constitutive. Two such constitutive mutations lie in a gene upstream of the *MAL63* gene, referred to as *MAL64*, and the two constitutive alleles are called *MAL64-C2* and *MAL64-R10*. Wild-type *MAL64* is not essential for maltose fermentation.

MAL63 and *MAL64* are highly homologous. Both genes encode proteins which are 470 amino acids long and 85% identical. Analysis of *MAL64-C2* and *MAL64-R10* revealed nonsense mutations at codons 307 and 282,

respectively. Experiments here confirmed that the nonsense mutations in these constitutive alleles were responsible for activity.

Chimeric gene fusions between *MAL63* and *MAL64* fragments showed that residues 215-470 of *MAL63* encoded protein (MAL63p) are responsible for responding to the inducer, maltose. Also, deletion of a short acidic stretch of 14 amino acids at the C-terminal end of MAL63p results in an uninducible activator. Therefore, unlike the constitutive MAL64p, MAL63p requires residues in its C-terminal domain for activity.

A hemagglutinin epitope tag was placed at the 5'-end of *MAL63*, overexpressed off the *GAL10* promoter and this fusion protein was detected in cells. Overexpression in galactose of either the wild-type tagged protein or a mutant tagged protein lacking the final 14 C-terminal acidic residues did not result in a constitutive phenotype. This experiment excludes the possibility of the involvement of a limiting repressor protein since overexpression of MAL63p would titrate accessory factors and thus activate structural gene expression.

Based on homology to other yeast activators, the acidic final C-terminal residues of MAL63p could be involved in transcription activation. By use of *lexA-MAL63* gene fusions, these residues were required for the maltose-inducible transcription activation function of *MAL63*.

DEDICATION

This thesis is dedicated to my late grandfather, Michael Monteleone who would have loved to see me finally receive my degree after all these years of studying and research. I remember him most for all his encouragement throughout my college career but, most of all, I miss his unconditional sense of humor.

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INTRODUCTION

Eucaryotic transcriptional regulation

Transcription initiation is probably the most common control point involved in the regulation of gene expression (Struhl, 1989b). Positive and negative regulatory factors which bind to specific promoter DNA sequences can induce or repress gene transcription by interacting with RNA polymerase and/or other general transcription factors. Studies have indicated that there may be common, conserved molecular mechanisms of transcriptional regulation in eucaryotic organisms from humans to yeast. For instance, there is a significant conservation of the subunit structure of RNA polymerase II and its catalytic properties along with similar post-transcriptional modifications of the primary transcripts. Near the position of the mRNA initiation site is a sequence called the TATA box (TATAAA) which is found in most eucaryotic promoters. Most yeast and higher eucaryotic promoters contain enhancer or UAS (*u*pstream *a*ctivating *s*equence) elements which activate transcription even when located far from the mRNA start site. More importantly, yeast and higher eucaryotic cells contain structurally similar and functionally analogous transcription factors that recognize almost identical sequences (Struhl, 1987;

Bohmann et al., 1987; Angel et al., 1988; Harshman et al., 1988; Jones et al., 1988; Chodosh et al., 1988; Buratowski et al., 1988; Cavallini et al., 1988). Because of this similarity, yeast proteins can activate transcription in many higher eucaryotic organisms (Kakidani et al., 1988; Webster et al., 1988b; Fischer et al., 1988; Ma et al., 1988) and vertebrate proteins (Lech et al., 1988; Struhl, 1988; Metzger et al., 1988; Schena et al., 1988) can stimulate transcription in yeast cells. These functional similarities between yeast and higher eucaryotes (humans), enables one to study molecular mechanisms *in vivo* and *in vitro* using components from both yeast and humans (Struhl, 1989b).

Much of the knowledge of the molecular mechanisms involved in transcriptional regulation has come from studying *Saccharomyces cerevisiae*, baker's yeast. The yeast genome contains about 5000 protein-coding genes that are contained on 16 chromosomes (Struhl, 1989b). The average yeast gene is transcribed 5-10 times during each cell cycle resulting in a steady state level of 1-2 mRNA transcripts per cell but some yeast genes are transcribed at different rates depending on growth conditions and many times, groups of genes can be coordinately expressed. Yeast promoters contain three basic kinds of DNA sequence elements: upstream activation sequences (UASs), TATA box sequences, initiation elements and less commonly, operator elements.

UASs are short DNA sequences 10-30 basepairs long located far (~ 100-1500 basepairs) upstream of the mRNA initiation site (Struhl, 1989b). For yeast genes which are transcriptionally regulated according to physiological conditions, the UAS has a large influence in the regulatory properties of the promoter. Different genes can be coordinately controlled by the presence of similar UAS sequences. Yeast UASs are similar to mammalian enhancer sequences since they can function in both orientations and at long, variable distances with respect to other promoter elements and mRNA initiation sites. However, UAS elements and enhancers differ in that UAS elements do not activate transcription when they are placed downstream of the mRNA start site where enhancers can function in this position (Guarente et al., 1984a; Struhl, 1984).

TATA elements are also necessary for transcriptional initiation of most yeast genes since deleting these elements reduces the mRNA transcript level (Struhl, 1982; Guarente, 1983; Siliciano et al., 1984). TATA elements contain the sequence, TATAAA or a very related AT-rich sequence which can be located 40-120 bp upstream of the mRNA start site (Struhl, 1989b). This TATAAA sequence is conserved in many different kinds of promoters and is suggested to play a general role in transcription. Recent studies have suggested that functionally distinct classes of TATA elements could exist (Struhl, 1989b).

Initiator elements, located near the actual mRNA start site, determine where transcription begins (Struhl, 1989b). In yeast, accurate initiation is still observed even when the distance to the TATA element or UAS is varied (Nagawa et al., 1985; Chen et al., 1985; McNeil et al., 1986). However, for higher eucaryotic mRNA, start sites are determined not by specific DNA sequences but by the distance (25-30 bp) from the TATA element (Struhl, 1989b). Spacing between yeast initiation sites and TATA elements is flexible and larger, but there are limits to the distance over which a TATA element can act (~40-120 bp). Many different sequence variations in these initiator elements can carry out the same function but these elements are unimportant for determining the rate of transcription initiation (Struhl, 1989b).

A fourth type of element, called the operator, can repress transcription initiation (Brent, 1985). Yeast operators are short sequences which function bidirectionally and at variable distances upstream of TATA elements (Struhl, 1989b). Operators can repress transcription when located upstream of UAS elements, but repress more efficiently when located between the UAS and the TATA element (Siliciano et al., 1984; Johnson et al., 1985; Struhl, 1985).

Current thoughts regarding the mechanism of transcription activation suggest that the above types of DNA promoter elements are present in order to attract specific

DNA-binding proteins to interact with these sequences and with RNA polymerase (Struhl, 1989b). This binding of a factor to a DNA sequence is necessary in order for activation or repression of transcription to occur. Yeast contains a wide variety of specific transcription factors which recognize different upstream promoter elements. These factors were first identified by mutations that altered the transcription of a gene or set of genes (Struhl, 1989b). In many cases, these mutations revealed genes that encode proteins exhibiting DNA-binding activity. These mutant strains provided information about how a particular DNA-binding protein functions *in vivo* and made cloning of the genes easier.

Cloning of genes encoding yeast DNA-binding proteins has enabled the study of the structures and functions of these proteins. For a protein to be a transcriptional activator, it is likely to contain a DNA-binding domain, a short nuclear localization sequence (NLS), a transcriptional activation domain and possibly a regulatory domain activated or repressed by changes in the physiological or growth conditions of the cell. The regulatory domain may mediate the response to these changes by interaction with other regulatory proteins (Perlman and Hopper, 1979; Johnston and Hopper, 1982), by undergoing post-translational modification (Ninfa et al., 1986) or by interaction with small effector molecules (Majors, 1975). Some factors activate

transcription but do not possess DNA-binding activity, and it is suggested that these factors interact with another protein which provides this DNA-binding function. Many times, deletion analysis of a gene shows that a smaller region (<100 amino acids) is required for DNA-binding activity (Struhl, 1989b). Different DNA-binding structural motifs are present in these proteins such as the helix-turn-helix (Pabo et al., 1984), the cysteine-rich zinc-binding motifs (Miller et al., 1985; Struhl, 1989a), the leucine zipper (Landschulz et al., 1988) and others.

Transcriptional activation domains can commonly contain stretches of acidic residues (aspartate or glutamate) which are highly negatively charged and required for activation (Hope and Struhl, 1986; Ptashne, 1988) and may contain a short repeat structure such as an amphipathic α-helix (Giniger and Ptashne, 1987). More recent work on the activation domains present in two yeast activators, *GAL4* and *GCN4* has led to a reassessment of the importance of acidic residues and formation of α-helical structures within these domains (Van Hoy et al., 1993). Short synthetic peptides representing these acidic domains actually adopt a structure, determined by circular dichroism spectroscopy, which is not α-helical but almost 100% β sheet at pH 6. More importantly, by performing site-directed mutagenesis on residues in the activation domain of *GAL4* (residues 854-874), it was shown that deletion of acidic residues 870-874

(DDEDT), still allowed for transcription activation and replacement of residues 868-881 with a positively charged domain had actually increased activity of GAL4p (Leuther et al., 1993). Therefore, there may not be a stringent relationship between negatively charged residues and the ability to activate transcription. In addition to these common activation domains, others also exist which contain glutamine-rich or proline-rich regions (Courey and Tjian, 1988; Dreiver et al., 1989; Mermod, 1989).

Studies of transcriptional activators which are DNA-binding proteins has revealed a number of unique factors: *GAL4, HAP1, PUT3, LEU3, ADR1, PHO4* and the human glucocorticoid receptor. Most recently, adaptor molecules or coactivators have been described which are needed for the proper functioning of certain acidic activation domains found in yeast transcription factors (Berger et al., 1992). In the following sections, I would like to summarize the relevant features of these different regulated gene systems and transcription activators so that they might serve as model systems for my analysis of the *MAL*-activator proteins.

GAL4. *GAL4* encodes the transcription activator required for the utilization of galactose in *Saccharomyces*. The structural genes required for galactose fermentation are: *GAL1* (kinase), *GAL7* (transferase), *GAL10* (epimerase), *GAL5*(mutase), *GAL2* (permease), *GAL3* (inducer), and *MEL1*

(melibiose). Expression of these genes except for *GAL5* is induced by galactose and repressed by glucose and the GAL4 protein (GAL4p) which binds upstream of all of these genes regulates their expression (Johnston, 1987). *GAL80* encodes a negative regulator (GAL80p) which, under noninducing growth in glycerol, binds to DNA-bound GAL4p preventing it from activating transcription (Johnston, 1987; Johnston, S.A. et al., 1987). The true inducer in this system is believed to be an unidentified molecule whose synthesis is catalyzed by the product of the *GAL3* gene and which is suggested to prevent GAL80p from binding to GAL4p thereby releasing GAL4p to promote transcription by interacting with the general transcriptional machinery (Johnston, 1987). *GAL3* has been shown to be important for induction since *gal3* mutants induce at very slow rates (3-5 days) as opposed to 20 minutes which a wild-type strain is able to do. When glucose is present, *GAL* gene expression is repressed on three levels: transcription initiation of the structural genes, transcription initiation of the *GAL4* gene, and reduction of inducer levels as a result of low rates of galactose transport (Griggs and Johnston, 1991).

GAL4p is an 881 amino acid residue protein which binds to the UAS_{GAL} in the upstream regions of the genes it regulates (Johnston, 1987; Johnston and Davis, 1984; Yocum et al., 1984; West et al., 1984). The UAS_{GAL} sequence which is guanidine and cytidine rich, was first elucidated by

localizing the region of DNA between *GAL1* and *GAL10* which is able to confer GAL4p-dependent expression of a heterologous gene, either *CYC1* or *HIS3* (Guarente et al., 1982). The UAS_{GAL} consists of four, closely linked 17 basepair sequences exhibiting partial dyad symmetry suggesting that GAL4p binds as a dimer or tetramer to these sites (Johnston, 1987). GAL4p produced in *Saccharomyces cerevisiae* or *E. coli* was shown to bind *in vitro* and *in vivo* to DNA upstream of the *GAL* genes and has been shown to bind as a dimer (Johnston, 1987; Carey et al., 1989).

The functional domains of GAL4p involving DNA-binding, transcriptional activation, and interaction with GAL80p have been defined. DNA-binding activity resides in the N-terminal 74 amino acids and consists of a cysteine-rich zinc-binding domain (Johnston, 1987; Pan and Coleman 1989, 1990). This domain is highly homologous to the DNA-binding domain present in *MAL63*, a transcriptional activator involved in maltose fermentation in *Saccharomyces* (Kim and Michels, 1988).

There are two negatively charged, acidic regions in GAL4p capable of activating transcription, in amino acid residues 148-196 and 768-881 (Ma et al., 1987a). Residues 768-881 can even activate transcription when only fused to the DNA-binding domain of GAL4p (residues 1-147) or when fused to the DNA-binding domain of the bacterial repressor, LexA (Brent et al., 1985). The other region, residues 148-

196 has activation function when the C-terminal residues from 197 to 881 are deleted. This truncated GAL4p can activate transcription even though the C-terminal activation domain (residues 768-881) is deleted along with a substantial portion of the protein (Ma et al., 1987a). The activation domain present in residues 768-881 had previously been suggested to adopt an amphipathic α-helical structure where acidic and hydrophobic residues tend to be clustered along separate surfaces. Recent experiments by Van Hoy et al. (1993) using circular dichroism spectroscopy on a synthetic GAL4 peptide encoding the final 34 C-terminal residues has shown that at pH 6, this peptide is not α-helical in solution but adopts a β sheet conformation. In addition, at pH 6, this peptide is able to interact with GAL80p showing that this β sheet conformation is not inhibiting this interaction. Genetic analysis on *GAL4* by Leuther et al. (1993) using site-directed mutagenesis has shown that acidic residues 870-873 (DDED), suggested as being required for activation potential, can be deleted and GAL4p will still retain 40% of wild-type activity on a *GAL1-lacZ* fusion gene and its response to GAL80p. Also, two other acidic residues, Asp-862 and Asp-863 can be mutated to glycine and asparagine, respectively, without loss of transcription activation (provided acidic residues 870-873 are present) but can no longer respond to GAL80p. Most strikingly is the observation that positively charged amino

acids can replace residues 870-873 of the GAL4 peptide and exhibit as high as 145% of wild-type induced levels of *GAL1-lacZ* expression. The conclusion to these studies is that aspartate residues 862 and 863 are involved in GAL80p interaction and GAL4p's C-terminal activation domain lacking negatively charged residues 870-873 can still retain a significant amount of activation function (Leuther et al., 1993).

The interaction of the inhibitor GAL80p with GAL4p appears to require the last 30 C-terminal amino acids of GAL4p (Ma et al., 1987b). GAL4p containing alterations in these residues will not bind GAL80p and results in a constitutive phenotype. When *GAL4* is cloned into a high copy plasmid causing an overabundance of GAL4p in the cell, titration of GAL80p occurs (which must be present in limiting amounts) and constitutive expression of the *GAL* genes results (Johnston and Hopper, 1982). A mutation which deletes *GAL80 (gal80Δ)* also allows for constitutive expression of the *GAL* genes (Torchia et al., 1984). These findings suggest that the trans-activation domain in the C-terminal region of GAL4p is recognized by GAL80p and GAL80p masks this domain until inducer disrupts this association. However, for some time it was not clear whether or not GAL80p remains associated with GAL4p in the presence of inducer (Leuther et al., 1992). Results of experiments using a fusion of GAL80p to the activation domain of the

VP16 protein suggest an interaction between this fusion protein and a mutant GAL4p capable of only low levels of transcription activation in the presence or absence of galactose. These results are consistent with the idea that GAL80p remains associated with GAL4p even in the presence of galactose. To further support the above data, experiments were done by Parthun and Jaehning (1992) showing that GAL4p and GAL80p can be copurified from yeast protein extracts by DNA-affinity chromatography. These heterodimeric complexes isolated from either uninduced (glucose grown) or induced (galactose grown) cells are indistinguishable from each other as illustrated by velocity gradient sedimentation, gel filtration studies and DNA-mobility shift assays even though GAL4p from induced cells has been shown to be phosphorylated and uninduced GAL4p to be in a dephosphorylated state (see below). These findings support a new model suggesting that uninduced as well as induced GAL4p remains complexed to GAL80p and that galactose induction does not require dissociation of GAL80p to reveal the activation domain of GAL4p but could require a more subtle event which leads to GAL4p activating transcription.

Recently, GAL4p has been shown to be post-translationally modified (Mylin et al., 1989; Mylin et al., 1990b). In glycerol+lactate grown wild-type cells, GAL4p exists in two forms, $GAL4_I$ and $GAL4_{II}$ with apparent molecular weights of 100 kD and 103 kD, respectively. Upon galactose

addition, a phosphorylated species, $GAL4_{III}$ appears which migrates with an apparent molecular weight of 108 kD. Upon addition of glucose, $GAL4_{III}$ disappears and $GAL4_{II}$ reappears. It appears that $GAL4_{III}$ is converted to $GAL4_{II}$ by dephosphorylation upon glucose addition. Also, the gene *GAL11 (SPT13)*, which is required for the expression of the *GAL* genes, has been shown to be required for the accumulation or maintenance of the phosphorylated $GAL4_{III}$ protein species (Long et al., 1991).

Phosphorylation in response to changes in the environment has been shown to be a common mechanism in controlling the activities of transcriptional activators (Jackson, 1992). Changes in the environment may activate one or more intracellular signal transduction pathways leading ultimately to the activation or inactivation of a transcription factor(s) resulting in a change in gene expression.

Phosphorylation can regulate the DNA-binding activity of a transcription factor. The gene product of *c-jun* (c-Jun), a proto-oncogene, is phosphorylated on residues next to its DNA-binding domain resulting in reduced DNA-binding activity (Jackson, 1992). Specific dephosphorylation of these three residues adjacent to the DNA-binding domain will increase the DNA-binding activity of c-Jun. This dephosphorylation may be achieved by inhibiting a kinase or by activating a phosphatase that will act on c-Jun.

Interestingly, the oncogenic derivative of c-Jun (v-Jun) has a mutation at one of the phosphorylatable sites (Ser to Phe) that affects DNA-binding and could be the reason why v-Jun is a transforming protein (Jackson, 1992; Smeal et al., 1991). In serum response factor (SRF), an opposite effect of phosphorylation occurs. Phosphorylation of SRF in response to serum stimulation *in vivo* by casein kinase II increases its DNA-binding activity (Gauthier-Rouviere et al., 1991). Casein kinase II has an opposite effect on c-Myb where, upon phosphorylation, the DNA-binding activity of c-Myb decreases (Jackson, 1992; Luscher et al., 1990). Since phosphorylation of different factors can either increase or decrease DNA-binding activity, it is likely that these factors utilize quite different mechanisms to regulate their functional activity (Jackson, 1992).

Phosphorylation of a transcription factor can also affect how it interacts with the general transcription machinery. A factor whose activation function is positively regulated by phosphorylation is c-Jun (Jackson, 1992). In c-Jun, two sites in the transcriptional activation domain are phosphorylated in response to mitogens and phorbol esters mediated by mitogen-activated protein kinases (MAP kinases) (Smeal, et al., 1991; Pulverer et al., 1991). Positive regulation by phosphorylation also occurs in the STE12 protein and heat shock transcription factor (HSTF) in *Saccharomyces cerevisiae* (Song et al., 1991; Sorger et al.,

1988).

Another *Saccharomyces cerevisiae* protein, SWI5 is active in the nucleus during G1 phase but is localized to the cytoplasm during S, G2 and M phases of the cell cycle (Nasmyth et al., 1990). The nuclear localization signal (NLS) in SWI5 is phosphorylated at three sites while in the cytoplasm but the nuclear form is not phosphorylated at these sites (Moll et al., 1991). When these three sites are converted by mutation to nonphosphorylatable residues, SWI5 is constitutively nuclear localized, indicating that phosphorylation of SWI5 allows it to remain in the cytoplasm.

In summary, phosphorylation of transcriptional activators is an ideal mechanism for post-translationally regulating the functional activity of transcription activators. The level of phosphorylation is determined by the action of activated kinases and can be easily reversed by the enzymatic action of phosphatases (Jackson, 1992). Phosphorylation allows information to be integrated from the action of a single kinase on an activator protein to the regulation of gene transcription by alteration of the activator's DNA-binding function, subcellular localization, transcription activation function or perhaps other functions (Jackson, 1992).

HAP1 *HAP1* encodes the HAP1 protein (HAP1p), a 1483 residue

protein required to turn on the transcription of the yeast *CYC1* gene encoding iso-1-cytochrome c (Guarente et al., 1984b; Lalonde et al., 1986) and *CYC7* encoding iso-2-cytochrome c (Prezant et al., 1987; Zitomer et al., 1987). These proteins participate in the respiratory chain. HAP1p has been shown to specifically bind to two sites of dissimilar sequence, one in the UAS1 of *CYC1* and the other in the UAS of *CYC7* (Pfeifer et al., 1987 a,b). This binding is induced by the presence of heme which could act as a ligand required for DNA-binding (Guarente and Mason, 1983). Actually, recent experiments described below have shown that HAP1p can bind to its target sequences *in vitro* without the addition of hemin (Fytlovich et al., 1993).

The amino acid sequence of HAP1p contains a cysteine-rich region in residues 64-86 homologous to the zinc-dependent DNA-binding domain found in GAL4p that is required for DNA-binding of HAP1p (Pfeifer et al., 1989). Any alteration of the cysteine residues found in this region abolished binding to the *CYC1* and *CYC7* UASs. Amino acids at the base of the finger and amino terminal to the finger are involved in specific recognition of DNA at the UASs of both *CYC1* and *CYC7*. For example, the mutant allele *HAP1-18* contains a change in residue 63 from a Ser to Arg at the base of the finger resulting in an increase in *CYC7* transcription (Kim et al., 1989). It is possible that this mutant protein exhibits a higher affinity for a component of

the general transcriptional machinery and thus causes a higher level of activation over wild-type HAP1p. A similar finding where mutations in a DNA-binding domain can affect transcription activation is also seen in the glucocorticoid receptor (discussed below) where mutations in one of the zinc-fingers allows for DNA-binding to GRE elements but not transcription activation (Schena et al., 1989).

The middle portion of HAP1p (residues 280-420) contains a repeat sequence consisting of cysteine and histidine residues present in seven copies. This region could contain a heme-responsive domain since deletion of residues 247-447 creates a constitutive activator (Pfeifer et al., 1989). In addition, interactions that bind heme to the protein could involve paramagnetic interactions between the iron of heme, the sulfur of cysteine and the nitrogen of histidine. This repeated sequence could bind heme directly but this has not yet been demonstrated (Pfeifer et al., 1989).

Recent experiments by Fytlovich et al. (1993) have allowed for the creation of a new model for the heme-inducible activity of HAP1p. DNA-gel shift experiments have shown that HAP1p can interact with its target sequence without addition of hemin. A large protein-DNA complex is observed here which reflects a possible interaction between HAP1p and an unrelated factor(s) they call X. Hemin addition results in the formation of a smaller complex which could represent a multimer of the HAP1p. Most importantly,

when residues 247-444 are deleted containing the seven repeated sequences which could bind heme, the interaction between HAP1p and this factor(s) X does not occur *in vitro*. These results indicate that HAP1p and factor(s) X could interact through this repeat motif or that this deletion simply prevents HAP1p and factor(s) X from associating with each other.

HAP1p has been shown to contain nonessential sequences between residues 247 and 1308 since this region can be deleted resulting in the activity of the protein increasing 10-fold above wild-type for *CYC1* transcription (Kim et al., 1990). DNA-binding of this deletion protein was comparable at the *CYC1* and *CYC7* promoters but activation of *CYC7* was considerably decreased and could mean that this deleted portion of HAP1p contains a regulatory domain specific for its function at *CYC7*. This variation in activation could be due to HAP1p requiring proper protein conformational contacts with the DNA which ultimately determines whether or not HAP1p can interact with the general transcription factors.

The C-terminal region of HAP1p is highly acidic in nature. Residues 1385-1483 contain a net charge of -12 (Pfeifer et al., 1989). This acidic region in HAP1p has been shown to mediate activation, since truncation of residues 1308-1483 results in a protein which binds DNA but does not activate transcription *in vivo* (Pfeifer et al.,

1989). We see that HAP1p also contains a conserved arrangement of a DNA-binding domain near the N-terminus, a heme-responsive domain and regulatory domain in the middle portion of the protein and an acidic transcriptional activation domain in the C-terminus.

PUT3 Proline catabolism in *Saccharomyces* involves the functions of three genes. *PUT1* and *PUT2* encode the enzymes, proline oxidase and Δ^1-pyrroline-5-carboxylate dehydrogenase, respectively. *PUT3* encodes a transcriptional activator, PUT3p which binds to UAS elements upstream of both the *PUT1* and *PUT2* genes *in vitro* and *in vivo* in the presence or absence of proline but proline is required for PUT3p activation of *PUT1* and *PUT2* gene transcription (Marczak et al., 1989). There is a 21-basepair partially palindromic UAS upstream of both genes required *in vivo* for induction of both genes (Siddiqui et al., 1989). Extracts from a wild-type *PUT3* strain and a *PUT3*^{c} constitutive strain exhibit binding to the *PUT2* promoter fragment containing the UAS element whereas extracts from a noninducible strain (*put3-75*) fail to show any DNA-binding activity.

Wild-type *PUT3* was cloned by functionally complementing a strain carrying the recessive, noninducible *put3-75* mutation with a *Saccharomyces* yeast genomic DNA library from a *PUT3* strain (Marczak et al., 1989). The *PUT3* gene is not essential for viability since *put3*Δ deletion strains are

viable. In addition, when *PUT3* is placed on a high copy plasmid, it does not result in a constitutive phenotype so it appears that a titratable GAL80p-like repressor is not involved in the regulation of proline catabolism (Marczak et al., 1989).

The wild-type *PUT3* gene was shown to encode a 979 amino acid protein (Marczak et al., 1991). The N-terminal portion of the protein is rich in basic amino acids and contains 6 cysteine residues that may constitute the DNA-binding domain based on structural similarity to the DNA-binding domain of GAL4p. Adjacent to this basic region in PUT3p is an acidic domain with a net negative charge of -17 consisting of a stretch of nine aspartic or glutamic acid residues. Another acidic domain exists in the C-terminus having a net charge of -29. However, these residues have not been directly shown to be responsible for transcription activation.

Seven constitutive mutants in *PUT3* were isolated and the mutations were found to reside in the final C-terminal 76 codons of *PUT3* (Marczak et al., 1991). Three of these were missense mutations. The *PUT3^{c}-1594* allele possessed a nucleotide change in codon 903 (Leu to Arg), the allele *PUT3^{c}-1657* altered two consecutive nucleotides and changed an asparagine residue to isoleucine, and the *PUT3^{c}-68* allele replaced a serine with a phenylalanine residue at position 683. Four other constitutive mutations were nonsense mutations leading to the creation of a premature truncation

of the protein at residue 956. One noninducible allele *put3-75* was also sequenced and shown to contain a single point mutation within codon 409 which converts a glycine to an aspartate residue. This mutation lies in a region that is not homologous to any domain in other regulatory proteins and it is not clear as to whether this mutation causes the mutant protein to be unstable or stable but inactive. Even when this mutant allele (*put3-75*) was cloned on a high copy plasmid, it did not compensate for the inability to utilize proline in a *put3*Δ strain (Marczak et al., 1991).

PUT3p is proposed to induce the transcription of *PUT1* and *PUT2* in the presence of proline by either: 1) binding to proline and changing its conformation in order to interact with general transcription factors, 2) being post-translationally modified, possibly by phosphorylation, which would affect its activity or 3) interacting with other regulatory proteins which are relatively abundant in the cell since there does not seem to be a GAL80p-like equivalent involved in proline catabolism.

LEU3 The yeast activator protein LEU3 (LEU3p) is involved in regulating genes encoding enzymes for the *de novo* synthesis of leucine: *LEU1* (isopropylmalate isomerase), *LEU2* (β-isopropylmalate dehydrogenase) and *LEU4* (α-isopropylmalate synthase) (Baichwal et al., 1983). Alpha-isopropyl malate (α-IPM) is an intermediate in the

production of leucine whose synthesis is negatively regulated by leucine through feedback inhibition of the enzyme α-IPM synthase (Friden et al., 1989). α-IPM has been connected with the regulation of leucine biosynthesis since 1) levels of *LEU1* and *LEU2* gene products are sharply decreased in a strain which lacks a functional α-IPM synthase and 2) a strain harboring a feedback resistant α-IPM synthase produces high levels of α-IPM which increases the amounts of *LEU1* and *LEU2* gene products (Friden et al., 1989).

The gene *LEU3* was identified by mutations resulting in a Leu- phenotype due to decreased expression of *LEU1* and *LEU2* (Baichwal et al., 1983). *LEU3* has been cloned and characterized by complementation of a *leu3* mutation (Brisco et al., 1987). The gene was shown to encode an 886 amino acid protein that binds *in vitro* in the absence of α-IPM to a UAS_L decanucleotide sequence (CCGGNNCCGG) present in the upstream promoters of *LEU1*, *LEU2*, and *LEU4* (Friden et al., 1987; Friden et al., 1988). It is hypothesized that α-IPM acts as an inducer of the leucine biosynthetic genes because cells starved for leucine produce high levels of α-IPM, and strains unable to synthesize α-IPM are noninducible (Friden et al., 1989; Brisco et al., 1987). Recent experiments have been done to create an *in vitro* LEU3p-dependent transcription system of a *CYC1* TATA box linked to a "G-less cassette" bearing UAS_{LEU} elements in its upstream region (Sze

et al., 1992). Activation of transcription in this system was completely dependent on the presence of one or more UAS_{LEU} sequences, the concentration of LEU3p and the addition of α-IPM. In the absence of α-IPM, LEU3p acts as a repressor of transcription in this system so therefore, these investigators predict that α-IPM can act to transform LEU3p from a repressor into an activator protein by a possible change in its conformation.

The sequence of the LEU3p revealed a potential DNA-binding domain in the N-terminal residues 1-173 homologous to a six cysteine residue-type DNA-binding domain, however, sequences in the middle portion of the protein and in the C-terminal portion are also required for proper and stable binding (Zhou et al., 1990). LEU3p also contains a cluster of acidic amino acids between residues 678-697 and an acidic domain in the C-terminal 112 amino acids (Zhou et al., 1990). The region within residues 678-697 can be deleted without affecting transcriptional activation by LEU3p (Friden et al., 1989). However, a truncated LEU3p containing only residues 1-774 did not complement a *leu3Δ* strain but bound *in vitro* to the decanucleotide sequence as strongly or even stronger than wild-type LEU3p suggesting that the last C-terminal amino acid residues (775-886) are involved in transcription activation. A large internal deletion was made within *LEU3* which deleted 68% of the coding region and fused residues 1-172 to residues 775-886

(Friden et al., 1989). This internally deleted LEU3p allowed for constitutive expression of the leucine biosynthetic enzymes even in the presence of leucine suggesting that this deleted region could somehow be sensitive to the inducer, α-IPM. The fusing of the N-terminal residues 1-172 of LEU3p to its C-terminal residues 775-886 is similar to a fusion made between the DNA-binding domain and activation domain of GAL4p except that this LEU3p is leucine insensitive whereas the deleted GAL4p's activity is still regulated by galactose (Ma et al., 1987a).

Purification of LEU3p has recently been performed by Sze et al. (1993) who identified two forms of LEU3p of apparent molecular weights, 104 kD and 110 kD. It was shown that both species reacted with antibodies raised against LEU3p and the 110 kD species could be converted to the faster migrating 104 kD species by treatment with calf intestinal phosphatase. It appears then that the 110 kD LEU3p species represents a phosphorylated form of the protein. Further work needs to be performed to determine whether or not both forms of LEU3p are equally capable of being activated in the presence of α-IPM.

ADR1 The *ADH2* gene encodes the enzyme, alcohol dehydrogenase (ADHII) which converts ethanol to acetaldehyde. ADHII activity is repressed in glucose but removal of glucose results in a 500-fold increase in ADHII

activity (Blumberg et al., 1988). *ADH2* expression is positively regulated at the transcriptional level by *ADR1*, encoding a positive transcriptional regulator protein, (ADR1p) (Ciriacy, 1975; Denis and Young, 1983a). However, other regulatory factors are responsible for regulating *ADH2* expression (Ciriacy, 1977; Denis, 1984; Taguchi et al., 1987 a,b). *ADH2* contains a 22 basepair UAS with dyad symmetry in its 5'upstream promoter region which is believed to be the site for ADR1p binding, where it exerts its control on *ADH2* expression (Beier et al., 1985).

ADR1 mutations have been isolated with either a semidominant, constitutive phenotype (*ADR1*^{c}) which allows for partially constitutive *ADH2* expression in the presence of glucose or a recessive, noninducible phenotype (*adr1*) which prevents *ADH2* derepression in the absence of glucose (Ciriacy, 1975; Ciriacy, 1979). A gene disruption in *ADR1* (*adr1*Δ) results in the inability to derepress *ADH2* expression but disruption strains are viable indicating that *ADR1* is not an essential gene (Blumberg et al., 1988).

By increasing the gene dosage of *ADR1* on a high copy plasmid, partially constitutive *ADH2* expression results even in the presence of glucose and even higher levels of *ADH2* expression under derepressed conditions as compared to a strain with one genomic copy of *ADR1* (Blumberg et al., 1988). This result implies that a repressor of ADR1p could exist in limiting quantities under glucose growth conditions

thereby keeping ADR1p inactive and *ADH2* expression low. However, increased levels of ADR1p are able to bind to and titrate this repressor resulting in a constitutive phenotype (Blumberg et al., 1988).

The wild-type *ADR1* gene was cloned and sequenced and found to encode a protein of 1323 amino acids. The N-terminal residues 99-155 contain two cysteine-histidine zinc finger DNA-binding domains similar to that found in the *Xenopus laevis* TFIIIA protein and most mutations that inactivate *ADR1* occur in this region (Bemis et al., 1988; Blumberg et al., 1987). A truncated gene encoding the first 302 amino acids of ADR1p also causes partial constitutive expression of *ADH2* suggesting that the C-terminal 1021 amino acids could play another role not involved in the derepression of *ADH2* (Blumberg et al., 1988). By progressively deleting the 3'-end of the *ADR1* open reading frame, the ability of the truncated ADR1p to activate *ADH2* expression progressively decreases, appearing to require the full length protein for maximal expression of *ADH2* (Bemis et al., 1988). A region in ADR1p (residues 304-506) was also shown to be required for growth on glycerol as a carbon source and indicates that ADR1p could be a more global regulator of nonfermentative growth (Bemis et al., 1988).

Another important functional domain in ADR1p is the cAMP-dependent protein kinase (cAPK) phosphorylation site located between residues 227 and 231 (Kemp et al., 1977).

Constitutive mutations which alter ADR1p by making it resistant to glucose repression, that is, able to activate *ADH2* expression in the presence of glucose, are localized to this region (Cherry et al., 1989). *ADR1* mutations encoding only the first N-terminal 220 amino acids were functional and allowed for derepression of *ADH2* showing that these residues contained the DNA-binding and transcription activation functions (residues 29-40 are 50% acidic) but lack the phosphorylation site (residue 230) (Bemis et al., 1988). Various *ADR1*^{c} mutations which are constitutive for *ADH2* expression have been postulated to increase ADR1p function under repressed conditions by the dephosphorylation of ADR1p (Cherry et al., 1989). Increased expression on high copy plasmids of ADR1p N-terminal peptides (>220 residues) are capable of bypassing glucose repression and allow for increased *ADH2* expression (Bemis et al., 1988). This increase in expression could be due to tighter DNA-binding of ADR1p to the *ADH2* promoter or enhanced contacts with other regulatory proteins or transcription factors. Many *ADR1*^{c} mutations lie in the cAPK phosphorylation recognition sequence, RRASF, in which Ser-230 is the phosphate acceptor. Three *ADR1*^{c} mutations were identified as containing mutations within this sequence (Phe-231 to Ser, Arg-228 to Lys) and in one mutation, a nonphosphorylatable residue, leucine was substituted for serine at residue 230 (Cherry et al., 1989).

Serine-230 of ADR1p has also been shown to be a substrate for phosphorylation by either bovine or yeast cAPK *in vitro* (Cherry et al., 1989). Additionally, the ADR1p constitutive activators had diminished or undetectable amounts of phosphorylation at residue 230. Strains with unregulated cAPK activity, as a result of deletion of the *BCY1* gene encoding the regulatory subunit of cAPK, exhibit a 12-15 fold decrease in *ADH2* expression in ethanol showing that cAPK does regulate *ADH2* gene expression and the *ADR1*^{c} mutations bypass this effect of unregulated cAPK activity (Cherry et al., 1989). Taken together, this data suggests that the glucose repression of *ADH2* transcription involves the inactivation of ADR1p as a result of the phosphorylation of Ser-230 by cAPK (Cherry et al., 1989).

More recent evidence, however, does not support the hypothesis that *ADR1*^{c} mutations enhance ADR1p activity by preventing cAPK-mediated phosphorylation of Ser-230 (Denis et al., 1992). Firstly, by using an *in vitro* system with cAPK and synthetic ADR1-derived peptides, not all of the *ADR1*^{c} mutations that have been identified prevent phosphorylation of Ser-230 contained in the synthetic ADR1 peptides encoding the region surrounding Ser-230 and containing these various constitutive alterations. Secondly, when cAPK activity is lacking in an *ADR1*^{c} mutant strain containing a nonphosphorylatable amino acid at residue 230, ADR1p function increases under glucose

repressed conditions indicating that cAPK acts by a mechanism independent of Ser-230. Thirdly, when cAPK activity is lacking in an *ADR1* wild-type strain, an *ADR1*^{c} constitutive phenotype does not result *in vivo* as might be expected. Lastly, when the *ADR1*^{c} region is deleted (residues 220-262), a constitutive phenotype results suggesting that this region plays an inhibitory role in the function of ADR1p. These observations support a new model indicating that the *ADR1*^{c} mutations could alter ADR1p's structure, function or ability to bind to a repressor in such a way as to make the protein constitutively active and that this alteration is not dependent on the effect of the phosphorylation of residue Ser-230 by cAPK.

Recent experiments performed by Vallari et al. (1992) looked at the levels of ADR1p and its phosphorylated species under growth conditions in glucose or ethanol. What was found was that ADR1p was multiply phosphorylated *in vivo* when cells were grown in either glucose or ethanol. The rate of ADR1p synthesis was also examined by doing pulse chase experiments. ADR1p synthesis was shown to rise about 40-60 minutes after cells are allowed to grow in ethanol medium which agrees with the observation that ADR1p-dependent *ADH2* transcription begins within one hour after glucose depletion from the growth medium (Denis et al., 1981). Strains containing *ADR1*^{c} mutations did not contain higher amounts of ADR1p so another mechanism is responsible

for increasing ADR1p's activity other than increasing its synthesis rate (Vallari et al., 1992). Also, *ADR1* mRNA levels are only two-fold higher in ethanol grown cells as compared to glucose grown cells indicating that there must be some post-transcriptional mechanism by which ADR1p is synthesized at a higher rate in ethanol grown cells. A region of ADR1p (residues 262-642) was later found to be involved in the translational control of ADR1p. For example, when ADR1p was truncated at either residue 262 or 642 to encode truncated proteins, ADR1p-642 was detected in ethanol grown cells but ADR1p-262 was detected in equal abundance in either glucose or ethanol grown cells. It would appear then that residues 262-642 of ADR1p are involved in the accumulation of ADR1p under ethanol growth conditions.

In conclusion, Vallari et al. (1992) suggest that glucose repression of *ADH2* expression results from a combination of effects controlling *ADR1* mRNA abundance, translation rates of *ADR1* mRNA in glucose and ethanol and post-translational mechanisms affecting ADR1p activity.

PHO4 Repressible acid phosphatase (rAPase) is encoded by *PHO5, PHO10,* and *PHO11* (Rogers et al., 1982; Thill et al., 1983). *PHO4* encodes one transcription factor (PHO4p) involved in the positive regulation of the transcription of various genes in the phosphatase regulon of *Saccharomyces*

cerevisiae. It is proposed that in low phosphate medium, PHO4p activates transcription of the structural genes which encode rAPase. In high phosphate medium, the *PHO80* gene product (PHO80p), a negative regulatory factor, is proposed to interact with PHO4p and thereby prevent transcription of the genes in the phosphatase regulon.

PHO4p is 312 amino acids in length and has four functional domains (Ogawa et al., 1990). The C-terminal residues 228-312 are responsible for binding to the *PHO5* promoter region and has homology to the amphipathic helix-loop-helix DNA-binding motif found in *c-myc*; residues 163-202 are responsible for interaction with the negative regulatory factor PHO80p; the N-terminal residues 7-90 are rich in acidic residues and are required for transcriptional activation; and residues 203-227 are necessary for oligomerization of PHO4p.

Constitutive synthesis of rAPase in high or low phosphate media can occur by either increasing the gene dosage of *PHO4* suggesting that the overabundance of PHO4p titrates out PHO80p or by the creation of constitutive mutations in the *PHO4* gene (Parent et al., 1987). Constitutive mutations in the *PHO4* gene can occur in several different ways (Ogawa et al., 1990). Four particular *PHO4*^{c} mutations that have been sequenced are missense mutations resulting in the replacement of proline 174 by leucine (mutants *PHO4*^{c}-1,9) or serine (mutants *PHO4*^{c}-3,6). A

constitutive phenotype could also be achieved by a four amino acid (AGSG) insertion between residues 171 and 172 or by deletion of residues 163-171. The region between residues 167-173 is proposed to fold as an α-helical structure and proline 174 is the break point in this helix. If proline 174 is replaced by serine or leucine, the α-helical structure will begin at residue 167 and should continue to isoleucine 178. Hence, the α-helix of the *PHO4*^{c} mutants should be four amino acids longer than wild-type, the amino acid (AGSG) insertion would disrupt this α-helix and deletion of residues 163-171 would fully eliminate the α-helix. Since all of these mutations result in a constitutive phenotype, the α-helix between residues 167-173 could be critical perhaps for interaction with the negative regulator, PHO80p, implying that, when this helix is disturbed or deleted, PHO4p is unable to bind PHO80p and is then able to transcribe the *PHO* structural genes constitutively (Ogawa et al., 1990).

Human glucocorticoid receptor (hGR) Glucocorticoids, a class of steroid hormones, through interaction with an intracellular receptor protein, converts this receptor to a DNA-binding transcription activator. Glucocorticoid receptor is found in the cytoplasm in the absence of hormone. In the presence of glucocorticoid hormone, this protein will localize to the nucleus and activate genes

possessing enhancers called glucocorticoid response elements (GREs) (Godowski et al., 1987). The human glucocorticoid receptor (hGR) has been purified by its ability to bind steroid hormones such as dexamethasone. Antibody was made against the receptor and cDNA clones from a human fibroblast genomic library were screened for inserts containing the gene encoding the receptor. Two positive clones were found encoding a predominant alpha species (777 residues) and a βeta form (742 residues) which were identical in the first 727 residues but differed in their C-terminal residues (Hollenberg et al., 1985). It is presumed that the two forms are encoded by the same gene but alternative splicing of the mRNA determines their different C-terminal residues.

The functional domains of the hGR have been defined by looking at the effects of deletion mutants in the receptor on DNA-binding and/or transcription activation (Giguere et al., 1986; Hollenberg et al., 1987). These analyses have determined that there exists an immunogenic domain in the N-terminal region which is required for complete activity of the receptor since deletion of it causes a 3 to 20-fold reduction in activity. However, residues 1-403 of the hGR are not required for steroid-binding or DNA-binding. This immunogenic domain could play a structural role or be involved in the dimerization of receptor molecules.

The next domain that was looked at is located in the C-terminal region of hGR (Hollenberg et al., 1987). By

deleting a large region of the 3'-end of the open reading frame (codons 532-697), the encoded hGR protein was unable to bind steroid hormone resulting in a constitutively active receptor. Constitutive receptor mutants have also been characterized in the rat glucocorticoid receptor where deletions of 190, 200, 239 and 270 C-terminal amino acid residues result in constitutive receptors no longer requiring hormone for DNA-binding to GRE elements (Godowski et al., 1987). Therefore, the steroid binding domain of the hGR could exert a negative influence over the rest of the protein but binding of hormone relieves the inhibition of this domain on DNA-binding and/or transcription activation. The hormone-binding domain in hGR has been shown to contain an inducible transcription activation domain (Webster et al., 1988a). By fusing the DNA-binding domain of GAL4p to the C-terminal hormone-binding domain of the estrogen or glucocorticoid receptor, a transcriptionally active chimeric protein results which transcribes a GAL4p-responsive reporter gene in a hormone-dependent manner. Other experiments were done creating rearranged receptors which were able to maintain hormonal control over transcription activation. This argues against the possibility that the steroid-binding domain binds hormone and then unmasks other functional regions of the protein (Picard et al., 1988). Instead, this domain could repress most of the receptor's activities without regard to its position in the protein.

The DNA-binding domain and the transcriptional activation domain of hGR were shown to colocalize to the N-terminal region (Hollenberg et al., 1987). Deletion of the majority of the DNA-binding domain (residues 428-490 containing two zinc finger-like binding motifs which coordinate two zinc ions) did not allow the receptor to stimulate transcription although it was able to bind steroid. This data had delineated a core domain of 88 amino acids (residues 404-491) essential for both DNA-binding and transcription activation since mutations in this highly conserved region destroy both activities indicating that these two functions cannot be easily separated.

Mutations were later done in this core region by changing all invariant residues as well as conserved and nonconserved residues to glycines (Hollenberg et al., 1988). By altering only one of the nine invariant cysteine residues, activity was destroyed and critical amino acids were localized to amino acids in the interfinger region (residues 442, 444, 445 and 447). These nonfunctional mutants were tested for their ability to bind DNA even though they failed to activate transcription. The ability to activate transcription correlated well with the ability to bind DNA *in vitro*. One mutant called G442, converted the lysine following the first zinc-finger to a glycine and was able to bind DNA but unable to stimulate transcription.

Changes in the zinc-finger region can also change the

DNA-binding specificity of the hGR. For example, when Gly-Ser residues located between the second pair of cysteine residues in the first finger are changed to Glu-Gly (as found in the estrogen receptor), a hybrid protein results which activates transcription of an estrogen receptor element (ERE)-containing reporter gene and only weakly from a GRE-containing reporter gene (Danielson et al., 1989; Berg, 1989). Other mutations were made in the second finger domain and were shown to specifically impair transcription activation (Schena et al., 1989). For example, an arginine to lysine change in residue 489 or an arginine to glutamine change in residue 488 were fully defective in expression of a GRE-*lacZ* fusion but bound DNA normally. These mutant receptors were not shown to exhibit instability or reduced expression so these mutations definitely affected receptor function. Mutations of residues immediately C-terminal to each finger were found to abolish transcription activation but other mutations which abolished activation did not reside within a finger domain or at the C-terminal side of the finger.

Further analysis of the transcriptional activation domains in the hGR revealed a 30 amino acid region between the steroid-binding and DNA-binding domains (residues 526-556) which when fused to the GAL4p DNA-binding domain, generates a hormone-inducible activator (Hollenberg et al., 1988). This C-terminal activation sequence is acidic in

nature, like the activation domain in the N-terminal core region, and these autonomous transcription activation domains are both located outside the DNA-binding domain of the hGR.

The glucocorticoid receptor has been shown to associate with the 90 kD heat shock protein, HSP90p, based on coimmunoabsorption of HSP90p with the receptor when anti-hGR antibody is incubated with cytosolic extracts (Howard et al., 1988). HSP90p did not associate with the receptor when dexamethasone was added to the cell extracts suggesting that the hormone promotes dissociation of HSP90p-hGR complexes *in vivo*. This association of HSP90p with the glucocorticoid receptor has also been demonstrated for other steroid receptors including those for progestins, estrogens and androgens (Joab et al., 1984; Catelli et al., 1985; Dougherty et al., 1984; Schuh et al., 1985). The biological role of HSP90p in glucocorticoid receptor function has not been established. One hypothesis is that HSP90p blocks the DNA-binding site on the receptor, thus preventing the receptor from binding to DNA in the absence of hormone (Howard et al., 1988). However, when steroid hormone is present, the hormone will bind to the receptor, HSP90p will dissociate from the HSP90p-hGR complex and the DNA-binding site or nuclear localization signal on the receptor will be exposed. This could identify HSP90p as having a possible role in stabilization or nuclear localization of the

glucocorticoid receptor.

***ADA2* and other coactivators** A recent hypothesis has stated that non-DNA-binding proteins (adaptor molecules or coactivators) could exist which bridge the interaction between specific DNA-binding activators and general transcription factors (Berger et al., 1992). Previous hypotheses about how eucaryotic activators work involved the thought that protein-protein interactions existed between specific transcription factors bound at the UAS or enhancer sequences and general factors bound at the TATA box upon looping out of intervening DNA (Ptashne, 1988). This possibility has been ruled out since several coactivators have been identified.

Coactivators or mediators of transcriptional activation have been isolated in *Drosophila* which are associated with the TATA box binding protein (TBP) as a tight protein complex called the TFIID fraction (Dynlacht et al., 1991). However, in yeast, the TBP is not part of a stable multisubunit complex like that seen in *Drosophila* and humans. The multimeric TFIID complex in *Drosophila* consists of TBP and at least six tightly bound TBP-associated factors (TAFs) having approximate molecular weights of 32, 40, 60, 80, 110 and 150 kilodaltons which can be dissociated from TBP by urea treatment. All TAFs coprecipitate with TBP when anti-TBP antibody is used. One or more of these proteins

are required as a coactivator(s) for activated transcription *in vitro* of a *Drosphila* gene containing binding sites for the *Drosophila* transcription factor, NTF-1. Addition of a recombinant form of TBP alone is not sufficient to allow for activation but addition of the purified TAFs restores NTF-1 activity.

Other proteins that exhibit coactivator function have been isolated on the basis of interference or "squelching" between two activator proteins by the sequestration of a factor(s) that mediates the transcriptional effects of the activator protein (Kelleher et al., 1990). Sequestration would result from the binding of the interfering activator to this common factor, making this factor unavailable for interaction with the second activator.

A yeast *in vitro* RNA polymerase II transcription system utilized the potent fusion activator GAL4-VP16 (containing the GAL4p DNA-binding domain fused to the activation domain of the herpes virus protein VP16) and the yeast T-rich binding factor. The binding site for this T-rich binding factor is a thymidine-rich upstream sequence of the *DED1* gene (Lue et al., 1989; Buchman and Kornberg, 1990). When GAL4-VP16 is added to this transcription assay containing T-rich binding factor and a promoter containing T-rich upstream elements, it will inhibit transcription (Kelleher et al., 1990). This inhibition can be relieved by addition of a partially purified yeast component believed to function

as a mediator in activation. Addition of RNA polymerase II or TBP did not relieve the inhibition by GAL4-VP16 indicating that a general transcription factor was not responsible. A nonspecific effect of this purified yeast component binding to the activation domain of GAL4-VP16 was ruled out since the same levels of GAL4-VP16 were required for stimulation of transcription as well as for inhibition so, since stimulation is specific, then inhibition should be as well. This mediator component was later enriched from a yeast extract and shown that its activity could not stimulate basal transcription, that it was heat labile and that its activity was abolished by treatment with proteinase K (Flanagan et al., 1991). Another observation was that as with GAL4-VP16, stimulation by the yeast activator protein GCN4 (GCN4p) was lost when this component was not added but GCN4p activity was restored when the mediator was added back to the *in vitro* transcription assay. Since this mediator supports activation by both GCN4p and the hybrid activator GAL4-VP16, this could be a common mediator required by acidic activation domains such as those present in GCN4p and VP16 proteins.

The *ADA2* gene has been isolated recently as a gene encoding an "adaptor" protein necessary for the functioning of certain transcriptional activators (Berger et al., 1992). To isolate a gene(s) coding for adaptor molecules, experiments were done using the fusion gene *GAL4-VP16*, as

described above, fused to the *ADH1* promoter. This fusion protein is a very strong activator and becomes toxic to yeast cells but yeast cells which are resistant to this toxicity could lack a factor which would normally allow for the high level of activation exhibited by the GAL4-VP16 fusion protein. For inhibition of toxicity to occur, it was proposed that the VP16 target would be sequestered and unavailable to other cellular activators. This inhibition could not be reversed by addition of TFIID or RNA polymerase suggesting that the VP16 target was not a general transcription factor.

Selection of mutations in yeast genes encoding adaptor proteins was then undertaken by selecting for viable, slow growing mutants resistant to GAL4-VP16 toxicity (Berger et al., 1992). It was hypothesized that mutations in an adaptor molecule would weaken or destroy interactions with VP16 or other general factors, thereby restoring growth. These mutations were recessive and fell into three complementation groups referred to as *ADA1*, *ADA2* and *ADA3* (**a**lteration/**d**eficiency in **a**ctivation). The *ada2* and *ada3* mutant strains were tested for protein expression of the GAL4-VP16 fusion and were shown to contain levels of *ADH1* mRNA comparable to wild-type indicating that reduced expression of GAL4-VP16 was not the reason for the resistance to toxicity. The *ADA2* gene was deleted and shown to be nonessential but *ada2*Δ cells were slow growers and

resistant to GAL4-VP16 toxicity. *ADA2* was cloned on the basis of restoring normal growth and sensitivity to GAL4-VP16 in the *ada2-2* mutant using a yeast genomic library. *ADA2* contained an open reading frame of 434 codons not homologous to any known yeast gene or any gene from another organism in the data base. The N-terminal residues from amino acids 7-34 contain seven cysteine residues but their spacing does not resemble any known class of cysteine-rich DNA-binding domains. The cysteine-rich region is followed by a region (residues 65-95) with homology to the oncogene *myb* motif (Lane et al., 1990). Lastly, the region between residues 89-113 of ADA2 protein (ADA2p) shows homology to a region of the enzyme peptidyl-glycine monooxygenase (PGM) (Stoffers et al., 1989).

The *ada2* mutant was further studied to see if wild-type ADA2p was an adaptor for certain acidic activators and if the expression of particular yeast genes might be affected in the mutant (Berger et al., 1992). *LacZ* reporter plasmids were constructed which all contained the *CYC1* TATA box and mRNA initiation site coupled to either the UAS1 or UAS2 of *CYC1* (activated by HAP1p or HAP2/3/4 proteins) or the *HIS4* UAS (activated by GCN4p). In the *ada2* mutant, *lacZ* expression was found to be reduced 5 to 10-fold when harboring the *HIS4* UAS plasmid but there was very little or no reduction in *lacZ* expression when the mutant contained a *CYC1* UAS fused to the *lacZ* gene. It appeared that wild-type

ADA2p was necessary for the function of GCN4p but not for HAP1p.

In conjunction with the above experiments, yeast nuclear extracts from a wild-type and an *ada2* strain were used *in vitro* to transcribe a reporter plasmid, *CYC1-lacZ* containing upstream GAL4p and GCN4p binding sites with purified GAL4-VP16 or GCN4 proteins as transcription activators (Berger et al., 1992). The ability of GAL4-VP16 to activate transcription was abolished in the *ada2* mutant extract compared with that of the wild-type and the ability of GCN4p to activate transcription was reduced substantially in the mutant. Basal transcription was equivalent for the two extracts so the *ada2* mutation does not involve reduction in the activities of general transcription factors but has a more specific effect on activated transcription.

Given the regulated systems and transcription activators described above as models, we undertook this project to analyze the functional domains of the maltose-responsive transcription activator (the *MAL*-activator) which regulates the expression of the enzymes required for maltose fermentation in *Saccharomyces*. To accomplish this, we have compared the structure of the *MAL*-activator encoded by the *MAL63* gene to a unique class of constitutive *MAL*-activators encoded by the *MAL63* homologue, *MAL64*. The following sections describe the maltose fermentation genes of *Saccharomyces* and the identification and characterization of

MAL64 and the *MAL64*-constitutive alleles.

The maltose fermentation genes in *Saccharomyces cerevisiae*

Maltose fermentation in *Saccharomyces* yeasts requires the presence of at least one of five, unlinked *MAL* loci located at the telomeres of different chromosomes (in parentheses): *MAL1*(VII), *MAL2*(III), *MAL3*(II), *MAL4*(XI) and *MAL6*(VIII). Each genetic locus is a cluster of three genes required for fermentation. GENE 1 encodes maltose permease which transports maltose into the cell (Cohen et al., 1985; Chang et al., 1989; Cheng et al., 1989). GENE 2 encodes the enzyme maltase responsible for the intracellular hydrolysis of maltose (Dubin et al., 1985; Hong and Marmur 1986, 1987). GENE 3 encodes the *MAL*-activator, a transcriptional activator required for the maltose-inducible expression of GENEs 1 and 2 (Chang et al., 1988). Expression of GENEs 1 and 2 is regulated at the level of transcription and is induced by maltose and repressed by glucose (Charron et al., 1986; Needleman et al., 1984). The *MAL6* locus is shown in Figure 1 (Needleman et al., 1984). Each gene is named according to its function and the chromosomal locus to which it maps. For example, at the *MAL6* locus, the permease gene (GENE 1) is called *MAL61*, the maltase gene (GENE 2) is called *MAL62* and the *MAL*-activator gene (GENE 3) is called *MAL63*. GENES 1 and 2 are divergently transcribed. The UAS_{MAL}, the site for MAL63 protein (MAL63p) binding, was

Figure 1. Diagram of the *MAL6* locus on chromosome VIII of *Saccharomyces cerevisiae*. A restriction endonuclease map of the chromosomal region containing the *MAL6* locus is shown (Dubin et al., 1988). The approximate location of the open reading frames of each gene is shown above the map along with the direction of transcription (Charron et al., 1989; Cheng et al., 1989; Dubin et al., 1988; Kim et al., 1988; Needleman et al., 1984). The DNA fragments indicated as MAL63 and MAL64 are the yeast inserts used to subclone the *MAL63* and *MAL64* genes, respectively, into the various vectors for the experiments described here. Plasmid pMJC6ΔCla contains the indicated yeast insert in the vector YIp5ΔCla which was derived from YIp5 by deletion of the unique *Cla*I site (Charron et al., 1988). Genomic fragment pBamR10 is the fragment rescued from strain R10 which contained the *MAL64-R10* constitutive allele. Restriction endonuclease sites are as follows: A, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; Hp, *Hpa*I; M, *Mlu*I; N, *Nco*I; P, *Pst*I; R, *Eco*RI; S, *Sal*I; Sm, *Sma*I; Ss, *Sst*I; Xb, *Xba*I; Xo, *Xho*I.

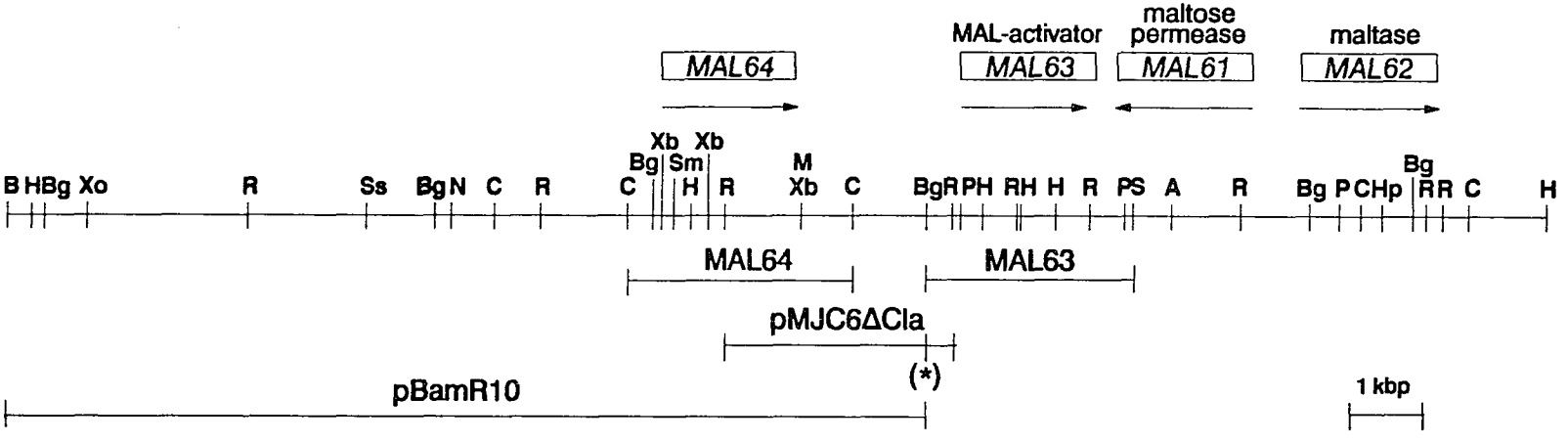


Figure 1: Diagram of the MAL6 locus on chromosome VIII of Saccharomyces cerevisiae

localized within the 874 basepair *MAL61-MAL62* intergenic region by deletion analysis and by DNaseI footprinting (Ni and Needleman, 1990; Levine and Michels, 1992).

The five *MAL* loci have been cloned and compared both structurally and functionally. All of the *MAL* loci show a high degree of homology based on restriction enzyme analysis and Southern analysis (Chow et al., 1983; Charron et al., 1989). Additionally, functional homology is exhibited among the loci based on complementation analysis (Charron et al., 1989). The most extensive functional comparison was made using the *MAL1* and *MAL6* loci where the *MAL6* homologues were shown to complement deletion/disruption mutations in the *MAL1* genes (Charron et al., 1986). In an analysis of all of the *MAL* loci, GENE 3 from each of the loci was shown to complement the *MAL1* structural genes and the structural genes (GENEs 1 and 2) from each locus was shown to complement *MAL13* (Charron et al., 1989). GENE 3 at the *MAL6* locus (*MAL63*) has been characterized by its ability to complement strains which only contain the structural genes encoding maltose permease and maltase. These strains are then able to activate the expression of the structural genes and to subsequently ferment maltose (Chang et al., 1988). The *MAL63* gene was cloned and sequenced and found to contain a cysteine-rich region near its N-terminal end which is highly homologous to the DNA-binding domain found in GAL4p (Needleman et al., 1984; Johnston and Dover, 1987; Kim and

Michels, 1988; Solliti and Marmur, 1988). A truncated MAL63p (containing the first N-terminal 111 residues) was expressed in *E. coli* and used to perform gel-shift assays (Jeong Kim, Ph.D. thesis, 1991). Utilizing 40 basepair double-stranded oligonucleotides whose sequence was derived from the UAS_{MAL} of the *MAL61-MAL62* intergenic region, the truncated MAL63p was shown to bind this DNA in a gel-shift experiment illustrating that MAL63p is a DNA-binding protein. The results of this study, those of Ni and Needleman (1990) and Levine and Michels (1992) taken together suggest that MAL63p binds to an 11 basepair sequence, 5'-GAAATTTTCGC-3' possessing partial dyad symmetry.

Mutations which result in the constitutive expression of maltase and maltose permease have been isolated using strains carrying different *MAL* loci. Selection of these mutations was done either indirectly by reverting nonfermenting mutant strains to maltose fermenters or, in one case, directly from maltose fermenting inducible strains. In the latter case, the constitutive mutations were isolated in inducible strains carrying the *MAL1*, *MAL2*, *MAL3*, and *MAL6* loci and all of the mutations were shown to be linked to the *MAL* locus (Needleman and Eaton, 1974). Two constitutive revertants of a *mal1* strain were described by Rodicio (1986). Both of the mutations were linked to *MAL1* and one was found to be partially dominant to the wild-type

MAL1 allele. Five *MAL2*-linked constitutive revertants of a *mal2* nonfermenting mutant strain were isolated by Zimmerman and Eaton (1974). Three of the five were resistant to glucose repression and all were partially dominant to the inducible *MAL2* allele. The *MAL4* locus was originally identified by Winge and Roberts (1950) as an X-ray induced revertant of a maltose nonfermenting strain. A strain carrying a *MAL4* locus was later shown to synthesize high, noninduced levels of maltase even in glucose grown cells (Khan and Eaton, 1971). The constitutive allele *MAL43-C* from this strain was cloned and later shown to encode an altered *MAL*-activator responsible for both the constitutive and glucose repression-insensitive phenotype (Charron and Michels, 1987; Andrew Gibson, unpublished results). The *MAL43-C* allele has been shown to be dominant to the wild-type inducible *MAL63* homologue. An inducible *MAL43* allele is not available in the stock collections.

The *MAL64*-constitutive mutations.

Constitutive mutations of a different type have been isolated in strains carrying the *MAL6* locus (ten Berge et al., 1973 a,b; Dubin et al., 1986). ten Berge and coworkers (1973, 1974) isolated maltose fermenting revertants in a nonfermenting *mal6* mutant strain, *mal6-13*. The *mal6-13* allele was shown to contain a substitution of an approximately 100 basepair region in *MAL63* (Jeong Kim,

personal communication). Most of these revertants constitutively synthesized maltase and maltose permease, were recessive to the wild-type allele and to the nonfermenting *mal6* mutants and were linked to *MAL6*. In their analysis of these constitutive mutants, they incorrectly proposed that the mutations were alleles of the *MAL63* regulatory gene. It was shown by Dubin et al. (1986) that the genetic alteration producing the constitutive phenotype in one of these revertants, strain C2, mapped to a gene outside the *MAL63-MAL61-MAL62* gene cluster which was called *MAL64*.

A derivative of the constitutive strain C2, called 8-2B, containing the *MAL6*-linked constitutive mutation was used by Dubin et al. (1986) for their molecular genetic analyses. Using gene replacement techniques, they showed that deletion of the *MAL63* sequences in strain 8-2B did not alter the constitutive expression of the structural genes illustrating that the constitutive mutation mapped outside of the *MAL63* gene and did not require the *MAL63* gene for constitutive activity.

The *MAL61* and *MAL62* genes were isolated from strain 8-2B by plasmid rescue and no major structural differences were evident between the wild-type *MAL61-MAL62* genes and the *MAL61-MAL62* genes isolated from strain 8-2B (Dubin et al., 1986). Therefore, alteration of these two structural genes such as insertion of a transposable element like Ty1 had not

occurred. In addition to containing the *MAL6* locus, strain 8-2B contained a partially functional allele of *MAL1* which contains an active copy of the maltase gene, *MAL12*. Deletion of the *MAL61* and *MAL62* genes produced a nonfermenter with reduced but significant levels of constitutively expressed maltase and this residual maltase expression was shown to result from expression of the unlinked *MAL12* gene. Therefore, the constitutive mutation in strain 8-2B was in a *MAL6*-linked *trans*-acting regulatory gene but clearly not in the *MAL63*, *MAL61* or *MAL62* genes. This constitutive, mutant allele was called *MAL64*-C2 and the wild-type allele, *MAL64*.

Another constitutive revertant strain, called R10, was isolated by Dubin et al. (1986) by reverting the nonfermenting strain A9 to a fermenter. Strain A9 contains a deletion/disruption of *MAL63*. Strain R10 was shown to retain the original *mal63::URA3* deletion/disruption and the constitutive mutation was found to be linked to the *MAL6* locus. The constitutive mutation in strain R10 was found to be partially dominant to wild-type *MAL63* and to the *mal63::URA3* deletion/disruption. Using molecular genetic analyses similar to those described for the C2 mutation, the R10 mutation was also shown to affect a *trans*-acting regulatory function. Since the constitutive mutations in strains C2 and R10 are linked to *MAL6*, epistatic to *mal63* and encode a *trans*-acting factor, it was very possible that

both mutations were alterations within the same *MAL64* gene. Given this, the *MAL64* allele of strain R10 was referred to as *MAL64-R10*. Using standard genetic analysis, the *MAL64-R10* constitutive mutation was shown to map 2.3 centimorgans upstream of *MAL63*. Additionally, *MAL64-R10 MAL63* recombinants obtained from this analysis were constitutive demonstrating that *MAL64-R10* is epistatic to both *mal63*Δ and *MAL63*.

MAL64 and its constitutive allele, *MAL64-C2* were cloned by Dubin et al. (1988) and the location of *MAL64* determined. Approximately 12 kbp of DNA to the left of *MAL63* was isolated from strain 332-5A carrying the wild-type *MAL6* locus and from strain 8-2B carrying the constitutive *MAL64-C2* allele by plasmid rescue. The *MAL64-C2* allele was localized within this region as follows. Using the sequences derived from strain 8-2B, an 11.5 kbp region to the left of *MAL63* was deleted from strain 8-2B creating a nonfermenter. A 3.4 kbp *Cla*I fragment derived from the 8-2B cloned sequences was shown to transform this nonfermenting strain to a constitutive strain when present in single copy. Additionally, integrative disruption of this fragment at a *Hin*dIII site within this sequence inactivated the constitutive allele. Therefore, the *MAL64-C2* gene mapped in the region of this site. Similarly, disruption of this 11.5 kbp region in strain R10 created a nonfermenter indicating the constitutive R10 mutation also

mapped to this region.

The role of the wild-type *MAL64* gene in maltose fermentation was investigated by disrupting this gene in a wild-type inducible *MAL6* strain 332-5A (*MAL63 MAL64*) (Dubin et al., 1988). The ability of the *mal64Δ MAL63* disruption strain to ferment maltose was unaffected and the *MAL61, MAL62* transcripts and proteins remain maltose-inducible. It appears then that the wild-type *MAL64* gene does not play an essential role in maltose fermentation in inducible *MAL6* strains nor does it encode an essential protein under these growth conditions. Clearly, only the *MAL61, MAL62* and *MAL63* gene products are required for inducible maltose fermentation. Each of the five dominant *MAL* loci contains the three genes which are structurally and functionally homologous to *MAL61, MAL62* and *MAL63*, but Southern analysis of the cloned *MAL* loci has shown that besides *MAL6, MAL3* contains a *MAL64* counterpart (Charron et al., 1989; Michels et al., 1992). This finding further supports the conclusion that *MAL64* is nonessential for maltose fermentation.

Dubin et al. (1988) used Southern analysis to determine the structural homology between *MAL63* and *MAL64*. Probes from both the *MAL61* and *MAL63* genes show homology to the DNA sequences containing *MAL64*. The results suggest that the chromosomal region containing *MAL64* represents a tandem duplication of the DNA sequences derived from *MAL63* and portions of *MAL61* but, because of a large number of sequence

variations, this duplicate copy is unable to function as an activator of the *MAL* structural genes. Even given these significant sequence differences, *MAL64* is clearly able to be activated by mutation to serve as a constitutive activator of *MAL* gene transcription. The genetic or evolutionary basis for this extensive variation is not clear.

Transcriptional expression of *MAL64* was investigated using Northern analysis. A wild-type *MAL6* strain (*MAL63 MAL64*), when grown under noninducing conditions, utilizing a *MAL64* specific probe, expresses low levels of a 2.0 kb transcript (Dubin et al., 1988). When grown in maltose, the levels of the 2.0 kb transcript increase and a 1.4 kb transcript appears. When *MAL64* was deleted (*MAL63 mal64*Δ) both transcripts were undetectable. Synthesis of the 2.0 kb constitutive and 1.4 kb maltose-inducible transcripts are dependent on *MAL63* since neither *MAL64* transcript is detected in a *MAL63* deletion/disruption strain (*mal63*Δ *MAL64*) grown in galactose or galactose plus maltose. Constitutive strain, R10 (*mal63*Δ *MAL64-R10*) constitutively expresses high levels of the 1.4 kb and 2.0 kb species and deletion of this allele results in loss of constitutive synthesis of both transcripts. These results indicate that the 2.0 kb and 1.4 kb transcripts are products of the *MAL64* gene. Since wild-type strains (*MAL63 MAL64*) require the presence of *MAL63* in order to detect *MAL64* transcripts but

constitutive strains do not, this suggests that the expression of *MAL64* constitutive gene transcripts are autoregulated. Two *MAL63* homologous transcripts are detected in inducible *MAL6* strains, a constitutive 1.6 kb transcript and a slightly maltose-inducible 2.0 kb transcript (Needleman et al., 1984). Deletion of the *MAL63* gene eliminates the 1.6 kb transcript but only reduced the level of the 2.0 kb transcript (Dubin et al., 1988). This shows that this 2.0 kb species is at least in part, derived from another gene besides *MAL63*. The homology between *MAL63* and *MAL64* raised the possibility that the 2.0 kb transcript detected by *MAL63* probes was actually the product of the *MAL64* gene. The results presented by Dubin et al. (1988) do not support this since normal levels of both the 2.0 and 1.6 kb *MAL63* homologous transcripts are present in a *MAL63* mal64Δ strain but when probed with *MAL64*-derived probes, no homologous transcripts are detectable. These results suggest that the 2.0 kb transcript is the product of another gene having a greater homology to *MAL63* than *MAL64* and whose expression is dependent on the *MAL63* gene product.

The goal of my thesis research was to determine the mechanism of constitutivity resulting from mutations in *MAL64*. For this, we sequenced the *MAL64*, *MAL64-C2* and *MAL64-R10* alleles. We found that the *MAL64* and *MAL63* gene products are both 470 amino acids long and are 85% identical. Surprisingly, the constitutive alleles contained

nonsense mutations at codons 282 (*MAL64-R10*) and 307 (*MAL64-C2*) creating constitutive activators by truncation of a substantial portion of their C-terminal regions. We constructed hybrid genes between *MAL63*, *MAL64*, and *MAL64-C2*. The results suggest that the maltose-responsive region of the *MAL63*-encoded activator is in the C-terminal half of the protein but a required function for this region was also uncovered. This region showed significant amino acid sequence variation between *MAL63* and *MAL64* in the final C-terminal residues 457-470. In *MAL63* and not in *MAL64*, this region is rich in acidic residues and contains a potential casein kinase II phosphorylation site. We have shown that a *MAL63* allele containing a nonsense mutation at codon 457, therefore deleting this site, is noninducible. For the first time in our study on *MAL63*, we have been able to detect MAL63p by overexpression in yeast and have shown that overexpression of this activator does not result in a constitutive phenotype. Lastly, we undertook experiments to localize the transcriptional activation domain of MAL63p by making *MAL63* gene fusions to the DNA-binding domain of the bacterial repressor gene, *lexA* and assaying the ability of these encoded fusion proteins to activate transcription of a reporter gene, *lacZ*, which contains LexAp binding sites in its upstream promoter region. We found that only the full length MAL63p, when fused to the LexA DNA-binding domain, activates transcription, provided maltose is present, from

the *lacZ* reporter gene. We were unable to create a transcriptionally active LexA fusion protein when either the N-terminal residues (1-215) or the C-terminal residues (216-470) of MAL63p were separately fused to the DNA-binding domain of LexA. However, we have shown that the final C-terminal residues of MAL63p are required in this LexA-MAL63p fusion for activity since deletion of these residues abolishes the maltose-inducible transcription activation function.

Materials and Methods

Yeast strains. The yeast strains listed below were utilized for various aspects of the work described in this thesis. Included are the genotype of each strain and a literature reference describing its origin.

8-2B (*MATa MAL64-C2 mal63-13 MAL61 MAL62 MAL12 ura3-52 leu2-3, 112 trp1 ade*) (Dubin et al., 1986)

R10 (*MATa MAL64-R10 mal63Δ:URA3 MAL61 MAL62 MAL12 leu2-3, 112 trp1 his*) (Dubin et al., 1986)

340-2A (*MATα mal13Δ MAL11-2 MAL12 ura3-52 ade*) (R.B. Needleman)

Sc340 (*AGT1 MAL12 MAL31 MAL32 his3 leu2-3, 112 ade1 MEL1 GAL10-GAL4-URA3* integrated at the *his3* locus) (Mylin et al., 1990a).

Sc490 (*AGT1 MAL12 MAL31 MAL32 his3 leu2-3, 112 ade1 MEL1 ura3-52 ADH1-GAL4* cassette) (Mylin et al., 1990a).

332-5AΔF1-5 (*MATa mal64:LEU2 MAL63 MAL61 MAL62 mal13 mal11 MAL12 ura3-52 trp1 his* (Dubin et al., 1988).

B16 (*MAL23 MAL21 MAL22 mal11 MAL12 mal13 ura3-52 his3*) (Our laboratory)

YPH500 (*AGT1 MAL12 MAL31 MAL32 ura3-52 his3 leu2*) (Roger Brent)

Bacterial and yeast strain growth. Yeast strains were grown at 30°C on either rich media, YP (1% wt/vol yeast extract, 2% wt/vol peptone) or minimal media, SM (0.67% yeast nitrogen base without amino acids) plus the appropriate carbon sources, amino acids and nitrogen base supplement as required (Sherman et al., 1986). The various carbon sources were added to this media as indicated in each experiment. Uninduced conditions is growth in 3% glycerol plus 2% lactic acid. To this, 2% maltose was added for induced conditions, or 2% glucose plus 2% maltose for repressed conditions.

E. coli strain, RR1 was grown in LB medium or LB medium plus ampicillin if harboring an ampicillin resistant plasmid. Bacterial strain JM109 *recA*-, used as a host strain for M13 phage constructs, was plated on M9 media [0.6% (wt/vol) $NaHPO_4.H_2O$, 0.3% (wt/vol) KH_2PO_4, 0.05% (wt/vol) NaCl, 0.1% (wt/vol) NH_4Cl, 1 mM $MgSO_4$, 0.2% (wt/vol) glucose, 0.1 mM $CaCl_2$ and 0.01 mg/ml B1 thiamine) and grown in liquid YT medium (0.8% tryptone, 0.5% yeast extract and 0.5% NaCl).

Recombinant DNA techniques. DNA preparations, restriction enzyme digestions and other DNA manipulations were done according to procedures given in Ausubel et al. (1989) and Maniatis et al. (1982). Several routine procedures involved the use of commercial kits. Single-stranded M13 DNA was prepared according to both Vieira et al. (1987) and

protocols described by Amersham. Sequencing was done by the dideoxynucleotide method using a kit from IBI (Sequenase Version 2.0) (Sanger et al., 1977). All *MAL64* sequences close to the primer were able to be read so as to overlap them at the centrally located *Eco*RI site and form a continuous sequence from the two gene fragments. Overlapping sequences were found by using the GAP program available from the Genetics Computer Group provided in their Sequence Analysis Software Package (Devereux et al., 1984). Both DNA strands of *MAL64* were sequenced in partially overlapping steps using a series of oligonucleotides complementary to previously sequenced regions. Oligonucleotides were synthesized using the ABI Oligonucleotide DNA synthesizer Model 380. The reagents and purification procedures used were from Applied Biosystems Inc. These *MAL64*-derived oligonucleotide primers were also used to sequence the constitutive alleles. Labelling of DNA was done by the random priming method using a kit from Promega and radioactive ^{32}P-dCTP from Amersham. Oligonucleotide-directed site mutagenesis was carried out using the M13 Muta-Gene *In Vitro* Mutagenesis kit from Biorad. Gene fragments used for cloning were often isolated from agarose gels with Gene Clean (Bio 101) before ligation with T4 DNA Ligase (New England Biolabs).

Yeast transformation. Yeast strains were transformed by

making cells competent with 0.1 M lithium acetate using the transformation method of Ito et al. (1983). Transformants were selected on SD minimal media lacking the appropriate amino acid(s). Integrative transformants were screened by Southern analysis (Southern, 1975) to demonstrate that insertion had occurred at the expected site and in single copy. Transformants carrying episomal plasmids were screened to show loss of the plasmid bourne nutritional marker under nonselective growth conditions, that is, YPD (1% wt/vol yeast extract, 2% wt/vol peptone and 2% dextrose).

Plasmid rescue of the *MAL64-R10* allele from constitutive strain, R10. The plasmid pMJC6ΔCla (Figure 1) was targeted to integrate at the *Bgl*II site 5′ of *MAL63* in strain R10 by digestion with *Bgl*II. Genomic DNA was isolated from a transformant carrying a single integrated copy of the plasmid (tested by Southern analysis), digested with *Bam*HI, and size-separated on a low-melting agarose gel. Fragments of approximately 10-20 kilobases in size were extracted from the gel, self-ligated and transformed into *E. coli*. A single ampicillin resistant clone was found which harbored plasmid pBamR10 containing a 12.5 kbp insert as shown in Figure 1. Based on the results of Dubin et al. (1988), this insert was expected to contain the *MAL64-R10* allele and genomic flanking sequences. The 3.5 kbp *Cla*I fragment shown

by Dubin et al. (1988) to contain *MAL64* was then subcloned from pBamR10 into the integrative plasmid, YIp5 (Rose and Broach, 1991); this construct is called pMAL64R10.

Construction of plasmid YCpMAL63ΔR. To simplify the construction of gene fusions between *MAL63* and *MAL64*, a 5′ upstream *Eco*RI site in *MAL63* had to be deleted. A *Sal*I fragment containing *MAL63* was cloned into the M13 vector mp19 and used for *in vitro* mutagenesis as described above. A 52-base long oligonucleotide (5′ACAATGCATATTACTATTGAGTTAAAATCAAATGCATCAATTGCGGGACCAT-3)′ complementary to the sequences flanking the *Eco*RI site upstream of *MAL63* but lacking the *Eco*RI sequence itself was used to delete this site. Deletion of this site was tested by loss of the *Eco*RI site by restriction enzyme digestion. The mutagenized *Sal*I fragment was then subcloned into the integrative plasmid, YIp5. To avoid sequencing the entire *MAL63ΔR* allele, the *Kpn*I-*Bam*HI fragment from this YIp5 clone containing the entire yeast insert except for an approximately 200 basepair region surrounding the deleted *Eco*RI site, was then replaced by an identical fragment which had not been subjected to mutagenesis. The *Sal*I fragment from this YIp5 clone was then subcloned into the yeast CEN vector, YCp50 (Rose and Broach, 1991). This construct is called YCpMAL63ΔR.

Placement of the hemagglutinin epitope tag on the N-terminus of MAL63p. Antibody directed against a peptide from the influenza virus hemagglutinin protein is commercially available (Berkeley Antibody Company, La Jolla, CA). A DNA sequence encoding this peptide was inserted at the 5′-end of *MAL63*, as follows, to provide an epitope-tagged allele of *MAL63* to be used for protein expression studies. For this construction, we used an allele of *MAL63* developed by Jeong Kim (1991, Ph.D. thesis) in which an *Nco*I site was created at the initiating methionine codon of *MAL63* and an internal *Nco*I site was deleted by oligonucleotide-directed *in vitro* mutagenesis. This allele is referred to as *MAL63/N*. The following oligonucleotides were made,

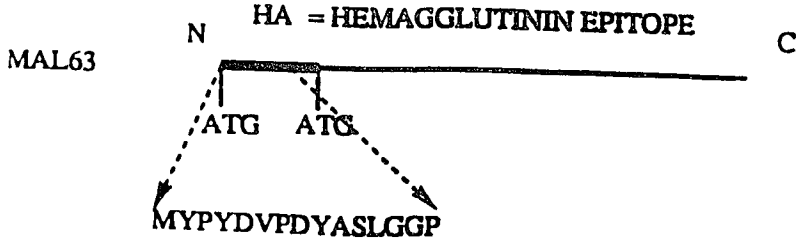
5′- TATCGATTAATACCATGGTTTAAATGTACCCATACGACGTTCCAGATTAC-3′

and

5′-TATCGATTAATACCATGGATCCGGGACCACCCAAGCTAGCGTAATCTGGAAC-3′

which upon annealing and extension encode the peptide MYPYDVPDYASLGGPGS. The first twelve residues of this peptide encode the hemagglutinin peptide epitope recognized by the 12CA5 antibody (Wilson et al., 1984; Field et al., 1988). The GGP residues are intended to create a hinge region so as to expose the epitope for better antibody recognition. The GS residues were added here to create a *Bam*HI site for identifying the presence of the epitope sequence. The oligonucleotides were designed so that the 12 bases at the 3′ ends anneal to each other and contain flanking *Nco*I sites

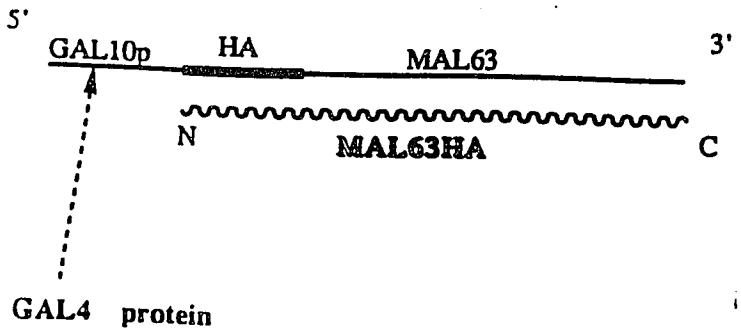
and this internal *Bam*HI site. The annealing was done *in vitro*, extended with all four deoxynucleotides using the Klenow fragment of DNA polymerase, the reaction product was run on a PAGE gel and an approximately 70 basepair fragment was isolated, eluted and purified from the gel according to Ausubel et al. (1989). Using a plasmid carrying the *Sal*I fragment containing the *MAL63/N* allele cloned into M13 mp19, the purified fragment was digested with *Nco*I and ligated to the *Nco*I site at codon 1 of *MAL63/N* and the ligation mix used to transform *E. coli* strain, MV1190. The resulting transformants were screened for the presence of plasmids containing the *Bam*HI site within the inserted fragment. The orientation of the epitope-encoding insert was determined by sequencing this region and demonstrating that only a single copy was present. The allele is referred to as *MAL63/HA*, shown below:



The *Sal*I fragment of this mp19 clone was subcloned into YCp50 to produce plasmid YCpMAL63/HA.

Expression of *MAL63/HA* from the *GAL10* promoter. In order to clone *MAL63/HA* downstream of the *GAL10* promoter in the overexpression vector, YEp51 (Broach et al., 1983), a *Xho*I

site was created 60 basepairs upstream of the coding region of *MAL63/HA* by *in vitro* mutagenesis of the mp19 cloned allele. The oligonucleotide used had the following sequence:5′-GTCTGTAAAGTGGAAAACTCGAGAACCCAGTTTTTTAGCTGCCTG-3′. Following the mutagenesis procedure, the full coding region of *MAL63/HA* was sequenced to confirm the creation of the *Xho*I site and no other unwanted mutations. An approximately 2.3 kbp *Xho*I-*Sal*I fragment including 60 basepairs upstream of the epitope-tagged *MAL63/HA* allele was then subcloned into YEp51 which had been digested with *Sal*I, placing *MAL63/HA* downstream of the *GAL10* promoter, to form plasmid YEpMAL63/HA:



Creation of *MAL63/HA-NS457*. The *Eco*RI fragment containing codons 215-470 of *MAL63* was cloned into the M13 vector mp18. A mutagenic primer creating a translation termination signal at codon 457 of *MAL63* and a downstream *Bam*HI site was used for *in vitro* mutagenesis of this fragment. The sequence of the oligonucleotide was 5′-GTCGTCTTCATCTTTGGAGGGATCCTTTAATTACAAAGGTTTAGAAATGGGCA-3′. The resulting clones were screened by digestion with *Ava*I

and *Bam*HI and sequenced from the internal *Cla*I site in this *Eco*RI fragment through the termination codon and the *Bam*HI site. A *Cla*I fragment in this mp18 clone containing this mutagenized region was used to replace the homologous fragment in plasmid YCpMAL63/HA (see above). This allele is referred to as *MAL63/HA-NS457* and the plasmid as YCpMAL63/HA-NS457. *MAL63/HA-NS457* was fused to the *GAL10* promoter of YEp51 for overexpression as follows. Plasmid YEpMAL63/HA was digested with *Bam*HI which cuts at the *Bam*HI site within the hemagglutinin tag sequence at the 5'-end of the coding region and in YEp51 vector sequences downstream of this *MAL63/HA* allele, and religated to produce YEpMAL63/HAΔBam. The homologous *Bam*HI fragment from YCpMAL63/HA-NS457 was then subcloned into plasmid YEpMAL63/HAΔBam at the *Bam*HI site to form plasmid YEpMAL63/HA-NS457.

Galactose-inducible expression of *MAL63/HA* and *MAL63/HA-NS457* in strains Sc340 and Sc490. The protocol of Mylin, et al. (1990 a,b) was used. Strain Sc340 (*URA3, his3, leu2, ade1*) contains a *GAL10-GAL4-URA3* cassette integrated at the *HIS3* locus and strain Sc490 (*ura3-52, leu2, his3, ade1*) contains an *ADH1-GAL4* cassette integrated into its genome. Both strains are nonfermenters and most likely contain *MAL1g* and *MAL3g* sequences since they are derived from strain S288C. This was confirmed by Southern analysis (data not

shown). Both strains are also complemented by functional *MAL63* clones, enabling them to ferment maltose, thereby confirming these findings. Strain Sc340 carrying either plasmid YEpMAL63/HA or YEpMAL63/HA-NS457 was grown to saturation in SM media lacking leucine containing 2% glucose. An aliquot of the cells was then transferred to SM media lacking leucine containing 3% glycerol and 2% lactate and grown to an OD_{600} of 0.4. Galactose was then added to a final concentration of 2% and the culture allowed to induce for 2-4 hours. Cell extracts were prepared (see below) from approximately 50 ml of this culture and the remaining cells were spun down, resuspended in 100 ml of SM media lacking leucine containing 2% maltose and allowed to grow for 1-2 hours. Cell extracts were prepared from 50 ml of this culture and the remaining cells were spun down and resuspended in SM media lacking leucine containing 2% glucose and allowed to grow for up to 2 hours. Cell extracts were prepared from this final culture. All extracts were analyzed by Western blotting. For strain Sc490 harboring either YEpMAL63/HA or YEpMAL63/HA-NS457, cells were grown in SM media lacking leucine plus maltose or glucose to an OD_{600} ~ 0.5-1.0. Extracts were prepared as before.

Preparation of protein extracts. Protein extracts were prepared according to Mylin et al. (1989). Cell culture

aliquots were chilled on ice for 10 minutes, pelleted at 4°C, washed once with cold water and once with breaking buffer (lacking protease inhibitors and reducing agent). Pellets could be stored at -70°C for less than 2 weeks. Cells were allowed to thaw on ice for 30 minutes and then resuspended in 0.3 ml of breaking buffer (50 mM $NaPO_4$ pH 7.2, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.2 mM Na_3VO_4, 2 μM pepstatin, 0.6 μM leupeptin and 20 μg/ml aprotinin). An equal volume of 0.45 mm glass beads was added and the cells were broken with 6, 20 second pulses on a vortex mixer with equal periods of rest on ice. Cell lysates were removed from the beads, the beads were rinsed with 0.2 ml of fresh breaking buffer and recovered extracts were combined. Electrophoresis sample buffer concentrate 5X (5% SDS, 50% sucrose, 50 mM Tris-Cl (pH 8.0), 5 mM EDTA, 160 mM DTT) was then added to a final 1X concentration and boiled for 5 minutes. The extracts were centrifuged for 5 minutes at room temperature and the supernatants were collected and stored at -70°C for Western blot analysis and protein estimation.

Protein determination. Protein concentrations in extracts containing SDS were estimated by the Lowry method according to Ausubel et al. (1989) using bovine serum albumen as a standard and excluding deoxycholate from the trichloroacetic acid precipitation step. Other protein extracts from β-

galactosidase assays or maltase assays were estimated using the BioRad Protein Dye Reagent using bovine serum albumin as a protein standard.

Protein-A Sepharose purification of the anti-hemagglutinin antibody from mouse ascites fluid. Mouse ascites fluid containing anti-hemagglutinin antibody, 12CA5 was purchased from the Berkeley Antibody Company (Babco). Purification of the antibody was performed as outlined by Dr. Jeff Field (Cold Spring Harbor; personal communication). Protein A-Sepharose (Pharmacia) was resuspended and equilibrated in 0.05 M Tris, pH 7.5 and 2 ml of this suspension was placed in a 3 cc syringe after placing a small piece of Whatman paper in the mouth of the syringe. Ascites fluid was loaded (0.5 ml) and allowed to flow through slowly and the flowthrough collected. This flowthrough was loaded onto the Sepharose column again, dripping slowly, collecting it again and storing this final flowthrough at -20°C in 0.2% NaN_3. The column was then washed with 10 ml of Tris buffer dripping slowly so as to allow for binding of the antibody and removal of any unwanted materials. Antibody was eluted with 0.1 M glycine, pH 2.9, fractions were collected (100 μl) and neutralized by addition of 100-200 μl of 1M Tris, pH 7.5. Aliquots of each fraction were dot blotted onto nitrocellulose and probed with secondary antibody, anti-mouse IgG peroxidase (Sigma) and developed by use of the ECL

detection system (Amersham). Fractions containing antibody were pooled together and dialyzed against 1X PBS buffer at 4°C overnight. These dialyzed fractions were stored in 0.2% NaN_3 and kept at 4°C.

Western blotting analysis using the ECL detection system. Aliquots of protein extracts and prestained protein markers (Bethesda Research Laboratories) were run on a PAGE gel by using the Laemmli method with an 8% separating gel and processed according to the methods in Ausubel et al. (1989). Protein gels were transferred to nitrocellulose for 1-2 hours at 80 volts in transfer buffer, pH 8, containing 20 mM Tris, 150 mM glycine and 20% methanol. Filters were rinsed with deionized, distilled water and stained with Amido Black (Sigma) to ensure that an approximately equal protein transfer had occurred since equal amounts of protein were loaded onto the PAGE gels for stability studies. Filters were destained in deionized water and blocked overnight at 4°C in blocking buffer (1X PBS from a 10X PBS stock containing 80 g/l NaCl, 2 g/l KCl, 6 g/l Na_2HPO_4, and 2 g/l KH_2PO_4 plus 10% nonfat dry milk and 0.1% Tween 20). Filters were then incubated at room temperature on a Hoefer Red Rocker for 2 hours with mouse anti-hemagglutinin antibody in blocking buffer at a concentration of 1/50. Filters were rinsed in 1X PBS buffer containing 0.1% Tween 20 for 15 minutes and then incubated with goat anti-mouse IgG (Sigma)

at a concentration of 1/1000 for 1 hour at room temperature. Filters were rinsed once again and then incubated with mouse PAP antibody (Sigma) at a concentration of 1/1000 for 1 hour at room temperature and rinsed for the final time for 15 minutes. An equal volume of each reagent contained in the ECL detection kit (Amersham) was reacted with the filters for 1 minute, drained of excess reagents, placed on a piece of Whatman paper and covered with a piece of Saran wrap. The filters were then exposed to ECL film (Amersham) for different time periods (from 15 seconds to 10 minutes) and developed.

Cloning of *MAL63* into the *lexA* fusion plasmid, pSH2-1. The approximately 1.0 kilobasepair *Eco*RI fragment of *MAL63* containing codons 215 to 470 plus downstream sequences was fused in frame to the DNA-binding domain of LexAp (codons 1-87) in plasmid, pSH2-1 (Hanes and Brent, 1989). This fusion was called pLexA:MAL63(215-470). It was also necessary to clone the full length *MAL63* gene downstream of the LexAp DNA-binding domain. This was done by excising a *Bam*HI-*Sal*I fragment from plasmid YEpMAL63/HA and cloning this into pSH2-1 digested with *Bam*HI and *Sal*I. This joined the *Bam*HI site within the hemagglutinin epitope of *MAL63/HA* to the *Bam*HI site in the polylinker region downstream of codon 87 of *lexA* but the proper reading frame was not achieved in this fusion. Therefore, the fusion plasmid was then

digested at the unique *Bam*HI site, the overhanging bases excised with S1 nuclease (IBI) and the blunt ends religated. Double-stranded sequencing (according to protocols from Promega) of the resulting clones which lost the *Bam*HI site was done to determine that a proper reading frame could now be established. One plasmid which contains codons 2-470 of *MAL63* (and none of the epitope residues) fused to codons 1-87 of *lexA* is referred to as pLexA:MAL63(2-470). By exchanging the C-terminal *Eco*RI fragment present in pLexA:MAL63(2-470) with the homologous fragment which had been mutagenized by creation of a nonsense mutation at codon 457 (see above), the clone pLexA:MAL63(2-456) was made encoding a truncated MAL63p (residues 2-456) fused to residues 1-87 of LexAp. Another fusion of *MAL63* to the DNA-binding domain of *lexA* containing only the N-terminal residues 1-215 was kindly constructed by Andrew Gibson in our lab by PCR techniques. Two oligonucleotide primers were used to amplify these residues from a *MAL63* clone: 5′-CGGAATTCATGGGTATTGCGAAACAGTCT-3′ (which anneals to the noncoding strand at codons 1-7) and 5′-TGAGTTGAAGTTCACCGA-3′ (which anneals to the coding strand at codons 219-224). In a PCR reaction, these oligonucleotides were able to amplify a 678 basepair fragment of *MAL63* containing codons 1-224. This fragment was then digested with *Eco*RI to generate a 651 basepair fragment of *MAL63* containing codons 1-215 and having *Eco*RI

sticky ends. This digested fragment was then ligated in frame into pSH2-1 digested with *Eco*RI. *E. coli* transformants containing the 651 basepair *Eco*RI fragment were screened by double-stranded DNA sequencing across the fusion junction using an available primer which anneals to the *MAL63* insert. By sequencing, the orientation and reading frame of the fusion was verified. This fusion was called pLexA:MAL63(1-215). Since residues 1-215 of *MAL63* do not contain an internal termination codon, upon fusion of these residues to LexAp, a termination codon was provided in the polylinker region of pSH2-1 downstream of the *Eco*RI site. This involved the addition of 24 residues (FPGIRRPAAKLIPGEFLMIYDFYY), derived from polylinker sequences, to the fusion pLexA:MAL63(1-215) before the terminator was reached. A positive control plasmid which activates reporter gene expression in this system, pLexA:SNF2 (Laurent et al., 1991) and the reporter plasmid (pSH18-18) containing the *GAL1-lacZ* fusion and six upstream LexAp binding sites was given to us by Brehon Laurent from the lab of Dr. Marian Carlson (Laurent et al., 1991; Hanes and Brent, 1989). Rabbit antiserum raised against the LexA protein was kindly provided by Roger Brent and used to assay the levels of expression of the various LexA fusion proteins. Plasmid pRSMAL63 was made in our lab by Andrew Gibson which contains the ~ 3.2 kbp *Sal*I fragment of *MAL63* cloned into the *Sal*I site of plasmid, pRS315, which contains

a *LEU2* marker (Sikorski and Hieter, 1989).

Enzyme assays. Maltase activity was assayed according to Dubin et al. (1985). β-galactosidase activity was assayed according to Ausubel et al. (1989) from crude extracts and the specific activity of the enzyme was calculated. Activities were calculated on three aliquots of extract and on two separately grown cultures. Variations in activity levels from individual cultures were no more than 15% for both types of assays.

Chapter I: Structural and functional comparison of *MAL63*, *MAL64*, and the *MAL64*-constitutive alleles

Results

Cloning and localization of *MAL64-R10*.

Figure 1 shows the structure of the *MAL6* locus located on chromosome VIII of *Saccharomyces cerevisiae* illustrating the location of the *MAL63* and *MAL64* genes and the genomic fragments used to subclone the two genes (Dubin et al., 1988). Dubin et al. (1988) cloned and characterized the constitutive allele, *MAL64-C2*. They showed that the *MAL64-C2* allele was contained within the 3.5 kbp *Cla*I fragment shown in Figure 1 and that *MAL64-C2* encoded a dominant, constitutive activator of the *MAL* structural genes. Using the same plasmid rescue procedure, we isolated plasmid pBamR10 (Figure 1) containing the *MAL64-R10* allele from strain R10 as described in Materials and Methods.

The homologous *Cla*I fragments from the *MAL6* locus of the wild-type strain and the two constitutive strains were each cloned into the integrative plasmid, YIp5 (Rose and Broach, 1991) and were called pMAL64 (containing the wild-type *MAL64* gene), pMAL64C2 (containing the *MAL64-C2* allele) and pMAL64R10 (containing the *MAL64-R10* allele). Transformants carrying a single copy of plasmids pMAL64, pMAL64C2 and

Figure 1. Diagram of the *MAL6* locus on chromosome VIII of *Saccharomyces cerevisiae*. A restriction endonuclease map of the chromosomal region containing the *MAL6* locus is shown (Dubin et al., 1988). The approximate location of the open reading frames of each gene is shown above the map along with the direction of transcription (Charron et al., 1989; Cheng et al., 1989; Dubin et al., 1988; Kim et al., 1988; Needleman et al., 1984). The DNA fragments indicated as MAL63 and MAL64 are the yeast inserts used to subclone the *MAL63* and *MAL64* genes, respectively, into the various vectors for the experiments described here. Plasmid pMJC6ΔCla contains the indicated yeast insert in the vector YIp5ΔCla which was derived from YIp5 by deletion of the unique *Cla*I site (Charron et al., 1988). Genomic fragment pBamR10 is the fragment rescued from strain R10 which contained the *MAL64-R10* constitutive allele. Restriction endonuclease sites are as follows: A, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; Hp, *Hpa*I; M, *Mlu*I; N, *Nco*I; P, *Pst*I; R, *Eco*RI; S, *Sal*I; Sm, *Sma*I; Ss, *Sst*I; Xb, *Xba*I; Xo, *Xho*I.

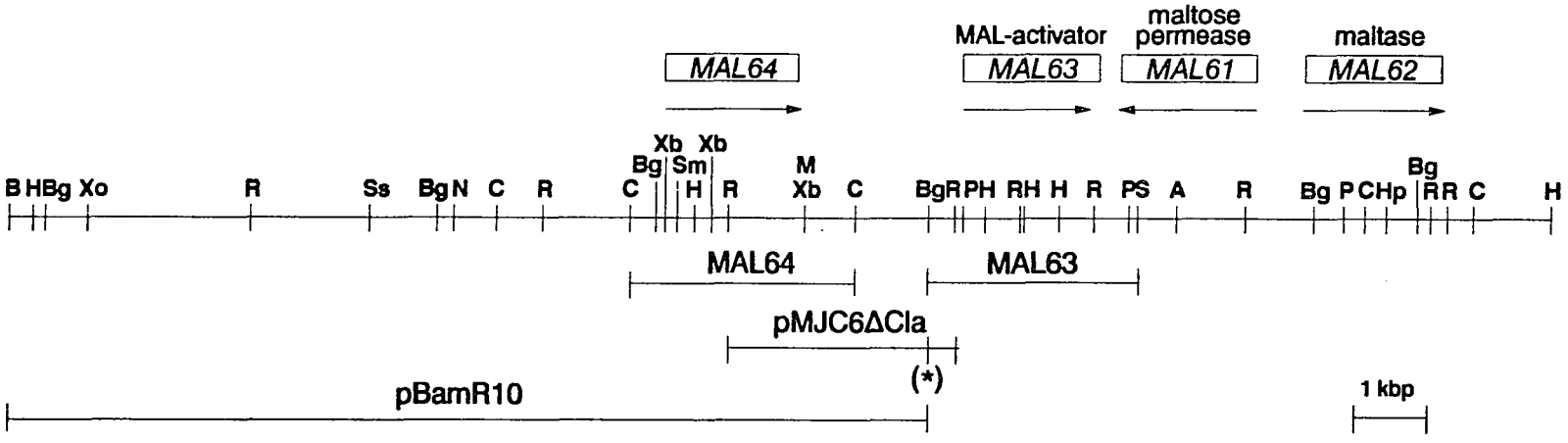


Figure 1: Diagram of the MAL6 locus on chromosome VIII of Saccharomyces cerevisiae

pMAL64R10 in strain 340-2A were obtained by integration of the plasmid at the chromosomal *ura3-52* locus. Strain 340-2A contains a natural variant of the *MAL1* locus containing only the structural genes encoding a maltose permease (the *AGT1* gene encoding a general α-glucoside transporter) and maltase (encoded by *MAL12*) and lacks sequences encoding a functional *MAL*-activator (Charron et al., 1988; Han et al., submitted for publication). The ability of these plasmids to turn on maltase expression in strain 340-2A are presented in Table 1. Clearly, pMAL64R10 contains the constitutive *MAL64-R10* allele. Both plasmids carrying the constitutive alleles complement strain 340-2A and maltase expression in these transformants is fully constitutive. Most notably, maltase expression in the 340-2A transformants containing an integrated pMAL64R10 plasmid under noninduced growth conditions (GL) is twice that under maltose-induced conditions. The basis for the "maltose repression" is not clear but a similar phenomenon is seen with the *MAL43-C* constitutive allele (Andrew Gibson, unpublished results). The results in Table 1 also confirm the finding that the wild-type allele of *MAL64* is unable to complement strains lacking a functional *MAL*-activator gene (Dubin et al., 1988). While some inducible maltase expression is seen for this allele, the level is inadequate to support fermentation. In addition, both constitutive alleles appear to be slightly insensitive to glucose repression. In 340-2A

Table 1. Activation of maltase gene expression by *MAL64*, *MAL64-C2*, *MAL64-R10* and hybrid genes.

Plasmids were constructed as described in Materials and Methods and were transformed into the yeast host strain 340-2A, genotype *AGT1 MAL12* (Charron et al., 1988; Han et al., submitted).

[a] Maltose fermentation was determined in Durham tubes containing YP medium (Sherman et al., 1986) plus 2% (wt/vol) maltose and is measured as the production of acid and gas 1-3 days after inoculation.

[b] Maltase activity was determined as the rate of release of p-nitrophenol from p-nitrophenyl-α-D-glucopyranoside (PNPG) and is expressed as nanomoles of substrate split per minute per milligram of protein at 30°C. GL indicates noninducing growth conditions on YP media plus 3% (vol/vol) glycerol and 2% (vol/vol) lactic acid; GL+M indicates inducing growth conditions on GL media plus 2% (wt/vol) maltose; GL+Glu indicates repressed growth conditions on GL media plus 2% (wt/vol) glucose; and GL+M+Glu indicates repressed growth conditions on GL media plus 2% (wt/vol) each of maltose and glucose.

Table 1. Activation of maltase gene expression by MAL64, MAL64-C2 and MAL64-R10 hybrid genes

Plasmid	Yeast Insert	Fermentation	Maltase activity			
			GL	MAL	GLU	M+GLU
None	-	-	16	38	4	3
pMAL64	MAL64	-	19	110	4	3
pMAL64C2	MAL64C2	+++	635	725	55	54
pMAL64R10	MAL64R10	+++	1725	930	94	107
pMAL64WT/C2	64 RI TGA C2	+++	580	690	40	63
pMAL64C2/64WT	64C2 RI 64	-	22	74	3	5
pMAL64WT/R10	64 RI TGA R10	+++	1600	937	120	107
pMAL64R10/64WT	64R10 RI 64	-	20	66	3	3
YCpMAL63	MAL63	++	30	639	4	3

transformants carrying the maltose-inducible *MAL63* gene on plasmid YCpMAL63, even though *MAL63* is not integrated in the genome but is on a centromere plasmid, maltase synthesis is induced approximately 30 to 35-fold by maltose and repressed 100 to 200-fold below the fully induced levels when glucose is added to the medium. Maltase synthesis in 340-2A transformants carrying the *MAL64*-constitutive alleles, however, is repressed only approximately 10-fold by glucose.

Sequences of *MAL64*, *MAL64-C2* and *MAL64-R10*.

Dubin et al. (1988) found that integration of a DNA fragment containing the *URA3* gene into the *Hin*dIII site of the 3.5 kbp *Cla*I fragment containing the *MAL64-C2* gene (Figure 1) disrupted its function, indicating that *MAL64* was located near this site. The 1.2 kilobasepair *Bgl*II-*Eco*RI fragment from *MAL64* containing this *Hin*dIII site and the adjacent 1.8 kilobasepair *Eco*RI fragment were each subcloned into mp18 and mp19 so that both strands could be sequenced (Yanisch-Perron et al., 1985).

Figure 2 shows the 2008 basepair sequence from the region surrounding the *Hin*dIII site of the wild-type *MAL64* allele beginning to the right of the 5'-*Bgl*II site shown in Figure 1 and extending toward the *MAL63* gene. Within this sequence, a single large open reading frame of 1410 basepairs is found which encodes wild-type MAL64p, a deduced protein of 470 amino acid residues in length as shown in

Figure 2. Nucleotide sequence of the *MAL64* gene and the deduced amino acid sequence of the MAL64 protein (MAL64p). The underlined region upstream of the initiating methionine codon shows homology to the proposed MAL63p binding site of the UAS_{MAL} found in the *MAL61-MAL62* intergenic region. The position of the *Eco*RI site at residues 215 and 216 used for the construction of the hybrid genes is shown. The positions of the nonsense mutations in the *MAL64-C2* and *MAL64-R10* constitutive alleles are indicated above the wild-type sequence.

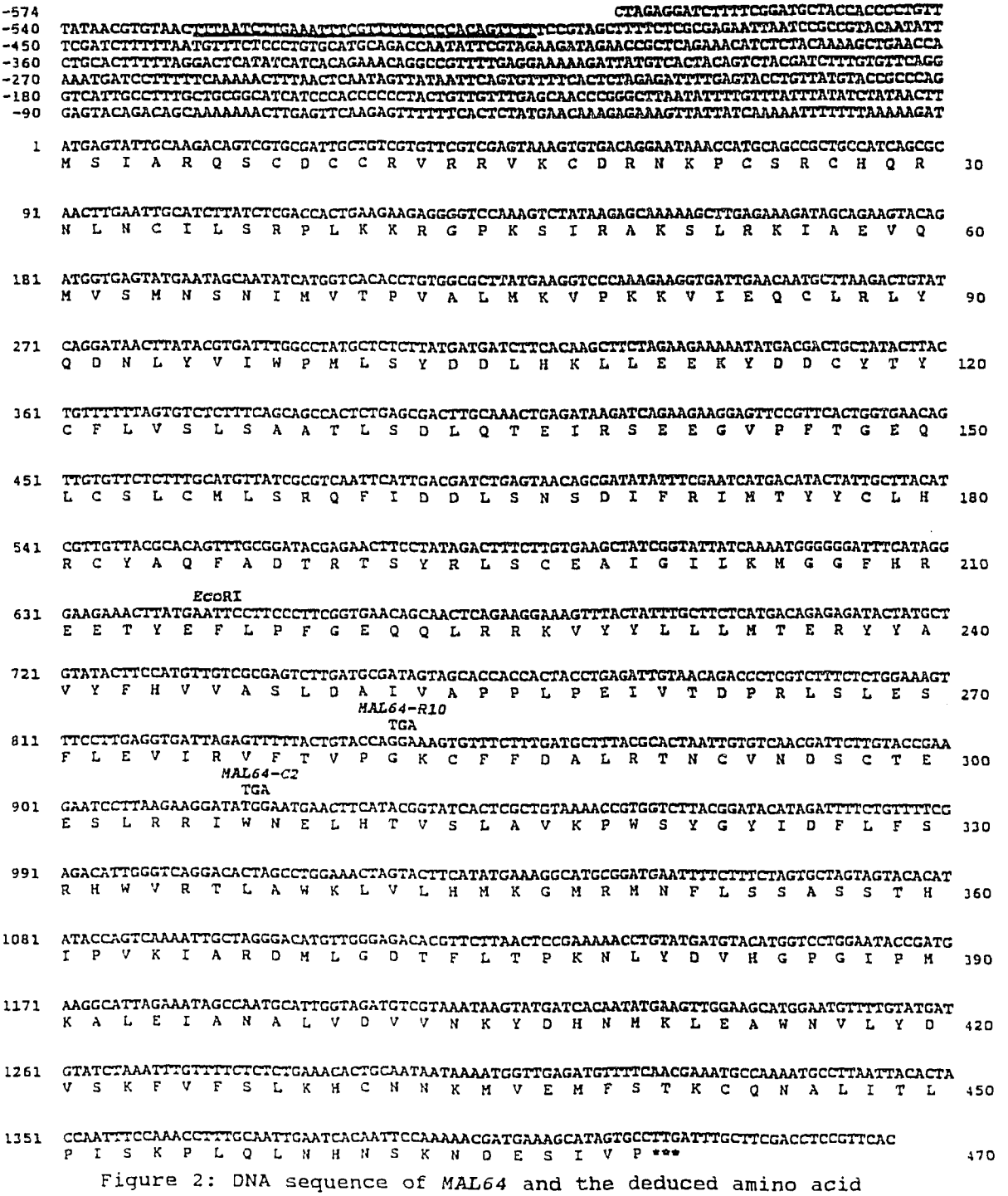
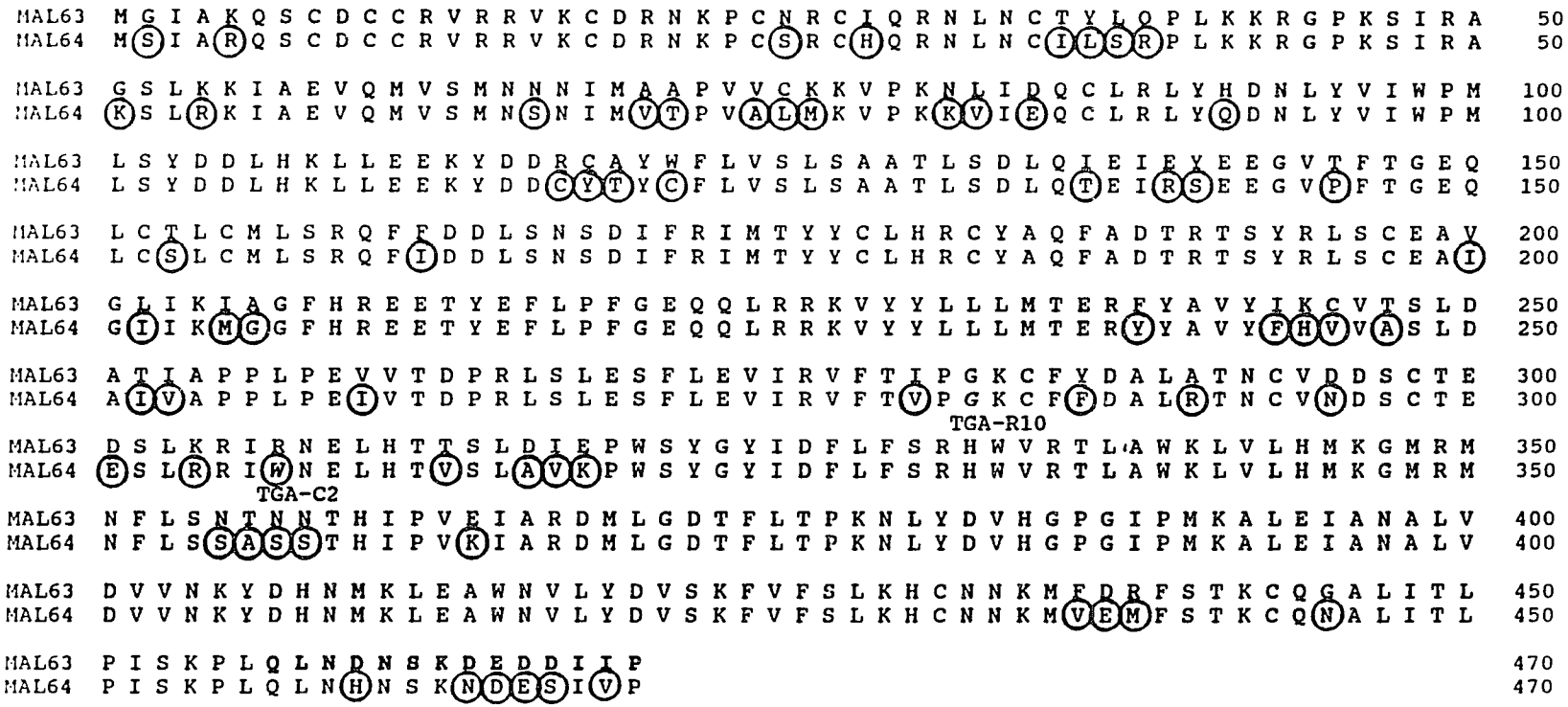


Figure 2: DNA sequence of *MAL64* and the deduced amino acid sequence

Figure 2. The sequence of the *MAL64* coding region is 85% identical on both the nucleotide and amino acid level to that of the *MAL63* gene (Kim et al., 1988) which encodes the inducible *MAL*-activator at *MAL6*. Figure 3 aligns the deduced amino acid sequences of MAL64p with that of the MAL63p. Both genes encode proteins of 470 amino acid residues in length. Variant residues in MAL64p are found scattered throughout the sequence but are usually clustered (see circled residues and residues in boldface type in Figure 3). Within the proposed DNA-binding domain of MAL63p (residues 8-34), differences in MAL64p are seen at residues 25 and 28 and a short cluster of variant residues is present in the region immediately adjacent to residue 34. The upstream sequences of the two genes are 58% identical (not shown) if mismatched and unmatched bases are both included in the calculation. Since transcription of *MAL64* is maltose-inducible and requires a functional *MAL*-activator (Dubin et al., 1988), a search of the 574 basepairs of the *MAL64* upstream sequence revealed a potential *MAL*-activator binding site at base positions -526 to -493 (Ni et al., 1990; see underlined sequence in Figure 2). This site shows a match of 22 out of 35 basepairs to a 34 basepair region containing the proposed upstream binding site of the *MAL*-activator in the *MAL61-MAL62* intergenic region with only a single one basepair gap. This 34 basepair sequence from the *MAL61-MAL62* upstream region includes the sequence of the *in*

Figure 3. Comparison of the deduced amino acid sequences of MAL63p and MAL64p. The deduced sequence of MAL63p was previously reported (Kim et al., 1988). Those residues in MAL64p which differ from MAL63p are circled. The final 14 C-terminal acidic residues are in boldface type. The italicized residues in the MAL64p sequence indicate the positions of the termination codons found in the *MAL64-R10* and *MAL64-C2* alleles.

Figure 3: Amino acid comparison of MAL64 and MAL63 proteins



vitro MAL63p footprint demonstrated by Ni and Needleman (1990) which, when deleted, eliminates maltose-inducible expression of *MAL61* and *MAL62* (Levine et al., 1992).

The position of the alterations in the *MAL64-C2* and *MAL64-R10* alleles were further localized within the 3.5 kilobasepair *Cla*I fragment by the swapping of genomic fragments from *MAL64*, *MAL64-C2* and *MAL64-R10*. For this, hybrid genes were constructed as shown in Table 1 using the conveniently located *Eco*RI site in approximately the middle of the 3.5 kbp *Cla*I fragment containing *MAL64* (or the constitutive alleles) and the *Bgl*II-*Sal*I fragment containing the *MAL63ΔR* allele. Sequence comparison of *MAL63* and *MAL64* (described above) demonstrated that this *Eco*RI site was in a homologous position in both genes. Plasmids pMAL64, pMAL64C2 and pMAL64R10 were all digested with *Eco*RI to delete the C-terminal *Eco*RI fragment and religated to produce the plasmids, pMAL64ΔRI, pMAL64C2ΔRI and pMAL64R10ΔRI. The 1.8 kilobasepair *Eco*RI fragments from the wild-type and constitutive alleles were then individually ligated into these constructs to produce the various hybrids shown in Table 1. For example, the *Eco*RI fragment containing the 3′ end of *MAL64* in plasmid pMAL64 was replaced with the homologous fragments from *MAL64-C2* and from *MAL64-R10* to form plasmids pMAL64WT/C2 and pMAL64WT/R10, respectively. Conversely, the *Eco*RI fragment containing the 3′-end of *MAL64-C2* and *MAL64-R10* in plasmids

pMAL64C2 and pMAL64R10 was replaced with the homologous fragment from wild-type *MAL64* to form plasmids pMAL64C2/64WT and pMAL64R10/64WT, respectively. These hybrid constructions were introduced into strain 340-2A in a single integrated copy at the chromosomal *ura3-52* gene and the phenotype of the transformants determined. As shown in Table 1, only transformants carrying the pMAL64WT/C2 and pMAL64WT/R10 hybrid plasmids fermented maltose and synthesized constitutive levels of maltase comparable to levels synthesized in transformants carrying the pMAL64C2 and pMAL64R10 plasmids, respectively. Transformants carrying the pMAL64C2/64WT and pMAL64R10/64WT hybrid plasmids did not ferment and only a slight (2-3 fold) increase in maltose-induced maltase expression was observed comparable to that observed for transformants carrying the pMAL64 plasmid. These results clearly indicate that the alterations producing the constitutive phenotype lie in the 3′ *Eco*RI fragment of each constitutive allele.

For the *MAL64-C2* allele, the entire coding region plus approximately 500 basepairs of the upstream region was sequenced. Only a single alteration from the wild-type *MAL64* sequence was found in *MAL64-C2*, a guanine to adenine transition at basepair +921 creating a translation termination site (nonsense mutation) at codon position 307 (Figure 2) located within the 3′-*Eco*RI fragment of *MAL64-C2*. This result is consistent with the results from the

MAL64/MAL64-C2 hybrids shown in Table 1. Based on the results in Table 1, only the coding region of the 3'-*Eco*RI fragment from the *MAL64-R10* allele was sequenced and compared to *MAL64*. This *MAL64-R10* fragment was shown to contain a single base change, a guanine to thymidine transversion at basepair +846 creating a premature termination codon at residue 282 (see Figure 2). Thus, in both constitutive alleles, *MAL64* has been activated to function as a constitutive transcription activator of the *MAL* structural genes as a result of the loss of a significant portion of the C-terminal region of the protein.

***MAL64/MAL63* hybrid genes**.

The results above suggest that the C-terminal portion of MAL64p inhibits its ability to function as a transcription activator. Moreover, in view of the fact that the C-terminal truncations produced a constitutive transcription activator, this region of MAL64p, and possibly also MAL63p, might respond to the inducer, maltose. In order to test this hypothesis, a series of *MAL64/MAL63* hybrid genes were constructed. To facilitate the construction of these hybrids, the upstream *Eco*RI site of the *MAL63* gene was deleted (as described in Materials and Methods) to produce the plasmid YCpMAL63ΔR. The deletion of this site had no effect on the inducible expression of *MAL12* in strain 340-2A (see Table 2). Using plasmids YCpMAL63ΔR

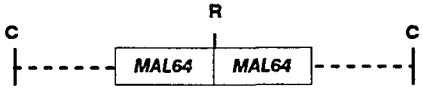
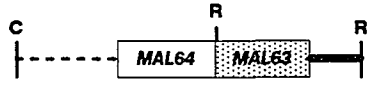
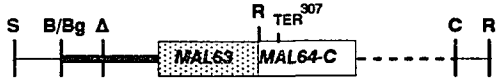
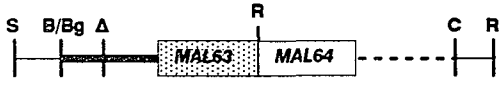
and YCpMAL64, hybrid gene fusions could be created by exchanging the 3′ *Eco*RI fragments of *MAL63* and *MAL64* encoding amino acid residues 215 to 470 as well as sequences 3′ to the *MAL64* coding region.

Plasmid YCpMAL64/63 carries the *Bam*HI-*Eco*RI fragment containing the promoter region and codons 1-215 of *MAL64* fused in frame to the 3′ *Eco*RI fragment of *MAL63*. This hybrid gene encodes a functional, maltose-inducible *MAL*-activator (Table 2) however, transformants carrying plasmid YCpMAL64/63 grow slowly on maltose and fermentation is very slow (5-7 days). In addition, the induced levels of maltase are about 40% of those seen in transformants carrying plasmid, YCpMAL63 expressing the wild-type *MAL63* activator. Because this hybrid activator gene is being expressed from the maltose-inducible *MAL64* promoter, it is likely to require auto-induction perhaps lengthening the time course of induction and fermentation and reducing levels of expression seen in mid-log phase of growth. This has not been studied further.

The inducible phenotype of the YCpMAL64/63 gene fusion clearly supports the proposal that the maltose-responsive domain lies in the C-terminus of the MAL63p. The construction containing the 5′ *Sal*I-*Eco*RI fragment of *MAL63* and the 3′ *Eco*RI fragment of *MAL64* (YCpMAL63ΔR/64) was unable to complement strain 340-2A. These transformants were noninducible, nonfermenters possessing similar levels

Table 2. Activation of maltase gene expression by *MAL64/MAL63* hybrid genes. Single copy yeast plasmid clones were constructed using the CEN plasmid YCp50 (Rose and Broach, 1991) containing *MAL64/MAL63* hybrid sequences as described in Materials and Methods and transformed into strain 340-2A. Cells were grown in SM medium lacking uracil and containing the indicated carbon source(s). Conditions for enzyme assays and fermentation tests were performed similarly as presented in Table 1.

Table 2. Activation of maltase gene expression by MAL64/63 hybrid genes

Plasmid	Yeast Insert	Maltose Fermentation	Maltase Activity (nM PNPG/min/mg protein) GL	GL+M	GL+Glu	GL+M+Glu
YCp50	None	-	16	39	4	3
YCpMAL63		++	30	639	3	4
YCpMAL63ΔR		++	22	677	2	3
YCpMAL64		-	22	67	2	3
YCpMAL64/63		+	20	260	2	3
YCpMAL63ΔR/64-C2		-	40	96	3	3
YCpMAL63ΔR/64		-	20	53	2	2

of maltase expression as those seen in transformants harboring the wild-type *MAL64* gene, plasmid YCpMAL64. This implies that *MAL64* encodes a nonfunctional activator possibly because the inhibitory effects of the C-terminal region cannot respond to maltose.

If the C-terminal region of MAL63p contained only a maltose-responsive domain then, based on the homology of MAL63p and MAL64p, truncation of the C-terminus of MAL63p at residues 282 or 307 should create a constitutive activator as was found for both MAL64C2 and MAL64R10 proteins. Plasmid YCpMAL63ΔR/64C2 fuses the 3′ *Eco*RI fragment of the *MAL64-C2* allele (containing a nonsense mutation at codon 307) to the 5′ *Sal*I-*Eco*RI fragment of *MAL63*. This hybrid gene product encodes a truncated protein with MAL63p sequence from residues 1 to 215 and MAL64p sequence from residues 216-306. Transformants carrying the YCpMAL63ΔR/64C2 plasmid did not ferment maltose and were only slightly inducible (Table 2) implying that the C-terminal region of MAL63p contains, in addition to the maltose-responsive domain, a domain which is required by MAL63p for function. We initially thought that the slight inducibility of this construct was significant and might be enhanced by increasing the gene copy number of this hybrid. The *MAL63ΔR/64C2* hybrid gene was cloned into plasmid pHR81, which carries a *LEU2*-defective allele and which amplifies to approximately 400 copies/cell in leucine-deficient medium.

This hybrid gene was amplified by PCR where *Bam*HI sites were created for subcloning into pHR81. This construct was not sequenced, however, upon amplification using PCR. Strain 340-2A carrying this plasmid was still unable to ferment maltose when grown in leucine-deficient media (Andrew Gibson, personal communication). The basis of this fundamental difference between truncated MAL63p and truncated MAL64p remains to be determined.

Discussion

The *MAL63*-encoded *MAL*-activator is a 470 residue protein required for the regulation of transcriptional expression of the structural genes encoding the maltose fermentative enzymes. As a maltose-responsive activator of transcription, it is expected that MAL63p, possibly in conjunction with other factors which interact with it, will be capable of localizing to the nucleus, binding to the UAS_{MAL}, responding to the presence of maltose and interacting with the transcriptional machinery. Regions of the *MAL*-activator show distinct homology to functional domains recognized in other fungal and higher eukaryotic activators of transcription (Guarente, 1987; Struhl, 1989b). The region from residues 8 to 34 in MAL63p is rich in cysteine and basic amino acids and is highly homologous to the well-characterized DNA-binding domain of GAL4p (Johnston, 1987; Johnston et al., 1986). In GAL4p, this region has been

shown to bind zinc and, reportedly, all six cysteines interact with two zinc ions to form a binucleate-metal-ion cluster responsible for DNA-binding (Pan et al., 1989, 1990). Recent studies in our laboratory confirm that this region of MAL63p is responsible for DNA-binding (Kim and Michels, unpublished results). In the MAL64 proteins, residues 8 to 34 differ from MAL63p at two sites, Asn-25 is a Ser and Ile-28 is a His (see Figure 3). However, DNA-binding of the MAL64p or constitutive MAL64p has not been demonstrated at the UAS_{MAL} but these changes in MAL64p could alter the function of MAL64p as compared to MAL63p at this binding site. A similar finding is seen in the *HAP1-18* allele as described previously. A mutation at the base of the DNA-binding domain results in a protein which increases transcription of *CYC7* (Kim et al., 1989). It is possible that this mutation in *HAP1* allows for the altered protein to bind with a higher affinity to a component(s) of the general transcription machinery. It is possible that this is the reason why MAL63p and possibly the constitutive MAL64p both bind to the UAS_{MAL} but activate maltase expression to different levels.

Nuclear targeting sequences have been identified in several proteins (Rihs et al., 1991; Robbins et al., 1991). These sequences appear to consist of two clusters of basic residues separated by approximately 10 other residues. The MAL63p sequence from residues 41 to 55 conforms well to the

reported consensus sequence and lies just C-terminal to the proposed DNA-binding domain. These 15 residues of the proposed nuclear targeting sequence vary between MAL63p and MAL64p at two sites. One is a conservative change of Lys-54 in MAL63p to an Arg in MAL64p and the second increases the number of basic amino acids in MAL64p by converting Gly-51 in MAL63p to a Lys. Single changes of this type reportedly do not have significant effects on the targeting ability of these sequences. It is interesting to note that many of the known nuclear localization signals have closely associated casein kinase II phosphorylation sites and that phosphorylation of these sites enhances the nuclear targeting ability of the protein (Rihs et al., 1991). A casein kinase II site is not located near the proposed NLS in MAL63p or MAL64p, however, the final C-terminal 14 residues of MAL63p contains a potential casein kinase II target site (see boldface residues in Figure 3; Kuenzel et al., 1987; Marchiori et al., 1988).

The results reported here localize the maltose-responsive domain of the *MAL63*-encoded activator to its C-terminal residues 216 to 470. Loss of this homologous region, as only seen in the *MAL64-C2* and *MAL64-R10* mutations, leads to a constitutive phenotype indicating that maltose induction involves the release from negative control. However, a similar truncation of the maltose-responsive domain in *MAL63* does not result in a constitutive

activator. In this regard, the *MAL64*-encoded activator is similar to HAP1p and LEU3p of *Saccharomyces* and the mammalian glucocorticoid receptor where large internal deletions have been shown to lead to a constitutive phenotype (Kim et al., 1990; Zhou et al., 1990; Godowski et al., 1987). Two alternate models for this negative control can be suggested. Either the maltose-responsive domain interacts with another region of the *MAL63* activator itself or with a negative regulatory factor encoded by another gene. In the absence of maltose, this interaction would prevent the *MAL63* activator from functioning as a transcription activator. In the presence of maltose, the *MAL63* activator could undergo a conformational change needed to release the inhibitory effects of the maltose-responsive domain or release the negative regulatory factor. Thus, deletions or other mutations in the maltose-responsive domain of *MAL63* would not exhibit this interaction and would be constitutive. However, we only see this effect in the MAL64 constitutive activators. Our results suggest that a *MAL*-specific negative regulatory factor is not involved in the regulation of maltose fermentation (see Chapter II results). Efforts by us and by investigators in other laboratories have failed to identify constitutive mutations affecting only maltose fermentation in genes unlinked to the *MAL* loci (Needleman and Eaton, 1974; Rodicio, 1986; A. Gibson, unpublished results). This could be a result of the

selection methods since most of the *MAL*-constitutives were obtained by the reversion of maltose nonfermenting strains. None the less, if a GAL80p-like equivalent existed, it is likely that it would have been identified since loss of function mutations are expected to be relatively frequent. Thus, we propose that either the maltose-responsive domain itself inhibits the functional activity of the *MAL63* activator or, if a negative regulatory factor does exist, it is an abundant and/or required gene function.

At this point we have not identified the function inhibited by this negative regulation but several could be suggested. The *MAL63* activator could be prevented from entering the nucleus in the absence of inducer by an inhibitor protein as is seen for the glucocorticoid receptor (Howard et al., 1988; Picard et al., 1987) and NF-κB (Ghosh et al., 1990) or by a regulated nuclear targeting signal as is the case for those proteins whose localization to the nucleus is enhanced by phosphorylation at a nearby casein kinase II phosphorylation site (Rihs et al., 1991). The negative regulation could prevent or enhance a post-translational modification process, such as phosphorylation or proteolysis. The c-Myb nuclear oncoprotein is phophorylated at a casein kinase II type site near its N-terminal end (Luscher et al., 1990). Deletion of this site leads to oncogenic transformation and allows the protein to bind DNA independent of casein kinase II. Phosphorylation

of GAL4p appears to correlate with its activity as an activator and missense mutations which lie outside of the DNA-binding domain affecting the phosphorylation of GAL4p are noninducible (Mylin et al., 1990b). Alternately, binding of MAL63p to the UAS_{MAL} or interaction with the transcriptional machinery could be affected. For example, the GAL80p interacts with the C-terminal acidic domain of GAL4p inhibiting interaction with the transcriptional machinery (Johnston, 1987; Ma et al., 1987b). Dominant constitutive mutations in this region have been isolated (Salmeron et al., 1990). Constitutive mutations were also obtained by Marczak and Brandriss (1989, 1991) in *PUT3*, the transcriptional activator of the proline degradation genes indicating that interference with transcription activation is occurring in repressed growth conditions but the constitutive mutations eliminate this repression.

The extensive homology between *MAL63* and *MAL64* suggested to Dubin et al. (1988) that the chromosomal region containing *MAL64* represents a duplication of *MAL63* and surrounding sequences. If this is the case, then the sequence variations between the two genes could have resulted from the accumulation of random mutations. Alternately, *MAL64* could be a related but functionally distinct activator perhaps involved in the fermentation of a different α-glucoside such as isomaltose and α-methylglucosidase. Actually, constitutive *MAL* strains have

been shown to contain high levels of α-methylglucosidase. Several *MGL* genes, for α-methyl glucoside fermentation, have been shown to be telomere-linked (Schild et al., 1992). The sequence homology between *MAL63* and *MAL64* genes, while suggestive of functional homology, might be misleading. The results reported here, in fact, indicate a fundamental difference between the truncated, constitutive MAL64 activator proteins and the nonfunctional, truncated MAL63p. The constitutive MAL64-C2p is functionally independent of the C-terminal region of the protein while MAL63p is not. None the less, MAL63p and most likely, the constitutive MAL64p binds to the same DNA sequence. An example of a similar phenomenon is seen in the mineralcorticoid (MR) and glucocorticoid (GR) receptors. MR and GR receptors exhibit sequence homology particularly in their DNA-binding domains (Amero et al., 1992) and both bind to a composite GRE element containing the GR/MR binding site and an AP-1 binding site but MR and GR have different physiological effects (Payvar et al., 1983). For example, GR can enhance transcription from this composite GRE site when AP-1 consists of c-Jun homodimers but represses transcription when AP-1 is composed of c-Jun/c-Fos heterodimers. MR fails to repress at this site (Diamond et al., 1990). Thus, at this composite GRE, the distinct behaviors of MR and GR could reflect differing interactions with AP-1 or other nonreceptor factors. This hypothesis is supported by the

finding that the N-terminal domain of GR can confer the ability of a GR-MR chimeric protein to repress transcription at this composite GRE site (Pearce and Yamamoto, 1993). Another example of members of a related family of factors exhibiting differential behavior but sharing a common DNA-binding site are the octamer binding proteins, Oct-1 and Oct-2. Only the Oct-2 C-terminal activation domain fused to either the Oct-1 or Oct-2 DNA-binding domain can allow for octamer-linked transcription of the β-globin gene (Tanaka and Herr, 1990). This shows that Oct-2 can utilize a region outside of its DNA-binding domain to activate transcription. In light of these findings, MAL63p and the constitutive MAL64p could also be operating in a similar fashion where each can bind to the UAS_{MAL} but interact with a different factor(s) leading to the differential levels of maltase gene expression we observe under different growth conditions.

Chapter II: Detection and overexpression of an epitope-tagged allele of *MAL63*.

Results

Construction of an epitope-tagged allele of *MAL63*.

In order to assay the presence of wild-type or mutant MAL63p under various growth conditions, it was necessary to develop a specific antibody directed against the activator protein. For this, we chose to use recombinant DNA methodology to place a highly antigenic peptide sequence (an epitope tag) at the N-terminal end of MAL63p. The epitope we used is a peptide fragment from the *H. influenzae* hemagglutinin protein (Wilson et al., 1984). An oligonucleotide sequence encoding the 17 amino acids, including the 12 amino acid hemagglutinin epitope, was inserted at the *Nco*I site at the 5'-end of the *MAL63* coding region according to the procedures outlined in Materials and Methods. The tagged allele is referred to as *MAL63/HA*.

The ability of the tagged allele to activate maltase gene expression was tested to insure that the proximity of the epitope to the DNA-binding domain did not interfere with specific binding or possibly, transcription activation. Table 3 compares the level of maltase activity in strains of 340-2A transformed with either the wild-type *MAL63* gene (YCpMAL63), the *MAL63/N* allele constructed by Kim (1991) by

Table 3. Activation of maltase gene expression by the epitope-tagged *MAL63/HA* allele. Fermentation tests and maltase assays were done as described in Table 1 except that cells were grown in SM media lacking uracil plus the indicated carbon source(s).

TABLE 3. Activation of maltase gene expression in 340-2A by *MAL63* before and after hemagglutinin epitope addition

		Maltase activity[b] (nM PNPG/min/mg protein)			
Plasmid	Fermentation[a]	GL	GL+M	GL+Glu	GL+Glu+M
YCp50	_	16	38	4	3
YCpMAL63	++	30	639	4	3
YCpMAL63/N	++	22	601	1	5
YCpMAL63/HA	++	31	460	2	4

placing an *Nco*I site at codon 1 of *MAL63* (YCpMAL63/N) and the *MAL63/HA* epitope-tagged allele (YCpMAL63/HA) all carried on a CEN plasmid and expressed from the *MAL63* promoter. All three strains are maltose fermenters and expression of maltase is clearly inducible. Induced levels of maltase are comparable in strains 340-2A[YCpMAL63] and 340-2A[YCpMAL63/N] but are slightly lower in the strain carrying the tagged allele indicating that the tagged MAL63/HA protein (MAL63/HAp) appears to have retained its maltose-inducibility but transcription activation is reduced perhaps resulting from affects on DNA-binding. Alternately, the epitope-tagged mRNA or protein could be slightly unstable.

The ability to detect the tagged MAL63/HAp in extracts from the strain, 340-2A[YCpMAL63/HA] was tested using Western analysis. Extracts were made from cells grown in SM media lacking uracil but containing 3% glycerol, 2% lactate and either 2% maltose or 2% glucose. Western blots were probed using the monoclonal anti-hemagglutinin antibody according to the procedures outlined in the Materials and Methods. No MAL63/HAp could be detected (results not shown). The inability to detect the protein could result from low abundance because of low levels of expression and/or rapid turnover of the activator, instability during the extraction procedure or a problem with detecting the epitope in this fusion protein. Other workers utilizing the hemagglutinin epitope have reported difficulties detecting

their tagged products, particularly with proteins of low abundance, and have solved this problem by inserting multiple copies of the epitope. We decided against this approach because the singly-tagged allele of *MAL63* had slightly reduced activity as a transcription activator (Table 3). Instead, we decided to increase the expression levels of the tagged allele.

Expression of *MAL63/HA* from the *GAL10* promoter.

For this, we utilized the galactose-inducible *GAL10* promoter which is induced 1000-fold over glucose-repressed levels by the addition of galactose to the growth media (Johnston, 1987). The process of cloning *MAL63/HA* downstream of the *GAL10* promoter in the high copy expression plasmid YEp51 is described in Materials and Methods. In summary, a *Xho*I site was created 60 basepairs upstream of the *MAL63/HA* open reading frame and the *Xho*I-*Sal*I fragment containing the full *MAL63/HA* gene was cloned into plasmid YEp51 at the *Sal*I site located within the *GAL10* promoter. This plasmid, referred to as YEpMAL63/HA, was introduced into either strain Sc340 or Sc490. Sc340 harbors a *GAL10-GAL4* cassette integrated into its genome which provides for the galactose-inducible, and very abundant, synthesis of GAL4p which in turn allows for the abundant expression of genes fused to the *GAL10* promoter, such as in our case, the *MAL63/HA* allele (Schultz et al., 1987). Strain Sc490

contains an *ADH1-GAL4* cassette integrated in its genome in addition to the normal wild-type copy of genomic *GAL4*. This strain allows for constitutive expression of *GAL4* which can then turn on the expression of another gene placed downstream of the *GAL10* promoter without the need for galactose. Strains Sc340 and Sc490 are otherwise isogenic to one another and were shown by Southern blot analyses to contain the maltose permease and maltase structural genes, *AGT1 MAL12* (*MAL1*-linked) and *MAL31 MAL32* (*MAL3*-linked), respectively. Both strains are maltose nonfermenters and apparently do not contain *MAL*-activator function. Plasmid YEpMAL63/HA was then transformed into strains Sc340 and Sc490. Interestingly, Sc340 transformants carrying YEpMAL63/HA were maltose fermenters in the absence of galactose indicating that galactose-independent expression of *MAL63/HA* was occurring, most likely from the *MAL63*-derived 60 basepairs of promoter sequences remaining in the construction.

Galactose-inducible expression of *MAL63/HA* was studied in strain, Sc340[YEpMAL63/HA]. Induction was carried out as described in Materials and Methods and expression of MAL63/HAp fusion was monitored by Western analysis. The predicted molecular weight of the MAL63/HAp fusion is 53,900 Daltons. As shown in Figure 4, detection of a protein with this approximate molecular weight was possible after 2 hours of galactose induction and the levels increased continuously

Figure 4. Overexpression of *MAL63/HA* by galactose induction off the *GAL10* promoter. Strains Sc340[YEp51] and Sc340[YEpMAL63/HA] were grown in SM media lacking leucine but containing 3% glycerol and 2% lactate to an OD_{600} of approximately 0.4, galactose was added to a final concentration of 2% and cell aliquots were taken at zero time (no galactose added), 2 hours, 4 hours and 6 hours after galactose addition. Proteins were extracted and a Western blot was performed using anti-hemagglutinin antibody. Lanes 1-6: 50μg of protein from Sc340[YEpMAL63/HA] grown in 3% glycerol and 2% lactate (Lane 1), after 2 hours of galactose induction (Lane 2), after 4 hours of galactose induction (Lane 3), after 6 hours of galactose induction (Lane 4), Lanes 5 and 6 identical to Lanes 3 and 4, respectively. Lanes 7-9: 50μg of protein from negative control strain Sc340[YEp51] grown in 3% glycerol and 2% lactate (Lane 7), after 2 hours of galactose induction (Lane 8) and after 4 hours of galactose induction (Lane 9).

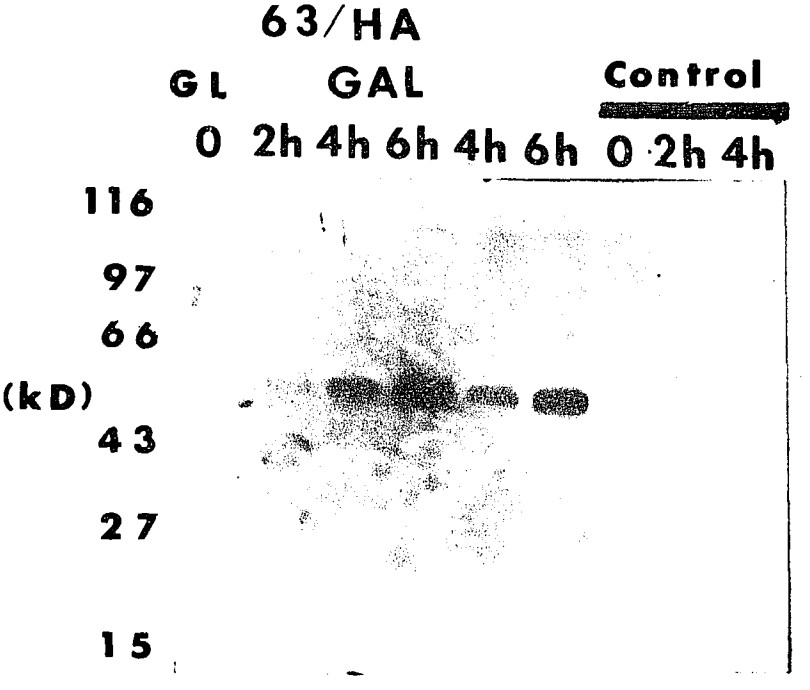


FIGURE 4

over the six hour induction period. No signal was detected in the control strain Sc340[YEp51], carrying the vector alone under similar conditions of galactose induction.

Stability of the MAL63/HAp under different growth conditions was tested. Cells were induced with galactose for 3.5 hours, transferred to maltose media for 1.5 hours with or without cycloheximide (100 μg/ml). The maltose grown culture was then transferred to glucose media and the maltose plus cycloheximideculture was transferred to glucose media plus cycloheximide and both were allowed to grow for an additional 1.5 hours. Cell extracts were prepared after 3.5 hours of galactose induction, after 1.5 hours of growth in maltose (+ or - cycloheximide) and also after 1.5 hours of additional growth in glucose (+ or - cycloheximide) and equal amounts of protein extract were analyzed by Western blot analysis. The results are shown in Figure 5A. It is clear from the maltose culture exposed to cycloheximide that the MAL63/HAp is unstable. According to Figure 5A, we can estimate the half-life of the protein to be approximately 1.5 hours in maltose media and perhaps somewhat shorter in glucose media. It is also apparent that, in the absence of cycloheximide, continued transcription and/or translation of *MAL63/HA* is occurring. Based on the studies of Mylin et al. (1991), the transcription of the *GAL10-GAL4* cassette is repressed following the addition of glucose to the media but translation of pre-existing transcript is likely to

Figure 5A. Western analysis of cell extracts from strain Sc340[YEpMAL63/HA]. Cells were induced in galactose for 3.5 hours, then resuspended in maltose media (+ or - 100 μg/ml cycloheximide) for 1.5 hours and then transferred to glucose media (+ or - 100 μg/ml cycloheximide) and extracts prepared. Lane 1: 60 μg of extract after 3.5 hours of galactose induction. Lanes 2-3: 60 μg of extract after cells were resuspended in maltose media for 1.5 hours without (Lane 2) or with cycloheximide (Lane 3) added to culture media. Lane 4: identical to Lane 1. Lanes 5-6: 60 μg of extracted protein after growth in glucose without added cycloheximide (Lane 5) or with cycloheximide (Lane 6). Lanes 7-8: identical to Lanes 5 and 6, respectively except 50 μg of extracted protein was loaded. Lane 9: identical to Lane 3.

Figure 5B. Western analysis of decreasing amounts of protein extract from Sc340[YEpMAL63/HA]. Cells were induced in galactose for 3.5 hours and protein extracts prepared. Lanes 1-6: 60 μg (Lane 1), 50 μg (Lane 2), 40 μg (Lane 3), 30 μg (Lane 4), 20 μg (Lane 5) and 15 μg (Lane 6) of protein extract. Lane 7: 20 μg of extract after addition of maltose for 1.5 hours without addition of cycloheximide (see Figure 5A above). Lanes 8-9: 50 μg (Lane 8) and 40 μg (Lane 9) of extract after growth in glucose for 1.5 hours without cycloheximide (see Figure 5A above).

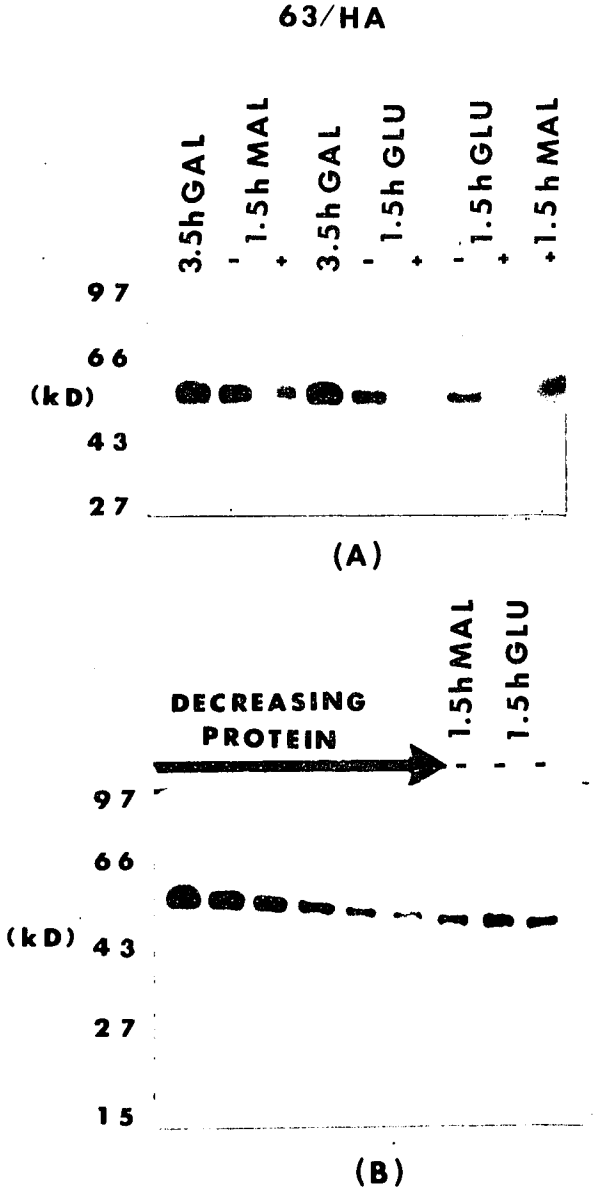

63/HA
3.5h GAL
1.5h MAL
3.5h GAL
1.5h GLU
1.5h GLU
+1.5h MAL
97
66
(kD)
43
27
(A)
DECREASING PROTEIN
1.5h MAL
1.5h GLU
97
66
(kD)
43
27
15
(B)

FIGURE 5

continue. In glucose, pre-existing or newly translated GAL4p would be converted to its dephosphorylated form and become inactive, unable to turn on expression of a gene from the *GAL10* promoter (Mylin et al., 1989, 1990). However, in maltose, the GAL4p is abundant enough and it is likely to remain active in promoting transcription of the *GAL10-MAL63/HA* fusion gene. Therefore, the levels of MAL63/HAp we are seeing in Lanes 2, 5 and 7 without added cycloheximide in Figure 5A are the result of a variety of factors (levels of active GAL4p and thus transcription rates of *MAL63/HA*, half-life of the *MAL63/HA* mRNA, half-life of MAL63/HAp in maltose versus glucose media). It should also be noted from Figure 5B that MAL63/HAp appears to represent a single protein species.

Constitutive expression of MAL63/HAp was studied in strain Sc490[YEpMAL63/HA]. The *ADH1-GAL4* cassette in strain Sc490 allows for constitutive expression of *GAL4* in any fermentable carbon source but not in lactate where expression decreases from 2 to 10 fold (Denis et al., 1983b). Protein extracts from the strain, Sc490[YEpMAL63/HA] were analysed by Western blot analysis following growth in selective media supplemented with maltose or glucose as a carbon source. Figure 6 clearly indicates that the MAL63/HAp is expressed in both maltose- and glucose-grown cells but that the level is slightly reduced in the glucose-grown cells. It is not clear whether

Figure 6. Detection of MAL63/HAp in strain Sc490. Strain Sc490[YEpMAL63/HA] was grown in SM media lacking leucine plus maltose or glucose. Protein extracts were prepared and subjected to Western analysis using the anti-hemagglutinin antibody. Lanes 1-2: 50 μg of protein extract from maltose-grown (Lane 1) or glucose-grown (Lane 2) cells. Lane 3: 8 μg of reduced anti-hemagglutinin antibody. Lane 4: 50 μg of protein extract from the control strain Sc490[YEp51] grown in selective media plus glucose.

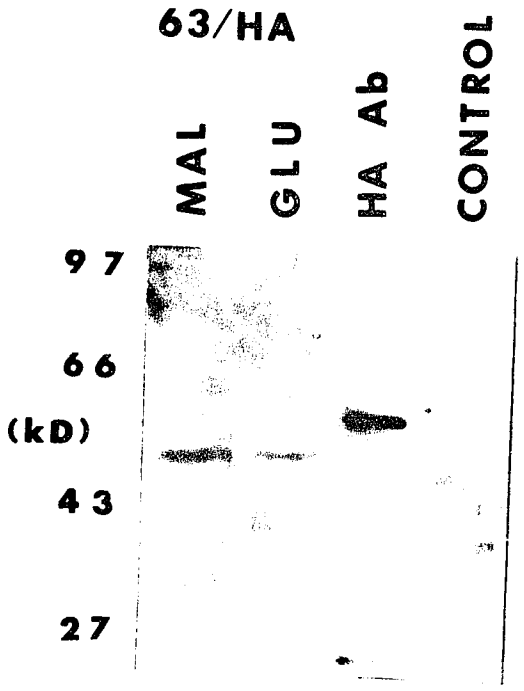


FIGURE 6

this is a reflection of possible differences in the functional activity of the GAL4p in these different growth conditions or differences in the half-life of MAL63/HAp.

Activation of maltase expression by *MAL63/HA*.

These two *MAL63/HA* expression systems can be used to characterize the regulation of transcription activation by the *MAL*-activator. In a number of yeast regulated systems, a specific repressor protein acts to prevent activation of the structural genes by interfering with the activator's function. This mechanism is seen in the interactions between GAL4p and GAL80p. In the absence of galactose, GAL4p interacts with GAL80p (present in limiting amounts) preventing transcription of the *GAL* genes but when galactose is present, GAL4p becomes phosphorylated and able to activate transcription even though it can still be associated with GAL80p (Ma et al., 1987b; Johnston et al., 1987; Parthun et al., 1992). Constitutive expression of the *GAL* genes can occur either by overproduction of the *GAL4* gene, resulting in titration of GAL80p or when *GAL80* is deleted (*gal80*Δ) (Johnston and Hopper, 1982; Torchia et al., 1984). Another yeast gene, *PHO4*, encodes a transcription factor which positively regulates the transcription of genes encoding repressible acid phosphatase (rAPase) (Rogers et al., 1982; Thill et al., 1983). When phosphate levels are low in the cell, PHO4p activates transcription of the

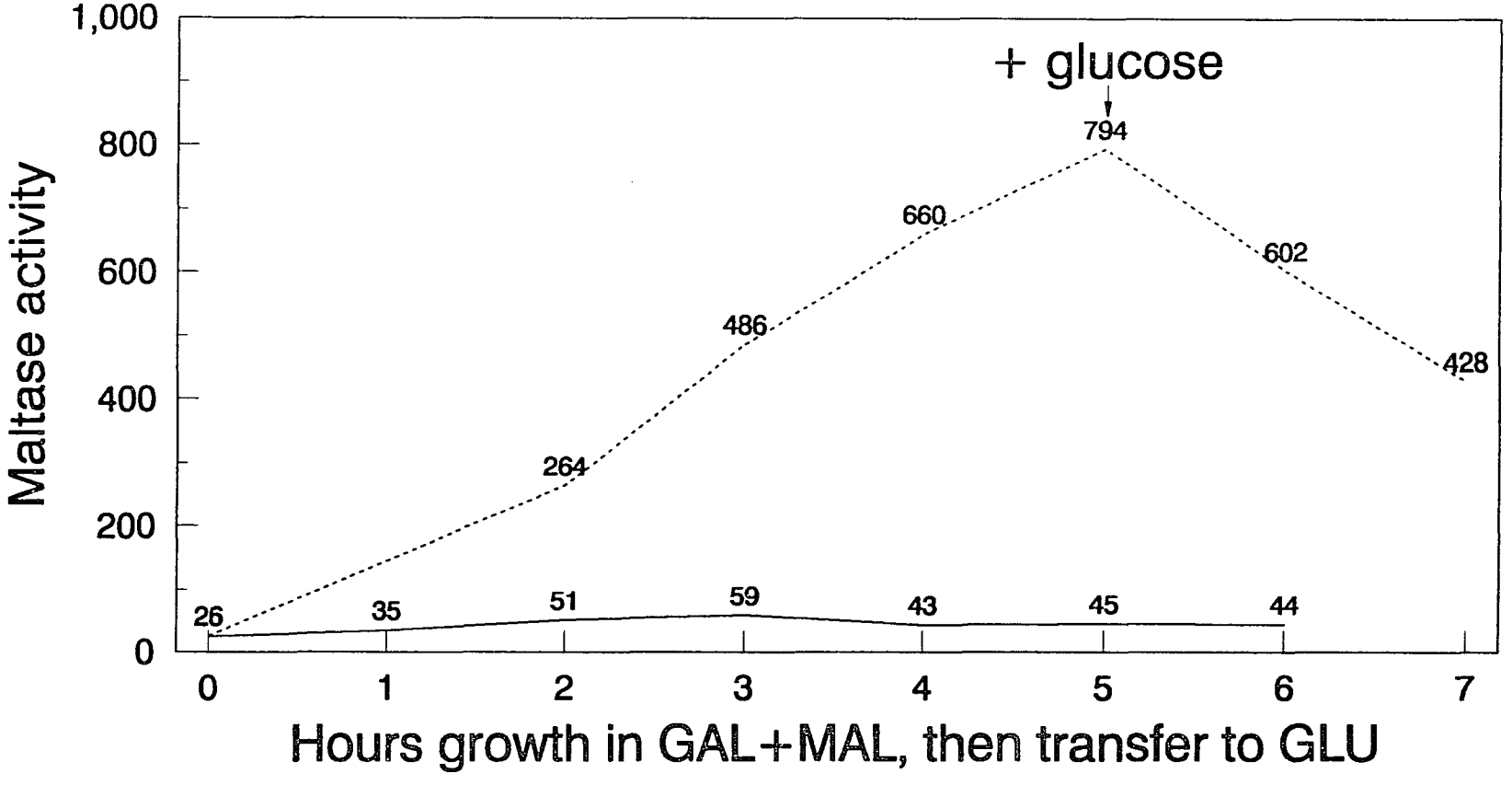
structural genes encoding rAPase. However, when phosphate levels are high, the PHO80p, a negative regulatory factor is proposed to interact with PHO4p, thereby preventing transcription of these structural genes (Ogawa et al., 1990). To test the possible presence of a negative regulator of the *MAL*-activator, we used the galactose-inducible *MAL63/HA* expression system described above.

Strains Sc340[YEp51] and Sc340[YEpMAL63/HA] were both grown to mid-log phase in glycerol-lactate media and expression of *MAL63/HA* induced by the addition of 2% galactose. Cell samples were taken at 1 hour intervals for six hours, total protein was extracted and the level of maltase activity determined. As shown in Figure 4, the level of MAL63/HAp increases continuously during the six hour induction period. Never the less, no significant expression of maltase is seen throughout the six hour induction with galactose as illustrated in Figure 7, indicating that abundant expression of MAL63/HAp does not allow for constitutive activation of the *MAL* structural genes.

The presence of the inducer, maltose, is required for *MAL* gene expression. Strains Sc340[YEp51] and Sc340[YEpMAL63/HA] were grown to mid-log phase and induced with galactose plus maltose (2% final concentration of each) for five hours. Following this, the cells were transferred to glucose (2%) and grown for an additional two hours.

Figure 7. Activation of maltase gene expression by Sc340[YEpMAL63/HA] upon galactose induction alone (solid line) and upon induction in the presence of galactose plus maltose (dashed line). Extracts were prepared as indicated in Table 1 and maltase activities were calculated.

Figure 7. Maltase activity in Sc340[YEpMAL63/HA] in galactose only or in the presence of maltose and subsequent transfer to glucose



Cell samples were taken after every hour and maltase assays performed. As shown in Figure 7, maltase activities in strain Sc340[YEpMAL63/HA] increased continuously during the five hours of induction in galactose plus maltose. The transfer of cells to glucose media leads to an approximately 50% decrease in the level of maltase activity. The levels of maltase activity were low throughout this study for the control strain Sc340[YEp51] (data not shown). Maltase enzyme is stable in the presence of glucose in the media (Cheng et al., 1991). The decrease in maltase enzyme levels probably reflects the continued growth of the cells in the absence of continued synthesis of maltase in the presence of glucose. This is supported by the results obtained in strain Sc490[YEpMAL63/HA] (see below).

Strains Sc490[YEpMAL63/HA] and Sc490[YEp51] were grown in selective media containing 2% maltose, 2% glucose, or 2% maltose plus 2% glucose. Extracts were prepared and maltase activities determined (Table 4). It is apparent that the MAL63/HAp is activated to express maltase only in the presence of maltose. Clearly, even though the levels of MAL63/HAp vary by only 2-3 fold in these different growth conditions (see Figure 6), there is no evidence of induction of the maltase gene in the presence of glucose, even in the presence of maltose. It appears then that the effect of glucose overrides that of maltose. It should be said that Figure 6 does not present a Western blot done on extracts

Table 4. Maltase activities of strains Sc490[YEp51] and Sc490[YEpMAL63/HA] when cells were grown in glycerol+lactate, maltose, glucose, and maltose plus glucose. Enzyme assays were performed as previously stated in Table 1.

TABLE 4. Maltase activities of strains Sc490[YEp51] and Sc490[YEpMAL63/HA] upon growth in maltose, glucose, and maltose plus glucose

	Maltase activity[b] (nM PNPG/min/mg protein)			
Strain	GL	MAL	GLU	MAL+GLU
Sc490 [YEp51]	17	17	1	1
Sc490 [YEpMAL63/HA]	19	1633	1	2

prepared from this strain grown in maltose plus glucose but it is presumed that levels of MAL63/HAp would be comparable to those found when cells were grown in glucose alone.

Discussion

We describe here the detection of the *MAL*-activator encoded by *MAL63* using epitope-tagging, the high level expression of this tagged allele from a heterologous promoter (*GAL10*) and the use of this expression system to investigate the regulation of the *MAL*-activator as a transcription activator. The epitope we used to tag the *MAL63*-encoded *MAL*-activator is derived from the *Haemaphilus influenzae* virus. In our case, a single copy of the epitope, a 12 amino acid residue sequence, was placed at the N-terminal end of the MAL63p. The tagged allele is referred to as *MAL63/HA*. The proximity of the epitope to the DNA-binding domain of the *MAL*-activator appears to have only a slight effect on the maltose-inducibility of the tagged protein as compared to the wild-type *MAL*-activator.

This hemagglutinin epitope has been successfully used to detect other yeast proteins such as the nuclear pore protein encoded by *NUP1*, adenyl cyclase encoded by *CYR1*, and subunit B of RNA polymerase II encoded by *RPBI* (Davis et al., 1990; Field et al., 1988; Kolodziej et al., 1990). In these three proteins, a single copy of the epitope was placed at either the N-terminus or in the middle of the protein. In one

case, three copies of the epitope were placed within the yeast cyclin B protein since one copy was not sufficient for immunodetection (Bruce Futcher, personal communication).

We were unable to detect the tagged MAL63/HAp when expressed from its own promoter even though protein levels were adequate to activate *MAL* gene expression. In order to express the MAL63/HAp at higher levels detectable by Western analysis, we placed the *MAL63/HA* gene fusion downstream of the galactose-inducible *GAL10* promoter in the multicopy plasmid, YEp51. The construction included 60 basepairs of the *MAL63* promoter. Expression of this construction was studied in two strains: Sc340 carrying a *GAL10-GAL4* cassette integrated in its genome where we were able to achieve abundant MAL63/HAp expression following galactose induction, and Sc490 carrying an *ADH1-GAL4* cassette integrated in its genome where constitutive MAL63/HAp expression was observed, albeit, at slightly lower levels than in the galactose-induced strain Sc340[YEpMAL63/HA]. We were able to use these two expression systems to explore the regulation of the *MAL63*-encoded *MAL*-activator as a transcription activator.

We used the galactose-inducible expression system to study the stability of the MAL63/HAp. The results indicate that MAL63/HAp is moderately unstable with a half-life of approximately 1.5 hours in maltose. The MAL63/HAp appears to be slightly more unstable in glucose. Our

immunodetection of MAL63/HAp indicates that only a single protein species is present during growth in galactose, maltose or glucose. However, it is possible that the methods used here (e.g. gel conditions) are not adequate to detect slight changes in protein mobility caused by changes in phosphorylation or perhaps other modifications. We cannot rule out the possibility that MAL63/HAp could be post-translationally modified under particular growth conditions as is seen with GAL4p. GAL4p is phosphorylated to a slower migrating species in the presence of galactose and is subsequently dephosphorylated in glucose (Mylin et al., 1989, 1990). A more detailed study must be undertaken.

The most significant result of these studies is the finding that extremely high levels of MAL63/HAp do not lead to constitutive maltase gene expression in the absence of maltose. This implies that a negative regulator of limited abundance which interacts with the *MAL*-activator, comparable to GAL80p, is not used to regulate maltose induction. This result is consistent with the fact that constitutive mutations have not been obtained in other genes besides GENE 3 or GENE 4 of the *MAL* loci (Needleman, Ph.D. thesis). If there were limiting levels of a negative regulator, the abundant expression of MAL63/HAp would titrate out this factor leading to constitutive expression of maltase. This finding with MAL63/HAp is similar to what was seen when the *PUT3* gene was placed on a high copy plasmid, that is, this

system remained proline-inducible (Marczak et al., 1989). For other yeast activators such as *ADR1*, *GAL4* and *PHO4*, increases in their gene dosages does result in constitutive or partially constitutive phenotypes since inhibitory proteins GAL80p and PHO80p are titrated out by the overabundant GAL4p and PHO4p, respectively (Blumberg et al., 1988; Johnston and Hopper, 1982; Parent et al., 1987). For another activator, *HAP1*, overexpression of this transcription factor reveals an interaction between HAP1p and an unidentified factor(s) termed X (Fytlovich et al., 1993). Upon hemin addition, this interaction is abolished resulting in the heme-regulated induction of transcription activation.

The inability of overexpressed MAL63/HAp to cause constitutive expression of maltase does not rule out the possibility that a negative regulator is present but, if so, this repressor is an abundant protein and is essential, since nonfunctional constitutive mutations have not been isolated. Another possibility is that the *MAL*-activator inhibits itself. We see an example of this type of inhibition with the *LEU3* activator. LEU3p binds to a UAS_{LEU} template independent of the presence of α-IPM as inducer (Sze et al., 1992). Only when α-IPM is added *in vitro* to extracts containing LEU3p will activation occur. In the absence of α-IPM, LEU3p will inhibit transcription from this UAS_{LEU} template (Sze et al., 1992). It is therefore

predicted that α-IPM works to convert LEU3p from a repressor to an activator by a possible conformational change. All of these possibilities for the functioning of the *MAL*-activator need to be studied further.

Studies with the constitutive expression of MAL63/HAp in strain Sc490 enabled us to look at the effects of glucose on the functioning of the *MAL*-activator. MAL63/HAp was detectable in strain Sc490[YEpMAL63/HA] under maltose or glucose growth conditions. The expression of maltase in this strain requires the presence of maltose as also seen in the galactose-inducible system. Glucose fully inhibits maltase gene expression in this strain even when maltose is also present in the medium. This could be the result of either inducer exclusion, inhibitory effects of glucose on the MAL63/HAp itself or effects of other factors involved in maltase gene expression (e.g. *MIG1*) (Nehlin et al., 1990). *MIG1* encodes a zinc-finger protein involved in glucose repression of various genes such as *SUC2*. While overexpression of *MIG1* inhibits the expression of *SUC2*, deletion of the *MIG1* gene interferes with glucose repression of *SUC2* (Nehlin et al., 1990). In order to see whether *MIG1* could play a role in the glucose repression of *MAL63*, overexpression of *MIG1*, even in the absence of glucose, was shown to inhibit maltose-induction of maltase implying that MIG1p competes with the *MAL*-activator for expression versus repression of the *MAL* genes (Zhen Hu and Corinne Michels,

unpublished results). Further studies are needed to determine if glucose acts directly on the *MAL*-activator by some post-translational modification such as phosphorylation or turn-over or if the effects are indirect and mediated by factors such as MIG1p.

Chapter III. Identification of a required functional domain of the *MAL*-activator.

Results

The transcription activation domains of several yeast transcription factors have been localized to the C-terminal region of the protein. These include *GAL4*, *LAC9*, *HAP1* and *LEU3* (Ma et al., 1987a; Pfeifer et al., 1989; Friden et al., 1989). Like GAL4p, the C-terminal region of MAL63p is rich in acidic residues and could form a β-sheet structure like the one recently predicted for the activation domain of GAL4p (Leuther et al., 1993; Van Hoy et al., 1993). Using a "Peptide structure" program from the GCG program package which predicts protein secondary structure according to the Chou and Fasman method, it is predicted that residues 446 through 460 in MAL63p are capable of forming a β-sheet structure (Devereux and Smithies, 1984). Additionally, residues 460 to 467 of MAL63p are rich in acidic residues with a serine residue at codon 462 which could represent a potential casein kinase II phosphorylation site (Kuenzel et al., 1987; Marchiori et al., 1988). For these reasons, the C-terminal region of MAL63p could be involved in transcription activation. We set out to test this hypothesis.

The *MAL63/HA-NS457* allele.

An allele of *MAL63/HA* containing a premature translation stop at codon 457 was constructed by *in vitro* mutagenesis. The allele was cloned into the yeast CEN vector, YCp50, transformed into strain 340-2A which lacks a *MAL*-activator and the ability to activate maltase gene expression was determined (Table 5). Clearly, the loss of the final 14 C-terminal residues are critical for function in the *MAL63*-encoded *MAL*-activator. Also, this plasmid was transformed into a *MAL6* strain, 332-5AΔF1-5 (*MAL63 mal64*Δ) to see if this mutant allele was recessive to the wild-type *MAL63* gene present in the genome of this strain. Indeed, this strain harboring the *MAL63/HA-NS457* allele remained a maltose fermenter and expressed comparable levels of maltase when grown in maltose media as did the untransformed strain, 332-5AΔF1-5 (data not shown). This mutant allele appears then to be recessive to the wild-type activator gene.

To confirm that the loss of activation was not due to the creation of a highly unstable mutant protein, we attempted to detect MAL63/HA-NS457p by Western blot analysis of protein extracts from the transformed strain. Expression of the mutant gene product in this construction was from its own promoter. Again, as was found for the *MAL63/HA* allele, we were unable to detect the mutant protein. It was necessary to utilize strains and plasmids capable of overexpressing the mutant allele so that maltase expression could be studied under conditions where *MAL*-activator

Table 5. Activation of maltase gene expression by *MAL63/HA* and *MAL63/HA-NS457*. Plasmid YCpMAL63/HA contains the approximately 3.2 kb *Sal*I fragment containing the *MAL63/HA* allele (described in Chapter II) in the vector YCp50. Plasmid YCpMAL63/HA-NS457 is identical except for the introduction of the termination signal at codon 457. Both plasmids were transformed into the maltose non-fermenting strain 340-2A (*AGT1 MAL12 MAL31 MAL32*). Fermentation tests and maltase assays were done as described for Table 1 except that cells were grown in SM media lacking uracil plus the indicated carbon source(s).

TABLE 5. Activation of maltase gene expression by *MAL63/HA* and *MAL63/HA-NS457*

		Maltase activity[b] (nM PNPG/min/mg protein)			
Plasmid	Fermentation[a]	GL	GL+M	GL+GLU	GL+GLU+MAL
YCp50	-	16	38	4	3
YCpMAL63/HA	++	31	460	2	4
YCpMAL63/HA-NS457	-	12	34	3	4

protein levels were detectable.

The mutant allele, *MAL63/HA-NS457* was fused to the *GAL10* promoter using the YEp51 plasmid as described in the Materials and Methods and this plasmid is referred to as YEpMAL63/HA-NS457. It should be noted that, as before, 60 basepairs of the native *MAL63* promoter are retained in this construction.

The expression of *GAL10-MAL63/HA-NS457* in strain Sc340 containing the galactose-inducible *GAL10-GAL4* cassette was studied. Strain Sc340 does not ferment maltose because it lacks a functional *MAL*-activator gene. It does contain *MAL1* and *MAL3*-linked genes encoding maltose permease and maltase (*AGT1 MAL12 MAL31 MAL32*). Strains Sc340[YEpMAL63/HA-NS457] and Sc340[YEpMAL63/HA] were induced with galactose and then transferred to media containing maltose or glucose. Protein extracts were prepared from cells following galactose induction and following the subsequent growth in maltose and glucose and analyzed by Western analysis. The results are shown in Figure 8. Mutant protein is clearly detected under these conditions, albeit at a lower level. (Note that five times as much total protein extract from the strain carrying the mutant allele was loaded per lane as compared to the extracts from the strain carrying *GAL10-MAL63/HA*. This lower level of expression could be the result of a reduction in expression from the *GAL10* promoter and might not be related to the instability of the mutant protein).

Figure 8. Western analysis of strains harboring the galactose-inducible *MAL63/HA* and *MAL63/HA-NS457* alleles. Plasmids YEpMAL63/HA and YEpMAL63/HA-NS457 carrying the *GAL10-MAL63/HA* and *GAL10-MAL63/HA-NS457* fusion genes were transformed into strain Sc340 carrying the galactose-inducible *GAL10-GAL4* cassette. The strains were induced with galactose as described in Materials and Methods for 3.5 hours and then transferred to media containing either 2% maltose or 2% glucose for 1 or 2 hours, as indicated in the figure. Lanes 1-3: 30 μg of protein extracts from Sc340[YEpMAL63/HA] after 3.5 hours of galactose induction (Lane 1), after transfer to maltose media for 1 hour (Lane 2) or glucose media for 1 hour (Lane 3). Lane 4: 15 μg of reduced anti-hemagglutinin antibody indicating the heavy (55 kDa) and light chain (25 kDa) polypeptides. Lanes 5-7: 150 μg of protein extracts from Sc340[YEpMAL63/HA-NS457] after 3.5 hours of galactose induction (Lane 5), 1 hour after transfer to maltose media (Lane 6) and 2 hours after transfer to maltose media (Lane 7).

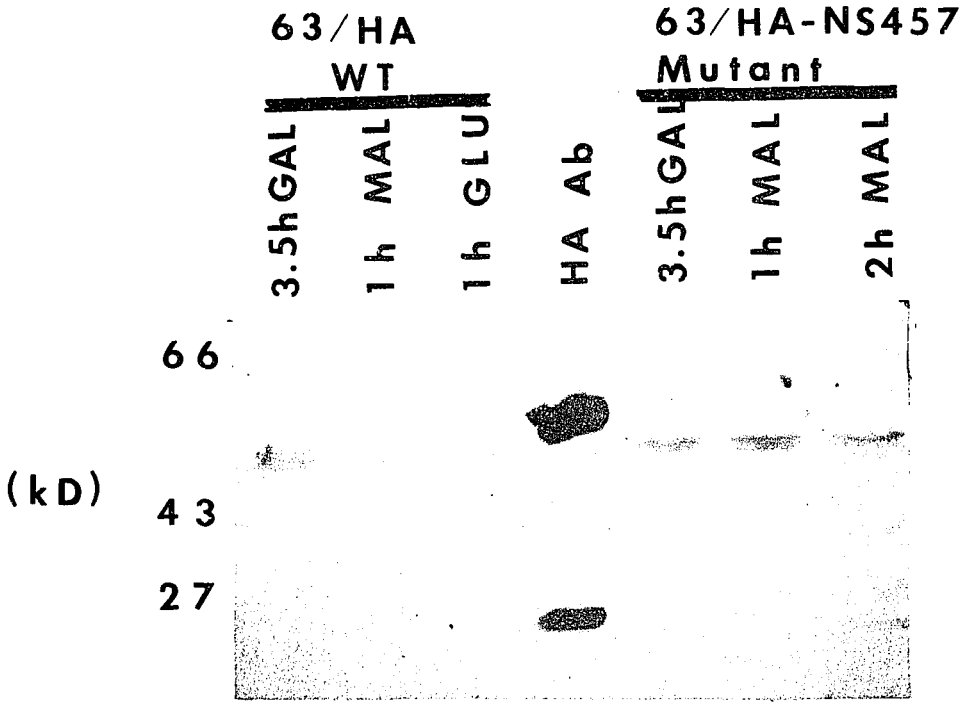

63/HA
WT
63/HA-NS457
Mutant
3.5hGAL
1h MAL
1h GLU
HA Ab
3.5hGAL
1h MAL
2h MAL
66
43
27
(kD)

FIGURE 8

Strain Sc340[YEpMAL63/HA-NS457] was induced with either galactose alone (2%) or galactose (2%) plus maltose (2%) and maltase gene expression was followed for up to 5 hours. No increase in maltase levels in this strain over those seen in strain Sc340[YEp51] carrying the vector alone was detected in either growth condition over the entire time course (data not shown). In a similar experiment, shown in Figure 7 of Chapter II, using strain Sc340[YEpMAL63/HA] carrying the epitope-tagged, wild-type *MAL63* allele, a 50-fold induction of maltase expression was seen at the end of 5 hours when both galactose and maltose were present in the media. These results clearly support the hypothesis that the C-terminal 14 residues of MAL63p are required for function.

As further evidence, we investigated the activation of maltase expression and *MAL*-activator protein levels in strain Sc490[YEpMAL63/HA-NS457]. Sc490 is isogenic with strain Sc340 except it carries the *ADH1-GAL4* cassette and produces GAL4p constitutively in media containing fermentable carbon sources. Cells were grown in maltose or glucose, protein extracts prepared and analyzed for *MAL*-activator levels by Western blotting (see Figure 9). Comparable levels of *MAL*-activator expression are seen in both the strain carrying the mutant allele *MAL63/HA-NS457* and the wild-type *MAL63/HA* allele. Maltase expression in strain Sc490[YEpMAL63/HA-NS457] is shown in Table 6 where no significant increase in maltase expression is seen in

Figure 9. Western blot analysis of strains constitutively expressing high levels of the mutant protein, MAL63/HA-NS457p and MAL63/HAp. Strains Sc490[YEpMAL63/HA] and Sc490[YEpMAL63/HA-NS457] were either grown in SM media lacking leucine plus 2% maltose or 2% glucose. Lanes 1 and 2: 150 μg of extract from strain Sc490[YEpMAL63/HA-NS457] grown in maltose. Lane 3: 100 μg of extract from strain Sc490[YEpMAL63/HA-NS457] grown in glucose. Lane 4: 100 μg of extract from strain Sc490[YEpMAL63/HA] grown in glucose. Lane 5: 100 μg of extract from strain Sc490[YEpMAL63/HA] grown in maltose. Lane 6: 100 μg of extract from negative control strain Sc490[YEp51] grown in glucose.

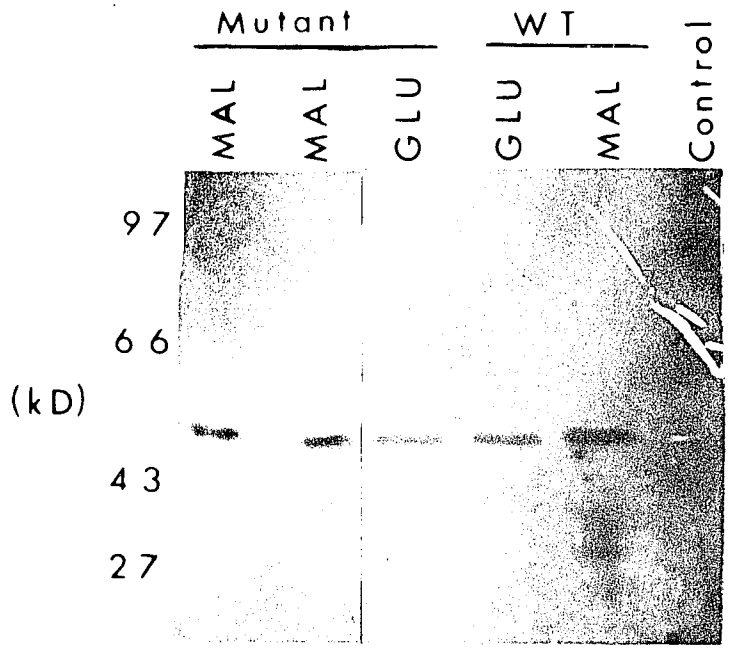


FIGURE 9

Table 6. Activation of maltase gene expression in strain Sc490[YEpMAL63/HA-NS457]. Maltase activities of extracts from strains Sc490[YEp51] and Sc490[YEpMAL63/HA-NS457] were determined as described in Materials and Methods. Cells were grown in SM media lacking leucine plus either 3% glycerol and 2% lactate, 2% maltose, 2% glucose, or 2% maltose plus 2% glucose.

TABLE 6. Activation of maltase gene expression in strain Sc490[YEpMAL63/HA-NS457]

	Maltase activity[b] (nM PNPG/min/mg protein)			
Strain	GL	MAL	GLU	MAL+GLU
Sc490 [YEp51]	17	17	1	1
Sc490 [YEpMAL63/HA-NS457]	18	20	1	1

maltose growth conditions. However, Table 4 of Chapter II shows an 85-fold induction of maltase expression in strain Sc490[YEpMAL63/HA] under these same conditions. Thus, the results of Figure 9 and Table 6 clearly indicate that truncation of the final C-terminal 14 residues of the *MAL63*-encoded *MAL*-activator removes sequences essential for its function.

***LexA-MAL63* gene fusions**.

To explore the possibility that this acidic region of MAL63p constitutes the transcription activation domain, we made use of a system developed by Hanes and Brent (1989). Their approach utilizes the DNA-binding activity of the *E. coli* repressor protein LexA. The *E. coli* LexA protein (202 amino acids) represses transcription of several bacterial genes involved in the SOS response (Brent and Ptashne, 1980). LexA protein (LexAp) binds as a dimer to its operator site (R. Brent, Ph.D. thesis, 1982). LexAp contains an amino-terminal domain (residues 1-87) that allows for binding to a short operator DNA sequence with weak dimerization contacts and a carboxy-terminal domain (residues 88-202) that contains a dimerization domain and a hinge region connecting the two domains (R. Brent, Ph.D. thesis, 1982). The N-terminal fragment of LexAp binds operator DNA with reduced efficiency since it is lacking a dimerization domain. As a result, this N-terminal fragment

induces the transcription of genes normally repressed by native LexAp in *E. coli* (Brent and Ptashne, 1985).

In yeast, one can utilize the ability of LexA protein to bind operator DNA by fusing the DNA-binding domain of LexAp (residues 1-87) to sequences from a eukaryotic protein believed to contain a transcription activation domain. Native bacterial LexA protein will repress transcription from a *GAL1-lacZ* reporter plasmid if it is bound to LexA operators positioned downstream of the UAS_{GAL} but upstream of the transcription start site (Brent and Ptashne, 1984). No repression is observed when the operator is close to, but upstream of the UAS (Brent, 1985). However, LexA fusion proteins which contain a transcription activation function from a heterologous protein will activate target gene transcription in yeast provided the gene contains one or more LexA operator sites positioned upstream of their transcription initiation sites (Lech et al., 1988). Function of these fusion proteins can then be monitored by following expression of a *lacZ* reporter gene containing up to six copies of the LexAp operator binding site within its promoter sequence. The multiple copies of the binding site enhances the ability of these LexA fusion proteins to activate transcription of the reporter gene. It is important to remember that residues 1 to 87 of LexAp lack a dimerization domain but that dimerization is required for the LexAp to bind DNA and thus this dimerization function

must be provided by the protein under study (Kim and Little, 1992).

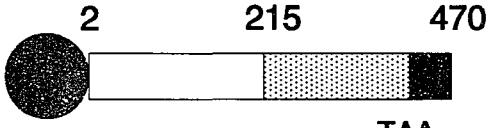
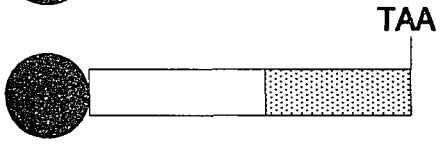
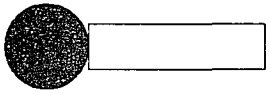
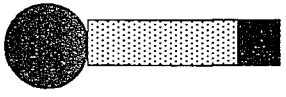
This method was first used to define the transcription activation domains of the GAL4p and GCN4p activators (Brent and Ptashne, 1985; Hope and Struhl, 1986). Fusions made between the DNA-binding domain of LexA to residues 74-881 of GAL4p or residues 1-125 of GCN4p are both able to induce the expression of the reporter gene, *lacZ* containing upstream LexAp binding sites in the promoter sequences. Other experiments have been done fusing the mammalian Fos oncoprotein to LexA showing that this is also a strong activator of transcription (Lech et al., 1988). Another yeast factor, *IME1*, required for expression of the gene *IME2* and other genes required for meiosis, was shown to encode a transcription activator in this system (Smith et al., 1993). The full length *IME1* gene was fused to the DNA-binding domain of *lexA* and shown to activate a *lacZ* reporter gene containing LexAp binding sites in its upstream region. Also, fusion of only the tyrosine-rich region present in IME1p to LexAp activates transcription of *lacZ* to 25% of the level seen with the full length fusion of *IME1* to *lexA*. Early studies showing that a 79 amino acid residue *E. coli* genomic sequence having a net charge of -10 but containing no yeast sequences, when fused to *lexA*, is able to turn on *lacZ* reporter gene expression in yeast led researchers to believe that an amphipathic acidic α-helical region was

required for interaction with the transcription machinery (Ma and Ptashne, 1987c). However, recent studies from Stephen Johnston and coworkers have not supported this conclusion and have shown that the activation domain in GAL4p is capable of forming a β-sheet structure which is functional in this conformation (Leuther et al., 1993; Van Hoy et al., 1993).

For our experiments, the *lexA* sequence encoding the DNA-binding domain (codons 1-87) was fused to the full length *MAL63* gene (codons 2-470), to the 5'-half of the *MAL63* open reading frame (codons 1-215), to the 3'-half of the *MAL63* open reading frame (codons 215-470) as well as to the *MAL63* mutant allele (codons 2-456) containing the translation termination signal at codon 457. These fusion genes and the *lacZ* reporter plasmid, pSH18-18 (containing 6 LexAp operator binding sites upstream of a *GAL1-lacZ* fusion gene) were doubly transformed into a maltose fermenting strain B16, containing a fully functional *MAL2* locus and a partially functional allele of *MAL1* with the genotype *mal11 MAL12 mal13*, and into the nonfermenting strain YPH500, which lacks a functional *MAL*-activator gene but contains the structural genes *AGT1 MAL12* and *MAL31 MAL32*. Expression of β-galactosidase was determined for each transformed strain and the results are shown in Table 7. Included in Table 7 as controls are the parent plasmid, pSH2-1, containing only the LexA DNA-binding domain and plasmid, plexA:SNF2, which

Table 7. β-galactosidase expression in the *MAL+* strain, B16 and the *MAL-* strain, YPH500 doubly transformed with the *lexA* fusion plasmids and the *lacZ* reporter plasmid. Assays were performed as stated in Materials and Methods. Cells were grown up in SM media lacking uracil and histidine (and leucine for the YPH500 transformant harboring the plasmid, pRSMAL63) and including the indicated carbon sources. Expression is given in units of specific activity. ND = not determined.

Table 7. Beta-galactosidase expression in B16 and YPH500

LexA Fusion		MAL2 Activator strain B16[pSH18-18]			MAL-activator deletion strain YPH500[pSH18-18]		
		GAL	MAL	GLU	GAL	GAL+MAL	GLU
	LexA	<1	<1	<1	3	3	4
	LexA :MAL63(2-470)	1	3	1	2	400	4
	LexA :MAL63(2-456)	ND	ND	ND	7	6	9
	LexA :MAL63(1-215)	<1	<1	<1	3	4	3
	LexA :MAL63(215-470)	<1	<1	<1	2	1	2
	LexA :SNF2	15	457	755	1200	3300	840

expresses a LexA:SNF2 fusion protein with transcription activation function (Laurent et al., 1991). Proper expression of the various LexA fusion proteins was monitored by Western blot analysis of total cell extracts from the strains above probing with anti-LexA antibody (given to us by Roger Brent). All of the LexA fusion proteins are expressed at high constitutive levels (data not shown).

The full length fusion of *MAL63* to *lexA* was able to provide activator function in the *MAL*-activator deletion strain, YPH500 in the presence of maltose. The fusion *lexA:MAL63(2-456)*, however, does not allow for the expression of *lacZ* under any growth condition and indicates that residues 457-470 are required for transcription activation and/or dimerization. Neither the 5'-half or 3'-half alone of *MAL63* fused to codons 1-87 of *lexA* was able to provide a similar function [see fusions *lexA:MAL63(1-215)* and *lexA:MAL63(215-470)*]. This could be because the activation domain is disrupted in each construction (perhaps the region from residues 200-300 is required intact for activation) or it could be due to the fact that residues of MAL63p which contain the activation domain do not provide a dimerization domain, thereby, preventing binding of these fusions to the LexAp binding site. To test this, these two halves of *MAL63* were fused separately to codons 1-202 of *lexA* which encodes the full length LexAp containing a dimerization domain and were tested for transcription

activation function. Neither of these constructions were able to activate *lacZ* gene expression (Andrew Gibson, unpublished results). Taken together, these results might indicate that the full length MAL63p is required intact for it to activate gene transcription.

Table 7 shows, most importantly, that in order for pLexA:MAL63(2-470) to activate transcription of *lacZ*, maltose must be present in the media since there is no activation when cells are grown in galactose or glucose. Therefore, maltose is required by MAL63p to activate transcription of the *lacZ* gene. This is not to imply that maltose itself binds to MAL63p. This would have to be investigated further.

None of the *MAL63* fusions to *lexA* are functional in the *MAL+* activator strain, B16 including the *lexA* fusion to the full coding region of *MAL63* (codons 2-470) (see Table 7). To determine if the inability of the LexA:MAL63(2-470)p fusion to activate reporter gene expression in the *MAL+* strain is related to the presence of the wild-type activator (*MAL23*) in strain B16 and not to differences in the backgrounds of strains B16 and YPH500, we transformed the wild-type *MAL63* gene controlled by its own promoter, cloned onto the pRS315 plasmid (pRSMAL63) (constructed by Andrew Gibson) into strain YPH500[pSH18-18] + [plexA:MAL63(2-470)] (Sikorski et al., 1989). This transformed strain was then tested for expression of the reporter gene, *lacZ* and found that *lacZ*

gene expression was activated, albeit at a slightly lower level. Whereas the strain containing *plexA:MAL63(2-470)* exhibits a β-galactosidase specific activity in maltose of 400 (see Table 7), addition of pRSMAL63 to this strain decreases the level of β-galactosidase activity in maltose to 240 (data not shown). This lowered level of activation could reflect the possible formation of LexA:MAL63(2-470)p/MAL63p heterodimers which are unable to bind to the LexAp binding site. However, this is not very likely since there is such a high constitutive level of LexA:MAL63(2-470)p expression compared to the wild-type MAL63p that formation of heterodimers should be at a low level. Alternately, this lowered β-galactosidase activity could reflect competition between the LexA:MAL63(2-470)p and wild-type MAL63p for a common factor found in limiting amounts but necessary for MAL63p to activate transcription, that is, a coactivator. *LacZ* expression was not seen in strain B16 harboring plexA:MAL63(2-470) but was only slightly reduced in strain YPH500 harboring plexA:MAL63(2-470) and pRSMAL63, indicating that differences in strain backgrounds could have led to the differences in *lacZ* expression levels exhibited in strains B16 and YPH500. However, another possibility is that a *MAL23*-specific effect could have inhibited the function of the LexA:MAL63(2-470)p in strain B16 (discussed below).

Discussion

Previous studies have demonstrated that *MAL63* as well as the other GENE 3 homologues, encodes a positive regulator required for the fermentation of maltose (Chang et al., 1989). DNA sequence analysis of *MAL63* identified a cysteine-basic amino acid rich region near the N-terminus (residues 8-27) that is highly homologous to the DNA-binding domains of other yeast transcription activators (Kim and Michels, 1988). An *E. coli* synthesized MAL63p fragment consisting of the N-terminal residues 1-111 was shown to bind to DNA sequences derived from the UAS_{MAL} of the *MAL61* and *MAL62* promoter region. These results identified MAL63p and cysteine residues Cys-11, Cys-24 and Cys-27 and proline-23 as being necessary for this binding. Clearly, MAL63p is a DNA-binding, transcription activator protein. In this study, we hoped to localize the region of the MAL63p involved in transcription activation and to investigate the function, if any, of the final C-terminal acidic region of the protein.

Acidic activation domains were originally thought to be made of "acid blobs" that interacted with their target factors through ionic interactions (Sigler, 1988). However, this model was not applicable in certain cases. Point mutations in nonacidic residues of the VP16 or GAL4 activation domains, in particular, can decrease activation significantly showing that there is no real correlation

between negative charge and activation potential (Leuther et al., 1993). Additionally, Ptashne and coworkers had suggested that amphipathic α-helices formed within the acidic activation domains allowing the acidic residues to lie on one face of the helix (Giniger and Ptashne, 1987; Ma and Ptashne, 1987c; Ptashne, 1988). In this way, the acidic residues were proposed to be positioned so as to enhance interaction with positively charged residues of target protein(s) in the transcription machinery. However, the idea that these acidic activation domains (AADs) could adopt an α-helical structure must be reevaluated due to the following findings. Recent work done with synthetic peptides representing the activation domains of GAL4p and GCN4p has shown that these peptides can form a β-sheet structure at pH 6 and are not α-helical as previously suspected (Van Hoy et al., 1993; Leuther et al., 1993). In addition, under these same *in vitro* conditions, the GAL4 C-terminal 34 amino acid peptide can block the association between GAL4p and GAL80p indicating that the β-sheet structure of this peptide is biologically functional. The final 25-35 C-terminal residues of MAL63p were analyzed using the "Peptide structure" program from the GCG package and residues 446 through 460 were found to have the potential to form a β-sheet type structure (Zhen Hu, personal communication). We felt that this C-terminal, acid-rich region of MAL63p might function as the activation

domain and our studies here hoped to test this hypothesis.

A nonsense mutation at codon 457 of *MAL63* which deletes the final C-terminal 14 residues is not functional as a transcription activator. Even when overexpressed, to abundant and easily detectable levels, and in the presence of maltose, no induced expression of maltase was observed. This result demonstrates that these C-terminal residues could be required for maltose-induced transcription activation. In an effort to determine if this region was involved specifically in transcription activation, we constructed a series of *lexA-MAL63* fusion genes using the *lexA* DNA-binding domain (codons 1-87) and monitored their function as transcription activators for the *lacZ* reporter gene containing LexAp binding sites in its upstream promoter sequences. It is clear that the fusion *lexA:MAL63(2-470)* is capable of providing a transcription activator for *lacZ* gene expression only in the presence of maltose. How maltose works to create this functional activator is unknown but could involve some conformational change or post-translational modification in the protein. This possibility needs to be looked at further. Once the C-terminal residues 457-470 are truncated from this fusion, however, the activation function is abolished (see *lexA:MAL63(2-456)* activity in Table 7). Therefore, these residues are required for maltose-induced transcription activation and/or dimerization by MAL63p which in turn, could be necessary for

activation. We were unable to localize the activation function in *MAL63* to its C-terminal region alone where these required residues are located. This could mean one of two things: 1) the C-terminal domain of MAL63p is necessary but not sufficient to activate transcription and also requires its N-terminal domain or 2) in order for the MAL63p to activate transcription, it must be in its full length form since a conformational change in the protein could be essential for it to interact with the general transcription machinery.

Our results might also be suggesting that an auxiliary factor could be required for transcription activation by MAL63p. Since the activity of LexA:MAL63(2-470)p fusion is abolished in strain B16 expressing a wild-type *MAL23*-activator at only single copy levels, this result could imply that a coactivator or adaptor protein is required by MAL63p but is unavailable since it also binds to the MAL23p. This coactivator might be sequestered and unavailable for binding to MAL63p, thereby, preventing activation by the LexA:MAL63(2-470)p. The various LexA:MAL63 fusion proteins are all expressed at high constitutive levels from the *ADH1* promoter and, as such, are present in much higher amounts than those of the wild-type MAL23p. Therefore, this effect is significant since it indicates that the LexA:MAL63(2-470)p cannot activate transcription on its own and requires some background factor not available in strain B16. To

confirm the presence of an adaptor molecule, function of the LexA:MAL63(2-470)p in strain B16 will have to be tested upon deletion/disruption of the genomic copy of *MAL23*. This remains to be tested.

Several examples of transcription coactivators or adaptors have been described. For example, the TATA box binding protein (TBP) of *Drosophila* exists as a tight complex called the TFIID fraction containing six TBP-associated factors (TAFs) where one or more of these TAFs is required as a coactivator(s) for activated transcription (Dynlacht et al., 1991). The *ADA2* gene encodes an adaptor protein which is required for the functioning of certain acidic activation domains present in VP16 and GCN4 proteins (Berger et al., 1992). HAP1p has recently been shown to bind to a factor(s) upon binding to its target sequences *in vitro* (Fytlovich et al., 1993). However, upon addition of hemin, a new complex forms with a reduced molecular mass. The function or identification of this factor(s) has not yet been determined. In addition to GAL4p interacting with its inhibitor GAL80p, a new factor has been identified which is required for GAL4p to bind to the UAS_{GAL} sequence *in vitro* (Parthun et al., 1992). This factor is highly basic (containing 21% of lysine and arginine residues) and encoded by the *EGD1* gene (enhancer of *G*AL4 *D*NA binding). This EGD1p was isolated in the flowthrough fraction upon affinity purification of the GAL4p. Purified GAL4p was not capable

of forming a stable complex with DNA by gel retardation assays but addition of this flowthrough fraction containing EGD1p restored complex formation. Deletion of *EGD1* results in a decreased rate and level of galactose induction of *GAL10* mRNA so this factor could very well be involved in GAL4p DNA-binding *in vivo*. Another factor which acts in B-cells, NF-EM5 binds to DNA at the immunoglobulin κ gene 3′ enhancer with the help of another protein, PU.1 (Pongubala et al., 1993). This factor, PU.1 needs to be phosphorylated at serine-148 (a consensus site for casein kinase II phosphorylation) in order to interact with NF-EM5, to allow NF-EM5 to bind to the κE 3′ enhancer probe and to enhance transcription activation.

The factor that could be required for MAL63p activation is unlikely to be required for DNA-binding since it is needed for *lacZ* reporter gene expression which uses LexAp binding ability to the LexAp binding sites and not MAL63p binding to the UAS_{MAL}. Most likely, our results imply that this factor could be required for transcription activation. The possible existence of this factor is being explored.

On the basis of all of the results presented in this thesis, I would like to propose a tentative model of how the *MAL63* activator might work to activate gene expression in response to maltose. The results of Chapter I show that a maltose regulable domain is present in the C-terminal half of MAL63p. Truncation of this domain, as seen in the

MAL63/MAL64-C2 gene fusion, does not lead to the creation of a constitutive activator as we might have expected based on the results with the constitutive *MAL64* alleles. Therefore, the C-terminal domain of MAL63p also appears to contain a domain required for maltose-regulated induction. Despite the high degree of homology between MAL64p and MAL63p, MAL64p does not require the C-terminal region for activation.

Figure 10 presents a diagram of the domains of the *MAL63* activator including the DNA-binding domain (Kim, 1991), the potential nuclear localization sequence (NLS), the required C-terminal acidic region (residues 456-470) and the region required for maltose induction. The results of Chapter II demonstrate that overexpression of MAL63p does not lead to the constitutive expression of the maltose fermentative genes indicating that a limiting negative regulatory factor, comparable to a GAL80-like repressor, is not involved in the *MAL* regulatory system. However, the participation of an abundant or essential factor cannot be ruled out. Additionally, maltose is required to induce the transcriptional activity of the MAL63p even when MAL63p is overexpressed. The results in Chapter III indicate that the final 14 C-terminal residues of MAL63p are essential for its transcription activation function and/or dimerization function. Activity of the *lexA-MAL63* fusion activator requires maltose for reporter gene induction indicating that

Figure 10. The functional domains of the *MAL63* activator. The confirmed DNA-binding domain, potential NLS and acidic regions are indicated. The acidic region in the final 14 C-terminal residues is indicated as being required for either transcription activation function and/or dimerization.

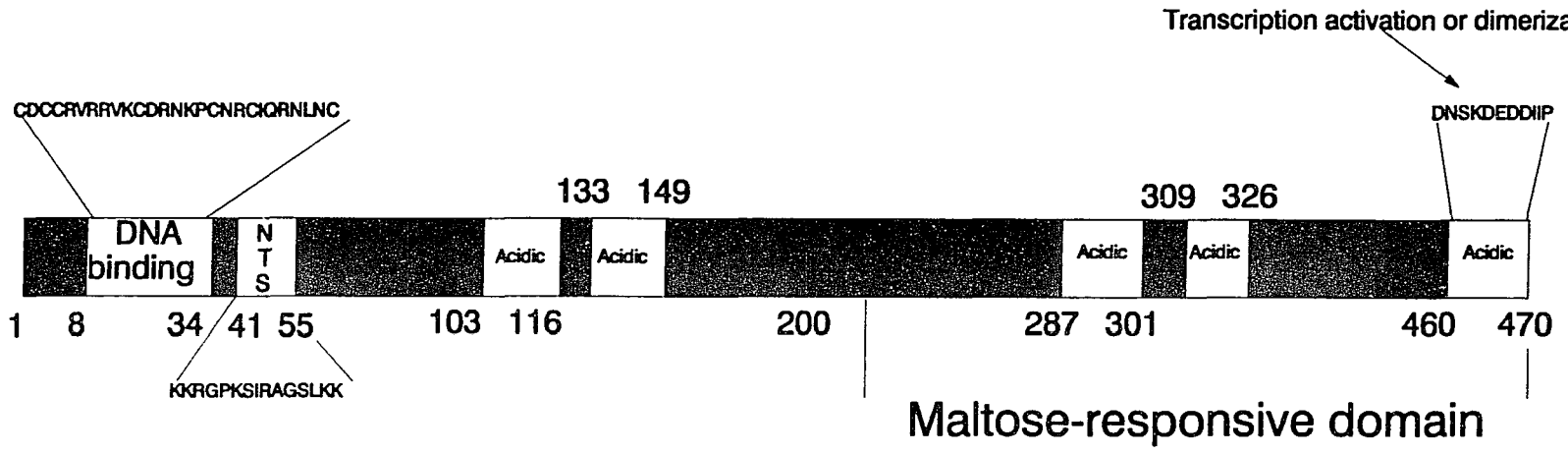


Figure 10: Domains of the MAL63 activator

maltose does not regulate nuclear localization or DNA-binding.

In summary, we propose the following model for the maltose induction of MAL63p transcription activation. Maltose, or a metabolite of maltose, either binds to MAL63p directly or creates an altered physiological state in the cell, such as a pH change or Ca^{+2} flux change, which leads to a post-translational modification of MAL63p or the binding of a signal molecule. This in turn induces a conformational change within MAL63p or the release of a repressor protein. If a repressor protein is involved, it must be either an extremely abundant protein, an essential protein or encoded by multiple genes. We do not favor the existence of this repressor. Maltose induction appears to enhance the interaction between MAL63p and the general transcription machinery at the UAS_{MAL} and not affect nuclear localization or DNA-binding. Our results did not allow us to localize the transcription activation domain of MAL63p. Perhaps several regions of the protein interact with one another in the presence of inducer to activate transcription. Alternately, the activation domain could be localized to a smaller region of the protein, for example, within the first 282 residues as was found for MAL64-R10p, but that MAL63p requires a functional domain found in the C-terminal half of the protein for proper formation/activation of the transcription activation function.

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